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Improved sample preparation ensure accurate quantification of multiple mycotoxins in maize by liquid chromatography-stable isotope dilution assay-tandem mass spectrometry (LC-SIDA-MS/MS)

By

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Abstract

The objective of this study was to develop and validate an improved sample preparation technique for accurate quantification of aflatoxin B_1 (AFB_1), AFB_2, AFG_1, AFG_2, deoxynivalenol (DON), fumonisin B_1 (FB_1), FB_2, Ochratoxin A (OTA), zearalenone (ZEN), HT-2 toxin and T-2 toxin in maize using liquid chromatography-isotope dilution mass spectrometry.

Mycotoxin contamination in agricultural commodities poses a threat to human health. Contamination of food is recognised as a source of food borne illness by the World Health Organisation (WHO). The toxicity of mycotoxins has been evaluated by the Joint Food and Agricultural Organisation (FAO)/WHO Expert Committee on Food Additives (JECFA) and the maximum levels (MLs) for the agricultural important mycotoxins have been established. Agricultural commodities need to be tested to ensure food safety prior to human consumption; this requires accurate analytical methods for identification and quantification of these mycotoxins at the regulatory levels.

Analytical methods based on liquid chromatography coupled to mass spectrometry have been developed for identification and quantification of mycotoxins. However, MS based analysis is affected by matrix effects that results from ionisation inefficiency of the target analyte due to co-eluting matrix components. Therefore, there is a need for improved sample preparation methods which can minimise, or possibly eliminate, matrix components prior to mass spectrometric analysis. "Dilute-and-shoot", Quick, Easy, Cheap, Efficient, Rugged and Safe (QuEChERS) and solid phase extraction (SPE) techniques were evaluated for matrix removal efficiency in multi mycotoxin determination in maize. Isotopically labelled mycotoxin standards were used to compensate for variations during the analysis. Spiked blank maize samples and matrix reference materials were used to evaluate the performance of each sample preparation technique.

Dilute-and-shoot technique was used as a first approach to estimate expected matrix effects and to verify whether isotopically labelled internal standards can compensate for matrix effects during the analysis. All the analytes were affected by the presence of matrix effects, signal suppression/enhancement (SSE) ranged between 88% - 194%. When %REC > 130% it was deemed enhanced. The QuEChERS method was ineffective in isolating mycotoxins from the matrix. Results from dilute-and-shoot and QuEChERS highlighted the need of a selective clean-up step to reduce matrix effects.

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Different SPE columns with different sorbents were evaluated for matrix removal efficiency and analyte retention performances. Columns with analyte(s) selective sorbents were effective in improving recoveries for those specific analytes. Also, minimum matrix effects were observed from these columns. However, for multi mycotoxin determination, an ideal clean-up step should yield good recoveries for all the mycotoxins with varying physicochemical properties. Hydrophilic-lipophilic balanced (HLB) SPE column gave good recoveries for most analytes despite relatively high matrix effects with respect to selective sorbents. A clean-up method based on HLB clean-up was optimised to improve matrix removal efficiency.

An accurate, precise and robust method for the determination of multiple mycotoxins in maize was developed and validated. This method is based on ultrasonic extraction, economical HLB SPE clean-up and ultra-high performance liquid chromatography-stable isotope dilution assay-tandem mass spectrometry (UHPLC-SIDA-MS/MS). Sample extraction based on two extraction steps using acidified methanol/water mixture and HLB SPE clean-up resulted in good analyte recoveries $57\% \leq \%REC \leq 142\%$ for most analytes. Fast polarity switching mode was used to determine all the analytes in one chromatographic run without compromising chromatographic resolution. Method performance results indicate that the method can be used to detect and quantify mycotoxins at the regulated levels.

Keywords: Mycotoxins, maize, stable isotope dilution assay, ultra-high performance liquid chromatography, tandem mass spectrometry.

List of scientific outputs

1. Poster presentation: W Nxumalo, Y Naudé and M Fernandes-Whaley "*Evaluation of Bond Elut Mycotoxin, Oasis HLB, MultiSep AflaZON+ SPE Cartridges Performance in Matrix Removal during Multi Mycotoxin Analysis*", Analitika 2014, awarded runner-up prize.

2. Poster presentation: W Nxumalo, Y Naudé and M Fernandes-Whaley "*Evaluation of Hydrophilic-Lipophilic Balanced (HLB) Solid Phase Extraction (HLB-SPE) Column Performance in Effective Matrix Removal for Multi-Mycotoxin Contaminant Determination in Maize*", SAAFoST Biennial Congress 2015. Poster presented by Dr Fernandes-Whaley.

3. Oral presentation: W Nxumalo, Y Naudé and M Fernandes-Whaley "*Evaluation of Bond Elut Mycotoxin, Oasis HLB, MultiSep AflaZON+ SPE Cartridges Performance in Matrix Removal during Multi Mycotoxin Analysis*", ChromSA Student Seminar 2015, awarded runner-up prize.

4. Poster presentation: W Nxumalo, Y Naudé and M Fernandes-Whaley "*Combining Ultrasonic Extraction (USE) and Solid Phase Extraction (SPE) for the Analysis of 11 Mycotoxins in Maize by Isotope Dilution Liquid Chromatography Tandem Mass Spectrometry (SIDA-UHPLC-MS/MS)*", Test and Measurement Conference 2015

5. Oral presentation: W Nxumalo, Y Naudé and M Fernandes-Whaley "*Improved Sample Preparation to Ensure Accurate Quantification of Multiple Mycotoxins in Maize by LC-SIDA-MS/MS*", 42nd SACI Convention.

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Declaration

I, Wonder Praise-God Nxumalo, declare that the dissertation, which I hereby submit for the degree MSc Chemistry at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

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W.P. Nxumalo

____ Day of _____ 2016

APPROVED FOR FINAL SUBMISSION

_____ _____
Supervisor: Dr Y. Naudé (PhD) **Co-Supervisor:** Dr M. Fernandes-Whaley (PhD)

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Abbreviations

ACN	Acetonitrile
CAC	Codex Alimentarius Commission
CCCF	Codex Committee on Contaminants in Foods
DNA	Deoxyribonucleic Acid
DON	Deoxynivalenol
CEN	European Committee of Standardisation
ESI	Electrospray Ionisation
EU	European Union
FB_1	Fumonisin B_1
FB_2	Fumonisin B_2
FLD	Fluorescence Detector
HSSE	Headspace Sorptive Extraction
IA	Itaconic Acid
IAC	Immunoaffinity Column
LC	Liquid Chromatography
LOD	Limit of Detection
LOQ	Limit of Quantification
MAE	Microwave Assisted Extraction
ME	Matrix Effects
MIP	Molecularly Imprinted Polymer
MS	Mass Spectrometry
MS/MS	Tandem Mass Spectrometry
NIV	Nivalenol
OTA	Ochratoxin A
PDMS	Polydimethyl Siloxane

PLE	Pressurised Liquid Extraction
SBSE	Stir-bar Sorptive Extraction
SFE	Supercritical Fluid Extraction
SIDA	Stable Isotope Dilution Assay
SLE	Solid Liquid Extraction
SPE	Solid Phase Extraction
SPME	Solid Phase Micro-Extraction
UHPLC	Ultra High Performance Liquid Chromatography
USE	Ultrasonic Extraction
ZEN	Zearalenone
%SSE	Signal Enhancement/Suppression (Percentage)

Chapter 1

Introduction

Mycotoxins are toxic secondary metabolites of fungi, with over 300 species yet to be identified. The production of mycotoxins in grains is largely affected by environmental factors such as temperature and moisture content during pre and/or postharvest. Mycotoxins are climate dependent and could also be affected by non-infections factors such as the bioavailability of micronutrients, insect damage that are determined by changing climatic conditions.[1] Only the agro-economic important mycotoxins are of concern to the regulatory agencies, producers and consumers.[2] Consumption of mycotoxin contaminated food may cause mycotoxicosis which can cause acute or chronic disease episodes. These conditions have a greater effect on human health in general as they stimulate various and potent toxic effects in test systems.[3] Mycotoxins can cause teratogenic, immunosuppressive, carcinogenic, mutagenic and/or estrogenic effects in humans and animals.[4] They can be categorised into *Aspergillus* mycotoxins (e.g. aflatoxins and ochratoxin), *Fusarium* mycotoxins (e.g. fumonisins, trichothecenes and zearalenone) and *Penicillium* mycotoxins (e.g. citrin).[5]

Mycotoxin contamination of grain-based food and feed is a world-wide occurrence. The World Health Organisation (WHO) recognises the contamination of food and feeds by the major mycotoxins, aflatoxins (AFB_1, AFB_2, AFG_1 and AFG_2), fumonisins (FB_1 and FB_2), ochratoxin A (OTA), deoxynivalenol (DON), zearalenone (ZEN), T-2 (T-2) and HT-2 Toxins (HT-2) as a significant source of food-borne illness.[6] Consequently, the JECFA have evaluated the toxicity of these mycotoxins and established food safety measures. In addition, several countries have implemented regulations with respect to the toxic effects of these mycotoxins. The European Commission has recently published the maximum permissible levels (MPLs) for mycotoxins of major concern in food.[7] However, it is a challenge to establish food safety in developing countries where people are exposed to mycotoxins which may have detrimental health effects. Most notably are the incidents of acute aflatoxicosis in Kenya during 1981, 2004 and 2005.[8, 9]

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Maize crops are vulnerable to the accumulation of mycotoxins. This is likely to be aggravated by climate change since drought-stressed crops are more vulnerable to mycotoxin accumulation.[10] Additionally, maize is the staple diet of the majority of South Africans. Agricultural commodities, for local and international trade purposes, need to be tested locally to ensure food safety prior to human and/or animal consumption. The accurate measurements of these contaminants are important for both the consumer and the producer. The Food and Drug Administration (FDA) estimates an annual economic loss of $ 932 million in food and feed due to mycotoxin contamination.[11] Mycotoxin contamination is severe and it requires multiple mycotoxins to be identified and quantified. It is therefore of utmost importance to have an accurate, precise and robust analytical measurement technique that is applicable at regulatory levels for the determination of relevant mycotoxins and commodities.

Current analytical techniques involve liquid extraction of the matrix followed by a clean-up step, chromatographic separation and detection. Although sample clean-up is generally required for chromatographic methods, some authors use the dilute-and-shoot approach, neglecting any sample clean-up.[12, 13] This approach is possible if the contamination levels are relatively high. However, the major drawback is that the dilute-and-shoot technique requires an instrument with high sensitivity to enable the analysis of the diluted extracts and it is prone to matrix effects. Matrix effects are the direct or indirect modification or interference in response due to the presence of unintended analytes (for analysis) or other interfering substances in the sample, as defined by Shah *et al*.[14] Matrix effects result from suppression or enhancement of the analyte signal during the ionisation process. Ion suppression or enhancement is caused by the presence of matrix components which may be co-eluting with the analyte of interest, resulting in decrease or increase of the ion intensity. In quantitative analysis, matrix effects significantly affect the reproducibility, linearity, and accuracy of the analytical method which results in a high level of uncertainty of measurement.[15]

Solid-phase extraction (SPE) and immunoaffinity columns (IAC) are the conventional tools for the purification of analytes in mycotoxin analysis. SPE and IAC are effective for analyte concentration and in minimising matrix interferences and they have been successfully used with LC-MS/MS enabling suitable sensitivity for analysis of complex matrices.[5, 13, 16-18] A major drawback to IACs is their selectivity for only one or a single class of analytes. A multi-analyte clean-up method is preferred in routine analysis.

QuEChERS (quick, easy, cheap, effective, rugged and safe) method is becoming a popular alternative to the dilute-and-shoot approach as a generic sample pre-treatment. QuEChERS

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based methods are being used to develop LC-MS/MS methods for the determination of multiple mycotoxins in food because they can cover different groups of mycotoxins with very distinct physicochemical properties.[19] However, significant matrix effects and modest recoveries have been reported if there was no post clean-up step.[20, 21] Stir bar sorptive extraction (SBSE), a modern sample preparation technique that employs a stir bar coated with a layer of polydimethyl siloxane (PDMS), was introduced as an improved sample preparation technique.[22] An advantage of SBSE is that a high concentration factor can be achieved; and can be used for liquid or semi-solid matrices, hence it has a potential for applications in food analysis.

Sample dilution, matrix matched calibration, internal standard and standard addition calibration are viable approaches to manage matrix effects. Sample dilution reduces the sensitivity of the analytical method towards the matrix; while matrix matched calibration is time consuming and increases the analysis cost. Additionally it is challenging to fully compensate for the matrix. Internal standards behave similarly to the analyte of interest but remain distinctive. Isotopically labelled compounds and structurally related compounds are the most commonly used internal standards. Stable isotopically labelled compound have been applied in the analysis of mycotoxin in food matrices.[2, 23, 24] This procedure is often referred to as stable isotope dilution assay (SIDA). SIDA is an appropriate method to overcome matrix effects, as the isotope behaves identically to the native analyte, thereby correcting the variations caused during sample preparation and instrumental analysis.

High performance liquid chromatography (HPLC) is perceived as the workhorse of mycotoxin analysis.[25] The strength of HPLC when coupled to MS/MS rather than conventional detectors such as ultra-violet (UV) or fluorescence (FLD), lies in the multi analyte determination within one analysis and its high selectivity and sensitivity. HPLC-MS/MS has been applied in analysis of multi mycotoxins in various food matrices; and recently, Ultra-High Performance Liquid Chromatography-MS/MS (UHPLC-MS/MS) has been investigated in this context.[25, 26] However, major problems relating to sample preparation have not been adequately addressed and solved. To date, there is no official method for multi mycotoxin analysis by LC-MS/MS within the European Committee of Standardisation (CEN) and Association of Official Analytical Chemist (AOAC).

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1. Aim of this project

This project aims to develop and validate an analytical method based on LC-MS/MS for determination of multiple mycotoxins in maize. In this work we investigate sample preparation techniques with the aim of minimising or possibly eliminating matrix effects associated with mass spectrometric analysis. Obtained results will be used with the objective of answering the following questions:

- Does eliminating matrix effects significantly improve accuracy and precision of measurement results?
- Does the use of stable isotope dilution assay compensate for variations during the analysis?

Solid-liquid extraction techniques will be used to extract mycotoxins from maize meal followed by a clean-up method. Different solid-liquid extraction techniques will be investigated to optimise analyte extraction efficiency. Also, different clean-up methods will be evaluated for matrix removal efficiency and analyte recovery performance.

Chapter 2

Literature review

2.1 Introduction

Mycotoxin contamination can have severe consequences. Several reports on the simultaneous occurrence of mycotoxins in food and feed commodities have been published. The simultaneous occurrence of mycotoxins have even more severe consequences as it has synergistic adverse effects on health and a larger negative impact on the economy. Therefore it is necessary to determine mycotoxin levels in food and feed. The simultaneous determination of several mycotoxins would be advantageous in terms of time and cost. Accurate identification and quantification of mycotoxin levels is required for legislative, health and economic reasons. Mycotoxin regulations can only be enforced if reliable, sensitive and accurate analytical methods are in place.

The determination of contaminants in food matrices frequently involves extensive sample preparation prior to instrumental analysis. Poor sample preparation often leads to inaccurate quantification of the target analytes. Thus, there is a demand for better sample preparation methods that minimises 1) the number of sample handling steps which consequently reduces the turnaround time required and 2) the sources of variability during the analysis. In addition, advances in liquid chromatography (LC) coupled to mass spectrometry (MS) offer the possibility to detect and quantify mycotoxins in complex matrices such as food samples.

This chapter critically reviews most aspects of mycotoxin analysis including sampling, sample preparation and accurate quantification approaches with a brief discussion on the chemistry and biological effects of mycotoxins. The sample preparation section covers both solvent and sorbent based extraction techniques whilst the use of SIDA is discussed in the accurate quantification approaches Section (3.3). The last section provides an overview of the LC-MS methods that have been applied in mycotoxin analysis, with emphasis on UHPLC-MS/MS. The combination of sample preparation and use of LC-MS methods is discussed in detail.

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2.2 Mycotoxin chemistry, biological effects and regulations

The production of these compound in grain is affected by environmental factors such as temperature and humidity pre and/or post-harvest. Therefore, during climate changes, mycotoxin production could be affected by non-infectious factors that results from climatic changes.[1] Consumption of mycotoxins have caused adverse human health effects for many centuries. Ingestion of mycotoxins results in mycotoxicosis which leads to acute and chronic episodes.[3] This depends on the type of toxin, dose, and the age of the exposed individual.[27]

2.2.1 Aflatoxins

Aflatoxins are produced by the three species of *Aspergillus*; *A. flavus*, *A.parasiticus*, and the scarce *A. nomius* which contaminate plants and plant products.[6] B aflatoxins are produced exclusively by *A. flavus* whereas the other two species produce both B and G aflatoxins. AFB_1 is the most potent mycotoxin carcinogen. Incidents of acute aflatoxicosis have been reported in Kenya during 1981, 2004 and 2005.[8, 28]

2.2.2 Fumonisins

Fumonisins are produced by *Fusarium*, particularly *F. verticillioides*, in maize and they were first described ad characterised in South Africa.[29] Unlike aflatoxins, fumonisins do not exhibit fluorescing properties and they are not soluble in organic solvents. Although fumonisins largely affect maize they have also been isolated from rice, sorghum, millet and various other food commodities. Consumption of fumonisin contaminated maize as a staple diet has been associated with oesophageal and liver cancer as well as neural tube defects.[30, 31] The 28 fumonisins analogs that have been characterised ca be separated into 4 main categories; fumonisin A, B, C, and P. Fumonisin B (FB) analogs comprises the toxicological FB_1, FB_2 and FB_3, and they are the most abundant naturally occurring fumonisins. FB_1 accounts for about 70% - 80% of the total fumonisin produced, whereas FB_2 make up 15% – 25% and FB_3 make up from 3% - 8% when cultured in maize, rice or in liquid medium.[32] Other fumonisins analogs may occur naturally contaminated maize at relatively low concentrations, less than 5% of the total fumonisin present.[32]

2.2.3 Ochratoxin

Ochratoxin A (OTA) was identified as a metabolite of *Aspergillus ochraceus* in 1965 by Van der Merwe and co-workers.[33] It is mainly found in oats, barley, wheat, coffee and other food

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commodities. OTA is also found in vine fruits at relatively low concentrations. Animal studies have reported OTA accumulated in certain organs, such as the kidneys. OTA is nephrotoxic in animals, in addition it also displays hepatoxic, immunosuppressive, teratogenic and carcinogenic effects.[34]

2.2.4 Trichothecenes

Trichothecenes are produced mainly by *Fusarium* as well as other genera such as *Trichoderma*, and *Trichothecium.* Trichothecenes are characterised by the presence of a tricyclic-12,13-epoxytrichothec-9-ene core structure. There are four types of trichothecenes (A-D). Deoxynivalenol (DON) and nivalenol (NIV) are type B trichothecenes, whereas T-2 toxin and HT-2 toxin (HT-2) and diacetoxyscirpenol (DAS) are type A trichothecenes.[35] DON is commonly found in grains such as maize, wheat, oats, rye, rice, sorghum, millet and barley. Ingestion of DON contaminated food causes nausea, vomiting and diarrhoea and is also known as vomitoxin.[36] Trichothecenes inhibit mitochondrial protein synthesis and interact with protein sulfhydryl groups.[37]

2.2.5 Zearalenone

Zearalenone (ZEN) is a phenolic resorcyclic acid lactone produced by *Fusarium graminearum*. ZEN is commonly found in maize but it can also be found in other commodities such as wheat, barley and sorghum. ZEN is biologically potent, however rarely toxic. Its structure is similar to that of 17β-estradiol and it can bind to an oestrogen receptor causing alterations of the reproductive system.[38] ZEN causes precocious development of mammals and reproductive problems have also been reported in bovine species.[39]

Table 2.1 lists class of mycotoxins that are of interest in this study. The fungal species that produce them, chemical structures and the health effects resulting from them are listed.

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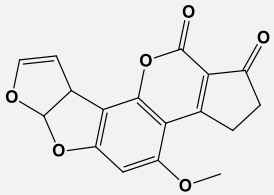
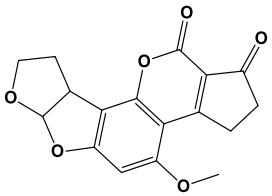
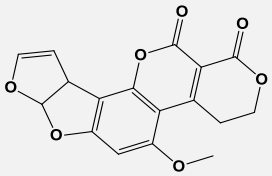
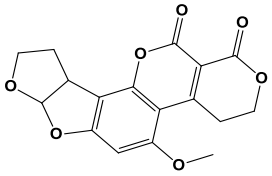
Table 2. 1. Major mycotoxin groups, chemical structure and health effects caused.[40]

Class	Name and chemical formula	Chemical structure	Major health effects
Aspergillus flavus* and *A. parasiticus	Aflatoxin B_1: $C_{17}H_{12}O_6$		Hepatotoxic, immunosuppressive, carcinogenic, teratogenic, mutagenic
	Aflatoxin B_2: $C_{17}H_{14}O_6$		
	Aflatoxin G_1: $C_{17}H_{12}O_7$		
	Aflatoxin G_2: $C_{17}H_{14}O_7$		

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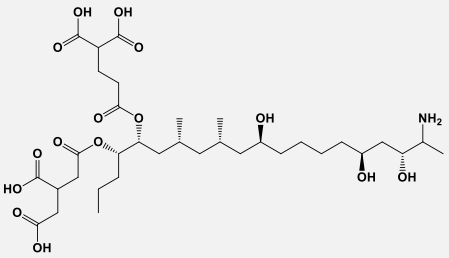
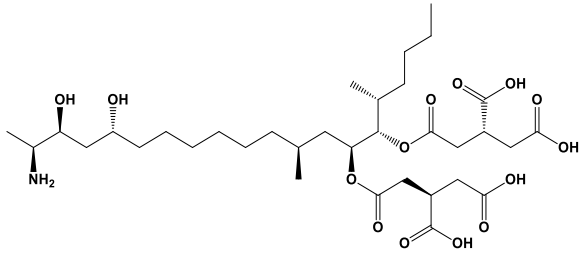
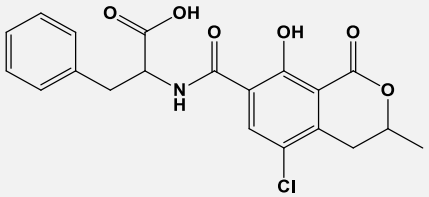
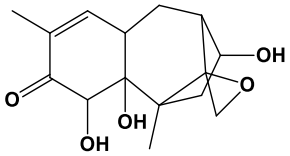
Fusarium verticilliodes and F. proliferatum	Fumonisin B_1: $C_{34}H_{59}NO_{15}$		Liver and kidney tumours, oesophageal cancer, lung oedema (swine), leukoencephalomacia (horses)
	Fumonisin B_2: $C_{35}H_{59}NO_{14}$		
Aspergillus ochraceus	Ochratoxin A: $C_{20}H_{18}ClNO_6$		Kidney and liver toxin, carcinogen; chronic toxicity as it accumulates in body
Fusarium graminearum	Deoxynivalenol: $C_{15}H_{20}O_5$		Food refusal and vomiting, kidney problems, immunosuppression (swine)

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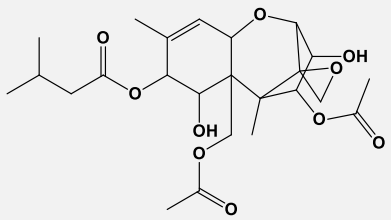
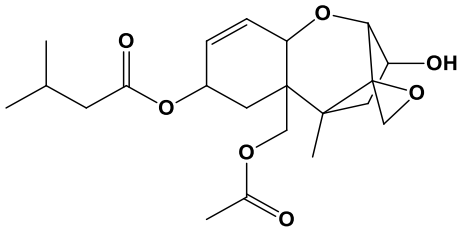
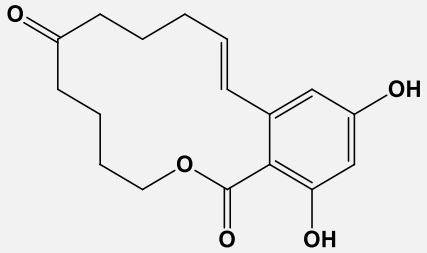
Fusarium sporotrichioides	T-2 Toxin: $C_{24}H_{34}O_{10}$		Weight loss, diarrhoea, dermal necrosis (poultry)
	HT-2 Toxin: $C_{21}H_{30}O_7$		
Fusarium graminearum	Zearalenone: $C_{17}H_{20}O_5$		Oestrogenic effects, reproductive toxicity

2.2.6 Regulations

Due to the adverse health effects associated with mycotoxin contamination, regulatory bodies such as the FDA and the European Commission[7] have set maximum permissible levels (MPLs) for mycotoxins of major concern in food. Aflatoxin is the most regulated mycotoxin worldwide and some countries only have legislation limits for aflatoxins. European Commission MPLs are listed in Table 2.2 with a special focus on maize or maize products intended for human consumption. There is a pending regulation for T-2 Toxin, as a sum of T-2 and HT-2 Toxins, in Europe for unprocessed cereals and cereal based products. South Africa (SA) has also published mycotoxin regulations, only two mycotoxins are considered, aflatoxins and patulin. Where no regulatory limits exists, SA defers to the WHO regulations.[41] The legal limit for AFB_1 is 5 µg/kg in all foodstuffs, but specifically peanuts and dairy milk, with a total aflatoxin limit of 10 µg/kg. A maximum legal limit for patulin in apple juice and apple juice-based products is set at 50 µg/L.

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Table 2. 2. Maximum permissible mycotoxin levels in maize and maize products.[7]

Mycotoxin	**Commodity**	**MPL/ (µg/kg)**	
Aflatoxins	Maize and rice to be subjected to sorting or physical treatment before human consumption or used as an ingredient in foodstuffs.	5.0 [AFB_1]	10.0 [$AFB_1 + B_2$, + $G_1 + G_2$]
	All cereal and all products derived from cereals, including processed cereal products.	2.0 [AFB_1]	4.0 [$AFB_1 + B_2$, + $G_1 + G_2$]
Deoxynivalenol	Unprocessed maize excluding unprocessed maize intended to be processed by wet milling	1750	
	Cereal intended for direct human consumption, cereal flour (maize flour, maize meal and maize grits)	750	
Fumonisins	Unprocessed maize	4000 [$FB_1 + B_2$]	
	Maize intended for direct human consumption including maize based foods	1000 [$FB_1 + B_2$]	
Ochratoxin A	Unprocessed cereals	5.0	
Zearalenone	Unprocessed maize excluding unprocessed maize intended to be processed by wet milling	350	
	Maize intended for direct human consumption, maize-based snacks and maize-based breakfast cereal	100	

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2.3 Sample preparation

2.3.1. Sampling

Analytical methods for mycotoxin analysis include sampling. The sample preparation procedure consists of 3 different stages: sampling, sample preparation (extraction, clean-up and concentration), and analysis. Improvements in sampling methods for mycotoxin detection in food remain a top priority within the regulatory agencies, international organisations and commodity industries around the globe.[42] In regulation enforcement correct decisions regarding the fate of commodities for export can only be made if the toxin concentration in the batch can be quantified with a high degree of accuracy and precision. The heterogeneity of mycotoxins varies with commodity; therefore sampling plans are often followed to obtain a representative sample. Thorough sampling strategies are essential for foodstuffs in particular, and any portion taken for analysis must represent the original bulk sample. Sampling strategies have been established for a few mycotoxins only; for instance, the European Commission stipulates the sampling strategy for the determination of aflatoxins in food (edible nuts and dried fruits).[43] The Codex Alimentarius Commission (CAC) has adopted the sampling plan that was proposed by the Codex Committee on Contaminants in Foods (CCCF) for aflatoxins in dried figs.[44]

Measurement uncertainty is a significant concern in analytical science. Although uncertainty arising from sampling is not considered part of the scope of this study it is important to emphasise that a generally higher high level of uncertainty is often inherent to the sampling plan. Accuracy and precision are the two kinds of uncertainties accompanying sampling plans.[45] Accuracy is correlated with bias, and biases have the potential to arise during the sample selection process. Precision is correlated with variability. Variability occurs with each step of mycotoxin analysis. The sampling, sample preparation and analytical steps of the mycotoxin analysis contribute to the uncertainty of the method. Whereas, the sampling step is often the largest source of variability related with mycotoxin analysis on a wide range of agricultural commodities. Establishment of an acceptable sampling protocol is the first significant step to ensure the reliability and trueness of the analytical results.[46]

Following sampling, the first step for a typical analytical method is isolation of the analytes of interest from the matrix. Sample preparation techniques are reviewed in the following sections.

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2.3.2. Sample extraction and clean-up techniques

Quantification of trace contaminants in various complex matrices often requires sample preparation before instrumental analysis. Sample preparation is matrix and analyte dependent. Selective extraction of distinct analytes is based on the difference of their physical and chemical properties; these may be molecular weight, polarity, solubility or differences in volatility.[45] Optimised sample preparation techniques are indispensable as they not only reduce the time taken for sample workup but also reduce the number of steps in the process, each of which becomes potential sources of error. Sample pre-treatment is usually necessary for solid samples; pre-treatment steps may involve sieving, grinding or drying. Drying is especially important when using non-polar extraction solvents as moisture can minimise extraction efficiency. Drying agents can be used to overcome this. Different extraction techniques suitable for different analyte and matrix types have been well established. During extraction, the analytes desorb from the matrix and dissolves into the solvent for which it has a greater affinity for. Thus, extraction is dependent on the analyte solubility, mass transfer, and particle size of the sample.[47] In the following sections solvent and sorbent based extraction techniques in mycotoxin analysis are reviewed.

2.3.2.1. Solvent-base extraction methods

Solvent extraction is the most frequently used sample preparation technique. Aqueous- e.g. water, buffers, organic- e.g. acetonitrile, methanol, ethyl acetate, chloroform etc. and aqueous-organic solvent mixtures are used. Liquid-solid partitioning to extract the analyte of interest from the matrix is often the first step in solid material preparation for mycotoxin analysis. A number of methods for extraction of foodstuffs; grain,[48] nut,[48], maize,[16, 18, 49], wheat,[38] and animal feeds,[5, 50, 51] has been established. Different approaches of solvent extraction techniques can be used, such as conventional Soxhlet extraction,[52] and sonication.[53] Modern techniques comprise of microwave-assisted extraction (MAE),[54] pressurised liquid extraction (PLE) and supercritical fluid extraction (SFE).

2.3.2.1.1. Solid-liquid extraction

Solid-liquid extraction (SLE) is a conventional sample extraction technique using solvents, and it is one of the most commonly used extraction techniques for mycotoxin extraction from grains, cereal food stuffs and other solid food matrices.[46] The choice of the solvent depends on the analyte to be extracted. Different mycotoxins have different functional groups which

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pose a challenge in multi mycotoxin analysis. Some mycotoxins, DON and HT-2 toxin, are highly soluble in polar solvents and others, ZEN and OTA, are highly soluble in non-polar solvents.[55] Hence, mixtures of organic solvents, with a small amount of acidified water or water are commonly used. Acidified water enhances the extraction efficiency, because water improves penetration of the solvent into the material while the acidic solution lowers pH aiding the extraction by breaking down the analyte-matrix interaction through protonation of the carboxylic groups present on the analytes.[56]

At present, acetonitrile (ACN) water (84:16 v/v) is the most frequently used solvent for multi mycotoxin extraction in maize.[16, 18, 49] Other solvent ratios have also been investigated in literature and include predominantly methanol: water (greener alternatives) and acidified methanol: water.[12] Double extractions (first organic, then aqueous) have also been investigated.[2] ACN/water mixture enables high recoveries for most toxins, with the exception of fumonisins which need acidification to improve analyte recovery. The four carboxylic groups of fumonisins are protonated at the low pH, and thus move out of water and into the organic phase. If not protonated it will be ionised (partially or in full) and remain in the water.[57]

In addition to the type of solvent used, sample/extractive solvent ratio, temperature and extraction time are other important parameters to be considered in order to obtain accurate quantification.[46]

2.3.2.1.2. Soxhlet extraction

The Soxhlet extraction technique has been applied in food analysis as a preliminary extraction technique particularly for solid samples. It is an exhaustive extraction technique that is not selective and further clean-up and concentration steps are necessary. A major drawback with this technique is that thermally labile compounds can degrade with possible production of artefacts due to the temperatures used.[58] However, this has not been shown for mycotoxins. Soxhlet extraction often requires long extraction times, and a significant amount of organic solvent is utilised, typically 50 – 200 mL for a 10 g sample. Modern automated Soxhlet extraction systems significantly reduce the time and the amount of solvent required; up to 6 samples can be extracted simultaneously.[59] Automated systems are capable of boiling, rinsing and recovering the solvent. Hartmann and co-workers (2008)[52] used the Soxhlet extraction method in the quantification of zearalenone in different matrices of agricultural importance such as plant organs and soil.

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2.3.2.1.3. Microwave-assisted extraction

The microwave-assisted extraction (MAE) technique is used to enhance the extraction efficiency of the classical solvent extraction technique. The sample is stirred and heated by microwaves in a tightly sealed vessel, allowing for elevated temperature and pressure- the combination of these two parameters drive the partitioning of analytes from a matrix into a solvent during extraction.[60] The solvent in use must absorb microwave energy; hence, mainly polar solvents are typically used with MAE. MAE is only applicable to thermally stable analytes because of the rise in temperature and pressure during extraction. Therefore, there is a limited number of published works applying MAE in mycotoxin analysis; also the equipment is comparatively expensive. An analytical method, using MAE and LC with fluorescence detection (FLD) for determination of OTA in bread samples, was developed by Paíga and co-workers (2013).[61] By means of a 2^4 composite design coupled to response surface technology, they were able to optimise the MAE parameters (extraction time, temperature, solvent volume and stirring speed) in order to maximise OTA recovery. The optimised conditions were: 10 min of extraction at 80 °C and stirring at maximum speed, using 25 mL of ACN. Most recently, MAE was used as a pre-treatment technique for the quantification of Aflatoxins B_1, G_1, B_2 and G_2 in grains and grain products, followed by SPE clean-up prior to LC-FLD.[54] The optimised MAE parameters are as follows: 12 mL ACN, heating at 80 °C for 15 min; and the relative recovery ranged from 90.7 – 105.7 % for aflatoxins in maize.

2.3.2.1.4. Pressured liquid extraction

Pressurised liquid extraction (PLE), also known as accelerated solvent extraction (ASE™),[62] uses solvent at elevated temperature and pressure to extract the analyte in solid or semi-solid matrices. The solubility of the analyte is improved by the gradual decrease in the viscosity of the extracting solvents at higher temperatures; this also reduces the analyte-matrix interaction and improves the diffusion of the analytes through the matrix.[46] Under these conditions, solvents have improved solvation power and increased extraction rates.[47] The sample is firstly dispersed with an inert material; then loaded into the extraction cell where the solvent is pumped in; followed by heating the cell to the temperature and pressure required. The extraction process can be performed statically, dynamically (flow-through systems) or as a combination of both modes. The pitfall of the dynamic mode is that it consumes a large amount of solvent, this leads to dilution of the analyte.[47] After extraction, the extract is flushed into

the collecting vial with a fresh solvent. The solvent is typically dried down and reconstituted in mobile phase prior to analysis.

In the mycotoxin context, a sensitive and reliable method employing PLE and LC/electrospray ionisation (ESI) tandem mass spectrometry with triple quadrupole (QqQ) mass analysers, was developed for the determination of fumonisins, $FB_{1,}$ FB_2 and $FB_{3,}$ in maize-based baby foods.[63] PLE instrument parameters were: 3g of sample was packed in 11 mL stainless steel cell and extracted with methanol at 40 °C at 34 atm in one cycle of 5 min at 60 % flush. Recoveries obtained were over 65 %.[63] In another work, Pérez-Torrado and co-workers (2010) used PLE to extract zearalenone (ZEN) from cereal flour prior LC-MS using MeOH/ACN (50:50 v/v).[59] Matrix matched calibration was used to compensate for the matrix effect. Zinedine *et al.* (2010) studied the influence of the extracting solvent in the analysis of OTA in breakfast and infants cereals by means of HPLC-FLD after PLE extraction.[64] In this study, it was proposed that a mixture of ACN/water (80:20 v/v) is optimal for the extraction of OTA as it gave better recoveries and less solubility of the matrix components. PLE can extract an excessive amount of the co-extracting matrix components which results in interfering signals in the LC-MS when using temperatures above 60 °C. However, operating the instrument at room temperature can yield modest recoveries, between 49 – 88 %.[65] A clean-up step after PLE can further reduce the presence of the matrix and consequently matrix-effects.

To conclude, PLE allows faster extraction with respect to conventional techniques, it is fully automated and uses less solvent. In addition to instrumentation procurement costs, PLE is rarely used in multi mycotoxin analysis due to the different physicochemical properties of the various mycotoxins, the complexity of the matrices and the highly observed matrix effects.[46]

2.3.2.1.5. Sonication extraction

Sonication is used to improve extraction. The solvent type or the mixture is selected to produce maximum extraction efficiency and selectivity. Sonication extraction (USE) allows several extractions to be performed simultaneously and the technique is relatively inexpensive as it requires no specialised laboratory equipment. A disadvantage of USE is that it is not automated and it is not suitable for volatile analytes.

Li *et al.* (2012) developed a method for the determination of FB_1 and FB_2 in maize by LC-ESI-MS/MS.[66] The analytes were extracted from maize (5 g sample) with 25 mL of MeOH/water (3:1 v/v) mixture by means of USE and analysis was carried out without a clean-up step. The extraction was performed for 10 min at room temperature, and the output power was set to 120

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W. Recoveries were 93 and 92.6 % for FB_1 and FB_2 respectively.[66] USE is limited by selectivity and sample pre-concentration capabilities. An analytical method based on sonication extraction and immunoaffinity column clean-up coupled with HPLC and post-column derivatization-fluorescence (USE-IAC-HPLC-PCD-FLD) was developed for simultaneous determination of multi mycotoxin in nutmeg samples.[67] AFB_1, AFB_2, AFG_1, AFG_2 and OTA were extracted from the matrix by USE using a MeOH/water (80:20 v/v) mixture followed by IAC clean-up. Chen *et al.* (2013) quantified *Fusarium* toxins in animal derived food by means of LC-MS/MS with USE and auto-solid phase clean-up.[60]

2.3.2.2. Sorbent-based extraction and clean-up methods

After solid-liquid extraction methods, the analyte is obtained in an organic or aqueous solution, which requires a concentration or additional clean-up step. The resulting liquid samples can then be directly treated by solvent-solvent extraction methods or by sorptive methods.[47] Solid phase extraction (SPE), improved-SPE, stir-bar sorptive extraction (SBSE) and Quick, Easy, Cheap, Efficient, Rugged and Safe (QuEChERS) methods have been applied to food analysis.

2.3.2.2.1. Solid phase extraction

Solid phase extraction is based on the liquid-solid partitioning of the analyte, whereby the extracting phase is a solid sorbent. SPE has been widely used to isolate and concentrate trace organic materials from liquid samples or solutions.[47] Currently, SPE is used for concentrating mycotoxins from both liquid and solid sample extracts. This technique is used as a clean-up and/or concentration step after an extraction procedure. The liquid sample is passed through a cartridge or a disk filled with a solid sorbent where the toxins are retained and then eluted with an organic solvent.[16] A wide selection of sorbent materials is available, using chemically/structurally different retention mechanisms to retain/extract the analyte(s) of interest. The sorbent selection depends on the food matrix, the analytes and the possible interferences.[47] A variety of sorbents has been used including C_8 and C_{18} bonded phases on silica, polymeric resins (polystyrene/divinylbenzene copolymer), Florisil® (activated magnesium silicate), alumina, charcoal and silica. Ionic functional groups, carboxylic acid and amino groups can be bonded to silica sorbent to produce ion-exchange sorbents. SPE is an efficient, safe and reproducible extraction technique. Limitations to SPE include the selectivity of a single cartridge to a single toxin or a class of toxins. Also, the method performance is significantly affected by changes in method conditions, such as pH, the type of solvent used and the ionic strength of the sample.[68] SPE batch-to-batch reproducibility has been in question.

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However, due to better understanding of surface chemistry, analyte retention and elution mechanisms this has been overcome.[69]

A clean-up procedure based on reverse phase SPE Oasis® HLB columns was used in the determination of trichothecenes in cereal and cereal based products by LC-MS/MS.[70] The Oasis® HLB column gave superior recoveries (72 – 94 %) compared to Mycosep® 227 (31 – 66%), particularly for nivalenol (NIV).[70] A method for determining T-2 and HT-2 toxins in a cereal by means of LC-MS/MS was developed.[71] Different SPE cartridges were evaluated for the reduction of matrix effects; Strata-XL (6 mL, 200 mg and 3 mL, 200 mg), Oasis® HLB (6 mL, 200 mg) and Strata-C-18-U (6 mL, 500 mg). The best recoveries for T-2 were obtained with Strata-XL (72.6%), while Oasis® HLB gave best recovery for HT-2 (120.2%). Conversely, Strata-C-18-U gave the best reproducibilities for both toxins.[71]

Mycosep® and MultiSep® columns are well-established clean-up columns/cartridges in the mycotoxin context. The Mycosep® 226 AflaZON+ and 227 Trich are aimed at aflatoxin and zearalenone and trichothecenes type A and B clean-up, respectively.[72, 73] In complex matrices. These cartridges are filled with adsorbents such as celite, ion-exchange resins, polymers, and charcoal, packed into a plastic tube between two filter discs.[46] With these columns, the interfering substances are retained in the solid phase while the purified toxin passes through the column. An advantage of these columns is that there is no need to pre-condition or wash the column; hence the process is simple and quick. However, the column is for single use only and it is designed for one analyte or a group of similar compounds. Furthermore, purification is not always effective and the columns do not allow for any analyte concentration.[68] Berthiller *et al.* (2005)[74] developed a method to simultaneously quantify *Fusarium* mycotoxins in maize by means of a reverse phase LC with atmospheric pressure chemical ionisation triple quadrupole MS (LC-APCI-MS/MS). Mycosep® 226 columns were used as a clean-up method prior to instrumental analysis. Recoveries ranged between 73% (T-2) and 89% (FUS-X) for all trichothecenes. However, recovery was only about 50% for NIV.[74] Also, Mycosep® 226 columns were used to clean-up rice extract for aflatoxins determination.[73]

Mycosep® 227 was used in a study to determine type-A and type-B trichothecenes in barley samples by GC-MS.[72] Montes *et al.* (2012)[75] used Mycosep® 227 for the analysis of trichothecenes in breakfast cereals bought from Spanish retail markets. Extraction efficiency of four clean-up procedures, Mycosep® 225 and 227, Oasis® HLB and IAC, was evaluated for the determination of DON in wheat flour extracts by LC-photodiode array (PDA).[76] Highest recoveries were obtained (99 %) using Mycosep® 225, while that of Mycosep® 227 was 65 %.

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DON recovery for IAC and Oasis® HLB were 53 and 42 % respectively. Monaci *et al.* (2011)[77] compared Mycosep® 227 and QuEChERS methods for the extraction of DON, HT-2 and T-2 toxin in bread samples prior to LC-Orbitrap-MS. The Mycosep® 227 procedure showed low matrix effects and better sensitivity. The development of methods applying SPE columns for the determination of mycotoxins is still minimal and should be encouraged.

2.3.2.2.2. Improved solid phase extraction methods

Mycotoxin analysis require selective clean-up techniques in order to improve analyte recoveries and minimise co-eluting components.[46] Most prevalent are those based on antibodies (immunoaffinity materials) and molecularly imprinted polymers (MIPs).

The antigen-antibody molecular recognition using antibodies has led to the development of highly selective immunosorbents.[78] These immunosorbents are either designed for a single analyte or a single class of analytes. Immunoaffinity columns (IACs) are the most utilised clean-up columns in mycotoxin analysis, owing to their high selectivity for target analytes.[40, 79] The column consists of a solid phase support fixed to specific antibodies which bind selectively to specific mycotoxins. When the sample extract is passed through the column, the mycotoxins bind selectively to the antibodies while the impurities are removed by a rinsing step. The mycotoxins are then eluted with a miscible solvent or by antibody denaturation.[46] The main advantages of IACs are the rapid and specific interactions between the antibody and the target analyte. In contrast, the analyte-antibody interaction can be disturbed by the sample matrix, thus leading to low extraction recoveries.[47] Also, the antibodies can be denatured by the presence of organic solvents, therefore the extract must be an aqueous solution containing little to no organic solvent.[80] In addition, IACs are expensive and only enable single application.

Methods for the analyses of mycotoxins have been developed and validated and are now commercially available.[81] Lattanzio and co-workers (2007) reported a multi mycotoxin immunoaffinity clean-up based on IAC containing antibodies for AFs, OTA, FBs, ZEN, DON, T-2 and HT-2 for the determination of the target mycotoxins in maize extracts prior to LC-MS/MS analysis.[16] An AOZFDT2™ column from Myco6in1® Vicam was used as a clean-up technique after PBS/MeOH liquid-solid extraction. Total recoveries ranging between 79% – 104% were obtained after optimizing the extraction and clean-up steps. More recently, Desmarchelier *et al.* (2014)[19] proposed a procedure that combines two clean-up strategies, the QuEChERS method and immunoaffinity clean-up, for multi mycotoxin determination by LC-

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MS/MS in various food matrices. The authors proposed that the inclusion of an IAC step allowed lower LOQs to be achieved, 0.05 µg/kg and 0.25 µg/kg for AFs and OTA in cereals respectively.

Synthetic molecularly imprinted polymers (MIPs) also mimic the antibody "lock-and-key" mechanism. Retention of the target analytes on the sorbent is achieved through shape recognition in the cavities or imprints. However, other physicochemical properties such as hydrogen bonding and hydrophobic interaction are also important.[47] MIPs provide an innovative synthetic extraction and enrichment material. Through molecular imprinting, cross-linked polymers are synthesised through free-radical co-polymerisation of functional monomers and cross-linkers in the presence of an analyte acting as template. After polymerisation, the template is washed away and selective three-dimensional cavities, matching in size, shape, and functionality to the template molecule remain in the polymer matrix.[25] However, some mycotoxins are too expensive or too toxic to be used in the production of MIPs. Other challenges encountered with MIPs include: inconsistent molecular recognition, polymer swelling in hostile solvents, slow binding kinetics and sample contamination by template bleeding.[40]

Imprinted polymers for the mycotoxins OTA,[82] DON and ZEN[83] have been reported in the literature for application as SPE using a non-covalent self-assembly imprinting approach. Pascale *et al.* (2008)[84] identified itaconic acid (IA) as a functional monomer with high affinity towards DON by means of molecular modelling and computational design. IA-based polymers were synthesised in dimethyl formamide using ethylene glycol dimethacrylate as a cross-linker and 1,1'-azo-bis(cyclohexane carbonitrile) as an initiator. IA-based polymers (without DON template) showed comparative results to DON MIPs and were successfully used as SPE adsorbents for clean-up of a pasta extract prior to HPLC-UV determination.

These preliminary results show promise for future applications of MIPs as a highly selective technique for separation and enrichment of mycotoxins in food matrices.

2.3.2.2.3. Stir-bar sorptive extraction

Stir-bar sorptive extraction (SBSE) was first introduced by Baltussen and co-workers in 1999 as an enhanced sample preparation technique to improve the extraction capacity of solid phase micro-extractions (SPME) membrane fibres.[22] The SBSE technique uses a stir-bar coated with a polydimethyl siloxane (PDMS) layer (about 0.5 mm – 1 mm thick) to give a larger surface area of stationary phase, allowing better recovery and analyte capacity.[47, 85] The stir-bar can

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either be thermally or liquid desorbed for GC or LC applications, respectively. Concerning liquid desorption, the choice of the solvent is of great importance. The solvent should have a high affinity for the extracting phase to ensure complete release of the analyte from the stir bar extracting material. PDMS coated stir-bars are most suited for non-polar analytes. However, the technique can be applicable to polar analytes through derivatization. A stir-bar coated with an adsorbent that has a high affinity for polar analytes would expand SBSE flexibility and selectivity while preserving its concentration capacity.[85] Numerous research groups are working on the development of polar coatings applicable for the extraction of polar analytes in order to expand its versatility.[86, 87] The key advantage of SBSE is the high analyte concentration factors that can be achieved.[47] High concentration factors for analytes having an octanol-water distribution higher than 100 (log $K_{ow} > 2$) have been reported.[88]

SBSE applications in food analysis are rising. However, owing to certain limitations, it is mainly used in non-fatty food matrices and non-polar analytes.[88] Recently, Nguyen and Ryu (2014) developed a simple and sensitive SBSE-HPLC-FLD method for the extraction of OTA in beer and recoveries greater than 83% were reported.[89] The analyte was extracted using the commercially available Twister® EG-Silicone prior to liquid desorption using MeOH.

2.3.2.2.4. QuEChERS method – combined extraction/clean-up

Quick, Easy, Cheap, Efficient, Rugged and Safe (QuEChERS) is becoming a popular alternative to the "dilute-and-shoot" approach as a generic sample pre-treatment technique.[42] This technique was originally developed by Anastassiades *et al.* (2003)[90] for pesticide analysis. The QuEChERS method involves micro-scale extraction with acetonitrile followed by a clean-up based on a dispersive solid-phase extraction (d-SPE). In the first step, magnesium sulphate ($MgSO_4$) and sodium chloride (NaCl) are used to reduce the water content. Thereafter, primary secondary amine (PSA) and C_{18} are used to remove co-extracted components such as sugars and fatty acids.[91]

Extraction of mycotoxins in food matrices requires adjustments to the fundamental procedure in order to ensure efficient extraction of the toxins. These adjustments may be the type of salts used,[92] the amount of sorbent (C-18), acidification of the extracting solvent,[93] or addition of water[94] or MeOH.[95] A modified QuEChERS method was used in the analysis of type-A and type-B trichothecenes in wheat flour. A MeOH/ACN (85:15, *v/v*) mixture was used to extract the wheat samples and recoveries ranged from 86% – 108%.[95] Sodium citrate dihydrate and sodium citrate sesquihydrate instead of $MgSO_4$ and NaCl were used to enhance extraction

efficiency of OTA in bread.[92] Lehotay *et al.* (2005)[96] proposed that buffering improved recovery of certain pH-dependent pesticides. A modified QuEChERS method for extraction of multi mycotoxins in dried fruits has been recently reported by Azaiez and co-workers (2014).[97] The method is based on a single extraction step using acidified acetonitrile prior to partitioning with salts and does not involve any additional clean-up step. Recoveries in the range of 60 – 135% were reported for spiked samples.[97]

Koesukwiwat *et al.* (2014)[98] evaluated a modified QuEChERS method for the analysis of multi mycotoxins in rice. In the evaluated method, the extract was cleaned up by d-SPE using $MgSO_4$, PSA, C_{18}, and alumina-neutral (Al-N) prior to UHPLC-MS/MS analysis. Recoveries between 70% – 98% were achieved.[98] QuEChERS extraction technique has been combined with IAC clean-up with the aim of minimising matrix effects and enhancing analyte recoveries.[99] The generic QuEChERS procedure was used with the IAC step dedicated specifically to the aflatoxins (AFLAs) and OTA. The additional IAC step resulted in LOQs as low as 0.05 and 0.25 µg/kg for AFLAs and OTA in cereal, respectively.[99]

The QuEChERS method is versatile; it can be applied over a broad pH range and can be easily modified to encompass a range of mycotoxins for analysis. However, it is not easily automated and the concentration factor is often low.[46] Therefore, an additional enrichment step is recommended.

2.4 Accurate quantification approaches

The origin of stable isotope dilution assays (SIDAs) dates back to the early 20th century when Soddy revealed the presence of isotopes.[100] Hevesy and Paneth (1913) determined the concentration of lead in rocks and the solubility of lead salts in water using radioactive isotopes.[101] The principle of SIDA is relatively simple. A compound exhibiting a natural isotopic distribution (unlabelled analyte) is combined with an isotopically different compound (labelled analyte), the naturally abundant isotopes are diluted in the resulting mixture, thus "dilution" in SIDA.[102] After equilibration of the labelled and unlabelled analytes, the ratio of the isotopologues is stable due to their almost identical chemical and physical characteristics. Mass spectrometry is then used to discriminate the isotopologues and, with the known amount of the internal standard, the amount of the unlabelled analyte can be calculated. Meaning, losses of the analyte are fully compensated for by the same losses of the isotopologues. Thus, the use of SIDA improves the specificity of the determination.[102] Uniformly (U-) [^{13}C] and [^{15}N]-labelled compounds are ideal in contrast to [^{2}H] or [^{18}O] labels. [^{13}C] and [^{15}N]-labels

are very stable as carbon and nitrogen are often part of the molecule backbone and C-C or C-N bonds cannot be cleaved easily.[2] In contrast, deuterated compounds will experience hydrogen-deuterium (H-D) exchange rapidly within the surrounding solution.[2] Compared to [^{13}C] and [^{15}N], deuterium labelled compounds are more vulnerable to so-called isotope effects where small physical or chemical differences of isotopologues can cause shifts in retention time.[83]

About 25 labelled analogues of the known mycotoxins have been synthesised. However, only 11 are commercially available. Several procedures have been established to determine mycotoxins in food using SIDA. Fumonisins were determined in maize samples using FB_1-D_6 as the internal standard,[82] whilst [$^{2}H_5$]-OTA was used to determine OTA in foods.[103] Asam and Rychlik (2007) synthesised four [^{13}C]-labelled type-B trichothecenes for the quantification of T-2, HT-2, diacetoxyscirpenol and monoacetoxyscirpenol in food and feeds.[104] [^{13}C]-DON was successfully used to determine DON in maize and wheat without a sample clean-up step.[24] Bretz and co-workers (2008)[84] synthesised 15-[$^{2}H_1$]-DON from its natural precursor 3-acetyldeoxynivalenol (3-AcDON) and used it as an internal standard in the determination of DON and 3-AcDON in cereals, whilst Häubl *et al.* (1999) [22] characterised and used U-[$^{13}C_{24}$] T-2 toxin in the analysis of maize. Recently, SIDA for aflatoxins, fumonisins, DON, OTA, ZEN, HT-2 and T-2 toxins using U-[^{13}C]-labelled analogues of all the target toxins prior to UPLC-MS/MS analysis was reported by Varga *et al*. (2012).[2] All the EU regulated mycotoxins were determined for the first time using this multi-targeted method.[2]

The application of labelled internal standards for LC-MS analysis of mycotoxins is increasing. This advancement is reflected by the rising supply of commercially available labelled mycotoxins. Nevertheless, many of these standards are relatively expensive particularly the [^{13}C]-isotopologues making it financially impractical to spike the isotopologue at the start of the extraction process. Isotopologues can only be applied prior to clean-up/analysis. It is expected that the price of labelled standards will remain very high in the near future. This poses a threat in multiple SIDAs as these toxins occur at different concentration and, hence, will require larger volumes of standard additions for the most abundant toxins.[102] Selectively combined SIDAs, rather than multiple SIDAs, are becoming more essential for multi mycotoxin analysis.[23]

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2.5 Analytical methods

Numerous analytical techniques have been implemented in the determination of mycotoxins. These comprise of enzyme-linked immunosorbent assay (ELISA),[105, 106] thin layer chromatography (TLC),[107] HPLC,[80] GC,[108] electrophoresis,[109] surface plasmon resonance (SPR) biosensors,[110] microfluidic chips[66] and microarrays.[111] Chromatographic techniques using standard detectors such as ultra-violet (UV) and fluorescence (FLD) are used for quantification; where mass spectrometric detection (LC-MS/MS or GC-MS) is often used to confirm the identity of the separated mycotoxins. This section provides an overview of the various LC-MS based approaches that have been used in mycotoxin analysis.

2.5.1. Liquid chromatography-mass spectrometry analysis of mycotoxin

Use of LC-MS/MS has long been considered the state-of the-art technique for mycotoxin analysis. The technique is becoming more widely accepted and applied, irrespective of the large capital investment and the need for skilled operators. LC-MS/MS offers the prospect of simultaneous identification and quantification of virtually all the mycotoxins at low concentration levels. The introduction of atmospheric pressure ionisation (API), specifically the electrospray ionisation (ESI) source, was a significant step for LC-MS/MS success in mycotoxin analysis. ESI is appropriate for the analysis of polar analytes; while atmospheric pressure chemical ionisation (APCI) and atmospheric pressure photo ionisation (APPI) are well suited for the analysis of medium to low-polar substances. Recently, combined interface (APCI/ESI) sources are common in modern instruments. However, the sensitivity is reduced in both modes. The great benefit of APCI/ESI is that both polar and non-polar analytes can be detected in a single run.[112] The majority of LC-MS/MS multi mycotoxin studies use the ESI interface in switching mode (applying both ESI+ and ESI -).[46] Sulyok *et al.* (2010)[113] reported the possibility of simultaneous analysis of 106 different mycotoxins by means of LC-MS/MS using the ESI interface. Using the ESI interface, both the protonated $[M+H]^+$ or deprotonated $[M-H]^-$ molecules can be generated. However, depending on the instrument conditions, some mycotoxins (OTA and ZEN) can be ionised at both ESI polarities enabling their analysis as protonated or deprotonated molecular ions.[114]

Selective sample preparation and chromatography allow the use of cheaper conventional detection techniques such as UV and FLD.[46] These simpler techniques are more accessible to third-world countries lacking the funding and other resources needed to maintain more complex instruments. Consequently, several HPLC-UV and HPLC-FLD methods have been

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accepted as official or standard methods by the International Association of Official Analytical Chemists (AOAC) and by the European Standardisation Committee (CEN) for analysis of mycotoxins in foods. However, HPLC-FLD methods are often optimised for a single analyte or a chemical group of analytes. OTA in cereal and cereal based products was determined by HPLC-FLD after extraction using the QuEChERS method[115], whilst fumonisins in maize were analysed by Marschik *et al.* (2013)[116] using HPLC-FLD. In spite of this, the use of these detectors is declining and being replaced with more current and advanced MS detectors, predominantly in first-world countries.

Ground-breaking work on mycotoxin analysis using MS was made in the early 1970s. Nowadays, unequivocal confirmation and quantification of mycotoxins can be promptly achieved with LC-MS. The co-existence of multiple mycotoxins in the same sample is of concern in mycotoxin analysis. Hence, the analytical method must be able to detect and quantify multiple mycotoxins in a single run. Advancement in LC and MS/MS techniques has been established to accomplish this purpose.[26] Currently UHPLC systems are preferred over HPLC due to their quicker separation capabilities, saving time and solvent and producing narrower/sharper peaks with better resolution and increased sample throughput. UHPLC displays superior chromatographic resolution and sensitivity while improving speed of analysis. Short columns with smaller particle size, less than 2 µm are used to improve separation efficiency thereby achieving fast analysis and better resolution. Also, less amount of solvent is required in UHPLC.

Han *et al.* (2010) [117] reported a UHPLC-MS/MS method using $^{13}C_{17}$-AFB_1 as the internal standard for the simultaneous quantification of aflatoxins in traditional Chinese medicines within 7 minutes, achieving a limit of detection (LOD) as low as 0.07 µg/kg with repeatability and reproducibility below 13.1%. Similarly, Han *et al.*(2010) also quantified OTA and OTB in traditional Chinese medicines using UHPLC/MS/MS and [$^{13}C_{20}$]-OTA as the internal standard.[118] A sensitive UHPLC-MS/MS method was used detect and quantify patulin in different fruit juices.[119] UHPLC-MS/MS has been used to determine multiple mycotoxins in rice[98] and maize[2].

UHPLC systems are often combined with triple quadrupole (QqQ) or combined quadrupole linear ion trap (QTRAP) mass filters to enable MS/MS. In MS/MS, the second selective MS step improves the signal-to-noise ratio, thereby improving the LOD. QqQ instruments perform MS/MS in the selective reaction monitoring mode (SRM). Following the initial fragmentation in the first quadrupole (Q_1), a selected precursor ion is fragmented in a collision cell (Q_2)

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through collision induced dissociation (CID) using a collision gas, typically Ar or He, to generate exclusive ion fragments which are filtered in the third quadrupole (Q_3) and monitored in multiple reaction monitoring (MRM) data acquisition mode.[120] By means of QqQ-MS/MS, Han and co-workers (2010) simultaneously detected five type-B trichothecenes in traditional Chinese medicines using [$^{13}C_{15}$]-DON as the internal standard.[121] Recently, Varga *et al.* (2012)[2] reported a UHPLC/ESI-QqQ-MS/MS method to determine 11 mycotoxins in maize. This method met the recovery and precision criteria set by EU regulation.

LC-MS/MS techniques display numerous advantages such as improved selectivity, sensitivity, reproducibility and robustness. With the improved specificity, multiple mycotoxins can be analysed in a single run with reliable detection. Conventional detectors, such as FLD and UV, are similarly appropriate in samples lacking analyte complexity. These detectors are typically used when selective clean-up is applied (e.g. IAC) where only targeted analytes are analysed, with little possibility of co-eluting/interfering analytes occurring. Table 2.3 shows some of the most commonly used analytical methods to quantify multi mycotoxins in various matrices.

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Table 2. 3. Overview of analytical methods used to multi mycotoxins in food, feed and beverages

Analytes	**Matrix**	**Sample pre-treatment (extraction/clean-up)**	**Type of LC/MS detection**		**Validation**		**References**
			Ionisation/ion selection	**Scan Mode**	**Precision (%RSD)**	**Recovery range (%)[a]**	
AFB_1, AFB_2, AFG_1, AFG_2, DON, FB_1, FB_2, HT-2, OTA, ZEN, T-2, DAS	Rice, wheat, maize, peanut, pistachio and almond	Acetonitrile/water (85:15); direct injection	ESI (+)QTRAP	MRM	3 – 20	84 – 104 (n=12)	Liao *et al.*, 2013
AFB_1, AFB_2, AFG_1, AFG_2	Maize	Tri-portions of acetonitrile/water (80:20); clean-up with Carbograph-4 cartridge	API(+)QqQ	MRM	2 – 12	87 – 101 (n=6)	Cavaliere *et al.*, 2007
AFB_1, AFB_2, AFG_1, AFG_2, DON, FB_1,	Maize	Acetonitrile/water/acetic acid (79:20:1); cleaned with SPE Oasis HLB cartridge	ESI(±)QTRAP	MRM	1.8 – 8.2	68 – 94 (n=3)	Wang *et al.*, 2013

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OTA, ZEN, T-2							
NIV, T-2, FUS-X, 3ADON, DON, 15ADON, ZEN, AFB_1, AFB_2, AFG_1, AFG_2,	Maize, wheat, cornflakes	Acetonitrile/water (85:15); followed by MultiSep 226 cartridge clean-up	APCI-TOF		2.1 – 7.1	71 – 133 (n=5)	Tanaka *et al.*, 2006
AFB_1, AFB_2, AFG_1, AFG_2, AFM_1, AFM_2 and OTA	Animal derived foods	Acetonitrile/hexane (50:50); followed by MycoSep 226, Oasis HLB and Bond Elut Mycotoxin clean-up columns	ESI(+)QqQ	MRM	4 – 18	68 – 105 (n=5)	Chen *et al.*, 2013
AFB_1, AFB_2, AFG_1, AFG_2	Animal feed	Methanol/water (80:20); direct injection	ESI(+)QqQ	MRM	2 – 15	78 – 122 (n=5)	Li *et al.*, 2011
AFB_1, AFB_2,	Maize	Double extraction, acetonitrile/water/formic acid	ESI(±)QqQ	MRM	4 – 11	88 – 105 (n=5)	Varga *et al.*, 2012

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AFG_1, AFG_2, DON, FB_1, FB_2, OTA, ZEN, T-2, HT-2		(80:19.9:0.1) and acetonitrile/water/formic acid (20:79.9:0.1); direct injection					
ZEN	Cereal flours	Pressurised Liquid Extraction, methanol/acetonitrile (50:50); direct injection	ESI(±)QqQ	MRM	2.9 – 4.1	70 – 78 (n=5)	Pérez-Torrado *et al.*, 2008
FB_1, FB_2	Maize	Ultrasonic extraction, methanol/water (67:33); IAC	ESI(+)QqQ	MRM	3.8 – 8.6	82.6 – 94.4 (n=3)	Li *et al.*, 2012
AFB_1, AFB_2, AFG_1, AFG_2, OTA,	Cereal, cocoa, coffee and nuts	QuEChERS; IAC	ESI(+)QTRAP	MRM	< 33	78 – 120, interlaboratory study	Desmarchelier *et al.*, 2014
OTA	Beer	Stir bar sorptive extraction (SBSE), liquid desorption	HPLC-FLD		< 10	81 – 84 (n=3)	Nguyen *et al.*, *2014*

[a] Recoveries were determined using spiked samples.

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2.6 Conclusion

A modern movement in chemical food safety control is shown by continuous efforts to perform multi mycotoxin analysis in a single run. Integrating LC with MS/MS has proven to be a robust instrument to identify and quantify multiple mycotoxins in complex matrices. Efforts are being made to develop universal extraction methods to simultaneously determine different classes of mycotoxins using LC-MS/MS. Despite detection methods becoming more selective and sensitive, there is still a need for better sample preparation techniques. Sample preparation is a critical step as it can account for a considerable amount of variability in the accurate determination of analyte concentration for a specific method.[47] Thus, the sample preparation technique should be as simple and effective as possible.

Solid-liquid extraction is still widely used for mycotoxin analysis. However, it is not selective enough to satisfy the needs of food regulatory requirements. Novel sampling extraction and clean-up methods are being developed and applied to mycotoxin analysis. However, these methods are analyte-matrix dependent and they still need to be evaluated for multi mycotoxin applications. For food analysis applications, integration of different methods is often required in order to meet the performance requirements such as sensitivity, accuracy and precision.

Hyphenating UHPLC to MS has been shown in multiple mycotoxin analysis to improve sensitivity and specificity. UHPLC-MS/MS methods boast full advantages such as enhanced sensitivity and accuracy. However, LC-MS methods have several drawbacks, most notably, matrix effects resulting in analyte signal enhancement or suppression during analysis. Specific sample preparation and clean-up methods can significantly minimise matrix effects by reducing the amount of co-eluting matrix components. The use of internal standards, in particular isotopically labelled internal standards, is a viable approach to deal with matrix effects. The application of stable isotope dilution assay (SIDA) has improved the reliability of mycotoxin LC-MS/MS analysis. Several labelled analogues of the known mycotoxins are commercially available.

In light of the above, it is proposed that an improved sample preparation procedure will significantly improve the accuracy and precision of the measurement results and also reduce uncertainty of the analytical method by minimising and possibly eliminating matrix effects during the analysis. It is envisaged that at the end of this project, an improved sample preparation method will be established and implemented in the quantification of multiple mycotoxins in maize. This improved sample preparation method, followed by LC-MS/MS

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analysis, may allow simultaneous quantification of toxins in maize with a high level of accuracy and good precision.

Chapter 3

Materials and methods

The following sample preparation methods were evaluated in this study:

- Dilute-and-shoot approach
- Quick, Easy, Cheap, Efficient, Rugged and Safe (QuEChERS)
- Solid phase extraction (SPE)

Matrix removal and analyte recovery efficiencies were used to evaluate the performance of each method. Orbital shaking, homogenisation and sonication extraction methods were evaluated for analyte extraction efficiency. Sample preparation methods were developed from a combination of extraction and clean-up techniques. The optimised method was validated with performance parameters

3.1. Chemicals and reagents

All standards including the unlabelled AFB_1, AFB_2, AFG_1, AFG_2, FB_1, FB_2, OTA, ZEN, DON, HT-2 and T-2 toxins, and the U-[^{13}C]-labelled AFB_1, AFG_1, FB_1, FB_2, OTA, ZEN, DON, mycotoxins were purchased from Romer Labs GmbH (Tulln, Austria). All the standards were individual stock solutions in acetonitrile or acetonitrile/water (1:1, *v/v*). Working solutions, for both the unlabelled and labelled mycotoxins, were prepared gravimetrically from the stock solutions in acetonitrile (Romil-SpS Super Purity Solvent, Romil). The working solution for the unlabelled mycotoxins had the following concentrations: AFB_1, AFG_1, 100 ng/g; AFB_2, AFG_2, 25 ng/g; FB1, DON, HT-2, T-2 Toxin, 5000 ng/g; FB2, ZEN, 2500 ng/g; OTA, 250 ng/g. The working solution for the labelled mycotoxins had the following concentrations: AFB_1, AFG_1, 10 ng/g; FB_1, DON, 500 ng/g; FB_2, OTA, 250 ng/g; ZEN, 300 ng/g. Individual stocks and the two working solutions were stored at -20 °C. Prior to usage, the working solutions were brought to room temperature and mixed thoroughly.

ACN and methanol (MeOH), both HPLC grade, were purchased from Merck (Darmstadt, Germany). Acetic acid (HOAc) (98% - 100% purity) and formic acid (HCOOH) (98% - 100% purity) were obtained from Fluka (Steinheim, Germany). Ultra-pure water was produced from distilled water using a Milli-Q system (Millipore Corp., Bedford, MA, USA). Anhydrous

magnesium sulphate ($MgSO_4$), sodium chloride (NaCl) and ammonium formate (NH_4COOH) (≥ 99% purity), were from Sigma-Aldrich (Steinheim, Germany). Polyvinylidene fluoride (PVDF) syringe filters, 0.22 µm were purchased from Membrane Solutions (Texas, US).

QuEChERS kit was purchased from Restek. MultiSep AflaZON+ 226 SPE columns (500 mg) (Romer Labs GmbH, Tulln, Austria) were donated by Tega Marketing. Bond Elut Plexa and Bond Elut Mycotoxins SPE columns (30 mg) (Varian) were donated by Chemetrix (Pty) Ltd. Oasis HLB (500 mg) SPE columns were from Waters Corp. (Milford, MA, USA) and Supelco Select HLB SPE (500 mg) columns were from Sigma-Aldrich (Steinheim, Germany).

Reference materials (RM) for mycotoxins in maize with well-defined analyte concentrations were sourced from Trilogy and Food Analysis Performance Assessment Scheme (FAPAS). Trilogy maize had the following concentrations: AFB_1, 18.8 ng/g; AFB_2, 0.9 ng/g; AFG_1, 2.4 ng/g; FB_1, 28.3 mg/g; FB_2, 7.1 mg/g; OTA, 5.57 ng/g; ZEN, 352 ng/g; DON, 2.6 mg/g; HT-2, 523 ng/g; T-2 Toxin 263 ng/g. FAPAS maize had the following assigned value concentrations: AFB_1, 8.01 ng/g; OTA, 4 ng/g; ZEN, 344 ng/g; DON, 1.79 mg/g.

3.2. Mycotoxin extraction and clean-up

3.2.1. The dilute-and-shoot approach

Ground and homogenised maize samples (5.00 ± 0.01 g) were weighed into 50 mL polypropylene tubes (Thermo Fischer). Double extraction was performed, the first extraction was performed with 20 mL of extraction solvent 1, ACN/H_2O/HCOOH (80:19.9:0.1, *v/v/v*), on an Orbishake rotary shaker (Labotec) for 60 min at room temperature. After extraction, the tubes were centrifuged for 5 min (3 500 *g*) using a Multifuge X3R centrifuge (Thermo Scientific) and the extract was decanted into a clean polypropylene tube. The residue was extracted for the second time with 20 mL of solvent 2, ACN/H_2O/HCOOH (20:79.9:0.1, *v/v/v*), on the rotary shaker for 30 min at room temperature. Thereafter, the samples were centrifuged again for 5 min (3 500 *g*) and the supernatant was combined with the first extract.

The combined extracts were centrifuged again for 5 min (3 500 g). An aliquot (750 µL) of the centrifuged extract was filtered using 0.2 µm nylon syringe filter and then transferred into an HPLC vial and dried down under a gentle stream of nitrogen (N_2) at 50 °C. The dried residue was reconstituted with 250 µL of the [^{13}C]-labelled working solution and 750 µL of the starting mobile phase (MP) composition, (35:65, *v/v*) MeOH/Milli-Q water both containing 5 mM

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NH_4COOH (65:35, *v/v*), was added. The content of the vial was mixed by vortex (Vortex Genie 2, Scientific Industries) and a 10 µL aliquot was used for LC-MS/MS analysis.

3.2.2. QuEChERS extraction methods

3.2.2.1. QuEChERS

Ground and homogenised maize samples (5.00 ± 0.01 g) were weighed into 50 mL polypropylene tubes and mixed with 10 mL of Milli-Q H_2O. After shaking for 1 hour, 15 mL of ACN:HOAc (99/1, *v/v*) and QuEChERS extraction mixture (4 g $MgSO_4$, 1 g NaCl, 1 g trisodium citrate dehydrate (TSCD) and 0.5 g disodium hydrogen citrate sesquihydrate (DHS)) (Restek) was added. The resulting slurry was vigorously hand-mixed and shaken on a vortex mixer for 5 min and centrifuged for 5 min (3 500 *g*). Thereafter, the ACN layer was transferred into a 15 mL PTFE tube containing QuEChERS d-SPE salts (1200 mg $MgSO_4$, 400 mg primary secondary amine (PSA) and 400 mg C_{18}) (Restek). The tubes were vigorously hand-shaken for 1 min and centrifuged again for 5 min (3 500 *g*). The supernatant was evaporated to dryness under a gentle stream of N_2 at 50 °C. To the dried residue, 250 µL of the [^{13}C]-labelled working solution was added, mixed and the solvent was evaporated to dryness under a gentle stream of N_2 at 50 °C. The dried residue was reconstituted in 250 µL of the starting MP composition, filtered through a 0.2 µm PVDF syringe filter and then transferred into an HPLC vial. The solution was mixed and a 10µL aliquot was used for LC-MS/MS analysis.

3.2.2.2. Modified QuEChERS

Ground and homogenised maize samples (5.00 ± 0.01 g) were weighed into 50 mL polypropylene tubes and mixed with 10 mL of Milli-Q H_2O. After shaking for 1 hour, 15 mL of ACN/HOAc (99.5:0.5, *v/v*) was added and shaken for 30 min. $MgSO_4$/NaCl salt mixture, 5 g, (4:1, *w/w*) was added. The resulting slurry was vigorously hand-mixed and shaken on a vortex mixer for 5 min and centrifuged for 15 min (3 500 *g*). Thereafter, 5 mL of the ACN layer was transferred into a 15 mL PTFE tube and 5 mL hexane was added and shaken for 20 min, centrifuged for 5 min (3 500 *g*), and the hexane upper layer was discarded.

3.2.2.2.1. Dispersive-SPE clean-up

A 2 mL aliquot , of the defatted extract was transferred into a 15 mL PTFE tube containing QuEChERS d-SPE salts, defined above, (Restek). The tubes were vigorously hand-shaken for 1 min and centrifuged again for 5 min (3 500 *g*). The supernatant was evaporated to dryness

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under a gentle stream of N_2 at 50 °C. The dried residue was reconstituted in 250 µL of the starting MP composition, filtered through a 0.2 µm PVDF syringe filter and then transferred into an HPLC vial. The solution was mixed and a 10µL aliquot was used for LC-MS/MS analysis.

3.2.2.2.2. Supelco HLB clean-up

A 2 mL aliquot, of the defatted extract was evaporated to dryness under a gentle stream of N_2 at 50 °C. The residue was dissolved in 2 mL, 5% MeOH in Milli-Q water and loaded into a pre-conditioned Supelco HLB column. The analytes were eluted with 2 mL MeOH, in two steps of 1 mL each, and collected in a silanized test tube. The eluate was evaporated to dryness under a gentle stream of N_2 at 50 °C. The dried residue was reconstituted in 250 µL of the starting MP composition, filtered using 0.2 µm PVDF syringe filter and then transferred into an HPLC vial. The solution was mixed and a 10µL aliquot was used for LC-MS/MS analysis.

Isotopically labelled analytes were not used in this section. As the samples were first analysed using HPLC-FLD with on-line derivatization then with UHPLC-MS/MS, no labelled standards were used as co-elutions would occur during HPLC-FLD analysis.

3.2.3. Extraction with orbital shaking and SPE clean-up

Ground and homogenised maize samples (5.00 ± 0.01 g) were weighed into 50 mL polypropylene tubes. Double extraction was performed, the first extraction was performed with 20 mL of extraction solvent 1, ACN/H_2O/HCOOH (80/19.9/0.1, *v/v/v*), on a rotary shaker for 60 min at room temperature. After extraction, the tubes were centrifuged for 5 min (3 500 *g*) in a Multifuge X3R centrifuge (Thermo Scientific) and the extract was decanted into a clean polypropylene tube. The residue was extracted for the second time with 20 mL of solvent 2, ACN/H_2O/HCOOH (20:79.9:0.1, *v/v/v*), on the rotary shaker for 30 min at room temperature. Thereafter, the samples were centrifuged again for 5 min (3 500 *g*) and the supernatant was combined with the first extract. The combined extracts were centrifuged again for 5 min (3 500 g).

3.2.3.1. Varian Bond Elut Mycotoxin

An aliquot, 4 mL, of the combined extract was passed through a Bond Elut Mycotoxin column and collected in a silanised test tube. The eluate was evaporated to dryness under a gentle stream of N_2 at 50 °C. The dried residue was reconstituted in 750 µL of the starting MP

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composition and 250 µL of the [^{13}C]-labelled working solution was added, the solution was mixed and a 10µL aliquot was used for LC-MS/MS analysis.

3.2.3.2. Waters Oasis HLB – undiluted extract clean-up

An aliquot, 4 mL, of the combined extract was evaporated to dryness under a gentle stream of N_2 at 50 °C. The residue was dissolved in 5% MeOH in Milli-Q water, 4 mL, and loaded into a pre-conditioned Oasis HLB column. The analytes were eluted with 4 mL MeOH and collected in a silanised test tube. The eluate was evaporated to dryness under a gentle stream of N_2 at 50 °C. The dried residue was reconstituted in 750 µL of the starting MP composition and 250 µL of the [^{13}C]-labelled working solution was added, the solution was mixed and a 10µL aliquot was used for LC-MS/MS analysis.

3.2.3.3. Romers MultiSep AflaZon+ 226

An aliquot, 4 mL, of the combined extract was passed through MultiSep AflaZON+ 226 column and collected in a silanised test tube. The eluate was evaporated to dryness under a gentle stream of N_2 at 50 °C. The dried residue was reconstituted in 750 µL of the starting MP composition and 250 µL of the [^{13}C]-labelled working solution was added, the solution was mixed and a 10µL aliquot was used for LC-MS/MS analysis.

3.2.3.4. Varian Bond Elut Plexa

An aliquot, 0.3 mL, of the combined raw extract was passed through the Bond Elut Plexa column and collected in a silanised test tube. The eluate was evaporated to dryness under a gentle stream of N_2 at 50 °C. To the dried residue, 250 µL of the [^{13}C]-labelled working solution was added, mixed and the solvent was evaporated to dryness under a gentle stream of N_2 at 50 °C. The dried residue was reconstituted in 250 µL of the starting MP composition, filtered through a 0.2 µm PVDF syringe filter and then transferred into an HPLC vial. The solution was mixed and a 10µL aliquot was used for LC-MS/MS analysis.

3.2.3.5. Waters Oasis HLB – diluted extract clean-up

An aliquot, 5 mL, of the combined extract was diluted to 50 mL with Milli-Q water. The diluted mixture was loaded into a pre-conditioned Oasis HLB column. The analytes were eluted with 4 mL MeOH and collected in a silanised test tube. The eluate was evaporated to dryness under a gentle stream of N_2 at 50 °C. To the dried residue, 250 µL of the [^{13}C]-labelled working solution was added, mixed and the solvent was evaporated to dryness under a gentle

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stream of N_2 at 50 °C. The dried residue was reconstituted in 250 µL of the starting MP composition, filtered through a 0.2 µm PVDF syringe filter and then transferred into an HPLC vial. The solution was mixed and a 10µL aliquot was used for LC-MS/MS analysis.

3.2.3.6. Supelco Select HLB

An aliquot, 5 mL, of the combined extract was diluted to 50 mL with Milli-Q water. The diluted mixture was loaded into a pre-conditioned Supelco HLB column. The analytes were eluted with 4 mL ACN/MeOH (50:50, *v/v*) and collected in a silanised test tube. The eluate was evaporated to dryness under a gentle stream of N_2 at 50 °C. To the dried residue, 250 µL of the [^{13}C]-labelled working solution was added, mixed and the solvent was evaporated to dryness under a gentle stream of N_2 at 50 °C. The dried residue was reconstituted in 250 µL of the starting MP composition, filtered through a 0.2 µm PVDF syringe filter and then transferred into an HPLC vial. The solution was mixed and a 10µL aliquot was used for LC-MS/MS analysis.

3.2.4. Homogenisation and sonication

3.2.4.1. Supelco HLB – sonication extraction

Ground and homogenised maize samples (5.00 ± 0.01 g) were weighed into 50 mL polypropylene tubes. Double extraction was performed, the first extraction was performed with 20 mL of extraction solvent 1, ACN/H_2O/HCOOH (80:19.9:0.1, *v/v/v*), on a sonication bath (Branson 8800) for 60 min at room temperature, output power set at 280 W. After extraction, the tubes were centrifuged for 5 min (3 500 *g*) and the extract was decanted into a new polypropylene tube. The residue was extracted for the second time with 20 mL of solvent 2, ACN/H_2O/HCOOH (20:79.9:0.1, *v/v/v*), on the ultrasonic bath for 30 min at room temperature. Thereafter, the samples were centrifuged again for 5 min (3 500 *g*) and the supernatant was combined with the first extract. The combined extracts were centrifuged again for 5 min (3 500 g).

An aliquot, 5 mL, of the combined extract was evaporated to dryness under a gentle stream of N_2 at 50 °C. The residue was dissolved in 5% MeOH in Milli-Q water, 2 mL, and loaded into a pre-conditioned Supelco HLB column. The analytes were eluted with 5 mL ACN/MeOH/HCOOH (50:49.9:0.1, *v/v*) and collected in a silanised test tube. The eluent was evaporated to dryness under a gentle stream of N_2 at 50 °C. To the dried residue, 250 µL of the [13C]-labelled working solution was added, mixed thoroughly and dried under a gentle

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stream of N_2 at 50 °C. To the dried residue, 250 µL of the [^{13}C]-labelled working solution was added, mixed and the solvent was evaporated to dryness under a gentle stream of N_2 at 50 °C. The dried residue was reconstituted in 250 µL of the starting MP composition, filtered through a 0.2 µm PVDF syringe filter and then transferred into an HPLC vial. The solution was mixed and a 10µL aliquot was used for LC-MS/MS analysis.

3.2.4.2. Supelco HLB – homogenisation extraction

Ground and homogenised maize samples (5.00 ± 0.01 g) were weighed into 50 mL polypropylene tubes. The maize sample was first extracted with 20 mL of ACN/H_2O/HCOOH (80:19.9:0.1, *v/v/v*) by homogenisation at high speed for 3 min using a commercial blender (Waring) at room temperature. The sample was then centrifuged for 5 min (3 500 *g*) and the extract was decanted into a new polypropylene tube. The residue was extracted for the second time with 20 mL of ACN/H_2O/HCOOH (20:79.9:0.1, *v/v/v*) using the blender at high speed for 3 min. Thereafter, the samples were centrifuged again for 5 min (3 500 *g*) and the supernatant was combined with the first extract. The combined extracts were centrifuged again for 5 min (3 500 g).

An aliquot, 5 mL, of the combined extract was evaporated to dryness under a gentle stream of N_2 at 50 °C. The residue was dissolved in 5% MeOH in Milli-Q water, 2 mL, and loaded into a pre-conditioned Supelco HLB column. The analytes were eluted with 5 mL ACN/MeOH/HCOOH (50:49.9:0.1, *v/v*) and collected in a silanised test tube. The eluate was evaporated to dryness under a gentle stream of N_2 at 50 °C. To the dried residue, 250 µL of the [^{13}C]-labelled working solution was added, mixed and the solvent was evaporated to dryness under a gentle stream of N_2 at 50 °C. The dried residue was reconstituted in 250 µL of the starting MP composition, filtered through a 0.2 µm nylon syringe filter and then transferred into an HPLC vial. The solution was mixed and a 10µL aliquot was used for LC-MS/MS analysis.

3.3. UHPLC-MS/MS conditions

For LC-MS/MS, an Acquity Ultra High Performance Liquid Chromatography (UHPLC) system interfaced with a Micromass Quattro Premier XE triple-quadrupole mass spectrometer (Waters Corp, MA, USA) was used. MassLynx software version 4.1 (Waters Corp, MA, USA) was used for instrumental control, and data acquisition and processing. LC separation was achieved using an Acquity UPLC BEH C_{18} column (2.1 mm i.d. x 100 mm; 1.7 µm particle

size) (Waters Corp.). The column and auto sampler tray temperature were controlled at 30 °C and 4 °C, respectively. The injection volume was 10 µL and the mobile phase flow rate was 0.35 mL min^{-1}. The mobile phase composition was (A) 5 mM NH_4COOH in MeOH: Milli-Q (95:5, *v/v*) and (B) 5 mM NH_4COOH in Milli-Q, pH 3 corrected using formic acid. UHPLC elution conditions are listed in Table 3.1.

Table3. 1. Elution conditions for the UHPLC separation of 11 mycotoxins using an Acquity BEH C_{18} column with a flow rate of 0.35 mL/min.

Time (min)	% Solvent A	% Solvent B
Initial	35	65
1.00	55	45
5.00	52	48
10.00	62	38
11.00	80	20
12.00	98	2
12.01	35	65
16.00	35	65

Chromatographic separation was also done using a Raptor ARC-18 UHPLC column (2.1 mm i.d. x 100 mm; 2.7 µm particle size) (Restek) to evaluate the method's robustness. UPLC conditions for the column are given in Table 3.2 below.

Table3. 2. Elution conditions for the UHPLC separation of 11 mycotoxins using Raptor ARC-18 column with a flowrate of 0.35 mL/min.

Time (min)	% Solvent A	% Solvent B
Initial	35	65
3.50	55	45
3.60	60	40
9.90	60	40
10.00	62	38
12.00	80	20
13.00	98	2
15	35	65

The MS instrument was operated in electrospray ionisation (ESI) in fast polarity switching mode. Ten of the analytes were analysed in ESI positive (ESI+) mode, whereas, ZEN was detected in ESI negative (ESI-) mode. Settings for the ESI probe are shown in Table 3.3.

Table3. 3. Mass spectrometer electrospray ionisation settings.

Parameter	ESI(±)
Capillary voltage (kV)	2.5
Cone voltage (V)	20
Extractor (V)	3
RF lens (V)	0
Source temperature (°C)	120
Desolvation temperature (°C)	300
Desolvation gas (L hr^{-1})	700
Cone gas (L hr^{-1})	50
Collision gas flow (mL min^{-1})	0.3

Analyte specific MS/MS parameters were obtained by direct infusion of each standard solution into the ESI source with the mobile phase connected via a T-piece. The two most abundant product ions generated from each precursor ion were chosen as the multiple reaction monitoring (MRM) transitions of each analyte. This MS/MS method contains seven windows and the MS/MS parameters are listed in Table 3.4.

Table3. 4. MS/MS parameters for mycotoxin detection by the multiple reaction monitoring (MRM) method.

Analyte	**ESI mode**	**Window[a]**	***m/z* precursor ion**	***m/z* product ions (CE in V)[b]**
AFB_1 **$[^{13}C_{17}]$-AFB_1**	+	2	312.9 $[M+H]^+$ 330.1 $[M+H]^+$	285 (24), 241 (30) 301 (30), 284.5 (30)
AFB_2	+	2	314.9 $[M+H]^+$	287 (27), 259.1 (30)
AFG_1 **$[^{13}C_{17}]$-AFG_1**	+	2	328.9 $[M+H]^+$ 346.0 $[M+H]^+$	243 (27), 283.1 (30) 211.8 (42), 257.0 (42)
AFG_2	+	2	330.9 $[M+H]^+$	245 (30), 217 (32)
DON **$[^{13}C_{15}]$-DON**	+	1	297 $[M+H]^+$ 312.2 $[M+H]^+$	249 (10), 231 (12) 263 (17), 245 (17)
FB_1 **$[^{13}C_{34}]$-FB_1**	+	4	722.0 $[M+H]^+$ 756.2 $[M+H]^+$	352.15 (40), 334 (40) 356.2 (40), 374 (40)
FB_2 **$[^{13}C_{34}]$-FB_2**	+	7	706.15 $[M+H]^+$ 740.5 $[M+H]^+$	336.2 (38), 318.2 (38) 358.0 (40), 340.2 (40)
OTA **$[^{13}C_{20}]$-OTA**	+	6	403.9 $[M+H]^+$ 424.1 $[M+H]^+$	238.9 (25), 340.9 (30) 250.0 (30), 232.0 (30)
ZEN **$[^{13}C_{18}]$-ZEN**	-	5	317.0 $[M-H]^-$ 335.2 $[M-H]^-$	174.8 (24), 130.8 (30) 185.0 (25), 290.2 (25)
HT-2 Toxin	+	3	442.2 $[M+NH_4]^+$	215 (22), 263 (27)
T-2 Toxin	+	4	489.0 $[M+Na]^+$	245.1 (22), 387.1 (27)

[a] Window 1: 0.00 – 1.50 min Window 2: 1.00 – 3.00 min

Window 3: 3.00 – 4.20 min Window 4: 4.00 – 8.00 min

Window 5: 7.00 – 9.00 min Window 6: 7.50 – 10.00 min

Window 7: 10.00 – 15.00 min

[b] Values are given in the order of quantifier ion, qualifier ion (in parentheses are the corresponding collision energy (CE) settings in volts).

3.4. HPLC-PDA-FLD conditions

HPLC coupled to fluorescence detector (FLD) and photodiode array (PDA) detectors was used for mycotoxin determination after the QuEChERS sample preparation technique. Separation was achieved on a RPC_{18} Symmetry HPLC column (4.6 mm x 150 mm, 3.5 µm particle size) (Waters). The column and auto sampler tray temperature were set at 30 °C and 4 °C, respectively. The injection volume was 25 µL and the mobile phase flow rate was 1.00 mL min^{-1}. The mobile phase compositions were (A) ACN, (B) Milli-Q water, (C) 0.1% H_3PO_4 (aq) and (D) MeOH.

The HPLC eluent flows through the PDA detector (208 nm and 220 nm), then through a photochemical reactor (PHRED) (Waters) enhancing the fluorescence response of the eluting aflatoxins, to a post column reaction coil and then to the FLD. An additional binary pump connected to the post column reaction coil controls the introduction of the derivatization reagents, *ortho*-phthaldehyde (OPA) and 2-mercaptoethanol (2ME), via a t-piece to the post column reaction coil. The flow rate for introduction of the derivatization reagent is 0.5 mL/min, at a specific time interval which allows the derivatization of the eluting fumonisins. Gradient conditions are listed in Table 3.5 and FLD wavelengths are listed in Table 3.6.

Table3. 5. HPLC-FLD elution gradient for the separation of 11 mycotoxins.

Time (min)	Flow (mL/ min)	%A	%C	%D
	1.00	15.0	85.0	0.0
4.00	1.00	15.0	85.0	0.0
5.00	1.00	15.0	60.0	25.0
16.00	1.00	15.0	60.0	25.0
17.00	1.00	30.0	40.0	30.0

30.01	1.00	15.0	85.0	0.0

Table3. 6. Fluorescence detector excitation and emission wavelengths.

Time (min)	Mycotoxin	Excitation	Emission
0.1 – 20	Aflatoxins	365 nm	455 nm
20 – 25.5	Fumonisins	329 nm	465 nm
25.5 – 28.6	Zearalenone	276 nm	460 nm
28.6 – 30	Ochratoxin A	329 nm	460 nm

3.5. Method validation

The determined method performance parameters include accuracy, precision, working range, limit of detection (LOD) and limit of quantification (LOQ), linearity and robustness.

Matrix matched calibration standards were prepared from blank maize extracts by spiking 5 mL aliquot with 250 µL of the neat calibration standards to prepare a nine point calibration curve. A dried aliquot of the blank extract was dissolved in 250 µL of the neat working solution and 250 µL of the [^{13}C]-labelled working solution was added. This mixture was dried under a gentle stream of nitrogen gas (N_2) at 50 °C and reconstituted in 250 µL of the starting mobile phase composition MeOH/Milli-Q water both containing 5 mM NH_4COOH (65:35, *v/v*).

In order to evaluate matrix effects, signal suppression/enhancement (SSE) of each analyte was estimated by calculating using the following equation:

$$\%SSE = \frac{slope\ of\ matrix\ matched\ internal\ calibration}{slope\ of\ neat\ solvent\ internal\ calibration}\ x\ 100 \quad (1)$$

If %SSE = 100, no matrix effect; if %SSE > 100, there is a signal enhancement and if %SSE < 100, there is a signal suppression.

Accuracy and precision of the method were validated by evaluating analyte recoveries from spiked maize samples at three concentration levels in triplicate. Recovery experiments were done by spiking blank maize samples (5.00±0.01 g) with the appropriate amount of spiking solution (unlabelled mycotoxins) at three concentration levels in triplicate before extraction.

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Spiked samples were stored uncapped overnight at room temperature to allow solvent evaporation and to achieve equilibrium between the analytes and the matrix. Prior to extraction, the samples were capped followed by a short shaking to ensure a homogeneous distribution of the spiked maize. Also, maize reference materials with a well-defined analyte concentrations from two different suppliers, Food Analysis Performance Assessment Scheme (FAPAS) and Trilogy, were analysed (n = 3).

Linearity, limit of detection (LOD) and limit of quantification (LOQ) were determined through linear regression analysis. For robustness analyte separation was carried out on a Raptor ARC-18 UHPLC column (2.1 mm i.d. x 100 mm; 2.7 µm particle size) (Restek) to evaluate the effect of a column from a different manufacturer.

Chapter 4

Results and discussion

Mycotoxin contamination can have severe consequences. Several reports on the simultaneous occurrence of mycotoxins in food and feed commodities have been published. The simultaneous occurrence of mycotoxins have even more severe consequences as it has synergistic adverse effects on health and a larger negative impact on the economy. Therefore it is necessary to determine mycotoxin levels in food and feed. The simultaneous determination of several mycotoxins would be advantageous in terms of time and cost. Accurate identification and quantification of mycotoxin levels is required for legislative, health and economic reasons. Mycotoxin regulations can only be enforced if reliable, sensitive and accurate methods are in place.

4.1. UHPLC-MS/MS method development

Analyte specific MS/MS parameters, including the determination of precursor and product ions and corresponding collision energies, were obtained by direct infusion of single unlabelled and labelled analyte solutions into the ESI source. Transitions for all compounds were evaluated in positive and negative mode using Waters MassLynx™ software. With the exception of ZEN, which displayed highest sensitivity when applying the $[M-H]^-$ ion as the precursor; most of the compounds displayed the $[M+H]^+$ ion as the most abundant precursor. For T-2 and HT-2 toxins, the $[M+NH4]^+$ and $[M+Na]^+$ ions were used respectively as the precursor ions. Two mass transitions with the highest abundance were selected for each analyte to serve as quantifier and qualifier ions during the analysis. Fast polarity switching mode was used to include all the compounds in a single analytical run. Table 4.1 lists MS/MS parameters obtained during infusion experiments.

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Table 4. 1. MS/MS parameters for mycotoxin detection by the multiple reaction monitoring (MRM) mode.

Analyte	Retention time (min)	Spiking level (ng/g)	*m/z* precursor ion	*m/z* product ions (CE in V)[a]
DON	1.07	1 500	297 $[M+H]^+$	249 (10), 231 (12)
$[^{13}C_{15}]$-DON		300	312.2 $[M+H]^+$	263 (17), 245 (17)
AFG_2	1.79	12	330.9 $[M+H]^+$	245 (30), 217 (32)
AFG_1	1.95	30	328.9 $[M+H]^+$	243 (27), 283.1 (30)
$[^{13}C_{17}]$-AFG_1		10	346.0 $[M+H]^+$	211.8 (42), 257.0 (42)
AFB_2	2.10	12	314.9 $[M+H]^+$	287 (27), 259.1 (30)
AFB_1	2.30	30	312.9 $[M+H]^+$	285 (24), 241 (30)
$[^{13}C_{17}]$-AFB_1		10	330.1 $[M+H]^+$	301 (30), 284.5 (30)
HT-2 toxin	3.95	1 500	442.2 $[M+NH_4]^+$	215 (22), 263 (27)
T-2 toxin	6.10	1 500	489.0 $[M+Na]^+$	245.1 (22), 387.1 (27)
FB_1	6.90	1 500	722.0 $[M+H]^+$	352.15 (40), 334 (40)
$[^{13}C_{34}]$-FB_1		300	756.2 $[M+H]^+$	356.2 (40), 374 (40)
ZEN	7.85	750	317.0 $[M-H]^-$	174.8 (24), 130.8 (30)
$[^{13}C_{18}]$-ZEN		300	335.2 $[M-H]^-$	185.0 (25), 290.2 (25)
OTA	8.35	75	403.9 $[M+H]^+$	238.9 (25), 340.9 (30)
$[^{13}C_{20}]$-OTA		50	424.1 $[M+H]^+$	250.0 (30), 232.0 (30)
FB_2	11.89	750	706.15 $[M+H]^+$	336.2 (38), 318.2 (38)
$[^{13}C_{34}]$-FB_2		300	740.5 $[M+H]^+$	358.0 (40), 340.2 (40)

[a] Values are given in the order quantifier ion, qualifier ion (in parentheses are the corresponding collision energy (CE) settings in volts).

Chromatography was optimised to achieve baseline separation for the compounds including aflatoxins. The best chromatographic results in terms of peak shape, signal intensity, and reproducibility, were achieved with MeOH and Milli-Q water both containing 5 mM NH_4COOH (pH 3). This acidified mobile phase was used to yield stable retention times and better ionization efficiencies for the fumonisins. The use of Acquity BEH C_{18} UHPLC column helped to achieve a short run time and to increase chromatographic resolution. Figure 4.1 shows the extracted ion MRM chromatogram of a matrix matched calibration sample including all 11 unlabelled mycotoxins and 7 labelled mycotoxins (DON, AFG_1, AFB_1, FB_1, ZEN, OTA

and FB_2). Analytes were identified by their retention times and by two selected MRM transitions (1 quantifier, 1 qualifier). The gradient elution began with 35% MeOH because DON is poorly retained relative to other analytes on the column. All mycotoxins were detected within 12 min without any co-elutions providing rapid determination of multiple mycotoxins.

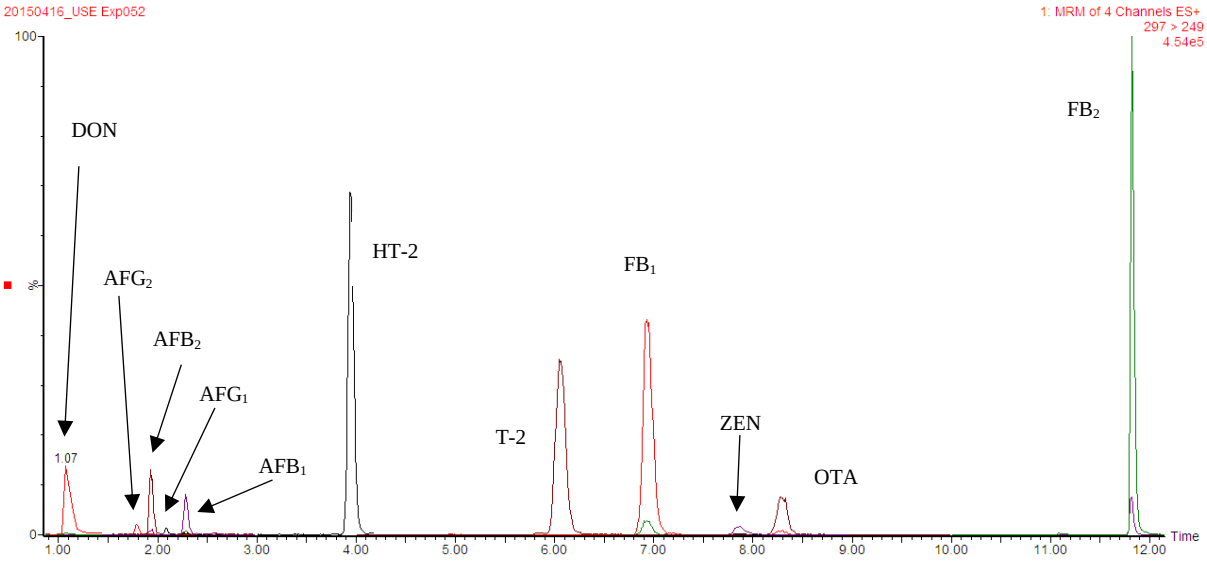

Figure 4. 1. Extracted ion chromatogram (XIC) of a blank maize spiked after extraction with labelled and unlabelled mycotoxins. Corresponding analyte spiking concentration and retention times are in Table 4.1.

4.2. Mycotoxin extraction and clean-up

The primary objective of this study was to develop a sample preparation method that will significantly reduce or preferably eliminate matrix effects by selectively isolating the target analytes from the matrix components. Different sample extraction and clean-up techniques were evaluated in this study and they are discussed below.

4.2.1. The dilute-and-shoot approach

The goal of this study was to apply the dilute-and shoot sample preparation method in multi mycotoxin analysis and to illustrate the need for a clean-up step before analysis as the presence of matrix effects leads to inaccurate, biased results.

A dilute-and-shoot sample preparation method for multi mycotoxin determination in maize has been used by Varga and co-workers (2012).[2] Sequential extraction using an acidified mixture of acetonitrile and water was used for extracting mycotoxins from maize using an orbital

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shaker. In the first extraction step, a high organic solvent mixture (ACN/H_2O/HCOOH, 80/19.9/0.1, *v/v/v*) was used to enhance extraction efficiency for non-polar compounds. Thereafter, a high aqueous solvent mixture (ACN/H_2O/HCOOH, 20/79.9/0.1, *v/v/v*) was used in the second extraction step to extract the more polar compounds like the FBs and DON. This two-step extraction is useful for extracting mycotoxins with different physicochemical properties and it is quick, the total extraction time is 90 minutes. However, a major disadvantage is the amount of matrix components extracted in the second extraction step. This then requires the sample extracts to be filtered to minimise matrix components prior to analysis.

For the evaluation of relative recovery and matrix effects, blank maize samples were spiked with unlabelled working solution at six different concentration levels before extraction. Recoveries were determined using external and internal matrix matched calibration. Enhanced recoveries, %REC ≥ 130 % were obtained for most mycotoxins using external calibration standards with the exception of AFG_1 and AFG_2, 116 % and 113 %, respectively. Using internal calibration standards, recoveries for aflatoxins, ZEN and HT-2 toxin ranged between 114 – 127%, whereas enhanced recoveries, %REC ≥ 130, were observed for DON, T-2 toxin, OTA and fumonisins. Table 4.2 shows obtained analyte recoveries and matrix effects on each analyte. Spiked samples do not mimic the conditions of naturally incurred mycotoxins in maize as the analytes do not penetrate the matrix but remains on the surface. Therefore, relatively high recoveries are characteristic for spiked samples.[122]

The presence of co-eluting matrix components during the ionisation step leads to suppressed or enhanced analyte signals. Signal suppression/enhancement (SSE), expressed as a percentage was determined as the ratio between the slopes of the curve obtained for the matrix matched standards and the slope of the curve for the solvent standard calibration curve. Using external calibration, %SSE ranged between 85 – 171% with DON and FB_1 being the most affected analytes at 162% and 171%, respectively. However, using internal standard values between 88 – 131% were obtained. This shows that the use of internal standards can compensate for resulting matrix effects. Analytes that were largely affected by matrix effects led to inaccurate recoveries illustrating the need for further sample clean-up prior analysis.

Table 4. 2. Comparison of analyte recoveries (n = 6) and matrix effects obtained using internal and external matrix matched calibration standards.

	SSE[a] int. (%)	Recovery,[b] x ± RSD (%)	SSE ext. (%)	Recovery,[c] x ± RSD (%)
AFB_1	115	128 ± 5	92	155 ± 12
AFB_2	106	118 ± 3	85	130 ± 7
AFG_1	101	120 ± 6	90	116 ± 10
AFG_2	101	114 ± 5	90	113 ± 9
FB_1	114	162 ± 10	171	394 ± 15
FB_2	109	147 ± 8	121	253 ± 12
OTA	131	152 ± 5	163	155 ± 17
ZEN	88	112 ± 3	119	133 ± 8
DON	96	134 ± 7	162	149 ± 5
HT-2	92	127 ± 3	108	158 ± 14
T2-Toxin	124	149 ± 11	132	181 ± 9

[a] $\%SSE = \frac{slope\ of\ matrix\ matched\ internal\ calibration}{slope\ of\ neat\ solvent\ internal\ calibration}\ x\ 100$

If %SSE = 100, no matrix effect; if %SSE > 100, there is a signal enhancement and if %SSE < 100, there is a signal suppression.

[b] Analyte recovery using internal calibration

[c] Analyte recovery using external calibration

4.2.2. QuEChERS extraction methods

QuEChERS is gradually becoming a popular alternative to the dilute-and-shoot approach as a generic sample preparation technique, particularly in environmental analysis.[42] The aim of this study was to apply the QuEChERS sample preparation technique in multi mycotoxin determination in maize.

A Restek™ generic procedure was initially applied without any modifications.[123] Results obtained were far from satisfactory, poor analyte recoveries and severe matrix effects were observed. Thereafter, the original method was modified with an aim to improve the method's performance. A Mg/NaCl salt mixture (4/1, *w/w*) was used in the first extraction step instead of the Restek mixture (4 g $MgSO_4$, 1 g NaCl, 1 g trisodium citrate dehydrate (TSCD) and 0.5 g disodium hydrogen citrate sesquihydrate (DHS)); salting out effect is used to improve

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extraction of analytes into the organic phase. A defatting step was also introduced prior to clean-up to remove lipophilic matrix components. Separate aliquots were taken for clean-up; dispersive solid phase extraction (d-SPE) and Oasis HLB were compared. Chromatograms obtained from HPLC-Photodiode array – Fluorescence detection (PDA-FLD) show severe matrix effects for samples treated with either clean-up method. Even a selective fluorescence detector could not be used to accurately quantify analytes.

Figure 4.2 shows PDA detector chromatograms of a solvent blank, blank matrix treated with d-SPE, a blank matrix treated with Oasis HLB, annotated 1A, 1B and 1C, respectively. The highest calibration concentration standard for each sample is shown below the blanks, 1D, 1E and 1F, respectively. The highest concentration standards had the following analyte's mass on column: AFB_1, AFG_1, 173 ng; AFB_2, AFG_2, 260 ng; FB_1, 4300 ng; FB_2, 2200 ng; OTA, 260 ng; ZEN, 2200 ng; DON, 4300 ng; HT-2 Toxin, T-2 Toxin, 2000 ng. Similarly, Figure 4.3 shows FLD detector chromatograms of a solvent blank, a blank matrix treated with d-SPE, a blank matrix treated with Oasis HLB, annotated 2A, 2B and 2C, respectively. The highest calibration concentration standards for each sample are shown below the blanks, 2D, 2E and 2F, respectively.

Despite the QuEChERS method modifications, it remained a challenge to isolate analytes from matrix components. Therefore, other sample preparation techniques were investigated.

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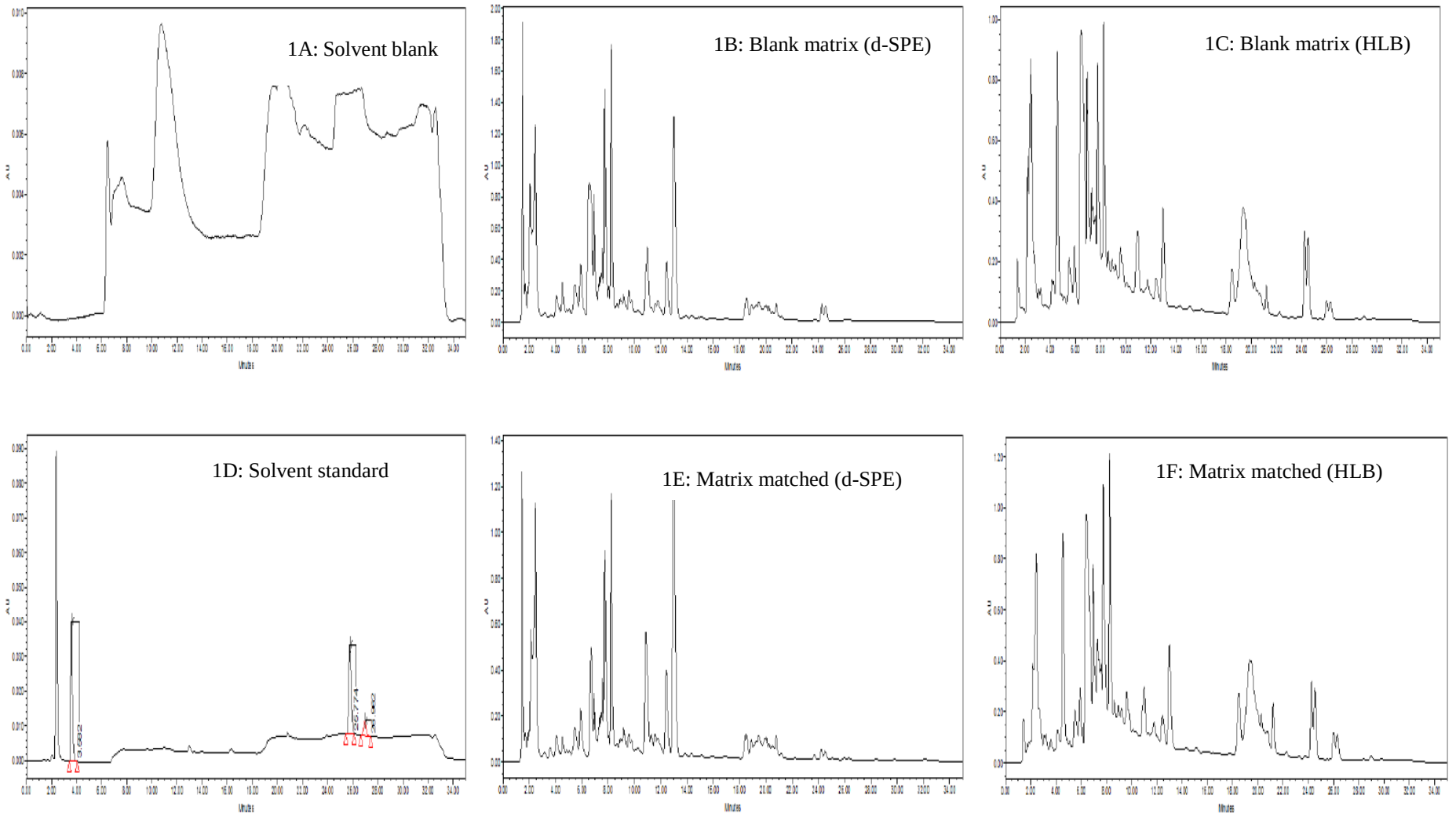

Figure 4. 2. Comparison of HPLC-PDA chromatograms for blank and spiked neat solvent and matrix matched calibration standards.

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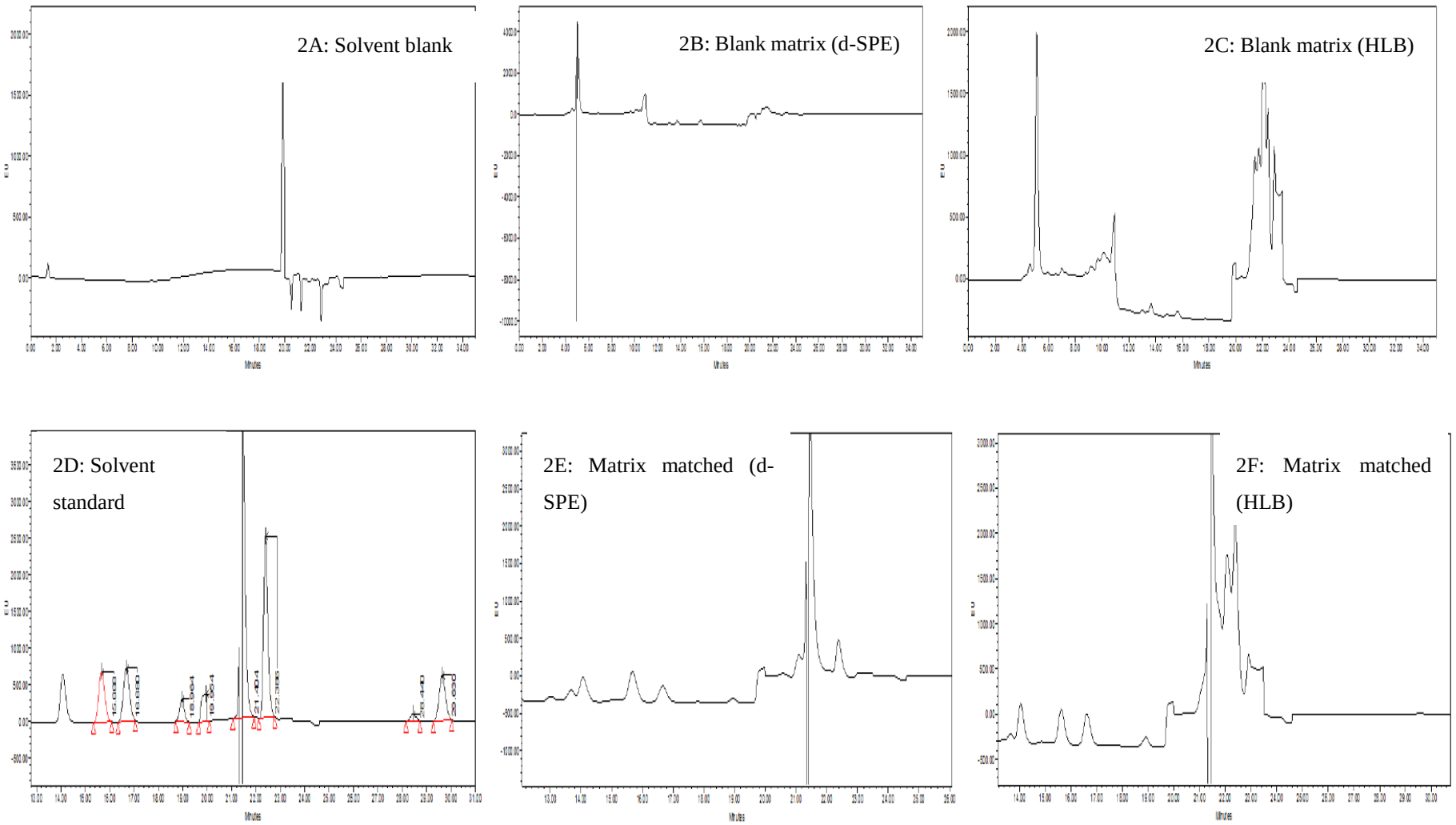

Figure 4. 3. Comparison of HPLC-FLD chromatograms for blank and spiked neat solvent and matrix matched calibration standards.

4.2.3. Extraction with orbital shaking and SPE clean-up

Results obtained using the dilute-and-shoot and QuEChERS methods confirmed that a clean-up step is important to reduce matrix effects. Solid phase extraction (SPE) clean-up prior to analysis is an indispensable tool to minimise or possibly eliminate matrix effects associated with mass spectrometric techniques resulting in inaccurate analyte quantification. In this study three commercially available SPE cartridges namely; Varian Bond Elut Mycotoxin, Waters Oasis HLB and Romer's MultiSep AflaZON+ 226, were evaluated for matrix removal performance during multi mycotoxin analysis.

Bond Elut Mycotoxin cartridges use a sorbent which cleans up food extracts for improved trichothecenes and zearalenone analysis, this sorbent is a proprietary silica-based ion exchange material.[124] MultiSep AflaZON+ adsorbent are designed for analysis of aflatoxins and zearalenone in complex commodities.[125] Both Bond Elut Mycotoxin and MultiSep AflaZON+ act in a selective non-retention mechanism, toxin analytes pass through the cartridge while food matrix components are retained. Oasis HLB is a hydrophilic/lipophilic-balanced (HLB) reverse phase sorbent used for acidic, neutral and basic compounds.[126] Oasis HLB SPE columns uses the conventional way of analyte retention mechanism; analytes and matrix components are retained by the activated sorbent material, washing with aqueous solution elutes the retained matrix components, then analytes of interests are eluted with an organic solvent.

An acidified acetonitrile/water mixture was used to extract the toxins from maize, using the procedure in Section 4.2.2. For Oasis HLB column, 4 mL aliquot, equivalent to 0.50 g maize sample, of the combined extract was evaporated to dryness under a stream of nitrogen at 50 °C and the residue was dissolved in 5% MeOH in water before loading onto a pre-conditioned column. After washing, analytes were eluted using MeOH, dried under nitrogen and reconstituted in starting mobile phase composition ($MeOH/H_2O$, 5 mM NH_4COOH, 35:65, *v/v*) prior to LC-MS/MS analysis. When using Bond Elut Mycotoxin and MultiSep AflaZON+ 226 SPE columns, a 4 mL aliquot of the combined extract was passed through each column and collected in test tubes. The eluent was dried down under nitrogen and reconstituted in starting mobile phase composition prior to LC-MS/MS analysis.

Matrix removal efficiency was assessed by evaluating the absence/presence of matrix effects during the analysis. The ratio of the slopes of matrix matched internal calibration and solvent internal calibration curves was used to quantify the extent of matrix effects. Table 4.3 lists

matrix removal efficiency of each SPE column. AflaZON+ cleaned maize extract had minimum matrix effects, 71% ≤ %SSE ≤ 124%, for all the analytes. Signal suppression, %SSE ≤ 63%, was observed for AFG_2 from samples cleaned-up with both Bond Elut Mycotoxin and Oasis HLB SPE columns.

Table 4. 3. Comparison of matrix matched removal performance of three SPE cartridges by UHPLC-SIDA-MS/MS

		Neat Cal Std	MultiSep AflaZON+ 226		Bond Elut Mycotoxin		Oasis HLB	
Toxin	**Range**	**Slope**	**Slope**	**SSE[a]**	**Slope**	**SSE[a]**	**Slope**	**SSE[a]**
	$ng.g^{-1}$			**%**		**%**		**%**
AFB_1	0.1 - 25	0.2197	0.2728	124	0.1682	77	0.1780	81
AFB_2	0.25 - 50	0.1647	0.1914	116	0.1462	89	0.1123	68
AFG_1	0.1 - 25	0.0870	0.0914	105	0.0757	87	0.0644	74
AFG_2	0.25 - 50	0.1333	0.0943	71	0.0768	58	0.0712	53
FB_1	10 - 3000	0.0009	0.0010	110	0.0011	113	0.0012	128
FB_2	10 - 3000	0.0023	0.0024	103	0.0022	96	0.0024	104
OTA	0.1 - 150	0.0103	0.0092	90	0.0127	124	0.0081	79
ZEN	10 - 1500	0.0063	0.0046	73	0.0052	83	0.0039	62
DON	10 - 2500	0.0020	0.0023	115	0.0024	119	0.0025	123
HT-2	5 - 1500	0.0050	0.0054	108	0.0058	115	0.0057	113
T2-Toxin	2.5 - 500	0.0050	0.0051	102	0.0038	76	0.0041	82

[a] $\%SSE = \frac{slope\ of\ matrix\ matched\ internal\ calibration}{slope\ of\ neat\ solvent\ internal\ calibration}\ x\ 100$

If %SSE = 100, no matrix effect; if %SSE > 100, there is a signal enhancement and if %SSE < 100, there is a signal suppression.

For the evaluation of recovery, blank maize samples were spiked in triplicate with a known amount of unlabelled mycotoxins before extraction. Table 4.4 lists percentage recoveries obtained using matrix matched internal standard calibration. Low recoveries, %REC ≤ 62 for most analytes with the exception of AFB_2 (96%), were obtained for spiked samples when using MultiSep AflaZON columns. Recoveries between 22 – 99% were obtained with Bond Elut

columns. Good recoveries were obtained for trichothecenes (DON 99%, HT-2 Toxin 93% and T-2 Toxin 85%) and zearalenone (82%), whereas poor recoveries were obtained for aflatoxins and fumonisins. This is an expected observation as Bond Elut sorbent is designed for trichothecenes and zearalenone analysis. Recoveries between 88 – 113 % have been reported for trichothecenes and ZEN analysis in maize when using Bond Elut SPE columns.[122] Using Oasis HLB columns, recoveries between 69 – 116% were obtained, only AFB_1 and AFG_2 had low recoveries, 33% and 48%, respectively.

Matrix reference materials, FAPAS® and Trilogy® were used to evaluate the accuracy of the method. Results are listed in Table 4.3. In FAPAS® maize, recoveries between 36 – 96% were obtained using MultiSep AflaZON+ and between 45 – 95% with Oasis HLB. Whereas, using Bond Elut good recoveries were obtained for all the analytes; AFB_1, OTA, ZEN and DON, 80%, 89%, 102% and 85%, respectively. Mean recoveries for Trilogy® maize ranged from 23 – 110% fumonisins and T2-Toxin being the lowest; FB_1, FB_2 and T2-Toxin, 23%, 32% and 32%, respectively, for AflaZON+ treated samples. Whereas only fumonisins and AFB_1 recoveries were low for Bond Elut clean-up. In contrast, HLB cleaned-up samples had recoveries between 59 – 114% with the exception of AFB_1 (40%).

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Table 4. 4. Analyte recoveries obtained from spiked and naturally incurred mycotoxins maize samples after clean-up with AflaZON+ 226, Bond Elut Mycotoxin and Oasis HLB columns (*n* = 3).

	Spiked Samples				**Trilogy (MT-C-9999G)**				**FAPAS (T04209)**			
		Recovery				**Recovery**				**Recovery**		
Toxin	Spike Conc.	AflaZON+ 226	Bond Elut Mycotoxin	Oasis HLB	Reference value	AflaZON+ 226	Bond Elut Mycotoxin	Oasis HLB	Reference value	AflaZON+ 226	Bond Elut Mycotoxin	Oasis HLB
	ng/g	%			ng/g	%			ng/g	%		
AFB_1	16	49 ± 12	48 ± 8	33 ± 15	18.8	45 ± 18	42 ± 22	40 ± 15	8.0	96 ± 4	80 ± 9	88± 15
AFB_2	33	96 ± 5	69 ± 7	80 ± 9	0.9	110 ± 6	105 ± 9	82 ± 12		-	-	-
AFG_1	16	56 ± 15	42 ± 18	48 ± 7	2.4	101 ± 8	65 ± 20	60 ± 17		-	-	-
AFG_2	33	39 ± 23	22 ± 26	76 ± 8	-	-	-	-	-	-	-	-
FB_1	1627	54 ± 14	48 ± 10	116 ± 8	28300.0	23 ± 12	28 ± 18	83 ± 8		-	-	-
FB_2	1627	32 ± 22	39 ± 15	114 ± 4	7100.0	32 ± 15	38 ± 13	59 ± 12		-	-	-
OTA	98	48 ± 12	55 ± 10	105 ± 5	4.0	84 ± 14	86 ± 10	95 ± 5	5.6	70 ± 7	89 ± 10	45 ± 18
ZEN	980	48 ± 18	82 ± 5	69 ± 8	352.0	82 ± 5	74 ± 12	93 ± 7	344.0	36 ± 22	102 ± 6	53 ± 16
DON	1646	62 ± 14	99 ± 7	102 ± 4	2600.0	48 ± 15	90 ± 7	114 ± 6	1779.0	56 ± 12	85 ± 7	95 ± 10
HT-2	980	53 ± 13	93 ± 11	115 ± 5	523.0	58 ± 26	98 ± 10	105 ± 3	-	-	-	-

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T2-Toxin	329	34 ± 24	85 ± 6	94 ± 14	263.7	32 ± 16	79 ± 5	87 ± 12	-	-	-	-

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MultiSep AflaZON+ 226 SPE columns are efficient in removing matrix components in complex matrix such as maize. However, they also result in low recoveries for most analytes except aflatoxins and zearalenone. Good recoveries for trichothecenes and zearalenone were obtained using Bond Elut Mycotoxin SPE columns. Better analyte retention for most analytes was obtained from Oasis HLB columns owing to the sorbent capability of retaining analytes with different physicochemical properties.

The use of solid phase extraction clean-up step prior to instrumental analysis reduced matrix effects observed in the dilute-and-shoot approach and QuEChERS approaches. Also, hydrophilic-lipophilic balanced (HLB) SPE columns were shown to be better SPE columns in multi mycotoxin analysis. In this study two brands of HLB SPE, Waters Oasis and Supelco Select, columns and Bond Elut Plexa columns were compared for matrix removal performance and analyte recoveries. Agilent's Bond Elute Plexa uses a non-polar polymer that can extract acidic, neutral and basic analytes from different matrices.[127]

Mycotoxins were extracted from maize using the acidified acetonitrile water mixture using an orbital shaker. In order to minimise sample preparation steps from the previous section HLB clean-up procedure, Section 4.2.3, the dry down step was omitted. A 5 mL extract aliquot, equivalent to 0.63 g maize sample, was diluted with 50 mL Milli-Q water and the diluted extract was loaded onto a pre-conditioned HLB column followed by washing and eluting the analytes using 5% MeOH in Milli-Q water and MeOH, respectively. For Bond Elut Plexa 300 µL extract aliquot was diluted with 3 mL Milli-Q water and loaded into a pre-conditioned Bond Elut Plexa column, column wash and elution steps were the same as those for HLB columns. The mass of maize loaded on column for each clean-up was different.

Severe signal enhancements, %SSE > 130%, were observed for AFB_1, OTA, T-2 and HT-2 Toxin in all three SPE columns. Excluding AFB_1, OTA, T-2 and HT-2 Toxin, %SSE between 71 – 108 %, 59 – 113% and 54 – 114% were obtained for Bond Elut Plexa, Select HLB and Oasis HLB, respectively, for all other analytes. The rationale behind low matrix effects from samples prepared using Bond Elut Plexa is that very little amount of sample that was used owing to the smaller bed size of the column, 30 mg. Less amount of matrix components were loaded onto the column.

Spiked blank maize samples (n = 3) were used to evaluate analyte retention of each SPE column. Recoveries of mycotoxins in spiked maize were quantified using matrix matched internal standard calibration, as shown in Figure 4.4. Recoveries between 54 – 126%, 59 –

123% and 67 – 110% were obtained for Bond Elut Plexa, Select HLB and Oasis HLB, respectively. Recoveries in the range between 68 % and 94% were reported by Wang *et al* (2013) during the determination of 9 mycotoxins in maize using Oasis HLB columns prior to HPLC-MS/MS analysis.[18]

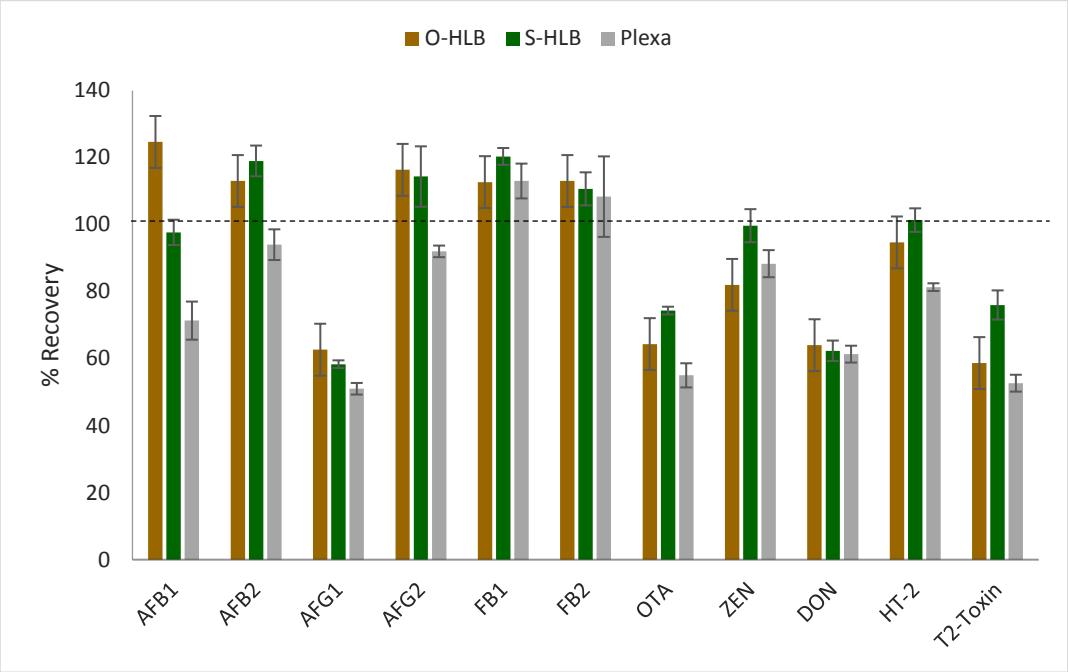

Figure 4. 4. Recoveries of spiked maize samples (n = 3). Spiking concentrations are listed in Table 4.4.

Recoveries obtained from Trilogy® matrix reference material, as shown in Figure 4.5, ranged from 52% - 112%, 46 – 121% and 54% - 122% for Bond Elut Plexa, Select HLB and Oasis HLB, respectively. In both spiked samples and reference materials, low recoveries for DON were observed. Deoxynivalenol is a polar compound, pK_{OW} = -1.41, that is highly soluble in aqueous solutions. In this work the sample extracts were diluted with large quantities of water prior to loading on the SPE columns, there is a possibility that DON was eluted during the loading and washing steps.

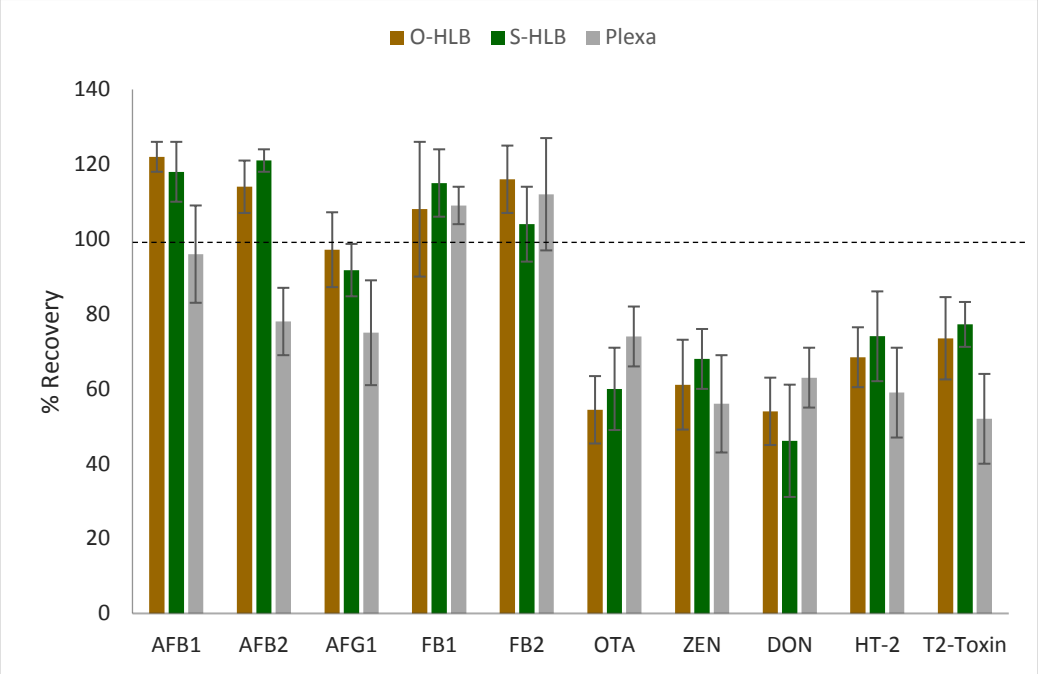

Figure 4. 5. Recoveries of naturally contaminated maize sample (Trilogy) (n = 3); concentration levels given in Table 4.4.

Oasis HLB and Select HLB recoveries are relatively comparable in both spiked and naturally contaminated maize samples. DON recoveries were statistically compared as shown in Table 4.5. There was no significant difference in the scores for Oasis HLB ($\bar{x}$ = 64, SD = 9) and Select HLB ($\bar{x}$ = 62.3, SD = 9.33) conditions; t(4) = 0.67, P = 0.05. Statistically there is no significant difference between recoveries obtained when using these clean-up columns, either HLB clean-up column can be used for multi mycotoxin analysis. Thus, Select HLB SPE columns were chosen for further method development based on cost effectiveness.

Table 4. 5. Statistical comparison of two HLB clean-up columns for DON recovery.

DON	O-HLB	S-HLB
Sample 1	64	63
Sample 2	61	65
Sample 3	67	59
t-Test: Two-Sample Assuming Equal Variance		
	Variable 1	Variable 2
Mean	64	62
Variance	9	9.3
Observations	3	3
Pooled Variance	9.17	
Hypothesized Mean Difference	0	
DF	4	
t Stat	0.67	
***P*(T<=t) one tail**		
t Critical one-tail	2.13	

Results obtained in this study illustrate that Bond Elut Plexa is efficient in removing matrix components compared to the tested HLB columns. However, these matrix-effect results are not conclusive as a smaller amount of maize sample was loaded onto the columns, also lower recoveries were obtained when using this column. Better analyte recoveries were obtained from HLB clean-up columns.

4.2.4. Effect of extraction technique during multi mycotoxin analysis

In the previous sections orbital shaking extraction method was used to extract mycotoxins from maize. In this section different extraction techniques; homogenisation and sonication extraction, were investigated with the aim to improve analyte extraction from the matrix prior to a clean-up step. Sample clean-up and pre-concentration was done using Select HLB column

for all the investigated extraction methods. Blending offers quick extraction times reducing the amount of time needed for sample preparation, whereas, pulsation from ultrasonication facilitates matrix breakdown to release bound analytes. Analyte recoveries were used to evaluate the extraction efficiency of homogenisation and sonication compared to orbital shaking.

Homogenisation extraction was used by Klötzel and co-workers[122] (2006) for trichothecenes determination in cereal and cereal based food. Homogenisation extraction method is relatively simple and there are few parameters that need to be optimised to enhance extraction efficiency. In this study the sequential extraction method, first ACN/H_2O/HCOOH (80/19.9/0.1, *v/v/v*) then ACN/H_2O/HCOOH (20/79.9/0.1, *v/v/v*), was used instead of the single mixture ACN/H_2O (80:20, *v/v*) used by Klötzel and co-workers. Each sample needs less than 15 min for extraction before the clean-up step granting this method a potential to be quick. However, with a single blender that can homogenise one sample at a time this potential fades away for routine analysis.

The sonication extraction technique allows several extractions to be performed simultaneously in a short amount of time. Li *et al* (2012) developed a method to determine fumonisins in maize, the extraction time was 10 min.[53] In this work we compared MeOH/H_2O and sequential ACN/H_2O/HCOOH extraction mixtures. MeOH/H_2O gave recoveries between 11 - 105% for aflatoxins, and 78% for DON while ACN/H_2O/HCOOH gave recoveries between 55 – 130% and 120% for aflatoxins and DON respectively in spiked maize samples, as shown in Figure 4.6. Aflatoxins and DON are polar compounds and their extraction efficiency is affected by the solvent polarity therefore they were chosen to evaluate extraction efficiency of the solvent mixtures. Also, aflatoxins are regulated at much lower levels than the other toxins, 5 ppb, thus emphasis was placed on the extraction solvent being able to extract aflatoxins with good recoveries (%REC ≥80%).

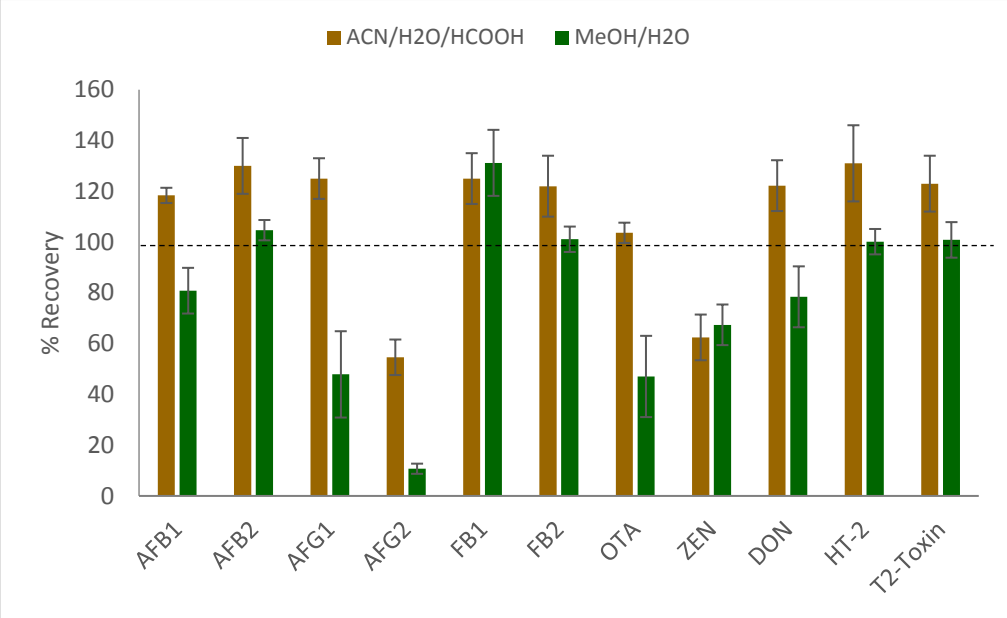

Figure 4. 6. Comparison of mycotoxin recoveries between ACN/H_2O/HCOOH (50:49.9:0.1, *v/v/*v) and MeOH/H_2O (70:30, *v/*v) extraction mixtures followed by Supelco-HLB column clean-up and LC-MS/MS analysis.

The ACN/H_2O/HCOOH mixture was chosen as the extraction solvent due to high extraction efficiency for most analytes. To optimise ultrasonic extraction time, different extraction times (10, 15, 30, 45, and 60 min) were tested to extract mycotoxins in Trilogy® matrix reference material. High recoveries were obtained between 30 and 60 min for most analytes, and thus 60 min was chosen for further analysis. It is easier to optimise sample preparation conditions for a single mycotoxin or a class of mycotoxins. With multi mycotoxin analysis the best condition is the one that gives optimal performance for most analytes with certain specific requirements, such as emphasis on Aflatoxin recoveries. Figure 4.7 shows the effect of extraction time on AFB1, DON and FB1 (secondary axis) peak areas.

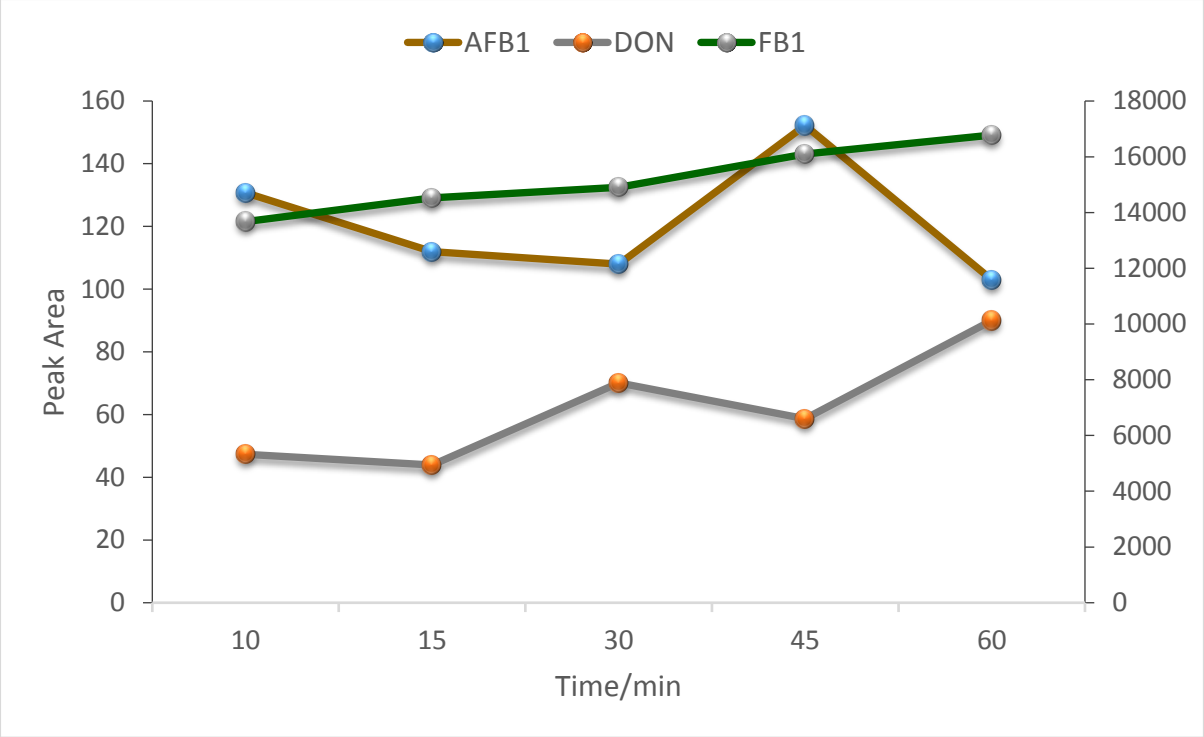

Figure 4. 7. Effect of extraction time on AFB_1, DON (primary axis) and FB_1 (secondary axis) peak areas using ultrasonic extraction and ACN/H_2O/HCOOH (50:49.9:0.1, *v/v*/v) mixture.

The combination of extraction technique and clean-up can significantly alter matrix effects. Minimum matrix effects, 72% ≤ %SSE ≤ 129% were observed for aflatoxins, trichothecenes, FB_2 and OTA; whereas, enhanced signals (%SSE ≥ 130%) were observed for FB_1 and ZEN using the blending extraction method. Using the ultrasonication technique, %SSE between 86 % and 116% were obtained for aflatoxins, fumonisins, OTA, ZEN, and trichothecenes excluding T2-Toxin (%SSE > 130%). In contrast to these two extraction techniques, signal enhancement was observed for all the analytes with the exception of AFB_2 (81%) which was suppressed using orbital shaking.

Analyte recoveries for each extraction method were determined using matrix matched internal standard calibration curves, summary shown in Table 4.6. Recoveries between 88% - 134%, 55% - 131% and 86% - 136% were obtained for homogenisation, sonication and orbital shaking, respectively, for spiked maize samples. Trilogy reference material had recoveries ranging from 76% - 120%, 70% - 130% and 70% - 132% and recoveries for FAPAs reference material were between 86% - 121%, 74% - 109% and 92% - 110% were obtained for homogenisation, sonication and orbital shaking, respectively. Recoveries obtained for DON, ZEN, HT-2 and T-2 toxins using the blender and HLB clean-up are in a similar range, 88% –

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113% to those obtained by Klötzel and co-workers (2006) using Bond Elut column after blending extraction.[122]

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Table 4. 6. Comparison of analyte recoveries in spiked and naturally contaminated maize samples using three different extraction techniques (*n*=3).

	Spiked samples				**Trilogy (MT-C-9999G)**				**FAPAS (T04209QC)**			
	Spike Conc.	Homogenisation	Sonication	Orbital Shaking	Reference value	Homogenisation	Sonication	Orbital Shaking	Reference value	Homogenisation	Sonication	Orbital Shaking
	$ng.g^{-1}$	%Recovery			$ng.g^{-1}$	%Recovery			$ng.g^{-1}$	%Recovery		
AFB_1	15	96 ± 4	118 ± 9	129 ± 10	18.8	114 ± 13	130 ± 22	95 ± 12	8	103 ± 7	106 ± 10	99 ± 12
AFB_2	5	111 ± 8	130 ± 12	124 ± 7	0.9	120 ± 18	124 ± 19	132 ± 24	-	-	-	-
AFG_1	15	134 ± 12	125 ± 19	133 ± 14	2.4	79 ± 10	78 ± 9	115 ± 10	-	-	-	-
AFG_2	5	107 ± 10	55 ± 10	136 ± 21	-	-	-	-	-	-	-	-
FB_1	1000	132 ± 23	86 ± 27	116 ± 6	28300	113 ± 16	121 ± 18	124 ± 18	-	-	-	-
FB_2	500	125 ± 25	122 ± 18	117 ± 12	7100	85 ± 5	118 ± 22	98 ± 8	-	-	-	-
OTA	50	118 ± 3	104 ± 7	127 ± 5	4	94 ± 9	113 ± 6	103 ± 7	5.6	121 ± 12	109 ± 14	110 ± 8
ZEN	500	88 ± 8	62 ± 12	132 ± 18	352	76 ± 18	70 ± 14	79 ± 14	344	89 ± 7	74 ± 11	106 ± 5
DON	1000	123 ± 11	122 ± 8	86 ± 12	2600	103 ± 4	115 ± 12	81 ± 8	1779	86 ± 10	105 ± 5	92 ± 9

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HT-2	1000	93 ± 7	131 ± 14	106 ± 2	523	96 ± 8	123 ± 7	70 ± 10	-	-	-	-
T2-Toxin	1000	114 ± 6	123 ± 15	110 ± 7	264	109 ± 11	125 ± 5	87 ± 15	-	-	-	-

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Each extraction technique has its advantages and limitations. Blending extraction is the quickest for all the investigated techniques per samples, each sample can be prepared just under 10 min. However, without a multi sample homogeniser sample preparation can take longer than either. Sonication or shaking can both accommodate multiple.

As far as we know sonication extraction has not been used in multi mycotoxin analysis of maize. However, it has been used for determination of fumonisins.[53] Therefore, this method was chosen for method validation over orbital shaking. With an aim to further minimise matrix effects, the clean-up step was modified. The sample aliquot was dried down prior to loading onto a pre-conditioned SPE column. Subsequent washing resulted in a yellowish band on the column which only moved with the eluting solvent. This resulted in a sticky residue on the collecting test tube after drying down the eluent; filtering the sample on a 0.22 µm does remove the sticky residue in the collecting test tube following drying down of the eluent.

Thus, the SPE clean-up step had to be modified in order to remove this yellow band. Washing solvent composition was changed from 5% MeOH in water in 5% increments to 20% MeOH in water. Eluent fraction was collected and analysed during the wash steps; from 10% MeOH up to 20% MeOH analytes started to elute. Pre-elution leads to poor analyte recoveries, and this was confirmed in the analysis. The washing composition was then maintained at 5% MeOH in water and different wash volumes were investigated. Wash volumes of 5, 10, 15 and 20 mL were used. The yellow band started to fade at 15 mL, however, due to the loss of DON and other relatively polar analytes associated with large amount of water during the wash step, 10 mL was chosen as the optimal wash volume.

The optimised sample preparation step uses ultrasonic extraction, drying the sample extract aliquot prior to SPE column loading and washing the SPE column with 10 mL of 5% MeOH in water. This optimised sample preparation method was used for the determination of eleven mycotoxins in maize using liquid chromatography and isotope dilution mass spectrometry.

4.3. Method validation

The determined method performance parameters are, accuracy, precision, working range, limit of detection (LOD), limit of quantification (LOQ) and robustness. Also, the presence/absence of matrix effects was determined.

Analyte recovery in spiked maize samples and matrix reference materials were used to evaluate method's accuracy, whereas, repeatability was calculated from triplicate analysis at the spiked

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levels. Blank maize sample were spiked with native mycotoxins at three concentration levels each in triplicate before extraction. Table 4.7 list recoveries determined using matrix matched internal standard calibration. Recoveries between 95% - 142%, 126% - 137%, 113% - 133%, 75% - 124% and 57% - 130% were obtained for aflatoxins, fumonisins, OTA, ZEN and trichothecenes, respectively. Enhanced recoveries, %REC $\geq$ 130%, were obtained for samples spiked at low levels for most analytes which were lower than the conservative LOD determined from the matrix matched internal standard calibration. Nonetheless, analytes spiked at low concentration levels were above the instrumental LODs. Also, high recoveries were obtained for fumonisins; the use of a universal extraction solvent can result in higher extraction recoveries for fumonisins.[2]

The absence/presence of matrix effects was evaluated by computing the signal suppression or enhancement, expressed as a percentage, of each analyte using the slope ratio of matrix matched internal calibration and solvent calibration curves. Signal suppression, %SSE < 100%, was observed for aflatoxins and fumonisins. AFG_2 was marginally affected by matrix effects with %SSE of 101%; OTA, ZEN and DON were enhanced at 119%, 109% and 122%, respectively. Severe analyte enhancement was observed for HT-2 and T-2 Toxins, 194% and 183%, respectively.

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Table 4. 7. Method performance parameters from different analytical columns in maize.

Analyte	Range	R^2		LOD		LOQ		Spike Conc.	Recovery ± RSD (n = 3)		SSE[c]	
		BEH[a]	ARC[b]	BEH	ARC	BEH	ARC		BEH	ARC	BEH	ARC
	ng/g			ng/g					%			
AFB_1	0.5 - 100	0.9972	0.9900	2.39	4.52	7.95	15.07	0.90	142 ± 25	154 ± 24	91	106
								6.96	114 ± 4	115 ± 33		
								21.70	131 ± 7	122 ± 17		
AFB_2	0.1 - 25	0.9942	0.9645	0.81	2.03	2.71	6.77	0.21	ND	106 ± 47	79	104
								1.64	141 ± 32	126 ± 26		
								5.10	134 ± 30	144 ± 20		
AFG_1	0.5 - 100	0.9848	0.9681	5.59	8.16	18.62	27.20	0.90	134 ± 40	165 ± 5	88	156
								6.94	127 ± 17	114 ± 6		
								21.64	134 ± 6	89 ± 12		
AFG_2	0.1 - 25	0.9777	0.9864	1.61	1.25	5.37	4.17	0.21	95 ± 16	74 ± 24	101	147
								1.65	130 ± 5	107 ± 35		
								5.13	125 ± 26	103 ± 14		
FB_1	10 - 1500	0.9742	0.9765	107.90	101.77	359.66	342.58	13.27	137 ± 18	128 ± 45	85	79
								102.26	126 ± 15	117 ± 11		

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								318.83	131 ± 10	122 ± 9		
FB_2	5 - 1000	0.9675	0.9840	73.40	51.11	244.68	170.36	8.01	ND	85 ± 4	82	93
								61.77	135 ± 15	113 ± 10		
								192.60	126 ± 14	117 ± 8		
OTA	1 - 250	0.9917	0.9832	8.87	12.65	29.55	42.18	1.94	133 ± 8	212 ± 25	119	130
								14.96	113 ± 18	132 ± 14		
								46.64	113 ± 7	121 ± 19		
ZEN	10 - 1500	0.9666	0.9834	100.89	71.54	336.29	238.46	10.87	124 ± 24	135 ± 5	109	96
								83.75	84 ± 6	148 ± 48		
								261.13	75 ± 15	128 ± 19		
DON	25 - 5000	0.9965	0.9782	116.54	294.09	388.47	980.30	39.42	78 ± 13	73 ± 32	122	84
								303.90	66 ± 16	83 ± 43		
								947.50	57 ± 7	51 ± 10		
HT-2	10 - 1500	0.9954	0.9974	37.98	28.38	126.60	94.61	11.20	127 ± 24	109 ± 2	194	159
								86.31	100 ± 8	161 ± 14		
								269.11	105 ± 8	109 ± 2		
T2-Toxin	10 - 1500	0.9970	0.9906	35.01	62.25	116.70	207.46	12.83	130 ± 10	180 ± 22	183	195
								98.88	103 ± 8	131 ± 7		

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								308.29	122 ± 7	80 ± 4		

[a] Waters Acquity UPLC BEH C_{18} column (2.1 mm i.d. x 100 mm, 1.7 µm particle size)

[b] Restek Raptor ARC-18 UHPLC column (2.1 mm i.d. x 100 mm, 2.7 µm particle size)

[c] $\%SSE = \frac{slope\ of\ matrix\ matched\ internal\ calibration}{slope\ of\ neat\ solvent\ internal\ calibration}\ x\ 100$

If %SSE = 100, no matrix effect; if %SSE > 100, there is a signal enhancement and if %SSE < 100, there is a signal suppression.

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Matrix matched calibration standards were prepared by spiking unlabelled mycotoxin standards into blank maize extracts at nine concentration levels: AFB_1, AFG_1, 0.5 – 100 ng/g; AFB_2, AFG_2, 0.1 – 25 ng/g; OTA, 1 – 250 ng/g; DON, 25 – 5000 ng/g; FB_2, 5 – 1000 ng/g; FB_1, ZEN, T-2, HT-2 Toxin, 10 – 1500 ng/g. The coefficient of determination (R^2) values were between 0.93 and 0.99. The LOD and limit of quantification LOQ were determined through linear regression analysis. The LOD was defined as the analyte concentration giving a signal equal to blank signal, y_B, plus three standard deviation of the blank, s_B: [LOD = y_B + 3s_B] and LOQ was defined as [LOQ = y_B + 10s_B]. The LOD and LOQ ranged between 0.8 – 116 ng/g and 3 – 390 ng/g, respectively. European Commission (EC) maximum permissible levels (MPLs) for AFB_1, FB_1, ZEN and DON in maize intended for human consumptions are 5 ng/g, 1000 ng/g, 100 ng/g and 1175 ng/g, respectively.[7] LODs for AFB_1 (2.4 ng/g) and ZEN (100 ng/g) are below their MPLs and LOQs for FB_1 (360 ng/g) and DON (390 ng/g) are below their MPLs; this method is applicable for detection and quantification of these regulated mycotoxins at the stipulated levels. Extracted ion chromatograms showing analyte peaks and the associated noise levels achieved with this method at the lowest matrix matched calibration standard are shown in the Appendix.

Since the LOD and LOQ were determined from the matrix-matched calibration curve, the R^2 is not as good as from a neat calibration, the instrument is also not operating at optimal sensitivity for the analytes, the error on the estimate from the calibration curve is therefore much higher as reflected through the elevated LOD and LOQ determined through regression analysis. LOD and LOQ determined using signal-to-noise ratios, indicate that much lower LOD and LOQ estimates are analytically achievable.

Currently there is no matrix reference material with a certified concentration for all the regulated mycotoxins. Hence, matrix reference materials with well-defined analyte concentrations were used to determine the trueness of the developed method. Table 4.8 summarises determined and assigned values for Trilogy and FAPAS matrix reference materials. Measured values were found to be within the given ranges of the assigned values, with the exception of AFG_1 and FB_1. AFG_1 assigned value is below the method LOD for AFG_1 and the measured value was overestimated, %REC ≥ 140%. Extracted ion chromatograms Trilogy and FAPAS matrix reference materials are shown in Figure 4.8 and Figure 4.9, respectively.

Table 4. 8. Comparison of measured and reference material assigned values determined by UHPLC-SIDA-MS/MS (*n* = 3) between different analytical columns.

Analyte	Measured value ± RSD		Assigned value		Recovery	
	ng/g				%	
Trilogy (TM-C-9999G)	**BEH[a]**	**ARC[b]**	**Reference value**	**Range**	**BEH**	**ARC**
Sum AFLAs	18.83	16.05	13.8	6.0 - 21.5		
AFB_1	16.8 ± 0.3	12.92 ± 7	12.4		136	104
AFB_2	1.45 ± 0.9	1.32 ± 0.9	1.1		132	120
AFG_1	0.58 ± 0.3	1.81 ± 0.5	0.3		193	605
AFG_2	-	-	ND		-	-
FB_1	6374.64 ± 489	5135.45 ± 540	4300	3600 - 5000	148	119
FB_2	1554.61 ± 183	1566.84 ± 63	1400	800 - 2000	111	112
OTA	56.56 ± 3.9	56.61 ± 7	62.1	8.5 - 115.7	91	91
ZEN	176.61 ± 37	196.75 ± 27	226.3	172 - 280.6	78	87
DON	1456.79 ± 52	1776.51 ± 187	1800	1500 - 2100	81	99
HT-2	182.59 ± 14	178.14 ± 25	248.1	179.2 - 317	74	72
T2 Toxin	176.16 ± 3	178.45 ± 8	206.8	126.1 -287.5	85	86
FAPAS (T04209QC)	**BEH**	**ARC**	**Reference value**	**Range**		
AFB_1	6.15 ± 1.4	5.06 ± 0.1	8.01	4.49 - 11.54	77	63
OTA	4.52 ± 0.6	5.45 ± 0.9	5.57	3.12 - 8.03	81	98
ZEN	243.33 ± 2.4	253.08 ± 22	344	214 - 473	70	74
DON	1707.4 ± 207	1783.74 ± 229	1779	1257 - 2301	96	100

[a] Waters Acquity UPLC BEH C_{18} column (2.1 mm i.d. x 100 mm, 1.7 µm particle size)

[b] Restek Raptor ARC-18 UHPLC column (2.1 mm i.d. x 100 mm, 2.7 µm particle size)

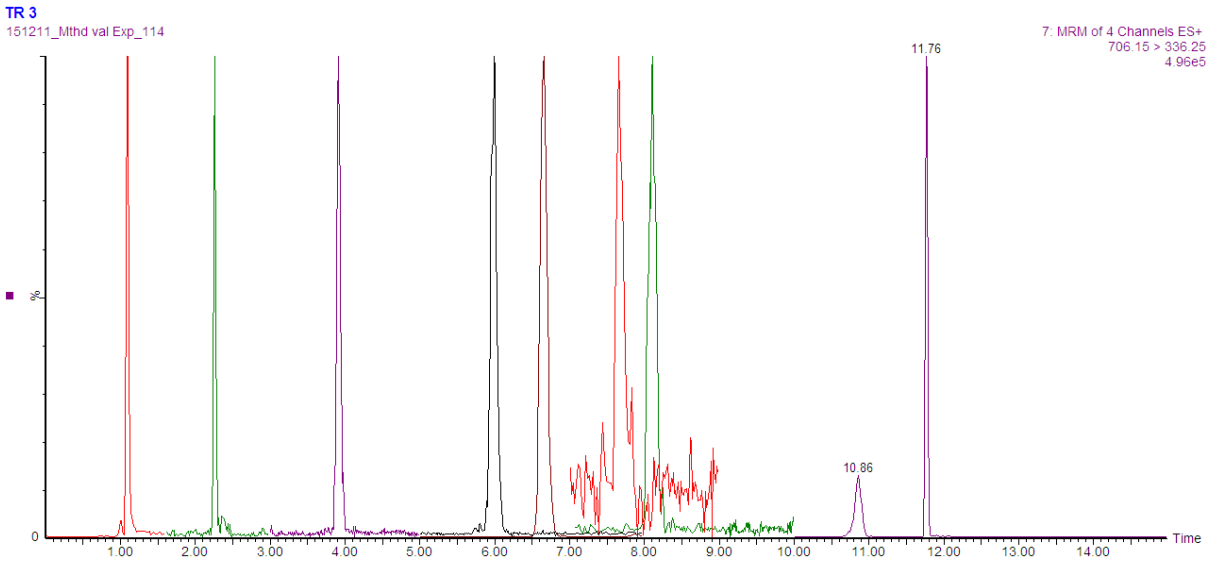

Figure 4. 8. Extracted ion chromatogram (XIC) of Trilogy matrix reference material. AFB_1, 12.4 ng/g; FB_1, 4300 ng/g; FB_2, 1400 ng/g; OTA, 62.1 ng/g; ZEN, 226.3 ng/g; DON, 1800 ng/g; HT-2 Toxin, 248.1 ng/g and T-2 Toxin, 206.8 ng/g. AFB_2 (1.1 ng/g) and AFB_2 (0.3 ng/g) are below the method's LOD for these analytes and they could be detected using smoothing function. Corresponding analyte and retention time are listed in Table 4.1.

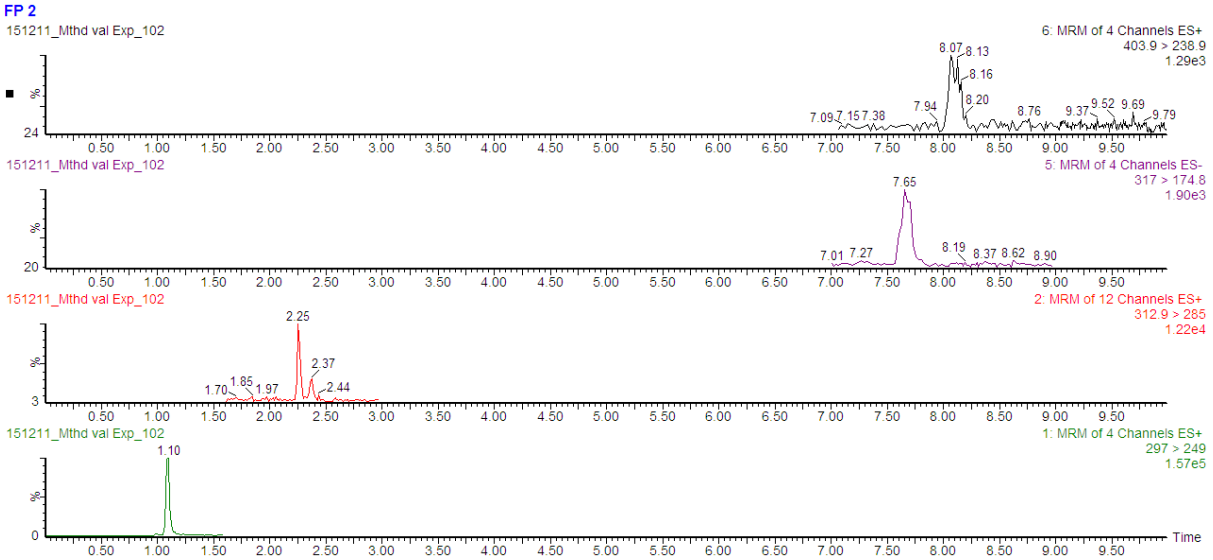

Figure 4. 9. Extracted ion chromatogram (XIC) of FAPAS matrix reference material. AFB_1, 8.01 ng/g; OTA, 5.57 ng/g; ZEN, 344 ng/g; DON, 1779 ng/g.

Matrix matching and internal standards are used to compensate for matrix effects to improve accuracy of an analytical method. However, it is a challenge to fully compensate for matrix effects as it is difficult to find blank matrix for spiking that is truly representative of the sample. Also, spiked maize samples do not exhibit similar characteristics as naturally occurring

mycotoxin contamination. In spiked samples the added analytes not bound to the matrix, whereas, in naturally occurring samples, the mycotoxins are bound to the matrix. Stable isotope dilution assay (SIDA) is a suitable method to overcome matrix effects associated with mass spectrometric analysis. The isotope behaves similar to the native analyte thus correction variations caused during sample preparation and during the analysis. However, due to the high costs of labelled standards, samples are often spiked prior to instrumental analysis thus only correcting variations occurring during the analysis.

In this study, samples were spiked with labelled standards before analysis. External and internal calibration curves were used to quantify the extent of matrix effects, shown in Table 4.9. Calculations using external calibration showed severe analyte suppression, whereas, minimal matrix effects were observed with internal calibration. This indicates that SIDA method can compensate for mass spectrometric variations during the analysis.

Table 4. 9. Comparison of obtained %SSE using internal and external calibrations.

Toxin	Internal calibration			External calibration		
	Slope neat solvent calibration standard	Slope matrix matched calibration standard	%SSE	Slope neat solvent calibration standard	Slope matrix matched calibration standard	%SSE[a]
AFB_1	1.9827	1.8125	91	203.7466	32.1608	16
AFB_2	1.6286	1.2866	79	167.7923	6.2086	4
AFG_1	1.8413	1.6224	88	168.5769	18.7156	11
AFG_2	0.8982	0.9078	101	80.8459	10.5611	13
FB_1	0.4894	0.4180	85	1.3860	1.2029	87
FB_2	0.7647	0.6289	82	4.2286	1.8634	44
OTA	1.2571	1.5003	119	25.2423	5.1031	20
ZEN	1.1438	1.2475	109	1.3917	0.1349	10
DON	2.2562	2.7528	122	3.3197	1.6193	49
HT-2	1.3000	2.5159	194	1.9167	1.4907	78
T2-Toxin	3.6677	6.7302	183	5.3878	3.9949	74

[a] $\%SSE = \frac{slope\ of\ matrix\ matched\ internal\ calibration}{slope\ of\ neat\ solvent\ internal\ calibration}\ x\ 100$

If %SSE = 100, no matrix effect; if %SSE > 100, there is a signal enhancement and if %SSE < 100, there is a signal suppression.

Method robustness was evaluated by changing the analytical column from Waters Acquity UPLC BEH C_{18} (1.7 µm, 2.1 mm i.d. x 100 mm) to Restek Raptor ARC-18 (2.7 µm, 2.1 mm

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i.d. x 100 mm). The flowrate was 0.35 mL/min for the BEH column and 0.5 mL/min for the ARC column to accommodate the larger particle size of the Raptor column resulted in significant shift in analytes elution times and co-elution between FB_1 and T-2 Toxin. The LC method was modified to minimise coelution of FB_1 and T-2 that was observed in initial experiments with the column. Isocratic elution was applied from 3.5 min to 9.0 min at 52% A; mobile phase composition (A) 5 mM NH_4COOH in MeOH/Milli-Q water (95:5, v/v) and (B) 5 mM NH_4COOH in Milli-Q water. There was an insignificant shift in retention times for most analytes except for FB_1, T-2, OTA and ZEN. FB_1 eluted before (6.00 min) T-2 Toxin which eluted at 6.8 min. Chromatographic separation of analytes was achieved in less than 12 min, shown in Figure 4.10. The Raptor ARC-18 method performance parameters are summarised in Table 4.7 above, the results are comparable to those obtained using Acquity UPLC BEH C_{18} column.

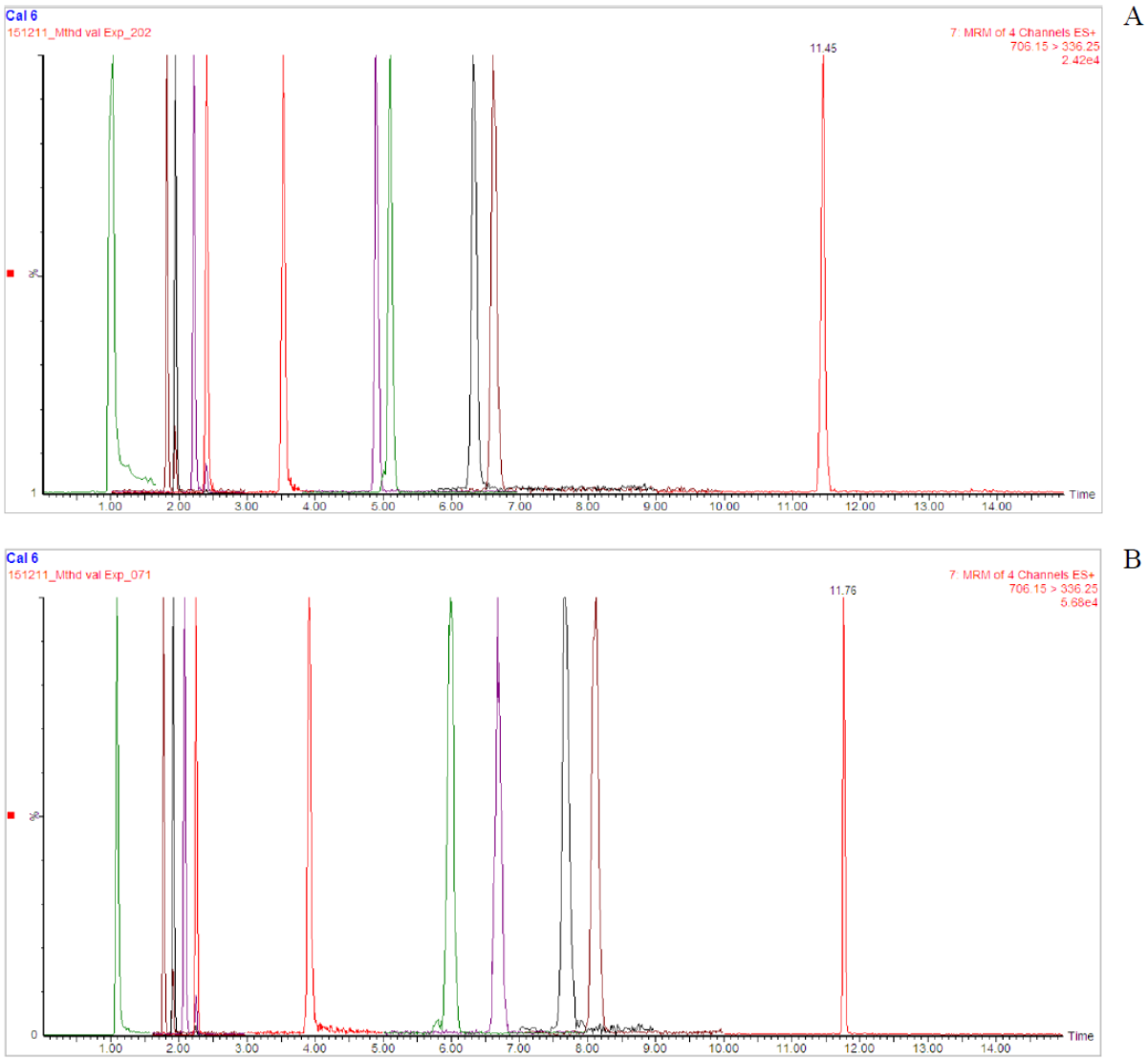

Figure 4. 10. Extracted ion chromatograms of solvent calibration standard obtained using Raptor ARC column (A) and Acquity BEH column (B). [^{12}C]-AFB$_1$, AFG$_1$, 30 ng/g; AFB$_2$, AFG$_2$, 12 ng/g; FB$_1$, 1500 ng/g; FB$_2$, 750 ng/g; OTA, 75 ng/g; ZEN, 750 ng/g; DON, 1500 ng/g; HT-2 Toxin, T-2 Toxin, 1500 ng/g. Corresponding analyte and retention time are listed in Table 4.1.

4.4. Conclusion

This study investigated sample preparation methods to improve quantification of multiple mycotoxins in maize using UHPLC-SIDA-MS/MS. The aim of this study was to develop a sample preparation method that minimise matrix effects associated with mass spectrometric analysis. Different sample preparation techniques were investigated to meet this objective.

The dilute-and-shoot sample preparation method was initially used as the matrix components can be diluted and the method is relatively quick. Results obtained using this method illustrated

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a need for a clean-up step prior instrumental analysis. The presence of severe matrix effects led to inaccurate analyte quantification in spiked samples and matrix reference materials. An alternative to the dilute-and-shoot method, the QuEChERS method, was then used with an aim to improve analytical results. Analyte peaks were severely suppressed using UHPLC-MS/MS. Therefore, a longer column was used in HPLC in an attempt to improve analyte separation from co-eluting matrix components and a fluorescence detector was used to further improve selectivity. However, results obtained from this method were far from satisfactory to be used in quantitative analysis.

Solid phase extraction based clean-up methods have a potential to minimise matrix components. Commercially available SPE columns with different sorbents and analyte-sorbent interaction were evaluated for matrix removal efficiency. MultiSep AflaZON+ SPE columns efficiently minimised matrix effects. However, they are only suitable for aflatoxins and zearalenone determination resulting in poor analyte retention for the other target analytes. Good recoveries were obtained using hydrophilic-lipophilic balanced (HLB) SPE columns; HLB sorbent can be used for both polar and non-polar analytes. HLB columns from different suppliers were compared in the analysis. Since no significant difference was obtained in analyte recovery performance, Select HLB columns were chosen based on cost effectiveness.

Orbital shaking, homogenisation and sonication extraction methods were compared. Good recoveries were obtained in all three methods. In addition to extraction efficiency, ease of use and time required to prepare multiple samples was the determining factor for an improved method. Sonication and orbital shaking can accommodate multiple sample extraction simultaneously, whereas, only one sample at a time can be extracted with a blender. Orbital extraction technique has been applied in multi mycotoxin determination in maize, therefore, sonication as a new approach was chosen for further method development and optimisation.

In the optimised sample preparation method; maize samples were extracted using a two-step sequential extraction using acidified acetonitrile/water mixture (HCOOH/ACN/H_2O, 0.1:50:49.9, *v/v/v*) in an ultrasonic bath for 60 min at 280 W prior to a clean-up step using HLB SPE columns. Analyte separation and detection was done using UHPLC-SIDA-MS/MS in fast polarity switching mode and multiple reaction monitoring (MRM). Analytes were detected within 12 min with good baseline resolution providing rapid determination of multiple mycotoxins. Accuracy, precision, linearity, limits of detection and quantification and robustness of the developed method were evaluated. Good recoveries between 95% - 142%, 126% - 137%, 113% - 133%, 75% - 124% and 57% - 130% were obtained for aflatoxins,

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fumonisins, OTA, ZEN and trichothecenes, respectively. LODs for AFB_1 and ZEN and LOQ's for FB_1 and DON were below the regulated levels in maize intended for human consumption, hence, this method is applicable for detection and quantification of these regulated mycotoxins in maize.

This study reports an improved sample preparation technique based on sonication extraction, economical HLB SPE clean-up columns and UHPLC-SIDA-MS/MS for the determination of multiple mycotoxins in maize.

Chapter 5

Conclusion

An accurate, precise and robust method for the determination of multiple mycotoxins in maize was developed and validated. This method is based on ultrasonic extraction, economical hydrophilic-lipophilic balanced (HLB) solid phase extraction (SPE) clean-up and ultra-high performance liquid chromatography-stable isotope dilution assay-tandem mass spectrometry (UHPLC-SIDA-MS/MS). Sample extraction based on two extraction steps using acidified acetonitrile/water mixture and HLB SPE clean-up resulted in good analyte recoveries for most analytes. Fast polarity switching mode was used to determine all the analytes in one chromatographic run without compromising chromatographic resolution. Method performance results indicate that this method can be used to detect and quantify mycotoxins at the regulated levels.

HLB SPE based clean-up method is limited by non-selectivity of the sorbent material, it can bind both matrix components and analytes of interests. Also, the use of a universal extracting solvent in multi mycotoxin determination can limit performance of the HLB based clean-up methods. Large amount of matrix components are extracted along with the analytes of interest and these compete with the analytes for sorbent active sites. Dilute-and-shoot approach by-pass this by diluting the matrix components. However, this approach also dilutes the analytes of interest and an instrument with superior sensitivity is then required for analysis

In contrast to expensive immunoaffinity columns (IAC) that are only selective for one class of mycotoxins, an improved sample preparation method based on HLB SPE clean-up was used to minimise co-eluting matrix components prior analysis thereby improving accuracy and precision of the measurement results.

Sample extracts were spiked with isotopically labelled standards prior to instrumental analysis. This was useful to correct and effectively compensate instrument variation during the analysis, but it does not correct variations arising from the extraction or clean-up step.

The method developed in this study was only applied to spiked and matrix reference materials. The method can be used further to analyse maize and maize based products that are available on the local market for possible mycotoxin contamination.

A limitation to multi mycotoxin analysis is the challenge to optimise sample preparation for analysis of compounds with varying physicochemical properties. Different class of mycotoxins were determined with this method but not all analytes were found to be in the ideal recovery range, 80% ≤ %REC ≤ 120%. Instruments with better sensitivity such as new generation mass spectrometers and improved LC systems should be used for analysis to improve analytical results.

The scope of this work did not cover uncertainty of measurement for the developed method. This is a requirement if a method is to be used for value assignment such as in the preparation of certified matrix reference materials.

Future work/recommendations

The proposed future work may therefore be summarised as follows:

- Investigate method performance when spiking the aliquots with isotopically labelled standards prior SPE to clean-up. It is not economically viable to spike samples with labelled standards prior to extraction as it requires large amounts of the labelled standards. It is proposed that spiking the sample extract aliquot with labelled standards prior to loading onto SPE column can correct for variations during the clean-up step. Minute amount of the labelled standards will be required at this stage as opposed to spiking prior extraction.
- Investigate method performance on alternative LC-MS/MS with better analytical performance. An analytical method can be developed for analysis of multiple mycotoxins in food matrices such as nuts, wheat and oats. The focus should be on the mycotoxins that are strictly regulated for that particular matrix, mycotoxins that have a high occurrence in the respective matrix and to those that are highly toxic at low concentration levels such as aflatoxin B_1.
- Compare method performance against immunoaffinity column clean-up

Estimate uncertainty of measurement contributors

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Appendix

Table 1. Summary of statistical comparison of two HLB clean-up columns for analyte recoveries, conditions t(4) = 2.13, P = 0.05.

	$\bar{x}$		Variance		t-stat	t-critical (one tail)
Analyte	O-HLB[a]	S-HLB[b]	O-HLB[a]	S-HLB[b]		
AFB_1	124	97	2	14	11.45	2.13
AFB_2	113	119	144	21	-0.819	
AFG_1	62	58	92.	1	0.7755	
AFG_2	116	114	1	81	0.381	
FB_1	112	120.	86	6	-1.37	
FB_2	113	110	27	24	0.564	
OTA	64	74	80	1	-1.917	
ZEN	82	100	13	24	-5.008	
DON	64	62	9	9	0.674	
HT-2	95	101	16	12	-2.156	
T2-Toxin	59	76	20	19	-4.787	

[a] Waters Oasis HLB

[b] Supelco Select HLB