Development, Chemometric-Assisted Optimization and in-House Validation of a Modified Pressurized Hot Water Extraction (PHWE) Method for Multi-Mycotoxin Analysis

By

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EXECUTIVE SUMMARY

Improvement in analytical methods, including extraction procedures has been a recurrent topic in mycotoxin research due to the fact that mycotoxins are ubiquitous, potent and notoriously difficult to eradicate from the food and feed supply chain. Effective management of these toxicants rely on stringent regulation and routine surveillance of food commodities via efficient analysis, hence the continuous need for improved methods. This research work describes for the first time the optimization, validation and application of a modified pressurized hot water extraction (PHWE) method for the simultaneous extraction of multiple mycotoxins and subsequent quantification on ultra-high performance liquid chromatography coupled to a tandem mass spectrometer (UHPLC-MS/MS). The research was performed in three phases which are presented as chapters (*i.e.*, Chapters Three to Five), in this thesis.

The first part of the research aimed to establish the thermal degradation patterns of mycotoxins and determine their relative stability under different temperature and time conditions. This was performed in order to determine the feasibility of the application of PHWE for the extraction of the mycotoxins since this extraction technique involves the use of heat. Using a set of statistically pre-determined experimental conditions (103 to 217 °C, and 6.72 to 63.28 min for temperature and time, respectively), simultaneous optimization of the thermal degradation of 15 different mycotoxins in pure form and when spiked into maize was achieved. The mycotoxins studied included aflatoxins (AFs), fumonisins (FBs), zearalenone and its analogues α-zearalenol and β-zearalenol (ZEAs), ochratoxins (OTs), T-2 toxin (T-2), alternariol monomethyl ether (AME) and sterigmatocystin (STEG). The global optimum degradation condition (*i.e.*, minimum temperature and time required for complete degradation of all mycotoxins) was computed as 217 °C/63.28 min and 211 °C/54.71 min for pure mycotoxin standards and spiked maize matrix, respectively. Based on the results obtained, it was inferred that the studied mycotoxins demonstrated sufficient thermal stability to undergo PHWE which typically employs temperatures ranging from 50 to 200 °C and extraction times ranging from 5 to 20 min.

The second part of this research work focused on the development, optimization and validation of a modified PHWE method for the simultaneous extraction of multi-mycotoxins from maize. This part of the research was aimed at providing technical solutions to some eminent challenges of conventional methods of mycotoxin extraction, such as, use of large volumes of harmful organic

solvents, high cost, long and tedious procedures as well as, as the inherent challenges of simultaneously extracting multiple mycotoxins in a single step. The adoption of advanced chemometric optimization models coupled with a co-solvent-modification of the PHWE system proved vital to achieve satisfactory recoveries across the analytes. The recovery values ranged from 71 to 124%, with the exception of ochratoxin A (OTA) and α-ZEL which had recoveries of 58 and 14%, respectively. These values were comparable to those of at least three other methods which are solvent-based, validated and well referenced in literature. The linearity (0.986 to 0.999), intra-day precision (0 to 27%) and inter-day precision (3 to 34%) values of the modified PHWE method were also good relative to European Commission (EC), *Codex Alimentarius* (CODEX) and the Association of Official Analytical Chemists (AOAC) guidelines for analytical method performance. Furthermore, a pilot-scale application of the method on 25 maize flour samples purportedly naturally contaminated with mycotoxins was successful.

The third phase of this research work describes the broader application of the modified PHWE method for the estimation of mycotoxin levels in different commercially available food commodities. Ninety-one (91) samples of maize, sorghum and millet obtained from 5 agroecological zones in Nigeria were analyzed for mycotoxins using the modified PHWE method. For further authentication, the same samples were analyzed using a validated solvent extraction method which served as a control for cross-validation. Scrutiny of the results using principal component analysis (PCA) and orthogonal projections to latent structures discriminate analysis (OPLS-DA) indicated that there were no significantly different patterns in the data obtained using the two methods. Accordingly, the results revealed that all of the maize samples (n=16), 32% (n=38) of sorghum and 35% (n=37) of millet samples were positive for at least one of the 15 tested mycotoxins. Amongst the toxins, fumonisins (FBs) were the most prevalent in terms of incidence rate and contamination levels. Aflatoxin (AF) contamination was also relatively high in the samples. In maize, mean contamination levels for aflatoxin B_1 (AFB_1) and sum of aflatoxins (ΣAFs) was 54 and 56 µg/kg, respectively. Eleven out of 16 of the maize samples were contaminated above the EC maximum level of 2 and 4 µg/kg for AFB_1 and ΣAFs, respectively. Generally, mycotoxins were more prevalent in maize, followed by sorghum and then millet. Co-occurrence of two or more mycotoxins, particularly AFs+FBs, were observed in some of the cereal samples, which re-affirmed the seriousness of the mycotoxin issue in Nigeria and the need for effective and sustainable intervention approaches to address this issue.

Overall, PHWE seems promising as a suitable and greener alternative to traditional methods of mycotoxin extraction. Research in this area is envisaged to allow bioanalytical scientists working in the field of mycotoxicology to conveniently and efficiently extract mycotoxins in various agricultural products without the need for harmful and expensive organic solvents, as well as, promote analytical techniques that are more environmentally friendly and sustainable.

Keywords: Mycotoxins, thermal stability, method development, chemometric-assisted optimization, pressurized hot water extraction.

DECLARATION

I, Sefater Gbashi, hereby declare that the composition of this doctoral thesis and the work herein described was carried out entirely by myself unless otherwise indicated. None of the material has been submitted previously, in whole or in part, for degree purposes at any other University or institution. All other sources used have been duly cited in text and acknowledged by complete references.

_____

Sefater Gbashi

DEDICATION

I dedicate this piece of work to God Almighty, the very essence of my existence and the sovereign ruler over the affairs of humanity. Also, to my family and late dad for the love and support.

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RESEARCH OUTPUT

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Journal articles

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Gbashi, S., Madala, N. E., De Saeger, S., De Boevre, and Njobeh, Changwa, R., Kagot, V., and P. B. (2019). A comparison of pressurized hot water extraction (PHWE) and a solvent-based extraction method followed by UHPLC-MS/MS analysis for the estimation of multi-mycotoxin levels in staple cereals from selected regions in Nigeria. (writing in progress).

Conferences

Gbashi, S., Madala, N., Adebo, O. A., and Njobeh, P. B. (2017). Development and validation of a pressurized hot water extraction method for the extraction of aflatoxin B_1 in maize. In: 7th Euro Global Summit on Clinical Microbiology and Mycotoxins. February 27-28, 2017 Amsterdam, Netherlands. Amsterdam, Netherlands.

Gbashi, S., Madala, N., Adebo, O. A., and Njobeh, P. B. (2017). Temperature-time numerical modelling of the thermal degradation of aflatoxin B_1 (AFB_1) using response surface methodology. In: Proceedings of the 1st MYCOKEY International Conference. Global Mycotoxin Reduction in the Food and Feed Chain. September 11-14 2017. Ghent, Belgium.

Gbashi, S., Madala, N. E., De Saeger, S., De Boevre, M., and Njobeh, P. B. (2018). Multivariate optimization of the thermal degradation of *Fusarium* toxins and ochratoxins in maize matrix. In: Proceedings of the Africa Food safety workshop 2018. Pretoria, South Africa.

Gbashi, S., Madala, N. E., De Saeger, S., De Boevre, and Njobeh, P. B. (2019). Development, chemometric-assisted optimization and in-house validation of a modified pressurized hot water extraction (PHWE) methodology for multi-mycotoxins in maize. Presented at the 2019 IUPAC Postgraduate Summer School on Green Chemistry. May 12-19 2019. Dar es Salaam, Tanzania.

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CONTENTS

Title **Page No.**

EXECUTIVE SUMMARY ..... **i**
DECLARATION ..... **iv**
DEDICATION ..... **v**
ACKNOWLEDGEMENT ..... **vi**
RESEARCH OUTPUT ..... **viii**
CONTENTS ..... **x**
LIST OF FIGURES ..... **xvi**
LIST OF TABLES ..... **xvii**
LIST OF APPENDICES AND ADDENDUMS ..... **xix**
LIST OF EQUATIONS ..... **xxi**
LIST OF ABBREVIATIONS ..... **xxii**
LIST OF SYMBOLS AND UNITS ..... **xxix**
THESIS OUTLINE ..... **xxxi**
CHAPTER ONE **1**
1. INTRODUCTION 1
1.1 BACKGROUND 1
1.2 PROBLEM STATEMENT 2
1.3 JUSTIFICATION 2
1.4 HYPOTHESIS 3
1.5 AIM 3
1.6 OBJECTIVES 3
CHAPTER TWO **4**
2. MYCOTOXINS 4
2.1 DEFINITION AND CONCEPT OF MYCOTOXINS 4
2.2 MYCOTOXIN DIVERSITY: STRUCTURAL, PHYSICOCHEMICAL AND TOXICOLOGICAL CHARACTERISTICS 5
2.2.1 Aflatoxins 5
2.2.2 Fumonisins 7

2.2.3 Ochratoxins 9
2.2.4 Zearalenone 10
2.2.5 T2-toxin and HT-2-toxin 11
2.2.6 Sterigmatocystin 13
2.2.7 Alternaria toxins 13
2.2.8 Other mycotoxins 15
2.3 EXPOSURE TO MYCOTOXINS AND THE SOCIO-ECONOMIC IMPACT .. 16
2.3.1 Exposure to mycotoxins 16
2.3.2 Socio-economic impact of mycotoxins 18
2.3.2.1 Impact on health 18
2.3.2.2 Impact on food security, agricultural trade and the economy 22
2.3.2.3 Commitment to research and the cost implications 23
2.4 ANALYSIS OF MYCOTOXINS 24
2.4.1 Sampling 24
2.4.2 Extraction of mycotoxins 25
2.4.2.1 Conventional methods of mycotoxin extraction 26
2.5 PRESSURIZED HOT WATER EXTRACTION (PHWE) 30
2.5.1 Concept and principle of PHWE 30
2.5.2 Instrumentation and mechanism of PHWE 32
2.5.3 Operational modes of PHWE 32
2.5.4 Factors affecting PHWE 33
2.5.4.1 Temperature 33
2.5.4.2 Pressure 34
2.5.4.3 Co-solvents and modifiers 34
2.5.5 Advantages and disadvantages of PHWE 35
2.5.5.1 Advantages of PHWE 35
2.5.5.2 Disadvantages of PHWE 35
2.5.6 Applications of PHWE 36
2.5.7 Prospects of PHWE of mycotoxins 36
2.6 CHROMATOGRAPHIC SEPARATION, DETECTION AND QUANTIFICATION OF MYCOTOXINS 37

2.6.1 Chromatographic separation of mycotoxins 37
2.6.2 Detection and quantification of mycotoxins 39
2.6.2.1 Mass spectrometry (MS) 40
2.6.3 Application of LC-MS/MS for mycotoxin analysis 42
2.7 DEVELOPMENT AND VALIDATION OF METHODS FOR MYCOTOXINS ANALYSIS 42
2.7.1 Method development 42
2.7.2 Validation of analytical methods 43
2.7.2.1 Accuracy 44
2.7.2.2 Specificity 44
2.7.2.3 Sensitivity 45
2.7.2.4 Linearity and range 45
2.7.2.5 Intra- and inter-day precision 46
2.7.2.6 Robustness 46
2.8 CHEMOMETRIC APPROACHES FOR OPTIMIZATION OF EXTRACTION METHODOLOGIES IN SAMPLE PREPARATION 47
2.8.1 Response surface methodology (RSM) 48
2.8.2 Principal component analysis (PCA) and orthogonal projections to latent structures discriminate analysis (OPLS-DA) 50
2.9 CONCLUDING REMARKS FROM THE LITERATURE REVIEWED 52
References 52

CHAPTER THREE 100

NUMERICAL OPTIMIZATION OF TEMPERATURE-TIME DEGRADATION OF MULTIPLE MYCOTOXINS 100
Abstract 100
Highlights 101
3.1 Introduction 101
3.2 Materials and methods 103
3.2.1 Materials 103
3.2.2 Methods 103
3.2.2.1 Experimental design 103

3.2.2.2 Sample preparation 106
3.2.2.3 Thermal treatment 106
3.2.2.4 Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) ... 106
3.3 Results and discussion 108
3.3.1 Thermal degradation of mycotoxins 108
3.3.2 Matrix effect on thermal stability of mycotoxins 113
3.3.3 Numerical modelling of the degradation of mycotoxins 114
3.3.3.1 Model fitting to experimental data 114
3.3.3.2 Factor effects 116
3.3.3.3 Optimization of the thermal degradation of multi-mycotoxins 118
3.3.3.4 Validation of model 121
3.4 Conclusion 122
Acknowledgement 123
References 124
APPENDIX 3 133

CHAPTER FOUR 138

DEVELOPMENT, CHEMOMETRIC-ASSISTED OPTIMIZATION AND IN-HOUSE VALIDATION OF A MODIFIED PRESSURIZED HOT WATER EXTRACTION METHODOLOGY FOR MULTI-MYCOTOXINS IN MAIZE 138
Abstract 138
4.1 Introduction 139
4.2 Materials and methods 141
4.2.1 Materials 141
4.2.2 Methods 142
4.2.2.1 Sampling 142
4.2.2.2 Experimental design 142
4.2.2.3 Pressurized hot water extraction (PHWE) 144
4.2.2.4 Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) ... 144
4.2.3 Validation of the modified PHWE method for multi-mycotoxins extraction 146
4.2.4 Comparative assessment of the recovery of PHWE with other conventional methods of mycotoxin extraction 147

4.3 Results and discussion 148
4.3.1 Method development and optimization 148
4.3.3 Validation of the optimized PHWE method performance characteristics 152
4.3.3.1 Matrix effect (ME) 152
4.3.3.2 Linearity, accuracy, LOD and LOQ 155
4.3.3.3 Intra-day precision (RSDr) and inter-day precision (RSD_R) 156
4.3.4 Comparison with other conventional mycotoxin extraction methods 156
4.3.6 Sample analysis 158
4.4 Conclusion 159
Acknowledgement 160
References 160
APPENDIX 4 168
CHAPTER FIVE 170
COMPARISON OF PRESSURIZED HOT WATER EXTRACTION (PHWE) AND A SOLVENT-BASED EXTRACTION METHOD FOR THE ESTIMATION OF MULTI-MYCOTOXIN LEVELS IN STAPLE CEREALS FROM SELECTED REGIONS IN NIGERIA 170
Abstract 170
5.1 Introduction 171
5.2 Materials and methods 173
5.2.1 Materials 173
5.2.2 Methods 174
5.2.2.1 Sampling and sampling preparations 174
5.2.2.2 Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) 177
5.2.2.3 Validation of the modified PHWE method for multi-mycotoxin extraction 179
5.2.2.4 Multivariate discriminant analysis 180
5.3 Results and discussion 181
5.3.1 Method validation and comparative evaluation of PHWE and solvent extraction 181
5.3.1.1 Validation of PHWE and solvent extraction 181
5.3.1.2 Comparative evaluation of PHWE and solvent extraction 185

5.3.1.2.1 Discriminatory analysis of PHWE and solvent extraction methods using PCA and OPLS-DA 185
5.3.2 Mycotoxin contamination of staple cereals 188
5.3.2.1 Mycotoxin levels and incidence rate in maize, sorghum and millet from Nigeria 188
5.3.2.2 Mycotoxin distribution patterns in maize, sorghum and millet from different agroecological zones of Nigeria 194
5.3.2.3 Simultaneous occurrence of multiple mycotoxins in maize, sorghum and millet from Nigeria as analyzed using PHWE followed by UHPLC-MS/MS 196
5.3.3 Significance of mycotoxin contamination of Nigerian staple cereals 198
5.4 Conclusion 199
Acknowledgement 200
References 200
APPENDIX 5 214
CHAPTER SIX 217
6.0 GENERAL DISCUSSION AND CONCLUSION 217
6.1 GENERAL DISCUSSION 217
6.2 CONCLUSION 220
References 222
ADDENDUM 227

LIST OF FIGURES

Figure No.	Caption	Page No.
Figure 2.1:	Chemical structures of (a) AFB_1 (b) AFB_2 (c) AFG_1 (d) AFG_2 (e) AFM_1 and (f) AFM_2	6
Figure 2.2:	Molecular structures of (a) FB_1 (b) FB_2 and (c) FB_3	8
Figure 2.3:	Molecular structures of (a) OTA, and (b) OTB.	9
Figure 2.4:	Molecular structures of (a) ZEA (b) α-ZEL (c) β-ZEL	10
Figure 2.5:	Molecular structures of (a) T-2 and (b) HT-2.	12
Figure 2.6:	Molecular formula of sterigmatocystin.	13
Figure 2.7:	Molecular structure of alternariol monomethyl ether	15
Figure 2.8:	Phase diagram of water as a function of temperature and pressure.	31
Figure 2.9:	Simple laboratory setup of a PHWE unit (Gbashi *et al*., 2016)	33
Figure 2.10:	Schematic representation of a typical HPLC unit	38
Figure 2.11:	Schematic representation of a typical triplequad MS instrument	41
Figure 2.12:	The central composite design of the response surface model for (a) $\alpha=\sqrt{k}$, where k=2, and (b) $\alpha=\sqrt{k}$.	49
Figure 3.1:	Compound response surface plots for thermal degradation for pure mycotoxins and mycotoxins spiked into maize matrix.	117
Figure 3.2a:	Pareto charts of standardized factor effects for model fit of pure mycotoxin standards.	119
Figure 4.1:	Response surface plots showing the relationship between mycotoxin recovery and extraction parameters (temperature and solvent composition)	150
Figure 4.2:	PHWE of multi-mycotoxins using CCD-optimized extraction conditions of 162 °C for temperature and 45% for solvent composition..	151
Figure 4.3:	Pareto charts of standardized factor effects for pressurized hot water extraction (PHWE) of multi-mycotoxins.	153
Figure 4.4:	Matrix-induced signal suppression/enhancement for mycotoxins using pressurized hot water extraction (PHWE)..	154
Figure 5.1:	OPLS-DA scores plot and variable importance plot (VIP) plot for discriminate analysis of PHWE and solvent extraction.	187

LIST OF TABLES

Table No.	Caption	Page No.
Table 2.1:	Mycotoxins and their toxic effects on human health.	20
Table 2.2:	Important electro- and physicochemical properties of water that changes with increase in temperature and pressure	31
Table 3.1:	Two-factor, 1 block standard order CCD experimental design for temperature-time degradation of mycotoxins	104
Table 3.2:	MRM transitions, optimized MS conditions and retention times of multi-mycotoxins.	107
Table 3.3:	Temperature-time degradation of pure multi-mycotoxin standards.	110
Table 3.4:	Temperature-time degradation of multi-mycotoxins spiked into maize matrix.	111
Table 3.5:	Regression model fit coefficients and validation indices for thermal degradation for pure mycotoxins standards	114
Table 3.6:	Regression model fit coefficients and validation indices for thermal degradation for mycotoxin standards spiked into maize flour	115
Table 4.1:	Two-factor, 1 block standard order CCD experimental design for temperature and solvent composition optimization during PHWE of multi-mycotoxins.	143
Table 4.2:	MRM transitions, optimized MS conditions and retention times of multi-mycotoxins	145
Table 4.3:	Accuracy or recovery efficiency (%), linear calibration function, coefficient of linearity (R^2), LOD and LOQ (µg/kg).	155
Table 4.4:	Comparison of method performance (recovery) between PHWE and conventional extraction methods	157
Table 4.5:	Mycotoxin contamination of maize meal from Ngwalemong village in Limpopo Province of South Africa.	158
Table 5.1:	Selected sampling locations for maize, sorghum and millet in Nigeria	175
Table 5.2:	MRM transitions, optimized MS conditions and retention times of mycotoxins.	177
Table 5.3:	Method validation parameters for PHWE and solvent extraction of multi-mycotoxin in maize, sorghum and millet matrices.	183

Table 5.4: Summary of PCA-X model and OPLS-DA models with the corresponding model validation parameters 186
Table 5.5: Mycotoxin contamination of maize, sorghum and millet from Nigeria using PHWE and solvent extraction 190
Table 5.6: Distribution of mycotoxins in maize, sorghum and millet across different agroecological zones in Nigeria as analyzed using PHWE followed by UHPLC-MS/MS 195
Table 5.7: Co-occurrence of different groups of regulated mycotoxins in maize, sorghum and millet samples from Nigeria. 196

LIST OF APPENDICES AND ADDENDUMS

APPENDICES

Appendix No.	Caption	Page No.
Appendix 3.A:	Single ion chromatogram of pure AFB_1 standard at different temperature-time conditions.	133
Appendix 3.B:	Single ion chromatogram of AFB_1 spiked into maize matrix at different temperature-time conditions.	134
Appendix 3.C:	Independent-samples t-test for average thermal degradation of pure mycotoxin and mycotoxins spiked into maize matrix.	135
Appendix 3.D:	CCD regression model fit factor effects for thermal degradation of mycotoxins	136
Appendix 3.E:	Individually optimized experimental conditions for pure mycotoxins and mycotoxins spiked into maize matrix	137
Appendix 4.A:	Pressurized hot water extraction (PHWE) of multi-mycotoxins at different levels of temperature and solvent composition based on the central composite design (CCD)	168
Appendix 4.B:	Model-fit regression coefficients and validation parameters	169
Appendix 5.A:	Linearity parameters and matrix effect of PHWE of multi-mycotoxins in maize, sorghum and millet.	214
Appendix 5.B:	Incidences of co-contamination of different classes of regulated mycotoxins in maize, sorghum and millet from Nigeria as analyzed using PHWE followed by UHPLC-MS/MS.	216

ADDENDUMS

Addendum No.	Caption	Page No.
Addendum A:	Picture of the homemade laboratory scale PHWE unit.	227
Addendum B:	Calibration curves for mycotoxins prepared in neat solvent.	228
Addendum C:	Maize matrix-matched calibration curves.	229
Addendum D:	Sorghum matrix-matched calibration curves.	230
Addendum E:	Millet matrix-matched calibration curves.	231

Addendum F: Selected single ion chromatograms (SIM) showing the peaks of the different mycotoxins prepared in neat solvent, maize-matrix, sorghum-matrix and millet-matrix..... 232

Addendum G: Selected single ion chromatograms (SIM) showing the peaks of the different mycotoxins for contaminated maize, sorghum and millet samples..... 233

LIST OF EQUATIONS

Equation No. **Page No.**

Equation 2.1 46
Equation 2.2 46
Equation 2.3 49
Equation 3.1 104
Equation 3.2 104
Equation 3.3 105
Equation 3.4 105
Equation 3.5 105
Equation 3.6 105
Equation 3.7 105
Equation 4.1 143
Equation 4.2 146
Equation 4.3 146
Equation 4.4 147
Equation 5.1 179
Equation 5.2 179
Equation 5.3 180

LIST OF ABBREVIATIONS

%+ve	Percentage of positive samples
%EtOH	Percentage ethanol
15-ADON	15-acetyldeoxynivalenol
3-ADON	3-acetyldeoxynivalenol
AAD	Average absolute deviation
A_f	Accuracy factor
AF	Aflatoxin
AFB's	Aflatoxin B_1 and Aflatoxin B_2
AFB_1	Aflatoxin B_1
AFB_2	Aflatoxin B_2
AFG's	Aflatoxin G_1 and Aflatoxin G_2
AFG_1	Aflatoxin G_1
AFG_2	Aflatoxin G_2
AFM_1	Aflatoxin M_1
AFM_2	Aflatoxin M_2
AFs	Aflatoxins
AME	Alternariol monomethyl ether
ANOVA	Analysis of variance
AOAC	The Association of Official Analytical Chemists
AOH	Alternariol
API	Atmospheric-pressure ionization

ARS	Agricultural Research Service (USDA)
BEA	Beauvericin
B_f	Bias factor
CCD	Central composite design
CE	Collision energy
CIT	Citrinin
CO_2	Carbon dioxide
CODEX	*Codex Alimentarius*
Corr.	Correlation
DALYs	Disability-adjusted life years
DAS	Diacetoxyscirpenol
DCM	Dichloromethane
DF	Desirability factor
DL	Desolvation line
DNA	Deoxyribonucleic acid
DON	Deoxynivalenol
DRS	Derived savannah
DTSC	Department of Toxic Substances Control (California)
EAs	Ergot alkaloids
EC	European Commission
ELISA	Enzyme-linked immunosorbent assay
ENNs	Enniatins
ESI^+	Electron spray ionization

EtOH	Ethanol
EU	European Union
FA	Formic acid
FAO	Food and Agriculture Organization (UN)
FB_1	Fumonisin B_1
FB_2	Fumonisin B_2
FB_3	Fumonisin B_3
FBs	Fumonisins
FDA	Food and Drug Administration (US)
FID	Flame ionization detection
FUS-X	Fusarenon-X
GC	Gas chromatography
GDP	Gross domestic product
HACCP	Hazard and critical control point
HBV	Hepatitis B virus
HLWE	Hot liquid water extraction
HPLC	High performance liquid chromatography
HRF	Humid rain forest
HRMS	High resolution mass spectrometry
HWE	Hot water extraction
IA	Immuno-affinity
IAC	Immuno-affinity column
IARC	International Agency for Research on Cancer

ICH	International conference on harmonization
ID	Inner diameter
IT	Ion trap
LC	Liquid chromatography
LC-MS/MS	Liquid chromatography coupled to tandem mass spectrometry
LLOQ	Lower limit of Quantification
LOD	Limit of detection
LOQ	Limit of quantification
MAE	Microwave-assisted extraction
ME	Matrix effect
MLS	Method of least square
MON	Moniliformin
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS/MS	Two mass spectrometers in tandem
N.D.	Not detected or Not determined
NA	Not applicable
NGS	Northern guinea savannah
NI	Not investigated
NIV	Nivalenol
NMISA	National Metrological Institute of South Africa
OD	Outer diameter
OPLS-DA	Orthogonal projections to latent structures discriminate analysis

OTA	Ochratoxin A
OTB	Ochratoxin B
OTC	Ochratoxin C
OTD	Ochratoxin D
OTs	Ochratoxin A and ochratoxin B
PA	Penicillic acid
PAT	Patulin
PCA	Principal component analysis
PCs	Principal components
PDA	Photo-diode array detector
PHW	Pressurized hot water
PHWE	Pressurized hot water extraction
PLPWE	Pressurized low polarity water extraction
Q1	Quadruple 1
Q1 Pre bias	Quadruple one pre-rod bias
Q2(cum)	Cumulative predictive capacity
Q3	Quadruple 3
Q3 Pre bias	Quadruple three pre-rod bias
qToF	Quadrupole time-of-flight
QuEChERS	Quick, Easy, Cheap, Effective, Rugged, and Safe
R^2	Coefficient of determination
R^2 **Adj**	Adjusted coefficient of determination
***R2*X(cum)**	Cumulative ratio of the variation of the X variables

***R2*Y(cum)**	Cumulative ratio of the variation of the Y variables
Ret. time	Retention time
RPM	Revolutions per minute
RSD	Relative standard deviation
RSD_R	Inter-day precision also referred to as between-day (intermediate) precision
RSDr	Intra-day precision
RSM	Response surface methodology
S/N	Signal to noise ratio
SFE	Supercritical fluid extraction
SGS	Southern guinea savannah
SHS	Sahel savannah
sIgA	Secretory Immunoglobulin A
SIM	Single ion monitoring
SOLV-DS	Solvent extraction followed by dilute and shoot injection
SOLV-EXT	Solvent extraction
SOLV-SPE	Solvent extraction followed by clean-up using different SPE cartridges
SPE	Solid phase extraction
STEG	Sterigmatocystin
SWE	Subcritical water extraction
T1L	Linear effect of temperature
T1L by T2L	Interaction effect temperature and time
T1Q	Quadratic effect of temperature
T-2	T-2 toxin

T2L	Linear effect of time
T2Q	Quadratic effect of time
THs	Trichothecenes
TLC	Thin layer chromatography
U$D	US dollars
UN	United nations
UHPLC	Ultra-high performance liquid chromatography
UPLC	Ultra-performance liquid chromatography
UV	Ultraviolet or Unit variance
VIP	Variable importance plot
ZAN	Zearalanone
ZEA	Zearalenone
ZEAs	Zearalenone, α-zearalenol, and β-zearalenol
α-ZEA	α-zearalenol
β-ZEA	β-zearalenol

LIST OF SYMBOLS AND UNITS

%	Percentage
±	Plus or minus
≤	Less than or equal to
≥	Greater than or equal to
°C	Degree Celsius
µg	Microgram
µg/g	Microgram per gram
µL	Microlitre
µm	Micrometer
C18	Octadecyl
C2	Ethyl
C8	Octyl
Channels/sec	Channels per second
CN	Cyanopropyl
Da	Dalton
eV	Electron volts
ε	Dielectric constant
g	Gram(s)
h	Hour(s)
H_2SO_4	Sulphuric acid
KCl	Potassium chloride
L/min	Litres per minute
m/z	Mass to charge ratio
Min	Minute(s)

mL	Millilitre
mL/min	Millilitre per minute
mM	Millimolar
mm	Millimeter
mm/yr	Millimeters per year
NH_2	Aminopropyl
psi	Pounds per square inch
r	Pearson's bivariate coefficient
S/N	Signal-to-noise ratio
sec	Second(s)
V	Volts
µg/g	Microgram per gram
µg/kg	Microgram per kilogram

THESIS OUTLINE

This thesis describes studies on the design, optimization, validation and applicability of a modified pressurized hot water extraction (PHWE) method for the analysis of mycotoxins in food commodities. The experiments described herein were conducted in the Department of Biotechnology and Food Technology, University of Johannesburg (South Africa) and partly at the Centre of Excellence in Mycotoxicology and Public Health, Department of Bioanalysis, Ghent University (Belgium). The thesis consists of six chapters; a brief outline of each of the chapters is presented below.

Chapter One

This introductory chapter provides a general overview of the research subject and rationale. The chapter starts off by presenting relevant background information, describing the problem under investigation and the context within which this study was conducted. Important terms used in the study are defined, while also highlighting the theoretical assumptions (hypothesis), aim and objectives of the study.

Chapter Two

This chapter reviews relevant literature within the context of the research focus, situates the research objectives in the framework of the wider academic community of mycotoxicology, and gives credence to the research rationale by identifying the knowledge gaps that the research seeks to explore. The concept of mycotoxins, their chemical characteristics, occurrence and significance is described. Various conventional methods of mycotoxin extraction including their pros and cons is reviewed. The principles and applications of mass spectrometry (MS) in mycotoxin analysis is also presented. The chapter further describes the process of method development and parameters that must be considered when validating an analytical method. The concept, mechanism and dynamics of PHWE, its advantages, disadvantages and applicability are also exhaustively appraised. The chapter ends by describing various chemometric tools applicable in the development of sample preparatory procedures and bioanalytical chemistry. Some aspects of this chapter have been published under two titles: the first is a book chapter titled *Subcritical water extraction and its prospects for aflatoxins extraction in biological materials*, which is published in InTech. The second publication is titled *The socio-economic impact of mycotoxin in Africa*, which is also published as a book chapter by the same publisher.

Chapter Three

Chapter Three describes experiments on a systematic study of the thermal stability and degradation patterns of multi-mycotoxins as a function of temperature and time using a numerical optimization approach called the central composite design. The experimental design, methodology and a detailed interpretation and discussion of the results thereof are clearly described. The work described in this chapter has been published online in Food and Chemical Toxicology.

Chapter Four

The chapter's primary focus is on the development, optimization and validation of a modified PHWE method for the extraction of multi-mycotoxins and subsequent analysis on UHPLC-MS/MS. The chapter describes the adoption of a chemometric optimization approach for this purpose. Method validation was performed according to standard guideline parameters by the European Commission (EC), *Codex Alimentarius* (CODEX), The Association of Official Analytical Chemists (AOAC), and the International Conference on Harmonization (ICH). Comparison of the validated PHWE method was made against a number of established solvent extraction methods. The Chapter further describes a pilot scale application of the validated PHWE method in the analysis of locally sourced food samples (*i.e.*, maize meal) for possible mycotoxin contamination.

Chapter Five

A comparative application of PHWE and a solvent-based extraction method for the quantitative estimation of mycotoxin levels in staple cereals (maize, sorghum and millet) from different agroecological zones in Nigeria is presented in Chapter Five. In addition to providing practical application for the PHWE method, inference is drawn from the results of this study to determine the current risk and situation of mycotoxin prevalence in the country.

Chapter Six

This chapter reaffirms the research focus (*i.e.*, problem statement and aim) of this thesis and puts the entire research experiments presented in Chapters Three to Five into perspective via an overall discussion of the issues addressed in the chapters. The contributions of the thesis to existing knowledge, recommendations and directions for future research is also provided.

CHAPTER ONE

1. INTRODUCTION

1.1 BACKGROUND

The pervasive contamination of agricultural commodities by mycotoxins has become a serious global concern because of their severe impact on health and the economy (Lizárraga-Paulín *et al*., 2011; Makun *et al*., 2012). These toxins are low-molecular weight secondary metabolites of fungi origins, principally those belonging to the genera *Aspergillus*, *Penicillium*, *Claviceps* and *Fusarium* (Njobeh *et al*., 2010; Zain, 2011; Garba *et al*., 2017). Prevalence of mycotoxins does not only constitute a public health hazard, it also affects livelihood, food security, income and causes significant economic losses (Daniel *et al*., 2011; Enyiukwu *et al*., 2014; Gbashi *et al*., 2018). These toxins have been identified as the most significant chronic dietary risk factor, superior to other natural inherent plant toxins, synthetic contaminants, food additives, or pesticide residues (Kuiper-Goodman, 1998; Bennett and Klich, 2003). In fact, one of the mycotoxins, aflatoxin B_1 (AFB_1) has been recognized as the most potent naturally occurring carcinogen known to man (Makun *et al*., 2012). In 2010, over 130 people died in Kenya and another episode in mid-2011 in South Africa over 220 dogs died after consuming respectively, maize and pet food contaminated with high levels of aflatoxins (AFs) (Lewis *et al*., 2005; Daniel *et al*., 2011; Mwanza *et al*., 2013).

About 25% of the global food and feed produce is contaminated by mycotoxins (Enyiukwu *et al*., 2014), and very often, contamination of agricultural commodities by mycotoxins results from a cumulative process, which begins from pre-harvest through post-harvest stage and continues throughout the entire food production chain (Wilson & Abramson, 1992; Gbashi *et al*., 2018). Some factors that drive mycotoxin contamination along the food and feed supply chain are the favorable climatic conditions for fungi growth, poor agricultural practices, poverty, limited data on their occurrence and effects, as well as inadequate regulatory mechanisms (Mejía-Teniente *et al*., 2011; Njobeh & Olotu, 2017). It has been estimated that approximately 4.5 billion people in the world are at the risk of being chronically exposed to mycotoxins (Bryden, 2007; Turner *et al*., 2007). As a result of their widespread proliferation and associated deleterious effects, there is a growing concern over their intake via consumption of contaminated food and feed by humans and animals alike. This has led to more stringent guidelines and regulatory limits of these toxins, especially with the globalization of the food supply chain, and consequently, necessitating the need

for routine surveillance of these toxicants in agricultural commodities (Krska *et al*., 2008; Rahmani *et al*., 2009). In this regard, there has been an ever increasing demand for more efficient and robust analytical methods for the determination of mycotoxins, particularly with respect to safer, cheaper and quicker methods (Rahmani *et al*., 2009; Gbashi *et al*., 2017c).

1.2 PROBLEM STATEMENT

Extraction is an important step in mycotoxin analysis. It is almost inevitable irrespective of the protocol adopted. Although different methods exist for extracting mycotoxins from food and feed such as solvent extraction, solid-phase extraction (SPE) and immuno-affinity column (IAC) extraction amongst others, there are concerns over human and environmental health regarding safety in their applications (Teo *et al*., 2010; Gbashi *et al*., 2017d). These conventional methods are also tedious and time-consuming (Choi *et al*., 2003), requiring relatively large volumes of organic solvents, which are expensive and hazardous (Santana *et al*., 2009; Teo *et al*., 2010). Bearing in mind these concerns, the design of a greener method that is efficient, cheap, fast and relatively easy to use is eminent. Pressurized hot water extraction (PHWE) seems promising in this regard. Better results, recoveries and effectiveness have been reported for PHWE as compared to other traditional methods for extracting different bioactive compounds (Hawthorne *et al*., 2000; Kubátová *et al*., 2001; Bart, 2005). It is intended herein to explore the possibilities of designing and adopting a modified PHWE method as an alternative to conventional methods for multi-mycotoxin extraction from various food commodities.

1.3 JUSTIFICATION

Analysis is an important component of mycotoxin control and management. The quality of analytical results has often been directly linked to the efficacy of the extraction process utilized. At present, available extraction methods for mycotoxins analysis remain largely limiting in many ways, particularly in terms of human and environmental safety, cost, time and labor intensity. This study sets out to address these issues by adopting an innovative, green and efficient extraction approach, PHWE, for mycotoxin analysis in agricultural commodities. To the best of our knowledge, there has been no report on the use of PHWE for mycotoxins analysis. However, the adoption of PHWE for multi-mycotoxin extraction has some envisageable challenges such as the possibility of degradation of some of the analytes due to the thermal conditions involved during

PHWE. Also, due to the diverse physicochemical properties of the different classes of mycotoxins, there may be limited extractability of some of the mycotoxins in a single-step multi-mycotoxin extraction procedure. In this regard, it is critical to adequately optimize the PHWE system using appropriate models for optimum performance of the method.

1.4 HYPOTHESIS

It has been hypothesized in this research work that: (1) using optimization models, it would be possible to establish the thermal degradation patterns and ascertain the thermal stability of mycotoxins within a range of temperatures relevant to PHWE; (2) by means of advanced chemometric tools, and perhaps, a co-solvent, it would be possible to design and optimize a PHWE method for the simultaneous extraction of multiple mycotoxins followed by quantification on ultra-high performance liquid chromatography and tandem mass spectrometry (UHPLC-MS/MS); (3) the optimized PHWE method would perform adequately relative to standard guideline parameters for validation of analytical methods; (4) practical applicability of the validated PHWE method would be achievable for the analysis of naturally contaminated food samples for possible mycotoxin contamination.

1.5 AIM

The aim of this research work was to develop, optimize and validate a modified pressurized hot water extraction (PHWE) method, and adopt same for multi-mycotoxin analysis in food commodities.

1.6 OBJECTIVES

The objectives of this research were:

i. To optimize the temperature-time degradation of multi-mycotoxins.
ii. To develop, optimize and validate a modified PHWE method for the extraction of multi-mycotoxins in maize.
iii. To adopt the validated PHWE method for the extraction and subsequent estimation of multi-mycotoxin levels in various commercially available food commodities from Nigeria and South Africa.

CHAPTER TWO

2. MYCOTOXINS

2.1 DEFINITION AND CONCEPT OF MYCOTOXINS

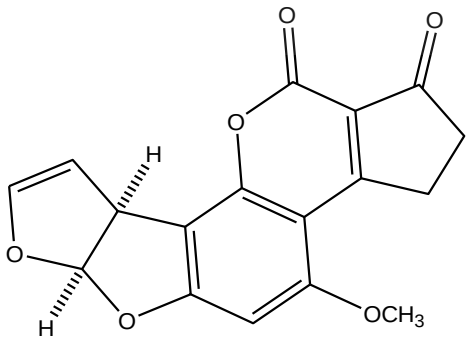
Mycotoxins can simply be described as toxic biochemical compounds produced by fungi (Adeyeye, 2016). These compounds are diverse secondary metabolites synthesized by certain fungi species that are ubiquitous in the food supply chain, contaminating pre- and post-harvest crops, as well as, processed food and feed commodities (Njobeh *et al*., 2010b; Abia *et al*., 2013; Chilaka *et al*., 2016). Fungal genera most frequently associated with mycotoxin production include *Aspergillus*, *Penicillium*, *Fusarium* and *Alternaria*, that are notable for their toxigenicity and disease-causing effects amongst humans and animals (Gbashi *et al*., 2018).

Production of mycotoxins by fungi depends on a number of factors including environmental conditions, physical damage to the crop, and the nature of the food substrate (Kokkonen *et al*., 2010; Magan *et al*., 2011; Pitt *et al*., 2013). A single fungal specie may synthesize more than one mycotoxin, while a specific mycotoxin may be produced by more than one fungal species all depending on favorable environmental conditions (Bayman & Baker, 2006; McCormick *et al*., 2011; Ismaiel & Papenbrock, 2015). Tropical climatic conditions such as in sub-Saharan Africa particularly favors the proliferation of mycotoxins. Over 300 mycotoxins have been characterized in literature, however, only a few have received significant attention due to their health and economic importance. Some of the economically important mycotoxins include aflatoxins (AFs), fumonisins (FBs), ochratoxins (OTs), zearalenone (ZEA) and its analogues α-zearalanol (α-ZEL) and β-zearalanol (β-ZEL), T-2 toxin (T-2), HT-2 toxin (HT-2), sterigmatocystin (STEG), alternariol monomethyl ether (AME), deoxynivalenol (DON), citrinin (CIT), and patulin (PAT). These mycotoxins and some of the emerging mycotoxins are described in detail in the succeeding sections of this chapter.

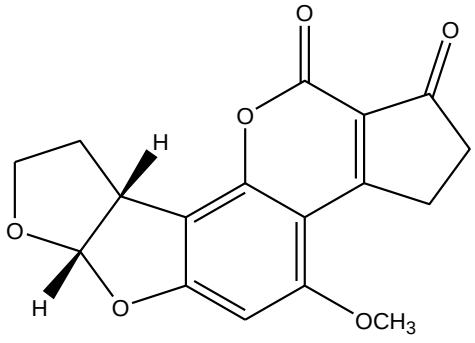
2.2 MYCOTOXIN DIVERSITY: STRUCTURAL, PHYSICOCHEMICAL AND TOXICOLOGICAL CHARACTERISTICS

2.2.1 Aflatoxins

Aflatoxins are the most perilous group of mycotoxins to humans and animals (Gbashi *et al*., 2017c). They are produced by toxigenic strains of *Aspergillus flavus, A. parasiticus* and *A. niger* (Mircea *et al*., 2008; Makun *et al*., 2012; Yang *et al*., 2014). At least 14 different types of AFs are known to exist in nature, however, the major ones of economic and health significance are AFB_1, aflatoxin B_2 (AFB_2), G_1 (AFG_1), G_2 (AFG_2), M_1 (AFM_1) and M_2 (AFM_2) (Figure 2.1). The molecular structure of AFs generally constitutes highly substituted coumarins containing a fused dihydrofurofuran moiety. The AFB's (*i.e.*,, members of the blue fluorescent series) generally feature a fusion of a cyclopentenone ring to the lactone ring of the coumarin moiety, while the AFG's possess a fused lactone ring (Kensler *et al*., 2011). Aflatoxin B_1 and G_1 contain an unsaturated bond on the terminal furan ring at the 8,9 position. Epoxidation at this position has shown to be essential for their carcinogenicity (Groopman & Kensler, 2005). Aflatoxin M_1 and M_2 are hydroxylated metabolites of AFB_1 and B_2, respectively, bio-transformed by the liver and found in milk, urine and other body fluids, being less harmful than their precursor toxins (Prado *et al*., 2008; Bbosa *et al*., 2013). The intensity of fluorescence (light) emission differs greatly among the four compounds. This property plays a significant role in their quantification by fluorescence techniques (Dhanasekaran *et al*., 2011).



(a)



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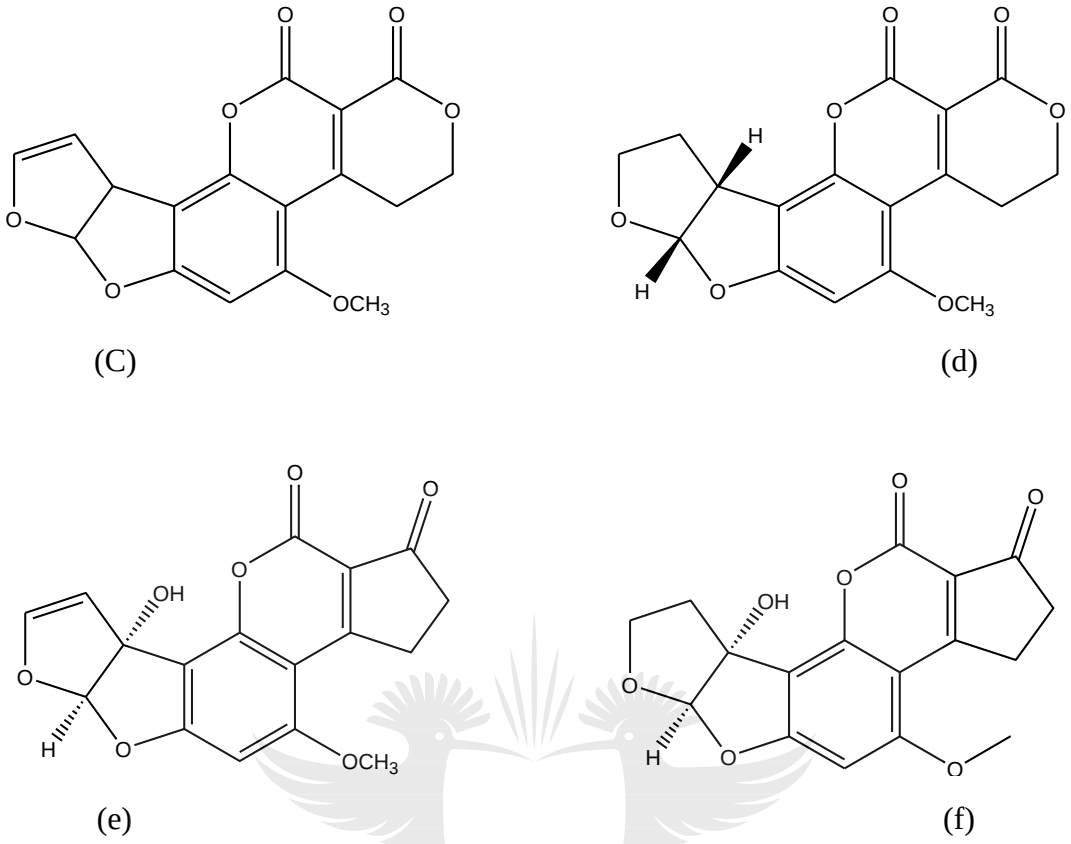
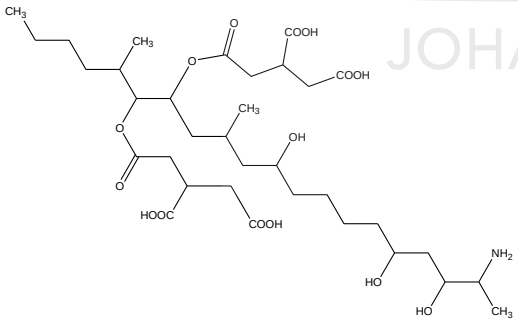


Figure 2.1: Chemical structures of (a) AFB_1 (b) AFB_2 (c) AFG_1 (d) AFG_2 (e) AFM_1 and (f) AFM_2 (Dhanasekaran *et al*., 2011).

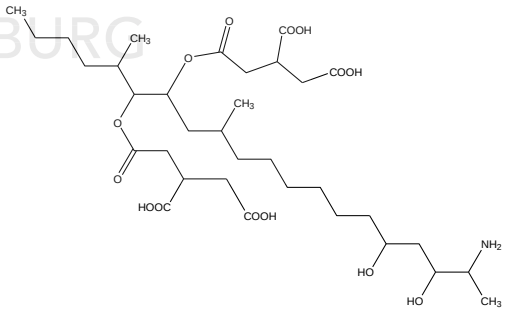
Aflatoxins are highly carcinogenic and equally recognized as being immunosuppressive. Among the AF group, AFB_1 is considered the most toxic. This one has been established as the most notorious naturally occurring carcinogen (IARC, 2002; D'Mello, 2003; Makun *et al*., 2012). For that reason, it has been classified as a group 1 human carcinogen by the International Agency for Research on Cancer (IARC) (IARC, 2002; Park *et al*., 2004a). Cereals such as maize are common crops that are contaminated by AFs. Additionally, crops such as oilseeds, including peanuts, different kinds of spices, figs and other dried fruit, are also familiar but most susceptible substrates. Aflatoxins are also very stable chemical compounds and notoriously difficult to eradicate in food commodities (Bullerman *et al*., 1984; Turner *et al*., 2009). They are chemically stable during processing and storage, even when heated at quite elevated temperatures such as those achieved during the production of breakfast cereals or baking of bread (Kabak, 2009; Turner *et al*., 2009). This necessitates the avoidance of conditions that favor their production, which is not always feasible in practice (Turner *et al*., 2009; Fernández-Cruz *et al*., 2010).

2.2.2 Fumonisins

The fumonisins group of mycotoxins are primarily produced by fungal species of the genera *Fusarium*, particularly *F. verticillioides* and *F. proliferatum* (Hussein and Brasel, 2001). About 28 different forms of FBs have been identified and sub-classified into A-series, B-series, C-series and P-series (Moreau *et al*., 2012). Of all the FBs, fumonisin B_1 (FB_1) is the most toxic, abundant and economically important, followed by fumonisin B_2 (FB_2) and fumonisin B3 (FB_3) (Peraica *et al*., 1999; Hussein and Brasel, 2001). Fumonisin B_1 has been classified as a group 2B carcinogen by the IARC (IARC, 2002). Figure 2.2 shows the molecular structure of FB_1, B_2 and B_3. Fumonisins are highly prevalent in African staple crops such as maize, sorghum, and millet, although they can occur in other agricultural commodities as well (Soriano & Dragacci, 2004; Stockmann-Juvala & Savolainen, 2008; Chilaka *et al*., 2016). Consumption of food contaminated with FBs has been linked with harmful health effects in humans. In Northeast Italy, consumption of foods contaminated with FBs resulted in upper gastro-intestinal tract cancer (Soriano and Dragacci, 2004). Moreover, FBs are also nephrotoxic, hepatotoxic, immunosuppressive, atherogenic and embryotoxic in experimental animal systems (Nair, 1998). They have been linked with esophageal cancer and spinal bifida (Missmer *et al*., 2005; Alizadeh *et al*., 2012). Fumonisins have also been associated with diseases in animals such as Leukoencephalomalacia in horses and pulmonary edema in swine (Haschek *et al*., 2001; Giannitti *et al*., 2011; Vendruscolo *et al*., 2016).



(a)



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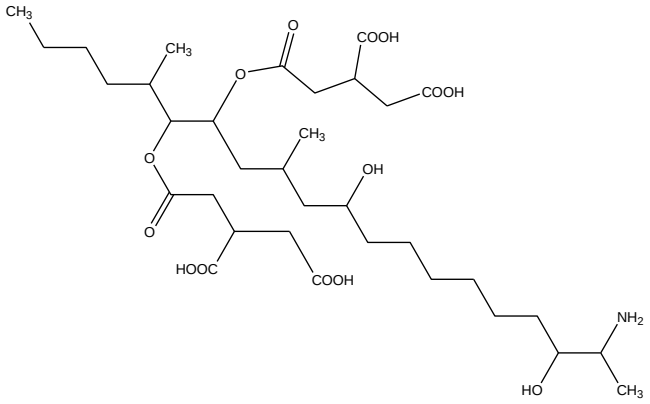


(c)

Figure 2.2: Molecular structures of (a) FB_1 (b) FB_2 and (c) FB_3 (Hussein and Brasel, 2001).

Fumonisins are generally thermolabile and have shown significant losses in most heat-based food processing operations (Castelo *et al*., 2001; Kabak, 2009; Patel *et al*., 2011). Jackson *et al*. (1996) investigated the effects of temperature and time on the stability of FB_1 in aqueous model systems (*i.e*. aqueous solutions at pH 4, 7, and 10). It was observed that the rate of FB_1 decomposition increased in proportion to increasing temperature. Thermal processing at ≤ 125 °C for 60 min resulted in a 27% loss of FB_1, after 60 min at 150 °C up to 18 to 90% of FB_1 was lost, depending on the pH buffer. Fumonisin B_1 was more thermally stable at neutral pH (i.e. pH 7), while the pH of 4 and 10 resulted in higher decomposition. The thermal processing condition of ≥ 175 °C for 60 min resulted in at least 90% decomposition regardless of the pH buffer. It has been shown that bound and hydrolysed forms of FBs are often formed during thermal processing of foods (Seefelder *et al*., 2003; Humpf & Voss, 2004; Park *et al*., 2004b). However, the occurrence of bound FBs is not limited to thermally processed food products (Patel *et al*., 2011). Seefelder *et al*. (2001) reported the formation of hydrolyzed FB_1 when samples containing FB_1 and sucrose were thermally processed. These forms of FBs are usually elusive to conventional analytical methodologies (Park *et al*., 2004b), as such, there is limited and inconsistent toxicological information about these compounds (Patel *et al*., 2011; Braun & Wink, 2018). Nonetheless, a few reports have indicated that some hydrolysed forms of FBs are less toxic compared to the parent compound (Humpf & Voss, 2004; Voss *et al*., 2017; Braun & Wink, 2018).

2.2.3 Ochratoxins

The ochratoxins are isocoumarin derivatives, occurring as ochratoxin A (OTA), B (OTB), C (OTC), D (OTD) and their methyl and ethyl esters (Haschek *et al*., 2002). This group of mycotoxins are produced by some *Aspergillus* species (mainly *A. ochraceus* and *A. niger*) and some *Penicillium* species, particularly *P. carbonarius* and *P. verrucosum* (Bayman and Baker, 2006; Kočube *et al*., 2013). Favored food substrates include cereals, coffee and grapes (Al-anati and Petzinger, 2006; El Khoury and Atoui, 2010). Similar to FB_1, OTA is a prevalent toxin, classified as a group 2B carcinogen to human (IARC, 2002; Malir *et al*., 2016). It accumulates in the organs and tissues of animals, including breast milk and human blood, thus, consumption of meat and meat products can constitute exposure to this toxin (Clark and Snedeker, 2006). In laboratory animals, OTA has been implicated for carcinogenicity (Mally and Dekant, 2009; Njobeh *et al*., 2010). This toxin is also mutagenic and nephrotoxic (Palma *et al*., 2007; Pfohl-Leszkowicz and Manderville, 2007). Figure 2.3 shows the chemical structure of OTA and OTB.

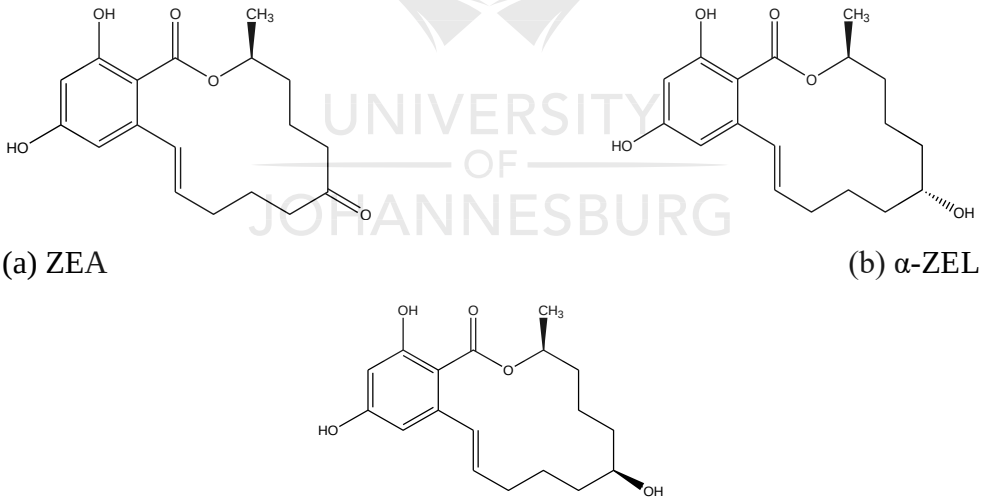
(a) (b)

Figure 2.3: Molecular structures of (a) OTA, and (b) OTB (El Khoury & Atoui, 2010).

Ochratoxins are very stable to heat treatment (Raters & Matissek, 2008; Vidal *et al*., 2015). This coupled to their widespread occurrence makes them very difficult to eradicate from the food chain (Kőszegi & Poór, 2016). Boudra *et al*. (1995) examined the stability of OTA under different moisture and temperature conditions. They observed that a temperature condition of 200 °C for 12 min could result in a 50% decomposition of OTA in dry wheat, while a temperature of 200 °C for 19 min was needed to achieve similar results when the wheat was heated under wet conditions. However, it was not possible to completely degrade OTA within the heating limits of the study i.e. conditions of 100-200 °C. This strongly indicates a high likelihood of detecting OTA in thermally processed food products, as such, it is recommended to adopt preventive measures in order to limit exposure to this mycotoxin.

2.2.4 Zearalenone

Zearalenone (ZEA) is a macrocyclic β-resorcyclic acid lactone metabolite (Figure 2.4) produced by certain *Fusarium* and *Gibberella* species, such as *F. culmorum, F. graminearum, F. roseum* and others (Atoui *et al*., 2012; Hueza *et al*., 2014). It has two major hydroxylated derivatives α- and β- zearalenol (α-ZEL and β-ZEL) (Figure 2.4), which are also lactone derivatives commonly found in food commodities (Gratz, 2017). However, many other derivatives and modified forms of ZEA occur in different agricultural commodities. Gromadzka *et al*. (2008) provides a detailed description and review of the ZEA and its metabolites, their occurrence, toxicity and detection in different food commodities. For the purpose of this review, emphasis will be placed only on ZEA, α-ZEL and β-ZEL due to their toxicological significance and widespread occurrence in cereals from Africa. Food contamination with ZEA, and α- and β-ZEL (ZEAs) has been reported worldwide and common food crop substrates include maize, millet, wheat, sorghum and rice (Atoui *et al*., 2012; Hewitt *et al*., 2012; Chilaka *et al*., 2016). It has been reported that ZEAs usually co-occurs with one or more of the trichothecenes (THs), because of the ability of its producing fungi to synthesize more than one mycotoxin (Grenier & Oswald, 2011).



(a) ZEA (b) α-ZEL

(c) β-ZEL

Figure 2.4: Molecular structures of (a) ZEA (b) α-ZEL (c) β-ZEL (Hussein and Brasel, 2001).

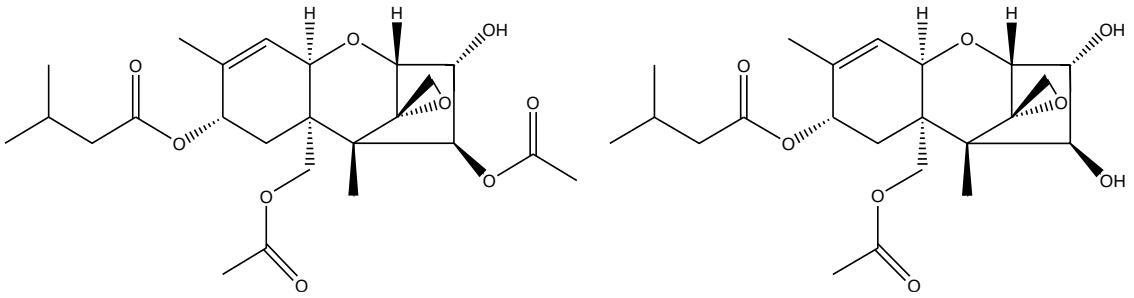
Zearalenone is soluble in alcohols, acetonitrile, benzene, chloroform, alkaline solutions but insoluble in water (Gromadzka *et al*., 2008). Zearalenone, α-ZEL and β-ZEL are chemically stable during oven heating, roasting a rotary gas-fired roaster, cooking, extrusion, milling and storage,

which may rationalize their quantification in processed food products such as beers, bread and processed animal feed (Yumbe-Guevara *et al*., 2003; Gromadzka *et al*., 2008; Hueza *et al*., 2014). Numanoglu *et al*. (2013) modelled the thermal degradation of ZEA in bread made from naturally contaminated maize during baking at temperatures ranging from 100 to 250 °C. The authors reported no degradation of ZEA at 100 °C. Maximum degradation of ZEA was 28% which was achieved at 250 °C (for 15 min). However, statistical analysis of the results revealed that none of the degradations at the studied temperature conditions was statistically significant ($p>0.05$). Foods contaminated with ZEAs have been linked to female reproductive changes due to their potent estrogenic activity, which exceeds that of many naturally occurring non-steroidal estrogens (Bennett and Klich, 2003). Exposure to high concentrations of ZEA in cattle feed have been linked with enlargement of the mammary gland, infertility, reduced milk production, vaginal secretions and vaginitis particularly in young dairy heifers (Zinedine *et al*., 2007). In swine, effects of ZEA include enlargement of the uterus, vaginal prolapse, swelling of the vulva, infertility, reduced litre size and embryonic death (Agag, 2004). These server effects of ZEAs in animals make its exposure a concern for human health.

2.2.5 T2-toxin and HT-2-toxin

T2-toxin (T-2) is a type A trichothecene mycotoxin, and consists of a tetracyclic sesquiterpenoid 12,13-epoxytrichothene ring system (Mbundi *et al*., 2014; Adhikari *et al*., 2017). Together with its analogue HT-2 toxin (HT-2), these toxins (Figure 2.5) are reported to be produced by *F. poae*, *F. sporotrichioides*, *F. acuminatum* and *F. equiseti*, which are the predominant pathogens of rice, wheat, maize and other cereal grains including their processed products (D'Mello, 2003; Galbenu-Morvay *et al*., 2011). T2-toxin and HT-2 have been reported to have different derivatives which exist in various modified forms such as glucosides (McCormick *et al*., 2015; Bryła *et al*., 2018; Schmidt *et al*., 2018). It has been suggested that wheat and oat may metabolize T-2 and HT-2 to T-2 3-O-glucoside (T-2Glc) and to HT-2-O-3-glucoside (HT-2Glc) (Busman *et al*., 2011). Several studies have reported the natural occurrence of glucosides of T-2 and HT-2 in maize, wheat, barley and oat grain (Veprikova *et al*., 2012; Nakagawa *et al*., 2013; Lattanzio *et al*., 2015). Lattanzio *et al*. (2015) performed the first quantitative analysis of T-2 and HT-2 glucosides. The ratio between the free toxins (i.e. sum of T-2 and HT-2) and their corresponding glucosylated forms (i.e. sum of T-2 and HT-2 glucosides) was found to range from 2 to 283%. The authors reported levels up to

163 µg/kg in 17 of the 18 analysed unprocessed barley grains for the glucosyl derivative of HT-2, whereas, much lower levels (and in fewer samples) were reported for the monoglucosyl derivative of T-2.



(a) (b)

Figure 2.5: Molecular structures of (a) T-2 and (b) HT-2 (Adhikari *et al*., 2017).

There is limited information in literature on the thermal stability of T-2 and HT-2. However, it is known that T-2 is non-volatile and relatively stable to adverse photo and thermal degradation conditions, as well as, during milling, storage, autoclaving, cooking, and other food processing conditions, though easily deactivated by strongly alkalinic or acidic conditions (Marin *et al*., 2013; Adhikari *et al*., 2017). Schmidt *et al*. (2017) systematically investigated the degradation of T-2 and HT-2 in naturally and artificially contaminated oats during industrial and laboratory extrusion cooking. There was a higher degradation in artificially contaminated oats compared to the naturally contaminated oats. The laboratory cooking conditions for fortified oats resulted in 35 and 22% degradation of H2 and HT-2, respectively. Generally, higher degradation was observed in T-2 as compared to HT-2. In another study, Kuchenbuch *et al*. (2018) investigated the heat stability of T-2 and HT-2 during the baking and roasting process for biscuit, crunchy muesli and toasted oat flakes-production. Again, the authors reported higher thermal instability for T-2 as compared to HT-2; up to 45 and 20% of T-2 and HT-2, respectively, were thermally degraded during the biscuit-making process.

Though insoluble in water, T-2 is soluble in ethanol, methanol, acetone, ethyl acetate, chloroform and propylene glycol (Adhikari *et al*., 2017). Its epoxide ring and several hydroxyl and acetyl functional groups on the side chains are responsible for its potent toxicity and biological activity (Vanhoutte *et al*., 2016). T2-toxin is known to be genotoxic (Horvatovich *et al*., 2013) and has been reported to induce apoptosis (Fang *et al*., 2012). In China, consumption of mouldy rice

contaminated with *Fusarium* and T-2 have been implicated in human mycotoxicosis (Wang *et al*., 1993).

2.2.6 Sterigmatocystin

Sterigmatocystin (STEG) is an AF-related substance produced during the biosynthesis of AFB_1 as an intermediate molecule (Nieto *et al*., 2018). Its chemical structure (Figure 2.6) consists of a bifuran structure to which a xanthone nucleus is attached (Chen *et al*., 2010). Sterigmatocystin is mainly produced by *A. versicolor* and *A. nidulans* (Nieto *et al*., 2018), and has been reported as a contaminant of a variety of poor-quality cereal crops and other food/feed products such as maize, wheat, animal feed, pecan nuts and hard cheese (Ferrante *et al*., 2012). There are indications that the toxicity of STEG is much similar to that of AFB_1, as such, it is considered a potent mutagen, carcinogen and teratogen (Veršilovskis & De Saeger, 2010; Huang *et al*., 2014b; Nieto *et al*., 2018). However, literature reports on the occurrence and prevalence of this mycotoxin is scarce. This lack of information may be due to limitations in analytical methods, and may be the reason for lack of specific regulatory limits for this toxin in food and feed (Stroka *et al*., 2004). Nonetheless, some European (EU) countries such as Czech republic and Slovakia have regulations for STEG ranging from 5-20 μg/kg depending on the food commodity (Stroka *et al*., 2004). There is currently no information in literature on the thermal stability of STEG.

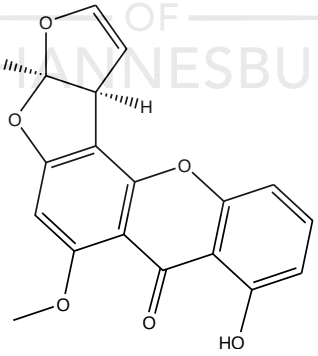


Figure 2.6: Molecular formula of sterigmatocystin (Nieto *et al*., 2018).

2.2.7 Alternaria toxins

Alternaria toxins are a group of possible health-endangering secondary metabolites produced mainly by the *Alternaria* fungal species (Escrivá *et al*., 2017; Hickert *et al*., 2017). In terms of

their chemical structure, these mycotoxins are related to FBs and are reported to contaminate mainly fruits and vegetables, however, they are equally found in other crops, especially cereals (Ostry, 2008; Escrivá *et al*., 2017). *Alternaria alternata* is the most important mycotoxin-producing species of the *Alternaria* species, and frequently contaminates a number of economically important crops (Li *et al*., 2001; Meena *et al*., 2017). Many important Alternaria toxins are known to naturally contaminate *Alternaria*-infected crops, some of which include alternariol, tenuazonic acid, altenuene, alternariol monomethyl ether (AME), and altertoxin I, however, for the purpose of this review, the focus would be on AME, due to its economic and health significance in various cereal crops from Africa.

The Alternaria toxin, AME, is a benzopyrone secondary metabolite, structurally elucidated as 3,7-dihydroxy-9-methoxy-1-methyl-6H-dibenzo[b,d]pyran-6-one (Figure 2.7) which is produced mainly by *Alternaria alternata* (Scott *et al*., 2012), though other Alternaria species (Ostry, 2008; Logrieco *et al*., 2009), and other fungi genera such as *Phomopsis strains* (Abreu *et al*., 2012) and *Stagonospora nodorum* (Tan *et al*., 2009) are reported to produce this mycotoxin as well. Alternariol monomethyl ether is a mycotoxin that is prevalent in the temperate regions of the world (Juan *et al*., 2016; Escrivá *et al*., 2017), however, recent reports show its increasing presence in more tropical countries, particularly in Africa (Van de Perre *et al*., 2015; Abass *et al*., 2017; Hickert *et al*., 2017). Favored food substrates include rice, maize, barley, sorghum, bread and other bakery products, fruits, vegetables and beverages (Asam *et al*., 2011; Escrivá *et al*., 2017). Despite being identified and characterized over 65 years ago (Raistrick *et al*., 1953), there are still limited studies on the toxicity of this compound. Alternariol monomethyl ether is reported to be mutagenic *in vitro* (Schrader *et al*., 2001; Yekeler *et al*., 2001; Scott *et al*., 2012). Podlech and Marko (Podlech & Marko, 2009) observed that AME and alternariol (AOH) induced breaks in DNA strands in cell cultures (Podlech & Marko, 2009). There is limited information in literature on the thermal stability of AME, however, it has been shown to be thermally stable during wet baking conditions (Siegel *et al*., 2010).

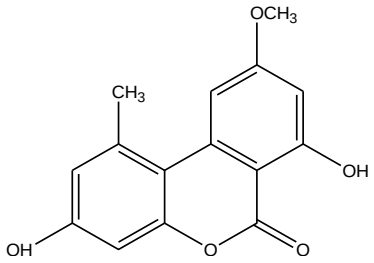


Figure 2.7: Molecular structure of alternariol monomethyl ether (Ostry, 2008)

2.2.8 Other mycotoxins

Aside from the afore-mentioned mycotoxins, many other mycotoxins exist which are equally of health and economic significance, such as citrinin (CIT), nivalenol (NIV), deoxynivalenol (DON), patulin (PAT), moniliformin (MON), ergot alkaloids (EAs), Fusarenon-X (FUS-X), penicillic acid (PA), including emerging mycotoxins such beauvericin (BEA), 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON), and the enniatins (ENNs). Nivalenol and FUS-X usually co-occur in nature and are some of the best known THs (Omurtag, 2008; Saengtienchai *et al*., 2014). Diacetoxyscirpenol (DAS) is prevalent in mixed feed samples and various cereal crops such as barley, maize and other grains around the world. The co-occurrence of T-2 and DAS in food and feed constitutes poses a concern to human and animal health in some parts of the world (Omurtag, 2008; Rodrigues *et al*., 2011). The fungal genera *Balarisia*, *Acremonium*, *Aspergillus* and *Claviceps* are responsible for the production of EAs (Panaccione *et al*., 2006; Schardl *et al*., 2006), which have been linked with a number of outbreaks of mycotoxicosis around the world (Schneider *et al*., 1996; De Costa, 2002; Naude *et al*., 2005). The prevalence of these mycotoxins in food crops have been reviewed extensively in literature (Cardwell, 2000; Darwish *et al*., 2014; Magoha *et al*., 2014; Misihairabgwi *et al*., 2017), and can be strongly associated with a number of socio-economic impacts (Gbashi *et al*., 2018).

Another important group of mycotoxins that are gaining increased popularity recently are the modified mycotoxins. Modified mycotoxins refer to any alteration of the basic chemical structure of the mycotoxin enabled by matrix-association, biological (plant, animal or fungi) or chemical (thermal or non-thermal processing) modifications (Rychlik *et al*., 2014). These modified forms of the mycotoxins are usually undetected during conventional analysis of mycotoxins (Chilaka *et al*., 2017; Freire & Sant'Ana, 2018). A case in point which stimulated interest in the study of

modified mycotoxins was reported by Gareis *et al*. (1990) where mycotoxicosis symptoms were observed in animals fed with feed containing relatively low levels of mycotoxins. The unexpected elevated toxicity which could not be correlated with the levels of the mycotoxins in the feed was credited to hidden (conjugated) forms of mycotoxins present in the animal feed. After ingestion, these modified mycotoxins are "unmasked" *i.e*. converted into free mycotoxins in the digestive system of the animals (Gareis *et al*., 1990). As a result of this finding and other subsequent corroborating reports in the literature (Ajandouz *et al*., 2016; Gratz *et al*., 2017), there has been growing interest in the prevalence and toxicity of modified mycotoxins (Galaverna *et al*., 2009; Freire & Sant'Ana, 2018). Advances in analytical methodologies such as the use of high-resolution mass spectrometry (HR-MS) coupled with ultra-high performance liquid chromatography (UPHLC) have enabled easier and more efficient study of these mycotoxin and the discovery of newer ones (Cirlini *et al*., 2012; Rychlik *et al*., 2014).

The occurrence of various modified mycotoxins have been reported in African commodities (Shephard *et al*., 1994; Chilaka *et al*., 2016, 2017). Zearalenone-4-sulfate (ZEN-4S) has been reported in Cameroonian maize beer in the range of 0.01 to 0.6 µg/kg (Abia *et al*., 2013). Deoxynivalenol-3-glucoside (DON-3G) and Hydrolysed FB_1 (HYD FB_1) have been reported in stored maize from Nigeria (Adetunji *et al*., 2014). Seventy-seven animal feed samples from Egypt were screened for the presence of multiple mycotoxins by Abdallah *et al*. (2017). All samples were contaminated with at least four toxins, the presence of modified mycotoxins such as deoxynivalenol-3-glucoside and other emerging mycotoxins such as beauvericin and fusaproliferin were established.

2.3 EXPOSURE TO MYCOTOXINS AND THE SOCIO-ECONOMIC IMPACT

The presence of mycotoxins in the food and feed supply chain is of much concern because it results in serious socio-economic problems.

2.3.1 Exposure to mycotoxins

Common route of exposure to mycotoxins include oral ingestion of mycotoxin-contaminated food and feed, however, dermal, inhalation (respiratory) and parental (placental and breastfeeding) exposure have also been reported (Boonen *et al*., 2012; Gbashi *et al*., 2017c). After assimilation

into the body, mycotoxins enter the blood stream and lymphatic system, and their actions are usually mediated in important body organs and systems such as the kidney, liver, lungs, immune system, endocrine and nervous systems (Bennett & Klich, 2003; CAST, 2003; Atanda *et al*., 2013). Exposure to mycotoxins can be chronic or acute, and the symptoms and severity of the resulting illness is dependent on the type of and concentration of the mycotoxin, as well as, the duration of exposure, age, sex, species and health status of the victim (Bennett & Klich, 2003).

The illness that results from consumption of mycotoxin-contaminated foods is termed mycotoxicosis (Richard, 2007). If there are evident symptoms within a period of seven days of exposure, it is termed "acute mycotoxicosis" and the victim may die if no proper medical attention is given. However, "chronic mycotoxicosis" has a longer window period of over seven days and the victim may survive though with protracted illnesses (Atanda *et al*., 2013). Mycotoxicosis resulting from AF poisoning is referred to as "aflatoxicosis" (Dhanasekaran *et al*., 2011), that resulting from poisoning by OTs is termed "ochratoxicosis" (Ibrahim *et al*., 2013), whereas mycotoxicosis resulting from exposure to ergot alkaloids is termed "ergotism" (Naude *et al*., 2005). A major public health concern of exposure to mycotoxins is that outbreaks of mycotoxicosis often remain unrecognized by health workers for an extended period of time, except when a large number of people are affected (Gbashi *et al*., 2017c).

Numerous cases of mycotoxicosis have been reported in literature. The most recently reported instance of mycotoxicosis was the 2016 outbreak of acute aflatoxicosis in the central part of Tanzania which resulted in 68 cases, 50% of which were below the age of 15 years (Kamala *et al*., 2018). Up to 30% of the cases ended in mortality. Highly contaminated home-grown maize (10 to 51,100 μg/kg) was implicated as the cause for the outbreak. In Ibadan, Nigeria, the death of some children who consumed mold-infested *kulikuli* was linked with aflatoxicosis (Atanda *et al*., 2013). An outbreak of hepatitis in 1974 in India that killed 100 people and caused ailment in hundreds of others was as a result of exposure to AFs from heavily contanminated maize (Montville and Matthews, 2008). Fumonisins have been reported to be the cause of hepatoxicity, carcinogenicity and nephrotoxicity in numerous animals (Howard *et al*., 2001; Voss *et al*., 2007; Stockmann-Juvala & Savolainen, 2008). One of the most epic episodes of mycotoxicosis reported in human history occurred in rural Kenya, of which 125 deaths and 317 cases of illnesses were reported (Lewis *et al*., 2005). The cause of this outbreak was deciphered to be consumption of maize

products contaminated with AFs (above the Kenyan regulatory limit of 20 µg/kg). Examples of well-known cases of mycotoxicosis and details on several other known mycotoxin related diseases in animals have also been provided by Pettersson (2004), Richard (2007) and Peraica *et al*. (2014).

2.3.2 Socio-economic impact of mycotoxins

Mycotoxin contamination has contributed significantly to the elusive sustainable development in many parts of the world. The socio-economic significance of mycotoxin prevalence has reached global proportions, with both the developed and developing nations incurring severe consequences, though the developing nations are the most affected.

2.3.2.1 Impact on health

The most significant impact of mycotoxin contamination has been demonstrated to be on human and animal health. Some of the significant health problems of mycotoxins have previously been described in Section 2.3.1, where different incidences of mycotoxicosis around the world, including those of aflatoxicosis in Nigeria, Kenya, India and Tanzania have been described. The 1993 World Bank report highlighted the various health problems modulated by exposure to mycotoxins accounting for up to 40% of lost disability-adjusted life years (DALYs) (Marechera & Ndwiga, 2014). In sub-Saharan Africa, about 250,000 deaths are caused by hepatocellular carcinoma annually and this can be linked to risk factors such as AFs and high prevalence of hepatitis B (Zain, 2011). AF contamination in groundnuts and maize in Nigeria contributed to 7,761 liver cancer cases, which results in a total burden of 100,965 DALYs (Atanda *et al*., 2015). In 2014, due to AF contamination, about 3,334 cases of hepatocellular carcinoma were reported in Tanzania, 95% of which ended as deaths resulting to a loss of 96,686 DALYs (Kimanya *et al*., 2012).

Based on several studies in Southern Africa, mycotoxin contamination has been strongly linked to child undernutrition, increased mortality and morbidity due to their negative effects on micronutrient absorption and immune function (Katerere *et al*., 2008). With immune disruption by AFs, it may aggravate health impacts of principal diseases plaguing Africa such as malaria, kwashiorkor and HIV/AIDS (Gnonlonfin *et al*., 2013). In Nigeria, posthumous autopsy of infants who suffered from kwashiorkor showed significant levels of AFs in their brains after consumption of AF-contaminated maize-based gruel (Oyelami *et al*., 1997). According to Jolly *et al.* (2007),

high levels of AFB_1 and acute aflatoxicosis symptoms were found within the Ghanaian population that also had abnormal liver function and high level of hepatitis B virus (HBV) infections. Turner *et al*. (2003) reported decreased levels of secretory immunoglobulin A (IgA) in Gambian children exposed to AFs. In Kenya, the mean birthweight of the children of women exposed to AFs prenatally was lesser than that of those who had not been similarly exposed (Hendrickse, 1999).

In the Gambia, maternal dietary intake was indicated to be an important factor in carcinogenic-induced damage in the unborn baby, due to a highly significant correlation between AF-albumin adduct levels in the mothers' venous and respective cord sera (Wild *et al*., 1991). In the same country, children with reduced levels of salivary Secretory Immunoglobulin A (sIgA) have been linked with exposure to AFs (Turner *et al*., 2003). The consumption of heavily FB-contaminated maize has been correlated to increased incidence of oesophageal cancer in some parts of South Africa (Wagacha & Muthomi, 2008) and Malawi (Ferlay *et al*., 2013). According to Ferlay *et al*. (2013), Malawi has the highest prevalence rate (24.2 per 100,000 persons) of oesophageal cancer in the world. Zearalenone as a naturally occurring endocrine-disrupting chemical, has been implicated in the manifestations of gynecomastia with testicular atrophy in rural males in Southern Africa (Shephard, 2008). Between 1977 and 1978, an outbreak of ergotism occurred in Wollo, Ethiopia wherein, 140 persons were affected, 4 children lost both or at least one leg and mortality as high as 34% (King, 1979).

In North Africa, particularly Tunisia and Egypt, cases of human nephropathies have been strongly associated with elevated exposure to OTA and cases of ochratoxicosis, *i.e*., illness due to OTA exposure (Maaroufi *et al*., 1995; Wafa *et al*., 1998; Zaied *et al*., 2011). Alpha-ZEA has been implicated as a potential risk factor for breast cancer in Tunisia (Belhassen *et al*., 2015). Likewise, high levels of OTA in Moroccan foods and other agricultural commodities have been linked to some chronic illnesses (Filali *et al*., 2002; Zinedine & Mañes, 2009). Table 2.1 shows some other mycotoxins and the toxic effects they provoke in humans. On the Asian continent, consumption of mouldy rice contaminated with *Fusarium* and T-2 was associated with the first human mycotoxicosis case in China, with an incidence rate of 59% and a latent period of only 10-30 min (Wang *et al*., 1993). Further studies are required to establish the association between other poorly investigated diseases and dietary exposure to mycotoxins (emerging, modified and multiple mycotoxins).

Table 2.1: Mycotoxins and their toxic effects on human health. Adapted from Capriotti *et al*. (2012).

Mycotoxins	Toxic effects	Reference
EAs	Ergotism: central nervous system disorder, gastrointestinal symptoms, & gangrene	Capriotti *et al*. (2012)
CIT	Hepatonephrotoxic	Capriotti *et al*. (2012)
PAT	Genotoxic, teratogenic, carcinogenic, & acute toxicity to kidney	Capriotti *et al*. (2012)
STEG	Carcinogenic, & hepatotoxic	Capriotti *et al*. (2012)
MON	Acutely toxic, & cardiac impairment	Capriotti *et al*. (2012)
AFs	Carcinogenic, & immunosuppressive	Gbashi *et al*. (2017)
OTs	Mutagenic, carcinogenic, & nephrotoxic	Clark & Snedeker, (2006); Palma *et al*. (2007); Pfohl-Leszkowicz & Manderville, (2007); Malir *et al*. (2016)
FBs	Carcinogenic, nephrotoxic, hepatotoxic, immunosuppressive, atherogenic, & embryotoxic	Nair, (1998); Soriano & Dragacci, (2004)
DON	Immunosuppressive, immunostimulative, & causes fertility problems	Bondy & Pestka, (2000); Pinton *et al*., (2010); Becker *et al*., (2011); Awad *et al*., (2013)
ZEAs	Infertility, reduced milk production, vaginal secretions, & vaginitis	Agag, (2004); Zinedine *et al*. (2007)
T-2	Cardiovascular defects, gastroenteritis, & alimentary toxic aleukia	Semple *et al*. (1989); Yuan *et al*. (2014)

Key: EAs – ergot alkaloids. CIT – citrinin. PAT – patulin. STEG – sterigmatocystin. MON – moniliform. AFs – aflatoxins. OTs – ochratoxins. FBs – fumonisins. DON – deoxynivalenol. ZEAs – zearalenone, α-zearalanol and β-zearalanol. T-2 – T-2 toxin.

Though tremendously difficult to estimate, the net monetarized impact of mycotoxins on human health in Africa [including physical pain, death (in severe cases), temporary or permanent impairment, loss of productivity, costs of diagnosis, treatment, hospitalization and health care (morbidity), cost of anxiety, misdiagnosis, suffering and reduced life quality, etc] could be enormous and demanding on national budget. A case in point is a study conducted in Gambia that observed diseases consistent with mycotoxin exposure (in particular, Hepatitis B and its associated medical complications) that resulted in a total monetized DALY worth over 94 million US$ of gross domestic product (GDP), which equals 9.4 % of the nation's GDP (ECOACAP, 2014). This

is a huge loss to the health of the populace and country's economy. Similarly, in Senegal, the cumulative cost in terms of health due to AFs is estimated at no less than 92 million US$ of the nation's GDP (ECOACAP, 2014). In 2014 in Tanzania, the economic impact (in monetary terms) of AFs was estimated between 6 million and 264 million US$ due to the resultant health impact (Kimanya *et al*., 2012).

For animals, very little work has been done on health impact of mycotoxins even though the term 'mycotoxins' was first coined in 1960 following the discovery that 'Turkey X' disease in which about 100,000 turkey poults died was caused by consumption of poultry feed which was contaminated by secondary metabolites of fungi origin (specifically AFs from *A. flavus*) (Bennett & Klich, 2003). This is understandable as the health effects and losses in animals (such as feeding efficiency, infertility, meat, milk and egg quality losses, susceptibility to diseases etc) are subtler to decipher. Moreover, in Africa, people have limited resources and may prioritize the care of humans above the 'waste of resources' on animals. To this effect, when mouldy cereals are too bad to be consumed, they are usually not disposed, but blended with non-mouldy ones and used as animal feed, or in some cases fed directly to the animals. However, monogastric farm animals such as poultry, swine and dogs are particularly at high risk, because their basal diet (feed) is made up of cereals (Bhat *et al*., 2010). Additionally, these animals also lack reservoir that harbours microorganisms that can break down secondary metabolites of fungi before they are absorbed into the blood stream.

In South Africa, there has been two episodes of aflatoxicosis among dogs through the consumption of contaminated dog food. The first occurred in 1987 where 10 cases of fatality were reported, and histopathological evaluation revealed chronic symptoms of necrosis, bile duct proliferation, hepatocellular fatty degeneration, fibroplasia, etc. were observed (Bastianello *et al*., 1987). The second episode occurred in 2011 wherein, over 220 dogs died, and several others were affected in the Gauteng province (Arnot *et al*., 2012). Subsequent clinical examinations revealed that the dogs were exposed to highly contaminated feed (with levels of AFs as high as 4,946 µg/kg), which is several folds above regulatory limits. In addition to AFs, other mycotoxins such as FB_1, ZEA, and OTA were all later implicated in this outbreak (Mwanza *et al*., 2013). Mwanza *et al*. (2007) evaluated the productivity and general health of domesticated animals in Limpopo Province of South Africa in relation to fungal and mycotoxin contamination, the results revealed that these

animals were at risk to mycotoxin contamination, which possibly plays an important role in abortion, low productivity, chronic and acute diseases, as well as reduced immunity in these animals. All these effects of mycotoxins reviewed herein, seriously impact negatively on food security, agricultural trade and the economy of any nation.

2.3.2.2 Impact on food security, agricultural trade and the economy

Mycotoxins have also been implicated as a major cause of food insecurity particularly in the developing nations. It has been estimated that at least 25% of global food and feed commodities is contaminated by mycotoxins; consequent food losses have been calculated at about 1 billion metric tons annually (Rahmani *et al*., 2009; Schmale & Munkvold, 2009; Rajani *et al*., 2012; Wilson *et al*., 2017). Such huge losses undoubtedly strain economic fortunes and stability. For example, losses linked with AF contamination of maize in the United States is estimated at around 1.68 billion U$D annually (Schmale & Munkvold, 2009; Mitchell *et al*., 2016). Due to high levels of AF contamination, about 2.3 million bags of maize worth over Ksh 3.2 billion *i.e.*, roughly 30 million U$D were declared unfit for human consumption by the Kenyan Ministry of Public Health and Sanitation in 2010 (Marechera & Ndwiga, 2014).

Globalization of trade has added to the cost and complexity of the situation. In Kitui, Kenya in 2009, it was reported that maize prices dropped by half from 1,800 to 900 Kenyan shillings due to concerns over possible contamination by AFs (Marechera, 2015). The enforcement of regulatory standards primarily by developed nations which are the main destinations of African agricultural export commodities have resulted in a more critical situation for the African agricultural trade (Wu, 2004; Gbashi *et al*., 2017c). It was estimated that adopting the EU standard limit of 4 µg/kg for AFs in peanuts would cost about 450 million U$D in annual losses on exports (Wilson & Otsuki, 2001; Schmale & Munkvold, 2009). Between 2000 and 2014, the cumulative economic loss on domestic and international trade in Gambia was about 23 million U$D, which amounts to a yearly loss of about 1.52 million U$D (ECOACAP, 2014). The International Institute of Tropical Agriculture (IITA) reported an annual global loss of 1.2 billion U$D due to AF contamination, with 38% of this loss (450 million U$D) incurred by African nations (Lamb et al., 2015).

In 2014, the Economic Community for West African States (ECOWAS) in collaboration with the African Union's Partnership for Aflatoxin Control in Africa (PACA) and other stakeholders

developed the "ECOWAS Aflatoxin Control Action Plan (ECOACAP)" which identified key actionable strategic interventions in order to combat the prevalence of AFs across ECOWAS member States. Policy 4.3 SO3 of this plan recommended that ECOWAS member states increase budgetary allocations and investments to at least 1% of national GDP for the development and enforcement of AFs control efforts (ECOACAP, 2014). An annual cost of 7.5 million U$D was calculated by member states of the African Groundnut Council (Mali, Nigeria, Gambia, Sudan, Niger and Senegal) for the implementation of an AF contamination reduction program (Atanda *et al*., 2013). The Maize Trust, an initiative principally funded by the government of South Africa, spends over 4 million U$D per annum on funding projects directly targeted at improving the South African maize industry, and one of the outlined key objectives is to combat mycotoxins in South African maize (Du Plessis, 2014).

2.3.2.3 Commitment to research and the cost implications

Mycotoxicological research is an important component of mycotoxin management and provides insight on the toxicity, level of exposure, and public health significance of mycotoxins. Such knowledge is critical for adoption of appropriate mitigation strategies and regulatory limits for the toxins. The lack of regulation in the developing countries may be due to limited data on the prevalence of mycotoxins in these countries, poverty and inadequate research infrastructure (Darwish *et al*., 2014). Two mycotoxin intervention initiatives, Aflasafe™ and Aflastop projects, were implemented to combat the prevalence of AFs in foods (particularly maize and peanuts). The former (*i.e*. Aflasafe™) being implemented in the West African countries of Nigeria, Ghana, Tanzania, Burkina Faso, Senegal, Kenya, Mali, and Zambia, whereas, the latter (*i.e*. Aflastop) was adopted in the East African countries of Kenya, Tanzania and Rwanda. The Aflasafe™ and Aflastop projects together with other mycotoxin projects described by Bowman *et al*. (2012) and CIMMYT (2013) costed about 15-20 million U$D in 2014 and 2-5 million U$D in 2010, sponsored by the US Government under the Feed the Future (FTF) - USAID Bureau for Food Security (Bowman *et al*., 2012; CIMMYT, 2013).

A study conducted in West Africa estimated annual costs averaging 466 million U$D from testing, regulatory enforcement, to other quality control measures (CAST, 2003). In 2000, the United States Department of Agriculture (USDA) Agricultural Research Service (ARS) instituted a

mycotoxin research program worth 17.7 million U$D primarily geared towards prevention of fungal contamination and toxin production in crops (Robens & Cardwell, 2003). On average, total value of commercially available test kits for AFs on the market is approximately 10 million U$D annually, whereas the cost for analysis of AFs alone is placed at 30 to 50 million U$D on annual basis (Robens & Cardwell, 2003).

2.4 ANALYSIS OF MYCOTOXINS

Due to the severe effects that mycotoxins elicit in humans and animals, and with the globalization of trade, several countries and economic unions have placed high priority on the safety of agricultural commodities marketed and consumed within their jurisdiction (FAO, 2004b; Van Egmond *et al*., 2007; Gbashi *et al*., 2017c). However, it is apparent that the complete elimination of mycotoxins from foods is unrealistic (Bennett & Klich, 2003). This has led to various interventions put in place to control and minimize exposure to them (Lopez-Garcia *et al*., 1999; Aldred *et al*., 2004). Adequate risk management has been identified as a critical frontline defense in the overall control of mycotoxins in food and feed (FAO/IAEA, 2001; Aldred *et al*., 2004; Toregeani-Mendes *et al*., 2011; Atanda *et al*., 2013; Gil *et al*., 2016).

Any good food safety management programme for naturally occurring toxicants such as Hazard Analysis of Critical Control Points (HACCP) assumes a holistic and multifaceted approach, such as determination of exposure levels, establishment of analytical capabilities, setting and ensuring compliance to regulatory limits, and establishment of surveillance programmes (Atanda *et al*., 2013; Gil *et al*., 2016). This positions analysis at the epicenter of mycotoxin management and risk control which is a global priority (Patel, 2004; Krska *et al*., 2008). Bearing in mind that decisions relating to regulatory issues or commercial arbitration need to be based on well-defined methods of analysis (Gil *et al*., 2016), it is thus vital to ensure that methods for mycotoxin analysis are sensitive, efficient and validated against standard guidelines (Pittet, 2005; Arroyo-Manzanares *et al*., 2014).

2.4.1 Sampling

Sampling is an important part of a good and accurate mycotoxin analytical method. This is because mycotoxins are often not evenly distributed in agricultural commodities (Krska *et al*., 2008; Rahmani *et al*., 2009), as such, if the sample fraction is not selected correctly, it can lead to large

errors when the result obtained for the selected sample is used to extrapolate for the larger population. It has been estimated that up to 76% of the total uncertainty in an analytical procedure is due to sampling error (Biomin, 2018). It is thus the main objective of sampling to obtain a sample fraction that is as representative as possible to the overall sample population.

The importance of sampling in mycotoxin analysis has been recognized by various international regulatory bodies, and various guidelines have been established in this regard (FAO, 2004b; Commission Regulation, 2006a, 2014; FDA, 2018). The general approach is to obtain a large number of small incremental samples from different locations distributed throughout the entire sampling population which helps to get a more representative sample (FSA, 2015). For example, for sampling of cereals and cereal products from lots < 50 tonnes, the EC Commission Regulation 401/2006 as amended by Commission Regulation 519/2014 requires that 10 to 100 small incremental samples be obtained, depending on the weight of the entire sample lot, resulting in a combined sample of 1 to 10 kg. If however the sample lot is very small i.e. ≤ 0,5 tonnes, then a lower number of small incremental samples may be obtained, notwithstanding, the combined sample should be at least 1 kg (Commission Regulation, 2006a, 2014).

2.4.2 Extraction of mycotoxins

The extraction and sample preparation part of an analytical process is often the most critical and difficult, both in terms of difficulty in extracting the desired analyte(s) from the matrix and the time involved, in addition to the fact that each sample matrix has its own unique challenges (Vaghela *et al*., 2016). In fact, it has been estimated that up to 70% and perhaps even more of the effort and time that goes into sample analysis comprises the extraction and sample preparation process (Rezaee *et al*., 2015). Many efforts have been geared towards developing suitable methods to quantitatively extract and detect mycotoxins in agricultural commodities. For any bioanalytical scientist, the goal is to develop methods with improved sensitivity and selectivity, while at the same time maintaining the credibility of the results, as well as reduce cost and time (Augusto *et al*., 2013).

A number of studies have aimed at designing methods for mycotoxin analysis that avoid a clean-up step, however, such easy-to-use methods often demonstrate lack of sensitivity or are too expensive (Rahmani *et al*., 2009). An efficient combination of these two characteristics (efficacy

and low-cost) in one method is rare and highly desirable. Proper design of the extraction process facilitates rapid, efficient and quality analytical results (Rezaee *et al*., 2015). In the succeeding section, a description of some widely referenced conventional methods of mycotoxin extraction will be provided.

2.4.2.1 Conventional methods of mycotoxin extraction

Solvent extraction

Of the available methods for mycotoxin extraction, solvent extraction is one of the oldest but still most frequently used method (Patterson & Roberts, 1979; Cigić & Prosen, 2009). Solvent extraction separates analytes based on their solubilities and mass transfers in organic solvent(s), often by adding the solvent to the sample containing the analyte of interest and shaking rigorously (Patterson & Roberts, 1979; Monbaliu *et al*., 2010; Kovalsky *et al*., 2016). The enriched solvent is called the extract. Common solvents used for solvent extraction include methanol, acetonitrile, chloroform, ethyl acetate, isooctane, ethanol and dichloromethane (Zabe *et al*., 2008; Cigić & Prosen, 2009; Chilaka *et al*., 2012). One of the earliest and frequently used solvent extraction method for mycotoxin analysis is the multi-mycotoxin extraction method of Patterson and Roberts, (1979). This method utilizes different organic solvents and reagents such as acetonitrile, isooctane, potassium chloride, dichloromethane and sulphuric acid. It has been widely favored because, it selectively extracts several mycotoxins in a single extraction. However, the application of this solvent extraction method, and perhaps other solvent-based extraction methods has been greatly limited because of the consumption of large quantities of organic solvents, which pose hazards to public health and the environment (Santana *et al*., 2009; Tabaraki & Nateghi, 2011).

These solvents are often toxic to humans, chlorinated, and reasonably contribute to greenhouse effects and other detrimental environmental impacts (Ismail & Hameed, 2013; Gbashi *et al*., 2017d). On the other hand, green solvents, are innocuous, biodegradable and generally derived from sustainable and renewable resources often naturally occurring (Sherman *et al*., 1998; Prat *et al*., 2013). Aside the development of harmless and effective procedures, efforts are made in adopting greener solvents, particularly in earliest stages of the design of the extraction processes (Bradley *et al*., 2015). For example, in the United States, the Pollution Prevention Act passed in 1990 encouraged the reduction or prevention of pollution at the source, hence limiting consequent

environmental issues before they happen (Sherman *et al*., 1998). The California Green Chemistry Initiative launched in 2008 approved two legislations encouraging green chemistry, one of which required California's Department of Toxic Substances Control (DTSC) to develop new regulations to ensure prioritization of "chemicals of concern" and promote the use of safer alternatives, the resulting regulations which took effect in 2013, initiating the DTSC's Safer Consumer Products Program (Cowan *et al*., 2014).

Another disadvantage of solvent-based extraction methods is that they often involve long extraction times and laborious procedures with the process extending up to 24 h or more (Patterson & Roberts, 1979; Chilaka *et al*., 2012). Moreover, solvents of the required purity tend to be expensive and there are often additional costs with proper disposal of wastes after use (Sapkale *et al*., 2010; Tabaraki & Nateghi, 2011).

Solid-phase extraction

Solid phase extraction (SPE) is another very commonly used extraction method for mycotoxins (Mata *et al*., 2015; Wang *et al*., 2016). It involves the separation of analytes between a liquid mobile phase and a stationary phase contained in a cartridge. Typical materials used as the solid adsorbent include ethyl (C2), octyl (C8), octadecyl (C18), cyanopropyl (CN), aminopropyl (NH2), and an ion exchange phase (Limsuwan, 2011). Non-specific SPE materials are commonly still employed in mycotoxin analysis, which is often used for the extraction of more than one mycotoxin (Cigić & Prosen, 2009). The use of more analyte specific stationary phases such as molecularly imprinted polymers (Pakade *et al.*, 2013), magnetic solid-phase extraction (Ibarra *et al.*, 2015), and immuno-affinity (IA) materials that contain specific antibodies that bind to the analyte of interest are also gaining much attention (Gam *et al*., 2003; Uchigashima *et al*., 2012; Arroyo-Manzanares *et al*., 2014). Although SPE techniques are relatively simple, have higher specificity and require little quantities of solvents, they are also very expensive, and the antibodies are not available for some mycotoxins and products.

Microwave-assisted extraction

Microwave-assisted extraction (MAE) involves the use of microwave energy to increase the thermal energy of solvents in contact with sample matrices in order to enhance the mass transfer of analytes from the sample matrix into the solvent (Devgun *et al.*, 2009). In recent times, there

has been an increased popularity in the use of MAE techniques in bioanalytical sciences (Devgun *et al.*, 2009; Sibiya *et al.*, 2013), possibly because of the obligation to reduce the quantity of organic solvents being used for extraction. Pallaroni *et al*. (2002) reported the use of MAE for the recovery of ZEA from maize, while Chen and Zhang, (2013) described a microwave-assisted extraction method for the analysis of AFs in grains and grain products. The fundamental difference between MAE and conventional solvent extraction is that MAE uses electromagnetic waves to alter the cellular structures of the sample matrices hence enabling partitioning of analytes from the matrix into the extraction solvent (Veggi *et al.*, 2013). Microwaves heat up microscopic traces of residual moisture in matrix tissues, resulting in expansion and pressure on the cell walls, and eventual rapture of the walls thus releasing analytes trapped in the cellular structures (Tatke and Jaiswal, 2011).

In contrast to other traditional extraction techniques, MAE requires less extraction time and reduced solvent consumption (Doughari, 2012; Veggi *et al*., 2013). A typical MAE procedure takes between 15–30 min, and uses a little amount of solvent (10 to 30 mL) which is about 10 times less than volumes required for conventional solvent extraction techniques (Eskilsson & Björklund, 2000). However, there are some disadvantages of this method. It is known that microwaves are ionizing electromagnetic waves that can catalyze or perhaps initiate irreversible chemical reactions or changes in some of the target analytes (Ghani *et al.*, 2008; Zhang *et al.*, 2011). The quantity of sample to be extracted is limited to 1 g, which is inadequate for a reasonable homogenous analysis (Shu *et al.*, 2000). Moreover, after extraction, there is need to remove the abundant solid residues of the matrix from the extracts via another sample preparatory step (Wang, 2010; Veggi *et al*., 2013).

Supercritical fluid extraction

Supercritical fluid extraction (SFE) is an extraction technique that employs high diffusion of CO_2 under supercritical conditions for the extraction of analytes from sample matrices (Co, 2010; Sapkale *et al*., 2010). A number of studies have described SFE methods for mycotoxin extraction (Josephs *et al*., 1998; Ambrosino *et al*., 2004; Zougagh & Rios, 2008). Under supercritical conditions, there are no distinct liquid or gaseous phases for the extraction fluid (Sharma, 2015), as such, the fluids have zero surface tension and can diffuse through solid sample matrices like gases while dissolving the analytes like solvents (Co, 2010; Attawood & Florence, 2012; Sofi *et*

al., 2013). Extraction by means of SFE takes between 10 to 60 min, is automatable and completely eliminates the use of toxic organic solvents, while leaving no trace of the extraction fluid in the extracts (Sapkale *et al*., 2010; Ayre *et al*., 2013). Despite the advantages, extraction using this method has some major setbacks. For example, the cost of the SFE equipment is very high, it is energy-intensive (CO_2 heating and compression) and handling the equipment requires technical expertise as well as rigorous precautions (Bulgariu and Bulgariu, 2015; Shine *et al*., 2015). Only analytes that are soluble in CO_2 can be extracted (Co, 2010).

Other methods for mycotoxin extraction

Several other methods exist for mycotoxin extraction aside from the ones discussed above, some of which include ultrasonic extraction (Kong *et al*., 2013), accelerated solvent extraction (Royer *et al*., 2004), aqueous two-phase (Pimentel *et al*., 2013), liquid-phase microextraction (González-Peñas *et al*., 2004), and many others reviewed in the literature (Cigić & Prosen, 2009; Rahmani *et al*., 2009; Anfossi *et al*., 2010; Arroyo-Manzanares *et al*., 2014; Turner *et al*., 2015). However, these methods have shortcomings, fundamentally being limited by cost, long and laborious procedures, issues of low recovery efficiency, and use of large volumes of organic solvents usage, some of which are well known for to be toxic and considered as environmental hazards (Henderson *et al*., 2011; Augusto *et al*., 2013; Sharma, 2015). Moreover, the emergence of novel mycotoxins and advancements in spectrometric analysis are pushing the limits of some of these conventional extraction techniques (Zhang *et al*., 2012; Augusto *et al*., 2013).

Based on the above described reasons, there is an eminent need for a greener approach that is cheap, fast and efficient in the extraction and analysis of mycotoxins (Augusto *et al*., 2013; Susanti *et al*., 2015). A green extraction method is one that is focused on limiting energy and solvent consumption, permits the use of safer alternative solvents from renewable sources, while ensuring high quality of the end product (Chemat *et al*., 2012). It is in line with this that we propose the adoption of pressurized hot water extraction (PHWE) as an alternative to conventional extraction methods. Pressurized hot water extraction has been in the spotlight as an efficacious and highly promising alternative to traditional techniques of extraction, whose successful applications in the biochemical, pharmaceutical and chemical engineering fields have been well documented in the literature (Wilson *et al*., 1993; Ibañez *et al*., 2003; Ozel *et al*., 2003; Gupta *et al*., 2012; Liang &

Fan, 2013; Shaddel *et al*., 2014; Gbashi *et al*., 2016, 2017d). A brief description of this method of extraction is presented in the succeeding section of this chapter.

2.5 PRESSURIZED HOT WATER EXTRACTION (PHWE)

Pressurized hot water extraction is a green, cheap and easy-to-adopt extraction technique that utilizes water at its subcritical state as the extraction solvent (Gbashi *et al*., 2017d). This method of extraction has often been represented under different names such as subcritical water extraction (SWE) (Liang & Fan, 2013), hot water extraction (HWE) (Paredes *et al.*, 2008), hot liquid water extraction (HLWE) (Wan and Li, 2011) and pressurized low polarity water extraction (PLPWE) (Cacace and Mazza, 2006; Guclu-Ustundag and Mazza, 2009). The use of pressurized hot water (PHW) as an extraction solvent was first reported by Hawthorne *et al*. (1994), when it was observed that sub- and supercritical water can be used for the recovery of organic pollutants from soil samples. Since then, numerous scientific researches have been carried out towards exploiting this novel technology.

2.5.1 Concept and principle of PHWE

The term pressurized hot water or subcritical water refers to liquid water between the boiling point temperature and critical point temperature of water (100 – 374 °C) (Figure 2.8) (Gbashi *et al*., 2017d). Pressure is applied to keep the water in liquid state. The phenomenon behind the extractability of PHW is based on the fact that when the temperature of water is raised and the pressure kept sufficient to maintain it in its liquid state (e.g. 250 °C and 50 bar), the dielectric constant of water decreases and the hydrogen bond and other intermolecular forces of water weakens, which greatly enhances its extractability (Table 2.1) (Alupului *et al*., 2012; Gbashi *et al*., 2017d).

At atmospheric temperature and pressure (25 °C at 1 bar), water has one of the highest dielectric constants amongst non-metallic liquids (ε=80) (Cabane & Vuilleumier, 2005). However, when the temperature and pressure of water is raised to 250 °C and 50 bar respectively, the dielectric constant falls (ε=27), which is around the range of non-polar solvents such as methanol (ε=33), acetone (ε=20.7), ethanol (ε=24), and acetonitrile (ε=37) (Teo *et al*., 2010; Alupului *et al*., 2012). As a result of the drop in dielectric constant of subcritical water, its surface tension and viscosity decreases, while its diffusivity increases (Teo *et al*., 2010; Gbashi *et al*., 2017d). As such, water

behaves like an organic solvent, dissolving a wide range of low and medium polarity analytes (Gbashi *et al*., 2017d).

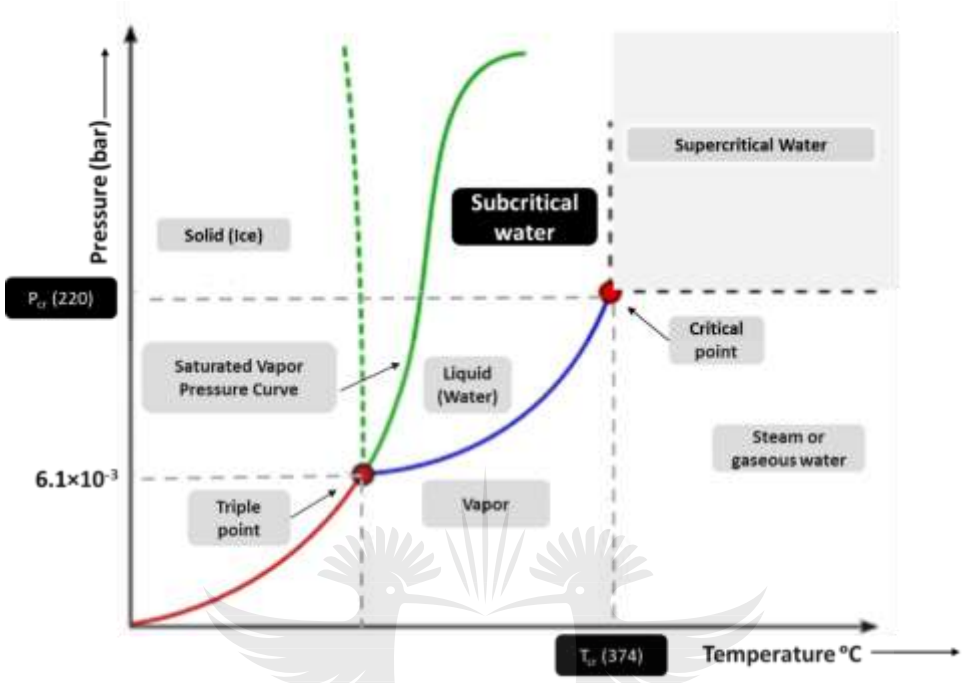


Figure 2.8: Phase diagram of water as a function of temperature and pressure (Gbashi *et al*., 2017c).

Interestingly, the extractability and selectivity of subcritical water can be easily maneuvered to extract a range of analytes by simply varying the temperature conditions of the water (Liang & Fan, 2013). Another theoretical explanation on the extractability of subcritical water bases this ability on the fact that, as the temperature of water increases, the average kinetic energy of the molecules of the mixture also increases. This thus disrupts the bonds that exist within and between the molecules, as such, increasing extraction rate.

Table 2.2: Important electro- and physicochemical properties of water that changes with increase in temperature and pressure (Gbashi *et al*., 2017c)

S/No	Property	Status	Reference
1.	Adhesion and cohesion	Decreases	Chaplin (2008)
2.	Collision frequencies	Increases	Buhler *et al.* (2002); Kruse and Dinjus (2007)
3.	Compressibility	Increases	Kruse and Dinjus (2007); Chaplin (2008)

S/No	Property	Status	Reference
4.	Density	Decreases	Kruse and Dinjus (2007); Chaplin (2008)
5.	Dielectric constant	Decreases	Kruse and Dinjus (2007); Chaplin (2008)
6.	Diffusivity	Increases	Kruse and Dinjus (2007); Teo *et al*. (2010)
7.	Electrical conductivity	Increases	(Hawthorne, 2000)
8.	Extraction rates	Increases	Teo *et al.* (2010); Gupta *et al*. (2012)
9.	Hydrogen bonding	Decreases	Yamaguchi (1998); Chaplin (2008)
10.	Miscibility	Increases	(Weingartner and Franck, 2005)
11.	Solubility	Increases	(Miller *et al.*, 1998)
12.	Surface tension	Decreases	(Chaplin, 2008)
13.	Viscosity	Decreases	(Kruse and Dinjus (2007); Teo *et al*., (2010)

Key: S/No.: serial number

2.5.2 Instrumentation and mechanism of PHWE

A typical setup of a laboratory scale PHWE unit comprises a source of water, temperature retention coil, a solvent pump, an oven and extraction cell, a backpressure valve and a condenser connected to the outlet (Figure 2.9). The grounded sample to be extracted is placed inside the extraction cell which is located inside the oven. The oven which usually has an automatic thermostat mechanism is set to the desired temperature, the backpressure valve is partially locked to maintain the desired pressure and water is pumped at a preset flow rate through the retention coil into the extraction cell. The extraction takes place in the extraction cell as the pressurized hot water flows through it and mixes with the sample. The hot water extract flows through the condenser and is collected at the outlet (Gbashi *et al*., 2017d). The extraction mechanism of PHWE involves the processes of heat convection, rapid fluid flow into matrix pores, analyte partition into solvent via diffusion and elution of analytes through bulk flowing of fluid (Asl & Khajenoori, 2013; Gbashi *et al*., 2017d). In addition, the high pressures enhance extraction by forcing the fluid into matrix cellular structures where low pressure water may not normally reach (Gbashi *et al*., 2017d).

2.5.3 Operational modes of PHWE

Extraction with PHW is performed in two common modes, static mode or flow-through (dynamic) mode. Extraction in the static mode involves retaining the sample in the extraction cell with subcritical water for short periods after which the fluid is allowed to flow out purging the extraction

cell and extract collected. It is important to optimize the retention periods to allow for an equilibrium to be reached between the solvent and analyte. The disadvantage of operating in this mode is that within a short time the analyte-fluid equilibrium is reached, hence no further extraction of the analyte occurs no matter how long the samples are retained in the extraction. On the other hand, extraction in the dynamic mode allows for a continuous flow of fresh fluid through the extraction cell which reduces or eliminates analyte-fluid equilibrium in a single operation when properly optimized. As such, recovery efficiency is higher in the dynamic mode, although, fluid consumption could be more, resulting in lower energy efficiency compared to the static mode (Teo *et al*., 2010; Gbashi *et al*., 2017d). In a study by Yang and Wyman, (2004), it was observed that extraction in dynamic mode resulted in higher recovery of lignin and hemicelluloses from maize stover cellulose than in the static mode.

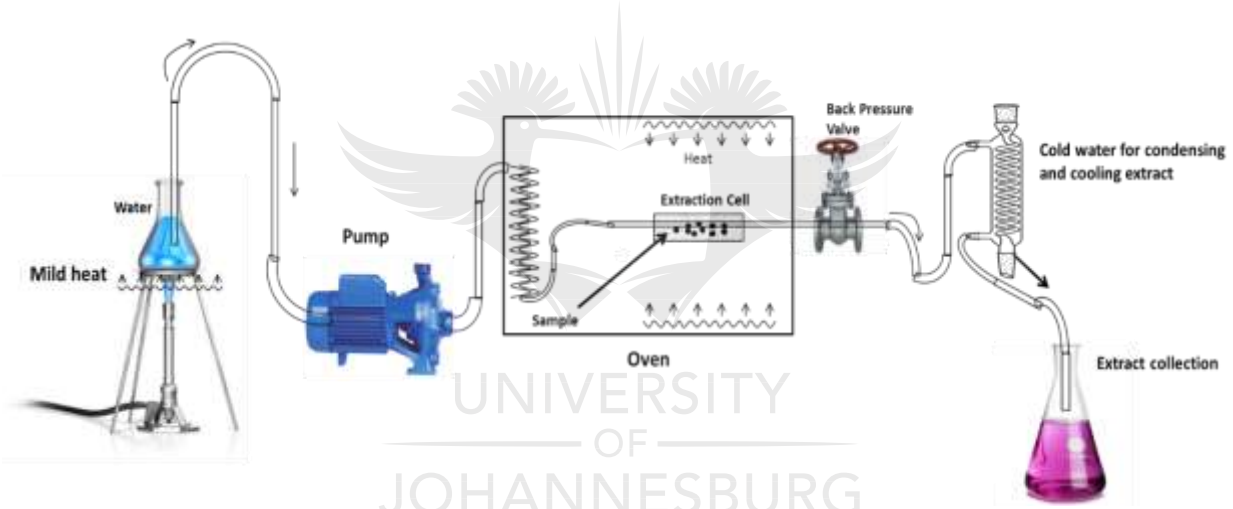


Figure 2.9: Simple laboratory setup of a PHWE unit (Gbashi *et al*., 2016)

2.5.4 Factors affecting PHWE

A number of factors such as temperature, flow rate, pressure, particle size, co-solvents and surfactants affect the performance of PHWE. Some of these factors are further described below.

2.5.4.1 Temperature

The extraction efficiency of PHWE is strongly affected by changes in temperature (Asl & Khajenoori, 2013). Generally, extraction efficiency increases with increase in temperature. A higher recovery of total antioxidants was achieved from grape pomace by increasing the extraction temperature (Vergara-Salinas *et al*., 2013). Despite the increase in efficiency by increase in

temperature, excess temperatures can result in degradation of thermolabile analytes, hence the need for optimization (Asl & Khajenoori, 2013; Khoza *et al*., 2015). The recovery of carvacrol and thymol from *Zataria multiflora* between 100 and 175 °C indicated that recoveries increased steadily with increase in temperature up until 150 °C, then a degradation phenomenon followed with a noticeable burning smell (Khajenoori *et al*., 2009).

2.5.4.2 Pressure

The effect of pressure on the extraction efficiency of PHWE has been described as insignificant (Hawthorne *et al*., 1994; Deng *et al*., 2004). In a study by Shalmashi *et al*. (Shalmashi *et al*., 2010), changes in pressure *i.e.,* 20, 30 and 40 bar during PHWE did not show any significant effect on the recovery of caffeine from tea waste. This is because water is fairly incompressible at temperatures below 300 °C, which implies that pressure has very little influence on the physicochemical properties of water, as long as it can maintain in a liquid state (Ramos *et al*., 2002; Carr *et al*., 2011). Nevertheless, increased pressure can compromise matrix tissue membranes and force the extraction fluid deep into matrix pores where water at lower pressure may not normally reach (Ong *et al*., 2006).

2.5.4.3 Co-solvents and modifiers

Co-solvents and solvent modifiers are often used to enhance the extractability of PHWE. Co-solvents are secondary solvents (usually organic solvents) that are added to subcritical water to enhance its solvation power (Williams *et al*., 2013; Gbashi *et al*., 2016). The incorporation of methanol during PHWE was observed to significantly ($p<0.05$) increase yield of flavonoids and di-acylated cinnamic acids from *Bidens pilosa* (Gbashi *et al*., 2016, 2017a). Solvent modifiers such as salts and other reagents can alter important physicochemical properties of water such as polarity, surface tension, and hydrogen bonding strength which results in an enhanced extractability (Curren & King, 2001b; Teo *et al*., 2010). Modifiers can also interact directly with the sample matrix, reducing the activation energy required for analyte desorption and diffusion (Plaza & Turner, 2015; Gbashi *et al*., 2017d). Curren and King (2001a) observed that the solubility of atrazine can be doubled when urea was added to subcritical water, and when ethanol was used, the solubility increased by over 10-folds.

In addition to the above described factors, other factors that influence the extractability of PHWE include solvent flow rate, physicochemical and functional characteristics of the sample matrix and analyte, matrix particle size and geometry of the extraction cell (Teo *et al*., 2010; Shitu *et al*., 2015; Gbashi *et al*., 2017d).

2.5.5 Advantages and disadvantages of PHWE

2.5.5.1 Advantages of PHWE

The major advantage of PHWE is that it is a green (*i.e.*, environmentally friendly) extraction method. The extractant is water, which is non-toxic, non-flammable and renewable. Moreover, water is readily available and cheap, and extraction with it does not generate harmful by-products (Chemat *et al*., 2012; Liang & Fan, 2013). In comparison with traditional extraction methods, PHWE is less time-consuming and much easier to perform with very few extraction steps, as such, human errors are greatly minimized. When put side-by-side with supercritical fluid extraction (SFE), PHWE edges on the basis of being a simple technology, hence, requiring much lower maintenance and engineering cost for equipment (Bart, 2005; Gbashi *et al*., 2017d). During extraction with PHW, the fluid can be maneuvered to selectively extract a range of analytes with different polarities by mere adjusting the temperature of the water, whereas SFE extracts only non-polar or light-weight compounds (Curren & King, 2001b; Liang & Fan, 2013). Further to this, PHWE is very compatible with various analytical instrumentations because water is colorless and may not interfere with sorts of photodetection such as ultraviolet (UV) detection or flame ionization detection (FID) (Khoza *et al*., 2016; Gbashi *et al*., 2017d).

2.5.5.2 Disadvantages of PHWE

A major setback of PHWE is the thermal degradation of some thermolabile analytes at elevated temperatures (Moreno *et al*., 2007; Khoza *et al*., 2014). When the temperature and pressure of water are extremely high (*i.e.*, above 374 °C and 221 bar), there is also the risk that water can become very reactive and could oxidize or catalyze hydrolysis of some compounds (Teo *et al*., 2010). However, optimization by means of the adoption of a co-solvent or modifier could ameliorate or eliminate these issues (Gbashi *et al*., 2016).

2.5.6 Applications of PHWE

In the last decade, PHWE has been widely investigated for the extraction of various nutritional constituents, organic pollutants, and pharmacoactive compounds from vegetal tissues, food products, soil residues and other ecological biomasses (Teo *et al*., 2010; Asl & Khajenoori, 2013; Duy *et al*., 2015; Gbashi *et al*., 2017d). Free fatty acids and other oils were extracted from spent bleaching earth using PHWE (Fattah *et al*., 2014). Likewise, it was possible to recover important metabolites from *Moringa oleifera* leaves using PHWE (Khoza *et al*., 2014). A similar extraction method was used for the recovery of proteins, carbohydrates and lignans from flaxseed meal (Ho *et al*., 2007), catechins and proanthocyanidins from grape seeds (García-Marino *et al*., 2006), flavonoids from aspen knotwood (Hartonen *et al*., 2007) and antioxidants from microalga *Spirulina platensis* (Herrero *et al*., 2004). The use of PHWE in various applications in different scientific disciplines has been reviewed (Ramos *et al*., 2002; Asl & Khajenoori, 2013; Liang & Fan, 2013).

2.5.7 Prospects of PHWE of mycotoxins

In a preliminary study, Gbashi *et al*. (2017b) developed and validated a PHWE method for the extraction of AFB_1 from maize followed by analysis on high performance liquid chromatography coupled to a Photodiode array detector (HPLC-PDA). Results obtained from that study revealed that PHWE could be suitable for the effective extraction of AFs from maize, with a recorded recovery rate of 115%. It has been stated earlier in Section 2.4 that more countries are enforcing stringent regulations limiting mycotoxins in food and feed, which is increasing the demand for their analysis. The speed, efficiency, simplicity, safety and low-cost implications of using PHWE are very attractive and compelling in this regard.

It is known that mycotoxins occur in a diverse manner and can be found deeply deposited inside the food matrices, and as such, their extraction usually requires a process that allows the solvent to penetrate all areas of the matrix to reach hidden toxins trapped in matrix pores (Scudamore, 2008). The high pressures involved in PHWE seem very suitable in meeting this requirement. Although issues with thermal degradation of some analytes have been a major limiting factor of PHWE, it is interesting to know that mycotoxins and most other mycotoxins are relatively thermally stable (Bullerman & Bianchini, 2007; Turner *et al*., 2009). Moreover, optimization using

co-solvents has been found effective in ameliorating this setback (Curren & King, 2001a; Gbashi *et al*., 2016). Based on these observations and other consulted literature reports, it is evident that PHWE is a viable alternative to conventional extraction methods for mycotoxins (Ong *et al*., 2006; Teo *et al*., 2010; Plaza & Turner, 2015; Gbashi *et al*., 2017d, 2017b). In the next section, a discussion on approaches used for the detection and quantification of mycotoxins after effective extraction will be presented.

2.6 CHROMATOGRAPHIC SEPARATION, DETECTION AND QUANTIFICATION OF MYCOTOXINS

After extraction of mycotoxins the next step is the detection (identification) of the specific mycotoxin and quantification of the amount of the mycotoxin present in the extracts. For this purpose, chromatographic equipment coupled with different types of detectors is being employed. Many of the official validated methods for mycotoxin detection and quantitation approved by regulatory authorities such as the Food and drug administration of the US (FDA), European Commission (EC) and The Association of Official Analytical Chemists (AOAC) are based on high-performance liquid chromatography (HPLC) coupled to an UV detector, fluorescence detector (FLD), or mass spectrometer (MS). Other detection and quantitation methods utilized for mycotoxins involve thin layer chromatography (TLC) followed by UV detection and gas chromatography linked to an MS (GC-MS) (Shephard, 2008a; Monbaliu *et al*., 2009).

2.6.1 Chromatographic separation of mycotoxins

Amongst chromatographic techniques employed for the analysis of mycotoxins in agricultural commodities, HPLC (Figure 2.10) is the most popular (Rahmani *et al*., 2009; Turner *et al*., 2015; Zhang *et al*., 2018). HPLC essentially relies on high-pressure pumps to convey the liquid mobile phase containing the sample extract or analyte(s) through a narrow column filled with a solid adsorbent material. The components present in the sample then interact with the adsorbent material in a slightly different manner depending on a number of factors such as polarity, chemical structure, size of molecule, functional groups present etc., causing different rates of flow for each component. This results in a partitioning of the components which then elutes from the column at a specific retention time (Kumar *et al*., 2013). The size of the sorbent particles packed into the

column directly affects partitioning of the analytes and their retention times; as the particle size decreases, the resolution and efficiency of the partitioning increases (Narwate *et al*., 2014).

The ultra-high performance liquid chromatography (UHPLC) is a superior evolution of the HPLC which preserves the basic principles of HPLC while increasing the overall combined elements of speed, sensitivity, and resolution (Wu & Engen, 2006). It uses finer particles (smaller than 2 µm in diameter), saves time and reduces solvent consumption compared to traditional HPLC (Wu & Engen, 2006; Srivastava *et al*., 2010). Some benefits of HPLC include low detection limits, small sample requirement and the ability to accommodate a vast range of analytes with various physicochemical characteristics. HPLC has numerous applications in mycotoxin analysis depending on the choice of analytical column, mobile phase, design of the gradient program and type of detector utilized (Rahmani *et al*., 2009; Roseanu *et al*., 2010).

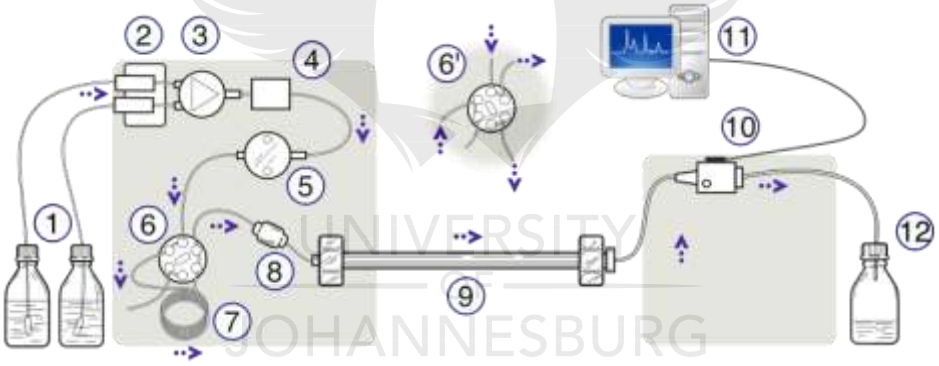


Figure 2.10: Schematic representation of a typical HPLC unit (Haider & Asif, 2011). (1) Mobile phase tanks, (2) Mobile phase degasser, (3) Gradient valve, (4) Vessel for mixing and delivery of the mobile phase(s), (5) Pressure pump, (6) Inject-positioned switching valve, (6') Load-positioned switching vale, (7) Injection loop, (8) Guard column (pre-column), (9) Chromatographic analytical column, (10) Detector (11) Data acquisition and processing, (12) Waste disposal or fraction collector.

The two broad categories of analytical columns utilized with HPLC are the normal-phase and the reversed-phase columns (Roseanu *et al.*, 2010). Normal-phase columns contain a polar sorbent (*i.e*. stationary phase) such as silica or alumina resins which separate analytes based on their ability

to engage in polar interactions such as hydrogen bonding, ionic, dipole-induced dipole, or dipole-dipole type interactions with the stationary phase. Normal phase columns are employed for the separation of analytes that are readily soluble in non-aqueous, non-polar mobile phases such as chloroform (Yang *et al*., 2013; Wierucka & Biziuk, 2014). Reversed-phased columns on the other hand involves the use of a hydrophobic stationary phase which is essentially the reverse of normal-phase columns (which uses hydrophilic sorbents), hence the term 'reversed-phased' columns. Generally, alkyl chains such as $C_{18}H_{37}$ or C_8H_{17} covalently bonded to the sorbent particles create a hydrophobic environment in reversed-phased columns, hence less affinity for hydrophobic compounds and stronger interactions with hydrophylic compounds. An aqueous (polar) or moderately polar mobile is employed for the separation of analytes on the reversed-phase column (Jandera, 2002; Yang *et al*., 2013; Wierucka & Biziuk, 2014). The reversed-phase columns are the most commonly used in mycotoxin analysis. In the past decade, almost all mycotoxins have been partitioned, detected and quantified by HPLC when coupled to a suitable detector (Arranz *et al*., 2004; Berthiller *et al*., 2007; Rahmani *et al*., 2009; Turner *et al*., 2009, 2015; Varga *et al*., 2013; Zhang *et al*., 2018).

2.6.2 Detection and quantification of mycotoxins

Detection and quantification is the final step in the analysis of mycotoxins aside data analysis and result interpretation. Detection is a qualitative process which confirms the presence of an analyte or group of analytes while quantification determines the amount of the analyte available in the sample extract. Detection and quantification are achieved by means of detectors that are linked to a chromatograph. The detectors convert the physical or chemical attributes of an analyte into measurable signals corresponding to elution time, identity and quantity of the analyte (Scott, 1996; Snyder *et al*., 2010).

There are many different detectors that have been effectively combined with HPLC instruments for the detection and quantitation of mycotoxins, which include FLD (Wen *et al*., 2014; Ali *et al*., 2015), UV detectors (Klinglmayr *et al*., 2010; Ramni *et al*., 2011), photo-diode array detector (PDA) (Maragos *et al*., 2008; Pascale *et al*., 2012), flame ionization detectors (FID) (Eke *et al*., 2004; Wu & Smith, 2007) and mass spectrometric detectors (MS) (Sulyok *et al*., 2006; Geary *et al*., 2016). A number of published literature have reviewed the principles and applications of the

different kinds of detectors utilized in chromatography and mycotoxin analysis (Scott, 1996; Swartz, 2010; Ramni *et al*., 2011; Sriveena *et al*., 2015). For the purpose of this thesis, only the MS detectors are discussed.

2.6.2.1 Mass spectrometry (MS)

Mass spectrometry is an analytical technique that relies on ionization of chemical species, fragmentation and sorting the ions based on their mass-to-charge ratios (Siuzdak, 2004; Sriveena *et al*., 2015). Achieving better accuracy, sensitivity and selectivity, MS is considered the standard method for detection and quantitation of mycotoxins (Cigić and Prosen, 2009; Rahmani *et al*., 2009; Turner *et al*., 2009). Key features of MS include the capacity to determine the (accurate) molecular mass, and generate information on elemental composition and structure of unknown substances (Siuzdak, 2004; Sriveena *et al*., 2015). MS is also used for verification of the identity and purity of known compounds, providing data on isotopic signature of a sample and quantification of the concentration of the analyte(s). Analysis by means of MS does not depend on the UV absorbance or fluorescence characteristics of the analyte, as such, the need for derivatization prior to detection may be eliminated (Rahmani *et al*., 2009; Sriveena *et al*., 2015). Additionally, MS has the ability to accommodate a wide range of analytes of various molecular masses and polarities, in addition to small sample amount requirements. MS also achieves lower detection limits and good repeatability even for complex sample matrices (Lattanzio *et al*., 2007, 2011; Ediage *et al*., 2011; Njobeh *et al*., 2012; Arroyo-Manzanares *et al*., 2018).

There are many variants of MS detectors such as ion trap (IT), quadrupole (Quad), time-of-flight (TOF) and Fourier transform-ion cyclotron resonance (FT-ICR), however, the Quad is the most widely utilized for mycotoxin analysis (Rahmani *et al*., 2009; O'Mahony *et al*., 2013; Adekoya *et al*., 2018). In a typical (triple) quadrupole MS system (Figure 2.11), the sample to be analyzed is conveyed into the source where it is ionized either positively or negatively depending on the nature of the analyte. Two kinds of ionization sources are typically used for mycotoxin ionization in MS, the atmospheric-pressure ionization (API) (Zöllner & Mayer-Helm, 2006) and the electron-spray ionization (ESI) (Ho *et al*., 2003). The ESI is however the most favored for mycotoxin analysis because it is suited for medium to high polar and medium to high molecular mass analytes (Rahmani *et al*., 2009). After ionization, the molecules acquire a charge and their masses are

measured with respect to their charge, *i.e.*, *mz*. The ionized species are then absorbed via a strong vacuum into the first quadrupole which functions as an ion filter, where the ions are filtered according to their *mz* allowing only the desired ions (precursors) to pass into the second quadrupole (Siuzdak, 2004; Sriveena *et al*., 2015).

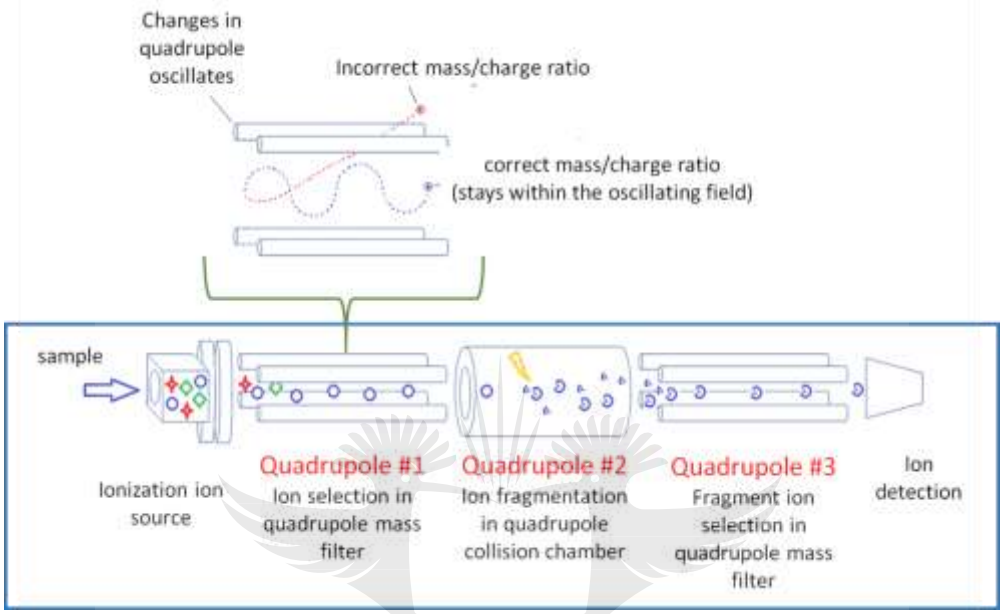


Figure 2.11: Schematic representation of a typical triplequad MS instrument (Adapted from Emerypharma, 2019)

In the second quadrupole also referred to as the collision cell, the precursor ions which are already structurally unstable by reason of bearing a charge are bombarded with a collision gas, typically argon gas. This causes the species to disintegrate into a number of charged fragments. The fragments are then accelerated into the third quadrupole where they are filtered, allowing only the pre-specified product fragments to pass onto the detector for identification and quantitation (Rahmani *et al*., 2009; Sriveena *et al*., 2015). The results from the MS detector are then translated into signals and displayed as mass chromatograms and spectrums. The identity of an analyte present in a sample is confirmed by observing the precursor *m/z* and its fragmentation pattern (*i.e.*, product ions) (Siuzdak, 2004), whereas the concentration is confirmed by correlating the abundance of the ions of a specific analyte to those of pure standards by means of a calibration curve.

2.6.3 Application of LC-MS/MS for mycotoxin analysis

Using a solvent extraction method followed by analysis on a triple quad MS detector, Chilaka *et al*. (2016) determined the incidence rates and levels of 16 *Fusarium* mycotoxins including FBs and hidden FBs, DON, ZEA and their metabolites, T-2, HT-2, FUS-X, NIV, neosolaniol (NEO) and DAS in 363 samples of maize, sorghum, millet and *ogi* from Nigeria. Ochratoxin A, AFM_1, ZEA and α-ZEL in milk have been determined based on a method using LC-MS/MS (Huang *et al*., 2014a). In a study by Varga *et al*. (2013), 191 mycotoxins and other fungal metabolites from hazelnuts, pistachios, peanuts and almonds were analyzed using a semi-quantitative method based on an LC-MS/MS. Elsewhere, 295 fungal and bacteria metabolites were simultaneously quantitatively analyzed using an optimized solvent extraction method followed by LC-MS/MS (Malachová *et al*., 2014).

Having described different methods for mycotoxin extraction, detection and quantification, in the next section, a discussion on the process of method development for mycotoxin analysis is presented. Also, the process of validating a newly developed analytical method is described.

2.7 DEVELOPMENT AND VALIDATION OF METHODS FOR MYCOTOXINS ANALYSIS

Development and validation of analytical methods play important role in the study of mycotoxins, particularly with regards to monitoring, policy and regulatory purposes.

2.7.1 Method development

Method development constitutes the process of designing an analytical procedure to enable the extraction, identification and/or quantification of an analyte of interest in a sample matrix (Arora & Gangadharappa, 2016). A number of reasons exists for developing new methods, some of which include (Arora & Gangadharappa, 2016); (1) lack of a suitable method for the analyte(s) of interest in a specific sample matrix; (2) existing methods may be inefficient and unreliable; (3) existing methods may be too expensive, tedious, energy intensive or time-consuming; (4) existing methods may not be compatible with analytical equipment, or may not provide adequate analyte selectivity, sensitivity and accuracy in samples of interest; (5) existing methods may be deficient in terms of human and environmental safety and sustainability; (6) an alternative method may be needed for

confirmation of data originally acquired using existing methods, for scientific or legal reasons. Literature evidence shows that safety considerations (human and environmental), cost, as well as, sample throughput are critical areas of interest in the development of new analytical methods for mycotoxin analysis (Zheng *et al*., 2006; Cigić & Prosen, 2009; Razzazi-Fazeli & Reiter, 2011; Arroyo-Manzanares *et al*., 2013, 2014).

When developing new methods that involve simultaneous extraction of more than one analyte, particularly those with diverse physicochemical characteristics, it is common practice to use a combination of solvents, as well as, manipulate different extraction conditions (such as temperature, pH etc.) in order to achieve the best extraction efficiency. This task can be very daunting, involving several experimental trials. An effective way to overcome this challenge is the adoption of chemometric optimization models which help in the selection of optimal sample preparation conditions (Díez *et al*., 2011; Khazaeli *et al*., 2016; Bhusnure *et al*., 2017). Examples of some of these chemometric models and their applications in the development and optimization of sample preparation procedures will be discussed in a subsequent section (Section 2.8) of this chapter. In the meantime, the process of determining the suitability of a newly developed analytical method (*i.e.*, method validation) will be discussed in the succeeding section.

2.7.2 Validation of analytical methods

Subsequent to method development is the validation of the developed method. Method validation is important in order to be confident in the reliability of the testing procedure. It is also a recommended process of any official and standard analytical procedure (Pittet, 2005; Hussain, 2011; Arora & Gangadharappa, 2016). The objective of the validation exercise is to demonstrate that the analytical procedure being employed for a specific analyte in a specific matrix is suitable, in terms of quality, consistency and reliability for its intended use (Anklam *et al*., 2002; Trombete *et al*., 2014; Arora & Gangadharappa, 2016). The validated method should also correspond to standard pre-established method performance criteria (Gilbert & Anklam, 2002; Arora & Gangadharappa, 2016).

A full method validation is expected when developing or implementing an analytical method for the first time, whereas a partial validation is required when minor modifications are made to an already validated method (Arora & Gangadharappa, 2016). Such minor modifications can include

changes in the extraction solvent, clean-up material, sample matrices, concentration ranges, analytical instrumentation, transfer of analytical methods between laboratories or amongst analysts. A partial method validation can range from a simple recovery or precision assay to an almost full validation performance (Arora & Gangadharappa, 2016). A cross-validation on the other hand implies a comparative assessment of the validation parameters of two or more methods that are used to generate data in a study or across different studies (Arora & Gangadharappa, 2016). For example, a cross-validation would be applicable where an original validated method is used as a control reference against a revised form of the original method or a newly developed method (Arora & Gangadharappa, 2016). Typical method validation parameters to be evaluated include, accuracy, linearity, sensitivity, selectivity, intra-day precision, inter-day precision, robustness and matrix effect (Anklam *et al*., 2002; Arora & Gangadharappa, 2016). The details about these validation parameters are described in the succeeding sub-sections of this chapter.

2.7.2.1 Accuracy

Accuracy, also referred to as recovery efficiency or trueness is arguably the most important validation parameter. It estimates the closeness of agreement between experimentally observed values and the accepted reference value (Huber, 2010). Accurate quantification of mycotoxins in food and feed commodities is essential in order to assess the compliance of the contamination levels of the toxin in the sample with respect to the legal limits (Anklam *et al*., 2002). The FDA recommends recovery values between 80 to 110% for concentrations ranging from 100 to 10,000 µg/kg (FDA, 2015). The EC recommends values between 60 and 130% (Commission Regulation, 2006a), the AOAC recommends 70 to 125% in foodstuff contaminated with 10 to 1,000 µg/kg of mycotoxins (AOAC, 2009), whereas, *Codex Alimentarius* (CODEX) recommends 80 to 110% for accuracy in agricultural commodities contaminated with mycotoxins depending on the type of mycotoxins and level of contamination (CODEX, 2015).

2.7.2.2 Specificity

Specificity or selectivity measures the ability of a method to differentiate and quantify an analyte of interest in the presence of other components in the sample (Anklam *et al*., 2002; Arora & Gangadharappa, 2016). This implies that no endogenous peaks should be present within 10% window of the retention time of the target analyte and an internal standard. In the event there is a

peak within this range of the retention time, its response should not be greater than 20% of the response of an extracted lower limit of quantification (LLOQ), and if any peak is present within the retention time of the internal standard, the response of such a peak should not exceed 5% of the extracted internal standard at a concentration of interest in the study (Anklam *et al*., 2002; Arora & Gangadharappa, 2016). Furthermore, the signal-to-noise ratio (S/N) should be ≥ 3. In case there is no noise, then a signal should be obtained in at least 5 subsequent scans. Target analyte peaks in the extracted ion chromatograms should overlap fully. Ion ratio should be within ±30% relative of average of the calibration standards from the sequence (EC, 2016).

2.7.2.3 Sensitivity

Sensitivity measures the ability of a method to demonstrate that two samples containing different amounts of an analyte of interest has indeed different amounts of the analyte. Sensitivity is essentially determined in terms of the limit of detection (LOD) and the limit of quantification (LOQ). The LOD estimates the minimum level from which an analyte of interest in a sample can be detected with a given certainty (e.g. 95%). The LOQ estimates the smallest amount of an analyte in a sample that can be accurately quantified by a method (Anklam *et al*., 2002). Usually the LOD and LOQ values are expected to be less than the maximum limits of the mycotoxin stipulated by regulatory agencies.

2.7.2.4 Linearity and range

Linearity measures the ability of an analytical method to, within a given range, provide instrumental responses that are proportional to the analyte concentration in the sample (Huber, 2010). The EC and the International Conference on Harmonization (ICH) recommends that coefficients of determination for linearity should be ≥ 0.95 (Shabir, 2005). The ICH further recommends a maximum of 2% for the y-intercept of the target concentration response (Shabir, 2005). Range on the other hand refers to the concentration interval of an analyte over which a validated method is considered to perform in a linear manner. The criteria for the linear range can be different. Generally, the linear range should cover 25 to 200% of the target (expected) analyte concentration.

2.7.2.5 Intra- and inter-day precision

Intra-day precision (RSDr) also referred to as repeatability or within-day precision expresses the closeness of results obtained under the same working conditions over a short interval of time, usually within the period of 24 h (Huber, 2010). The EC recommends RSDr values not exceeding the computed values of the Horwitz equation (Equation 2.1). Usually, for a mass fraction of ≥ 10 µg/kg to 100 µg/kg, this value should not exceed 20% (Rodríguez-Carrasco *et al*., 2016; Meerpoel *et al*., 2018). In the cases were the concentrations are lower than 100 µg/kg, care must be taken when calculating the Horwitz values as this could be too high (*i.e.,* >20%). In such situations, the RSDr values should be as low as possible (EC, 2002; Meerpoel *et al*., 2018). It should be noted that specific RSDr values have been established for some well-known mycotoxins: the AOAC recommends an RSDr of < 25% for each mycotoxin (AOAC, 2009), the EC recommends ≤ 20% for OTA, ≤25 for ZEA, ≤ 20% for FBs, ≤ 30 for T-2 (Commission Regulation, 2006a).

$$\text{RSDr} = {}^{2}/_{3}\left(2^{[1-0.5\log C]}\right) \qquad \textit{Equation 2.1}$$

Where C is the mass fraction expressed as a power of 10 (EC, 2002).

Inter-day precision also referred to as between-day (intermediate) precision (RSD_R), though similar to the RSDr, measures the relative standard deviation of repeated measurements for experiments conducted within a longer interval of time (usually over 24 h). The recommended values for the RSD_R values are similar to those of the RSDr, however, the Horwitz equation for calculation of RSD_R (Equation 2.2) is a little bit different than that of RSDr (EC, 2002; Meerpoel *et al*., 2018).

$$RSD_R = 2^{[1-0.5\log C]} \qquad \textit{Equation 2.2}$$

2.7.2.6 Robustness

Robustness measures the capacity of an analytical method to produce consistent results despite relatively small, but deliberate perturbations in analytical parameters (Shabir, 2005). In other words, robustness measures the stability of a method during little but deliberate variations in the analytical parameters. There are no specific criteria for robustness.

Further details on various other requirements for validation of methods for mycotoxin analysis as outlined by the EC, CODEX, AOAC, FDA, ICH can be found in the following literature (Anklam *et al*., 2002; Gilbert & Anklam, 2002; Shabir, 2005; Commission Regulation, 2006a; AOAC, 2009; CODEX, 2015; FDA, 2015). The next section of the chapter describes the importance of chemometric models in the development and validation of analytical methods. It also gives examples of some of these models and their applicability in the development of extraction methods.

2.8 CHEMOMETRIC APPROACHES FOR OPTIMIZATION OF EXTRACTION METHODOLOGIES IN SAMPLE PREPARATION

Chemometrics involves the use of mathematical and statistical tools for modelling of patterns, extraction of meaning information and interpretation of chemical and biochemical datasets (Bu, 2007; El-Gindy & Hadad, 2012; Ab Ghani *et al*., 2014). The application of chemometric tools in the design and optimization of sample preparation methodologies is gaining increasing popularity and leading to the development of more efficient analytical procedures (Ab Ghani *et al*., 2014; Li *et al*., 2017). Development and optimization of efficient sample extraction methods is often a very difficult task as very often the extraction efficiency is influenced by more than one analytical parameter. Achieving the best possible outcome usually requires repeated experimental trials under different conditions in order to select the best. For each experimental trial, there is a corresponding decision challenge to make as to whether the outcome is a feasible and optimal solution to the objective variable. When more than one objective variable is involved, the number of experimental trials required to make a logical inference on the optimal solution increases exponentially. The decision on the solution becomes very complex and often cannot be addressed using conventional statistical tools.

Chemometric optimization models such as the response surface methodology (RSM) are ideal for tackling such challenges of enhancing the performance of analytical procedures, typically an improved quantitative analytical response, and yielding maximum information (Gbashi *et al*., 2016; Adebo *et al*., 2018b). In terms of cross-validation of extraction methods or interpretation of multivariate datasets, the principal component analysis (PCA) and orthogonal projections to latent structures discriminate analysis (OPLS-DA) have found applicability (Yamamoto *et al*., 2009; Song *et al*., 2013; Bro & Smilde, 2014; Khoza *et al*., 2014). The application of chemometric tools

in the design and optimization of sample preparation methodologies is gaining increasing popularity and leading to the development of more efficient analytical procedures (Ab Ghani *et al*., 2014; Li *et al*., 2017). By proper use of the proposed mathematical models during method development and validation, a more efficient, robust and goal-specific method is possible. In the succeeding sub-sections of this chapter, a brief description of RSM, PCA and OPLS-DA will be presented.

2.8.1 Response surface methodology (RSM)

RSM employs mathematical algorithms in establishing approximate functional relationships between an objective variable(s) and a set of control variables by means of a series of pre-designed experiments, and then computing the value of the objective function using probabilistic analysis (Uma *et al*., 2010; Morshedi & Akbarian, 2014). When more than one responses exist, it becomes critical to find the compromise or global optimum that takes into consideration all the response variables (Adebo *et al*., 2018b). Examples of the commonly adopted approaches of RSM include the fractional and full factorial designs (Gunst & Mason, 2009; Rakić *et al*., 2014), Box-Behnken design (Maran *et al*., 2013), Plackett–Burman design (Robert *et al*., 2006), Doehlet design (Adebo *et al*., 2018b) and the Box-Wilson central composite design (CCD) (Mirzajani *et al*., 2010). Amidst these approaches, the CCD is the most widely used RSM technique. Bas and Boyaci, (2007), Bezerra *et al*. (2008) and Azami *et al*. (2012) have described in detail the mathematical principles behind these different approaches of RSM.

The CCD has been widely used for optimization of various sample preparatory methodologies (Nazari *et al*., 2007; Mirzajani *et al*., 2010; Shi *et al*., 2012). This model enables approximate estimation of second-degree polynomial models, with a reduced number of experimental runs (Goncalves *et al*., 2006; Tamhane, 2014). Typically, a set of experiments are statistically designed and conducted. The experiments consist of (1) levels of a factorial or fractional factorial design, (2) a set of replicated center points which represent median values for each factor used in the factorial section of the design. The number of center points depends on the specific characteristics desired by the design, (3) a set of star or axial points which are similar to the center points except that one of the factors takes values of α which are both above and below the median of the factorial levels (typically outside of the factorial range) which allows for the estimation of curvature. The

exact value of α depends on specific properties desired for the experimental design and the number of independent factors involved. In any case, a more common choice of α is $\alpha = \sqrt{k}$ which yields a spherical design (Figure 2.12), where k is the number of independent factors (STAT503, 2018).

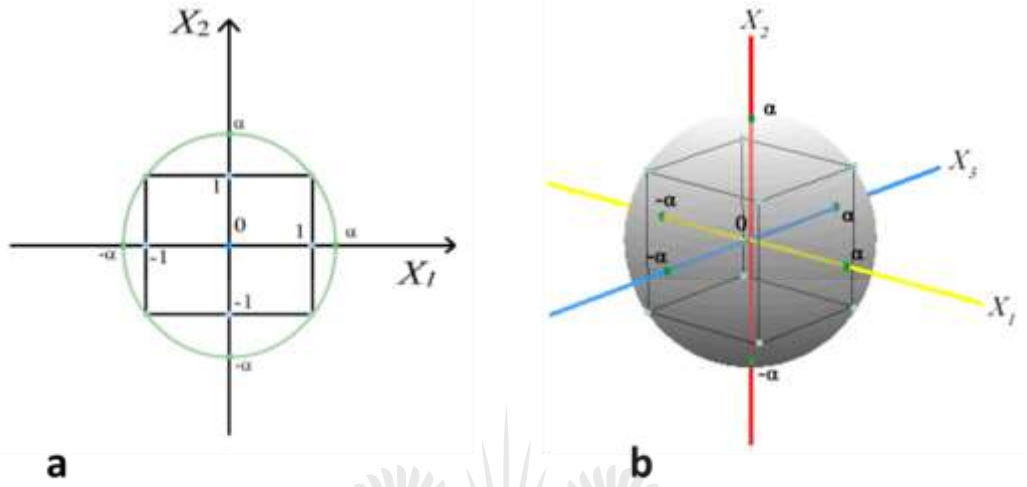


Figure 2.12: The central composite design of the response surface model for (a) α=√*k*, where *k*=2, and (b) *α*=√*k*, where *k*=3. *X*1=independent factor 1, *X*2= independent factor 2 and *X*3= independent factor 3.

After the design, experiments are performed based on the design and a linear regression approach is used for fitting the optimization model to the experimental data. The resultant quadratic models give the best approximate description of the patterns and behavior of the dataset, and thus can be maneuvered to obtain the best system performance *i.e.*, the optimal value of the response variable or to obtain a specific target value of the response variable (Bezerra *et al*., 2008). The quadratic models usually take the form of Equation 2.3.

$$Z = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i=1}^{k} \quad \sum_{j=1}^{k} \beta_{ij} x_i \, x_j \qquad \textbf{\textit{Equation 2.3}}$$

Where *z* is the response variable, x_i and x_j are the factors or independent variables, β_0 is the model constant, β_i, β_{ii} and β_{ij} are the coefficients for the linear, quadratic and interaction terms, respectively (Adebo *et al*., 2018b).

Further to this, the model-fit can be used to study the linear, quadratic and interaction effects of the control variable on the response variable (Uma *et al*., 2010). In more complex optimization

problems where more than one response exists, there is the possibility that the optimal value for one of the responses may not yield an optimal response for the other response(s). In this case, a global optimization approach is adopted which reduces variability in one or more of the responses while simultaneously optimizing or targeting a specific value for the other response(s).

2.8.2 Principal component analysis (PCA) and orthogonal projections to latent structures discriminate analysis (OPLS-DA)

The PCA (Bro and Smilde, 2014) and OPLS-DA (Song *et al*., 2013) are amongst the most widely utilized chemometric models for multivariate data interpretation in analytical and bioanalytical sciences (Daszykowski *et al*., 2003; Cordella, 2012; Worley & Powers, 2013). The PCA uses advanced mathematical algorithms based on a vector-space orthogonal (*i.e.*, uncorrelated) transformation to reduce the dimensionality of large datasets into a fewer number of latent variables also called principal components (PCs) while preserving as much of the variation in the data as possible (Daszykowski *et al*., 2007; Cordella, 2012; Worley & Powers, 2013). Examination of the compressed data allows for an easier and quicker determination of outliers and observation of the trends, patterns and overall structure of the dataset (Daszykowski *et al*., 2007; Worley & Powers, 2013).

An important feature of the PCA is that the PCs are chosen in order of magnitude in a manner that maximizes the variance of the projected points (Aliferis *et al*., 2010). The first PC explains as much of the total variation in the dataset as possible, and each other subsequent PC is chosen under the constraint that it is orthogonal to the previous PCs while explaining as much of the remaining variation in the data as possible (Panigrahi, 2014). Generally, the first few PCs are sufficient to describe the overall data structure and make inferential interpretations (Daszykowski *et al*., 2007). PCA is essentially an unsupervised model *i.e.*, it interrogates the dataset for patterns without making reference to the sample class labels in the dataset. As such, the PCA is helpful in exploring patterns in high-dimensional datasets when there is no specific information of class labels. However, the separation between data groups is not maximized nor is information provided on why the data groups are different.

The OPLS-DA on the other hand is somewhat similar to the PCA, however, it is a linear discriminant model which adopts a supervised approach (*i.e.*, makes reference to data class labels)

to minimize within-class variance and maximize the between-class variance in the data groups (Ballabio & Todeschini, 2009). OPLS-DA is particularly useful when there are two classification labels associated with the dataset, such as a control (*i.e.*, independent or predictor) group and a response (dependent) group. The objective is to predict a set of response variables (say X_1, …Xn) from a set of control variables (Y) by extracting a set of orthogonal latent variables (or PCs) which have the best predictive power. These PCs can then be used to create visual displays such as the score, loadings and variable importance plot (VIP) similar to those obtained by the PCA model (Abdi, 2010).

The score plot is a scatter plot that projects the data onto a two-dimensional subspace, emphasizing the variations in the data by highlighting similarities or differences between the various subsets by way of clusters. The loadings plot describes the relationship between original variables and subspace dimensions, showing the contribution of separate variables to a given PC. Whereas, the VIP shows the most important variables ranked in terms of significance in descending order by a mean decrease in Gini. Gini is a statistical measure of the degree of variation (or inequality) represented in a set of values of a frequency distribution. The top variables have higher predictive power and contribute more to the overall model. These dynamic and visually interpretable tools make PCA and OPLS-DA very attractive for multi-dimensional data analysis (Brereton, 2003; Daszykowski *et al*., 2003, 2007; Yamamoto *et al*., 2009; Tugizimana, 2012).

For both the PCA and OPLS-DA analysis, a data scaling and a data clustering approach is usually required. A number of data scaling approaches exists such as centering, unit variance, range, and Pareto scaling amongst others (Worley & Powers, 2013). The Pareto scaling is one of the most frequently utilized amongst the scaling methods because it aims to minimize the relative importance of large values while partially preserving the data structure. It stays closer to the original measurement than the unit variance scaling approach (Worley & Powers, 2013). In terms of data clustering methods, the Ward's method and the K-means are favorites, however, the Ward's method is regarded as being superior for data amalgamation because it adopts an analysis of variance (ANOVA) approach to evaluate the distances between the different data clusters. This approach is more efficient when the distance between clusters is estimated using the squared Euclidean method which is essentially robust to outliers or the addition of new objects to the analysis (Sima *et al*., 2017).

PCA and OPLS-DA have a wide range of applications in bioanalytical sciences. Some common applications include; data compression, de-noising signals, blind source separation, and cross-validation or authentication of data from different sources. PCA and OPLS-DA have been utilized for authentication, quality control and discrimination of different extraction methods (Tambellini *et al*., 2013; Cubero-Leon *et al*., 2014, 2018; Liu *et al*., 2016). Tambellini *et al*. (2013) demonstrated the use of PCA and OPLS-DA for the comparison of three different solvent extraction methods for the ability to simultaneously extract polar and non-polar yeast metabolites. It was shown that such an approach was a quicker and cheaper way to comparatively discriminate between extraction methods, and to identify the most efficient and reproducible method from the three methods.

2.9 CONCLUDING REMARKS FROM THE LITERATURE REVIEWED

From the various literature consulted and reviewed herein, it has been established that mycotoxins are potent natural toxins of fungi origin that constitute a significant nuisance to human and animal health as well as the economy. One way to adequately combat the prevalence of these toxic substances is by frequent monitoring of their prevalence along the food supply chain, in order to ensure conformity to established regulatory limits. This makes analysis as a critical element in mycotoxin management and control. Extraction being an important step during mycotoxin analysis hence requires constant improvement, as such, a priority in mycotoxicological research. At present, most of methods for mycotoxin analysis present a number of challenges such as being laborious, largely chemical-dependent and expensive, which are yet to be fully addressed. There is thus a continual quest for newer extraction methods that are fast, cheap, efficient and involve complete or partial replacement of organic solvents. Pressurized hot water extraction seems very promising in addressing all these requirements, if well exploited. Harnessing of PHWE using chemometric tools could yield a very efficient method for routine analysis of mycotoxins and other important fungal metabolites in foods and feeds.

References

Ab Ghani, T. F. T., Maarof, H., and Hassan, M. N. (2014). Classification of perfume products using gas chromatography-mass spectrometry and pattern recognition. *Malaysian Journal of Fundamental and Applied Sciences, 10*(3), 124–128.

Abass, A. B., Awoyale, W., Sulyok, M., and Alamu, E. O. (2017). Occurrence of regulated mycotoxins and other microbial metabolites in dried cassava products from Nigeria. *Toxins*, *9*(7), 207.

Abdi, H. (2010). Partial least squares regression and projection on latent structure regression (PLS Regression). *Wiley Interdisciplinary Reviews: Computational Statistics*, *2*(1), 97–106.

Abia, W. A., Warth, B., Sulyok, M., Krska, R., Tchana, A. N., Njobeh, P. B., Dutton, M. F., and Moundipa, P. F. (2013). Determination of multi-mycotoxin occurrence in cereals, nuts and their products in Cameroon by liquid chromatography tandem mass spectrometry (LC-MS/MS). *Food Control*, *31*(2), 438–453.

Abreu, L. M., Costa, S. S., Pfenning, L. H., Takahashi, J. A., Larsen, T. O., and Andersen, B. (2012). Chemical and molecular characterization of *Phomopsis* and *Cytospora*-like endophytes from different host plants in Brazil. *Fungal Biology*, *116*(2), 249–260.

Adebo, O. A., Njobeh, P. B., Mulaba-Bafubiandi, A. F., Adebiyi, J. A., Desobgo, Z. S. C., and Kayitesi, E. (2018). Optimization of fermentation conditions for ting production using response surface methodology. *Journal of Food Processing and Preservation*, *42*(1), e13381.

Adekoya, I., Obadina, A., Adaku, C. C., De Boevre, M., Okoth, S., De Saeger, S., and Njobeh, P. (2018). Mycobiota and co-occurrence of mycotoxins in South African maize-based opaque beer. *International Journal of Food Microbiology*, *270*, 22–30.

Adetunji, M., Atanda, O., Ezekiel, C. N., Sulyok, M., Warth, B., Beltrán, E., and Chilaka, C. A. (2014). Fungal and bacterial metabolites of stored maize (*Zea mays*, L.) from five agro-ecological zones of Nigeria. *Mycotoxin Research*, *30*(9), 89–102.

Adeyeye, S. A. O. (2016). Fungal mycotoxins in foods: a review. *Cogent Food and Agriculture*, *2*(1), 1213127.

Adhikari, M., Negi, B., Kaushik, N., Adhikari, A., Al-Khedhairy, A. A., Kaushik, N. K., and Choi, E. H. (2017). T-2 mycotoxin: toxicological effects and decontamination strategies. *Oncotarget*, *8*(20), 33933–33952.

Agag, B. I. (2004). Mycotoxins in foods and feeds 3-zearalenone. *Assiut University Bulletin for Environmental Researches*, *7*(2), 169–176.

Ajandouz, E., Berdah, S., Moutardier, V., Bege, T., Birnbaum, D., Perrier, J., Di Pasquale, E., and Maresca, M. (2016). Hydrolytic fate of 3/15-acetyldeoxynivalenol in humans: specific deacetylation by the small intestine and liver revealed using in vitro and ex vivo approaches. *Toxins*, *8*(8), 232.

Al-anati, L., and Petzinger, E. (2006). Immunotoxic activity of ochratoxin A. *Journal of Veterinary Pharmacology and Therapeutics*, *29*(2), 79–90.

Aldred, D., Magan, N., and Olsen, M. (2004). The use of HACCP in the control of mycotoxins: the case of cereals. In: N. Magan & M. Olsen (Eds.), *Mycotoxins in food: Detection and control* (pp. 139–173). Cambridge, England: Woodhead Publishing Ltd.

Ali, N., Hashim, N. H., and Shuib, N. S. (2015). Natural occurrence of aflatoxins and ochratoxin A in processed spices marketed in Malaysia. *Food Additives and Contaminants: Part A, Chemistry, Analysis, Control, Exposure and Risk Assessment*, just-accepted.

Aliferis, K. A., Tarantilis, P. A., Harizanis, P. C., and Alissandrakis, E. (2010). Botanical discrimination and classification of honey samples applying gas chromatography/mass spectrometry fingerprinting of headspace volatile compounds. *Food Chemistry*, *121*(3), 856–862.

Alizadeh, A. M., Roshandel, G., Roudbarmohammadi, S., Roudbary, M., Sohanaki, H., Ghiasian, S. A., Taherkhani, A., Semnani, S., and Aghasi, M. (2012). Fumonisin B1 contamination of cereals and risk of esophageal cancer in a high risk area in northeastern Iran. *Asian Pacific Journal of Cancer Prevention*, *13*(6), 2625–2628.

Alupului, A., Călinescu, I., and Lavric, V. (2012). Microwave extraction of active principles from medicinal plants. *U.P.B. Scientific Bulletin Series B*, *74*(2), 129–142.

Ambrosino, P., Galvano, F., Fogliano, V., Logrieco, A., Fresa, R., and Ritieni, A. (2004). Supercritical fluid extraction of beauvericin from maize. *Talanta*, *62*(3), 523–530.

Anfossi, L., Baggiani, C., Giovannoli, C., and Giraudi, G. (2010). Mycotoxins in food and feed: Extraction, analysis and emerging technologies for rapid and on-field detection. *Recent Patents on Food, Nutrition and Agriculture*, *2*(2), 140–153.

Anklam, E., Stroka, J., and Boenke, A. (2002). Acceptance of analytical methods for implementation of EU legislation with a focus on mycotoxins. *Food Control*, *13*(3), 173–183.

AOAC. (2009). A method for the determination of multiple mycotoxins in feeds and raw grains intended for feeds. *AOAC Agricultural Materials Task Force. Sub-Committee on Feed Additives and Contaminants* (pp. 2–3). Rockville, MD, USA: Association of Official Agricultural Chemists (AOAC).

Arnot, L. F., Duncan, N. M., Coetzer, H., and Botha, C. J. (2012). An outbreak of canine aflatoxicosis in Gauteng Province, South Africa. *Journal of the South African Veterinary Association*, *83*(1), 1–4.

Arora, K., and Gangadharappa, H. V. (2016). An Approach to bioanalytical method development and validation: a review. *International Journal of Pharmaceutical Sciences and Research*, *7*(6), 2291–2301.

Arranz, I., Baeyens, W. R., Van der Weken, G., De Saeger, S., and Van Peteghem, C. (2004). Review: HPLC determination of fumonisin mycotoxins. *Critical Reviews in Food Science and Nutrition*, *44*(3), 195–203.

Arroyo-Manzanares, N., De Ruyck, K., Uka, V., Gámiz-Gracia, L., García-Campaña, A. M., De Saeger, S., and Di Mavungu, J. D. (2018). In-house validation of a rapid and efficient procedure for simultaneous determination of ergot alkaloids and other mycotoxins in wheat and maize. *Analytical and Bioanalytical Chemistry*, *410*(22), 5567–5581.

Arroyo-Manzanares, N., Huertas-Pérez, J. F., García-Campaña, A. M., and Gámiz-Gracia, L. (2014). Mycotoxin analysis: new proposals for sample treatment. *Advances in Chemistry*, *2014*, 1–12.

Arroyo-Manzanares, N., Huertas-Pérez, J. F., Gámiz-Gracia, L., and García-Campaña, A. M. (2013). A new approach in sample treatment combined with UHPLC-MS/MS for the

determination of multiclass mycotoxins in edible nuts and seeds. *Talanta*, *115*, 61–67.

Asam, S., Konitzer, K., and Rychlik, M. (2011). Precise determination of the Alternaria mycotoxins alternariol and alternariol monomethyl ether in cereal, fruit and vegetable products using stable isotope dilution assays. *Mycotoxin Research*, *27*(1), 23–28.

Asl, A. H., and Khajenoori, M. (2013). Subcritical water extraction. In: H. Nakajima (Ed.), *Mass Transfer - Advances in sustainable energy and environment oriented numerical modeling* (pp. 459–487). Rijeka, Croatia: InTech.

Atanda, O., Makun, H. A., Ogara, I. M., Edema, M., Idahor, K. O., Eshiett, M. E., and Oluwabamiwo, B. F. (2013). Fungal and mycotoxin contamination of Nigerian foods and feeds. In: H. A. Makun (Ed.), *Mycotoxin and food safety in developing countries* (pp. 1–38). Rijeka, Croatia: InTech.

Atanda, O., Ndenn, J., and Diedhiou, P. (2015). The economic impact of aflatoxins in West Africa: The case of Nigeria, Gambia and Senegal. Retrieved February 5, 2018, from http://aflatoxinpartnership.org/uploads/2.3 - Economic impact of aflatoxin in West Africa.pdf

Atoui, A., El-Khoury, A., Kallassy, M., and Lebrihi, A. (2012). Quantification of *Fusarium graminearum* and *Fusarium culmorum* by real-time PCR system and zearalenone assessment in maize. *International Journal of Food Microbiology*, *154*(1), 59–65.

Attawood, D., and Florence, A. T. (2012). *Physical pharmacy* (2nd ed.). Philadelphia, USA: Pharmaceutical Press.

Augusto, F., Hantao, L. W., Mogollo, N. G. S., and Braga, S. C. G. N. (2013). New materials and trends in sorbents for solid-phase extraction. *Trends in Analytical Chemistry*, *43*, 14–23.

Awad, W., Ghareeb, K., Böhm, J., and Zentek, J. (2013). The toxicological impacts of the fusarium mycotoxin, deoxynivalenol, in poultry flocks with special reference to immunotoxicity. *Toxins*, *5*(5), 912–925.

Ayre, A., Ghude, K., Mane, P., Nemade, M., Gosavi, S., Pathare, A., and Lad, A. (2013). Supercritical fluid extraction - a green paradigm in the area of separation science. *Asian*

Journal of Biochemical and Pharmaceutical Sciences, *3*(23), 1–7.

Azami, M., Bahram, M., Nouri, S., and Naseri, A. (2012). A central composite design for the optimization of the removal of the azo dye, methyl orange, from waste water using the Fenton reaction. *Journal of the Serbian Chemical Society*, *77*(2), 235–246.

Ballabio, D., and Todeschini, R. (2009). Multivariate classification for qualitative analysis. In: D. W. Sun (Ed.), *Infrared spectroscopy for food quality analysis and control* (1st ed., pp. 95–97). Cambridge, Massachusetts: Academic Press Inc.

Bart, J. C. J. (2005). *Additives in Polymers: Industrial Analysis and Applications*. West Sussex, England: John Wiley and Sons Ltd.

Bas, D., and Boyaci, I. H. (2007). Modeling and optimization I: Usability of response surface methodology. *Journal of Food Engineering*, *78*, 836–845.

Bastianello, S. S., Nesbit, J. W., Williams, M. C., and Lange, A. L. (1987). Pathological findings in a natural outbreak of aflatoxicosis in dogs. *The Onderstepoort Journal of Veterinary Research*, *54*(4), 635–640.

Bayman, P., and Baker, J. L. (2006). Ochratoxins: A global perspective. *Mycopathologia*, *162*(3), 215–223.

Bbosa, G. S., Kitya, D., Lubega, A., Ogwal-Okeng, J., Anokbonggo, W. W., and Kyegombe, D. B. (2013). Review of the biological and health effects of aflatoxins on body organs and body systems. In: M. Razzaghi-Abyaneh (Ed.), *Aflatoxins - Recent advances and future prospects* (pp. 239–265). Rijeka, Croatia: InTech.

Becker, C., Reiter, M., Pfaffl, M. W., Meyer, H. H., Bauer, J., and Meyer, K. H. (2011). Expression of immune relevant genes in pigs under the influence of low doses of deoxynivalenol (DON). *Mycotoxin Research*, *27*(4), 287–293.

Belhassen, H., Jiménez-Díaz, I., Arrebola, J. P., Ghali, R., Ghorbel, H., Olea, N., and Hedili, A. (2015). Zearalenone and its metabolites in urine and breast cancer risk: A case-control study in Tunisia. *Chemosphere*, *128*, 1–6.

Bennett, J., and Klich, M. (2003). Mycotoxins. *Clinical Microbiology Reviews*, *16*(3), 497–516.

Berthiller, F., Sulyok, M., Krska, R., and Schuhmacher, R. (2007). Chromatographic methods for the simultaneous determination of mycotoxins and their conjugates in cereals. *International Journal of Food Microbiology*, *119*(1–2), 33–37.

Bezerra, M. A., Santelli, R. E., Oliveira, E. P., Villar, L. S., and Escaleira, L. A. (2008). Response surface methodology (RSM) as a tool for optimization in analytical chemistry. *Talanta*, *78*, 965–977.

Bhat, R., Rai, R. V, and Karim, A. A. (2010). Mycotoxins in food and feed: present status and future concerns. *Comprehensive Reviews in Food Science and Food Safety*, *9*(1), 57–81.

Bhusnure, O. G., Gandge, N. V, Gholve, S. B., Sugave, B. K., and Giram, P. S. (2017). Review on bioanalytical method development and validation by chromatographic techniques. *International Journal of Pharma Sciences and Research*, *10*(1), 76–97.

Biomin. (2018). 8 steps for taking the perfect sample for mycotoxin analysis. Retrieved March 23, 2019, from https://www.biomin.net/en/articles/8-steps-for-taking-the-perfect-sample-for-mycotoxin-analysis/

Bondy, G. S., and Pestka, J. J. (2000). Immunomodulation by fungal toxins. *Journal of Toxicology and Environmental Health, Part B*, *3*(2), 109–143.

Boonen, J., Malysheva, S. V, Taevernier, L., Diana Di Mavungu, J., De Saeger, S., and De Spiegeleer, B. (2012). Human skin penetration of selected model mycotoxins. *Toxicology*, *301*(1–3), 21–32.

Boudra, H., Le Bars, P., and Le Bars, J. (1995). Thermostability of Ochratoxin A in wheat under two moisture conditions. *Applied and Environmental Microbiology*, *61*(3), 1156–1158.

Bowman, J., Leslie, J., and Wu, F. (2012). How mycotoxins impact agriculture, nutrition and development. *USAID Bureau for Food Security*. Retrieved January 5, 2019, from https://agrilinks.org/sites/default/files/resource/files/USAID Agrilinks Ag Sector Council May -FINAL.pdf

Bradley, J. C., Abraham, M. H., Acree, W. E., and Lang, A. S. (2015). Predicting Abraham model solvent coefficients. *Chemistry Central Journal*, *9*(1), 12.

Braun, M. S., and Wink, M. (2018). Exposure, occurrence, and chemistry of fumonisins and their cryptic derivatives. *Comprehensive Reviews in Food Science and Food Safety*, *17*(3), 769–791.

Brereton, R. G. (2003). *Chemometrics: Data analysis for the laboratory and chemical plant*. West Sussex, England: John Wiley and Sons Ltd.

Bro, R., and Smilde, A. K. (2014). Principal component analysis. *Analytical Methods*, *6*, 2812–2831.

Bryden, W. L. (2007). Mycotoxins in the food chain: Human health. *Asia Pacific Journal of Clinical Nutrition*, *16*(1), 95–101.

Bryła, M., Waśkiewicz, A., Ksieniewicz-Woźniak, E., Szymczyk, K., and Jędrzejczak, R. (2018). Modified fusarium mycotoxins in cereals and their products—metabolism, occurrence, and toxicity: an updated review. *Molecules*, *23*(4), 963.

Bu, D. (2007). Chemometric analysis for spectroscopy: Bridging the gap between the state and measurement of a chemical system. *CAMO Software*, *Rockaway*, 3/26/2016. Retrieved June 24, 2019, from http://www.camo.com/downloads/resources/application_notes/Chemometric Analysis for Spectroscopy.pdf

Buhler, W., Dinjus, E., Ederer, H. J., Kruse, A., and Mas, C. (2002). Ionic reactions and pyrolysis of glycerol as competing reaction pathways in near- and supercritical water. *Journal of Supercritical Fluids*, *22*, 37–53.

Bulgariu, L., and Bulgariu, D. (2015). Biodiesel production from marine macroalgae. In: S. K. Kim & C. G. Lee (Eds.), *Marine bioenergy: Trends and developments* (p. 475). Boca Raton, Florida: CRC Press, Taylor and Francis Group.

Bullerman, L. B., and Bianchini, A. (2007). Stability of mycotoxins during food processing. *International Journal of Food Microbiology*, *119*, 140–146.

Bullerman, L. B., Schroeder, L. L., and Park, K. Y. (1984). Formation and control of mycotoxins in food. *Journal of Food Protection*, *47*, 637–646.

Busman, M., Poling, S. M., and Maragos, C. M. (2011). Observation of T-2 toxin and HT-2 toxin glucosides from Fusarium sporotrichioides by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). *Toxins*, *3*(12), 1554–1568.

Cabane, B., and Vuilleumier, R. (2005). The physics of liquid water. *Comptes Rendus Geoscience, Elsevier*, *337*, 1–23.

Cacace, J. E., and Mazza, G. (2006). Pressurized low polarity water extraction of lignans from whole flaxseed. *Journal of Food Engineering*, *77*(4), 1087–1095.

Capriotti, A. L., Caruso, G., Cavaliere, C., Foglia, P., Samperi, R., and Laganà, A. (2012). Multiclass mycotoxin analysis in food, environmental and biological matrices with chromatography/mass spectrometry. *Mass Spectrometry Reviews*, *31*(4), 466–503.

Cardwell, K. F. (2000). Mycotoxin contamination of foods in Africa: Antinutritional factors. *Food and Nutrition Bulletin*, *21*(4), 488–492.

Carr, A. G., Mammucari, R., and Foster, N. R. (2011). A review of subcritical water as a solvent and its utilization for the processing of hydrophobic organic compounds. *Chemical Engineering Journal*, *172*(1), 1–17.

CAST. (2003). *Mycotoxins: risks in plant, animal, and human systems*. *Task Force Report No. 139 January 2003*. Ames, Iowa, USA. Retrieved June 24, 2019, from http://www.international-food-safety.com/pdf/Mycotoxins - Risks in Plant, Animals and Human Systems.pdf

Castelo, M. M., Jackson, L. S., Hanna, M. A., Reynolds, B. H., and Bullerman, L. B. (2001). Loss of fuminosin B1 in extruded and baked corn-based foods with sugars. *JFS: Food Chemistry and Toxicology*, *66*(3), 416–421.

Chaplin, M. (2008). *Water's Hydrogen Bond Strength*. Retrieved June 24, 2019, from http:// arxiv.org/ftp/arxiv/papers/0706/0706.1355.pdf

Chemat, F., Vian, M., and Cravotto, G. (2012). Green extraction of natural products: Concept and

principles. *International Journal of Molecular Sciences*, *13*, 8615–8627.

Chen, J., Liu, D., Li, S., and Yao, D. (2010). Development of an amperometric enzyme electrode biosensor for sterigmatocystin detection. *Enzyme and Microbial Technology*, *47*(4), 119–126.

Chen, S., and Zhang, H. (2013). Development of a microwave-assisted-extraction-based method for the determination of aflatoxins B_1, G_1, B_2, and G_2 in grains and grain products. *Analytical and Bioanalytical Chemistry*, *405*, 1623–1630.

Chilaka, C. A., De Boevre, M., Atanda, O. O., and De Saeger, S. (2016). Occurrence of *Fusarium* mycotoxins in cereal crops and processed products (Ogi) from Nigeria. *Toxins*, *8*(11), 342.

Chilaka, C. A., De Boevre, M., Atanda, O. O., and De Saeger, S. (2017). The status of Fusarium mycotoxins in sub-Saharan africa: A review of emerging trends and post-harvest mitigation strategies towards food control. *Toxins*, *9*(1), 19.

Chilaka, C. A., Kock, S. De, Phoku, J. Z., Mwanza, M., Augustina, M., and Dutton, M. F. (2012). Fungal and mycotoxin contamination of South African commercial maize. *Journal of Food, Agriculture and Environment*, *10*(2), 296–303.

Choi, M. P. K., Chan, K. K. C., Leung, H. W., and Huie, C. W. (2003). Pressurized liquid extraction of active ingredients (ginsenosides) from medicinal plants using non-ionic surfactant solutions. *Journal of Chromatography A*, *983*, 153–162.

Cigić, I. K., and Prosen, H. (2009). An overview of conventional and emerging analytical methods for the determination of mycotoxins. *International Journal of Molecular Sciences*, *10*(1), 62–115.

CIMMYT. (2013). Tackling toxins with aflasafe^{TM}. International maize and wheat improvement center. Retrieved December 27, 2017, from http://www.cimmyt.org/tackling-toxins-with-aflasafe/

Cirlini, M., Dall'Asta, C., and Galaverna, G. (2012). Hyphenated chromatographic techniques for structural characterization and determination of masked mycotoxins. *Journal of Chromatography A*, *1255*, 145–152.

Clark, H. A., and Snedeker, S. M. (2006). Ochratoxin A: its cancer risk and potential for exposure. *Part B, Critical Reviews*, *9*(3), 265–296.

Co, M. (2010). *Pressurized fluid extraction of bioactive species in tree barks - Analysis using hyphenated elctrochemical mass spectrometric detection*. Doctoral thesis. Faculty of Science and Technology, Uppsala University, Sweden.

CODEX. (2015). General standard for contaminants and toxins in food and feed (CODEX STAN 193-1995). *CODEX Alimentarius. International Food Standards, CODEX STAN*, 25. Retrieved June 24, 2019, from http://www.fao.org/fileadmin/user_upload/livestockgov/documents/1_CXS_193e.pdf

Commission Regulation (2006). Commission Regulation (EC) No. 401/2006 of laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs. *Official Journal of the European Communities, L70*, 20–23.

Commission Regulation (2014). Commission Regulation (EU) No 519/2014 of 16 May 2014 - amending Regulation (EC) No 401/2006 as regards methods of sampling of large lots, spices and food supplements, performance criteria for T-2, HT-2 toxin and citrinin and screening methods of analysis. *Official Journal of the European Union*.

Cordella, C. B. Y. (2012). PCA: The basic building block of chemometrics. In: S. Krull (Ed.), *Analytical Chemistry* (pp. 1–46). Rijeka, Croatia: InTech.

Cowan, D. M., Kingsbury, T., Perez, A. L., Woods, T. A., Kovochich, M., Hill, D. S., Madl, A. K., and Paustenbach, D. J. (2014). Evaluation of the California Safer Consumer Products Regulation and the impact on consumers and product manufacturers. *Regulatory Toxicology and Pharmacology*, *68*(1), 23–40.

Cubero-Leon, E., Peñalver, R., and Maquet, A. (2014). Review on metabolomics for food authentication. *Food Research International*, *60*, 95–107.

Cubero-Leon, E., De Rudder, O., and Maquet, A. (2018). Metabolomics for organic food authentication: Results from a long-term field study in carrots. *Food Chemistry*, *239*, 760–770.

Curren, M. S. S., and King, J. W. (2001a). Ethanol-modified subcritical water extraction combined with solid-phase microextraction for determining Atrazine in beef kidney. *Journal of Agriculture and Food Chemistry*, *49*, 2175–2180.

Curren, M. S. S., and King, J. W. (2001b). Solubility of Triazine pesticides in pure and modified subcritical water. *Analytical Chemistry*, *73*, 740–745.

D'Mello, J. (2003). *Mycotoxins in cereal grains, nuts and other plant products: Food safety: Contaminants and toxins*. Wallingford, England: CAB International.

Daniel, J. H., Lewis, L. W., Redwood, Y. A., Kieszak, S., Breiman, R. F., Dana, W., and McGeehin, M. A. (2011). Comprehensive assessment of maize aflatoxin levels in eastern Kenya, 2005-2007. *Environmental Health Perspectives*, *119*(12), 1794–1799.

Darwish, W. S., Ikenaka, Y., Nakayama, S. M., and Ishizuka, M. (2014). An overview on mycotoxin contamination of foods in Africa. *Journal of Veterinary Medical Science*, *76*(6), 789–797.

Daszykowski, M., Walczak, B., and Massart, D. L. (2003). Projection methods in chemistry. *Chemometrics and Intelligent Laboratory Systems*, *65*, 97–112.

Daszykowski, M., Kaczmarek, K., Heyden, Y. Vander, and Walczak, B. (2007). Robust statistics in data analysis — A review: Basic concepts. *Chemometrics and Intelligent Laboratory Systems*, *85*, 203–219.

De Costa, C. (2002). St Anthony's fire and living ligatures: a short history of ergometrine. *The Lancet*, *359*(9319), 1768–1770.

Deng, C. H., Li, N., and Zhang, X. M. (2004). Rapid determination of essential oil in "*Acorus tatarinowii Chott*" by pressurized hot water extraction followed by solid-phase microextracton and gas chromatography-mass spectrometry. *Journal of Chromatography A*, *1059*(1), 149–155.

Devgun, M., Nanda, A., and Ansari, S. H. (2009). Microwave-assisted extraction - a promising extraction technique for natural products. *Pharmacological Reviews*, *7*, 87–94.

Dhanasekaran, D., Shanmugapriya, S., Thajuddin, N., and Panneerselvam, A. (2011). Aflatoxins and aflatoxicosis in human and animals. In: R. G. Guevara-Gonzalez (Ed.), *Aflatoxins - Biochemistry and molecular Biology* (1st ed., pp. 221–254). Rijeka, Croatia: InTech.

Díez, C., Barrado, E., and Rodríguez, J. A. (2011). Chemometric strategies for the extraction and analysis optimization of herbicide residues in soil samples. In: M. Larramendy (Ed.), *Herbicides, theory and applications* (pp. 345–368). Rijeka, Croatia: InTech.

Doughari, J. H. (2012). Phytochemicals: Extraction methods, basic structures and mode of action as potential chemotherapeutic agents. In: V. Rao (Ed.), *Phytochemicals – A global perspective of their role in nutrition and health* (pp. 1–32). Rijeka, Croatia: InTech.

Du Plessis, L. (2014). THE MAIZE TRUST: Custodian of the maize industry. Retrieved February 13, 2018, from http://www.grainsa.co.za/the-maize-trust:-custodian-of-the-maize-industry

Duy, L., Sephton, M. A., and Watson, J. S. (2015). Subcritical water extraction of organic matter from sedimentary rocks. *Analytica Chimica Acta*, *879*, 48–57.

ECOACAP. (2014). Aflatoxin control action plan for ECOWAS member states 2014-2024. *ECOWAS Aflatoxin Control Action Plan 2014*, 1–35. Retrieved June 24, 2019, from https://www.aflatoxinpartnership.org/sites/default/files/2018-06/ECOWAS%20Action%20Plan-%20FINAL%20DRAFT.pdf

Ediage, E. N., Di Mavungu, J. D., Monbaliu, S., Van Peteghem, C., and De Saeger, S. (2011). A validated multianalyte LC-MS/MS method for quantification of 25 mycotoxins in cassava flour, peanut cake and maize samples. *Journal of Agricultural and Food Chemistry*, *59*(10), 5173–5180.

Eke, Z., Kende, A., and Torkos, K. (2004). Simultaneous detection of A and B trichothecenes by gas chromatography with flame ionization or mass selective detection. *Microchemical Journal*, *78*(2), 211–216.

El-Gindy, A., and Hadad, G. M. (2012). Chemometrics in pharmaceutical analysis: An introduction, review, and future perspectives. *Journal of AOAC International*, *95*(3), 69–623.

El Khoury, A., and Atoui, A. (2010). Ochratoxin A: general overview and actual molecular status. *Toxins*, *2*(4), 461–493.

Emerypharma. (2019). Liquid chromatography mass spectrometry (LC-MS) analysis. Retrieved March 24, 2019, from https://emerypharma.com/chemistry/lcms-services/

Enyiukwu, D. N., Awurum, A. N., and Nwaneri, J. A. (2014). Mycotoxins in stored agricultural products: Implications to food safety and health and prospects of plant-derived pesticides as novel approach to their management. *Greener Journal of Microbiology and Antimicrobials, 2*(3), 32–48.

Escrivá, L., Oueslati, S., Font, G., and Manyes, L. (2017). Alternaria mycotoxins in food and feed: an overview. *Journal of Food Quality*, *2017*, Article ID 1569748.

Eskilsson, C. S., and Björklund, E. (2000). Analytical-scale microwave-assisted extraction. *Journal of Chromatography A*, *902*(1), 227–250.

European Commission (2002). The Commission of the European Communities. Commission Decision 2002/657/EC of 12 August 2002. Implementing Council Directive 96/23/EC, Concerning the Performance of Analytical Methods and the Interpretation of Results. Brussels. *Official Journal of the European Communities*.

European Commission (2016). Guidance document on identification of mycotoxins in food and feed. Supplementary guidance for Annex II of Commission Regulation (EC) No 401/2006. *European Union Commission Regulation*, *SANTE/1208*(Implemented by 01/01/2017), 1–4.

Fang, H., Wu, Y., Guo, J., Rong, J., Ma, L., Zhao, Z., Zuo, D., and Peng, S. (2012). T-2 toxin induces apoptosis in differentiated murine embryonic stem cells through reactive oxygen species-mediated mitochondrial pathway. *Apoptosis*, *17*(8), 895–907.

FAO/IAEA. (2001). Manual on the application of the HACCP system in mycotoxin prevention and control. *Joint Food and Agriculture Organization of the United Nations (FAO), and the International Agency for Atomic Energy (IAEA) Training and Reference Centre for Food and Pesticide Control. Rome, Italy. 2001*, (FAO Food and Nutrition Paper 73), 1–104.

FAO. (2004). *Worldwide regulations for mycotoxins in food and feed in 2003*. Food Quality and Standards Service (ESNS) No. 81. *FAO Food and Nutrition Paper 81*. Rome, Italy. Retrieved December 23, 2018, from http://www.fao.org/3/a-y5499e.pdf

Fattah, R. A., Mostafa, N. A., Mahmoud, M. S., and Abdelmoez, W. (2014). Recovery of oil and free fatty acids from spent bleaching earth using sub-critical water technology supported with kinetic and thermodynamic study. *Advances in Bioscience and Biotechnology*, *5*, 261–272.

FDA. (2015). Guidelines for the validation of chemical methods for the FDA FVM program. In: P. A. Orlandi, W. T. Flynn, D. L. Zink, P. E. Norris, V. K. Bunning, T. Mcgrath, & M. E. Torrence (Eds.), *US Food and Drug Administration. Office of Foods and Veterinary Medicine* (2nd ed.). Silver Spring, Maryland.

FDA. (2018). Investigative operation manual. *US Food and Drug Administration (FDA), Office of Regulatory Affairs. Washington (DC)*. Retrieved December 24, 2018, from https://www.fda.gov/downloads/ICECI/Inspections/IOM/UCM607759.pdf

Ferlay, J., Soerjomataram, I., Dikshit, R., and Eser, S. (2013). Mortality world wide: IARC CancerBase. *Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11*.

Fernández-Cruz, M. L., Mansilla, M. L., and Tadeo, J. L. (2010). Mycotoxins in fruits and their processed products: Analysis, occurrence and health implications. *Journal of Advanced Research*, *1*, 113–122.

Ferrante, M., Sciacca, S., and Conti, G. O. (2012). Carcinogen role of food by mycotoxins and knowledge gap. In: M. Pesheva (Ed.), *Carcinogen* (pp. 133–162). Rijeka, Croatia: InTech.

Filali, A., Betbeder, A. M., Baudrimont, I., Benayada, A., Soulaymani, R., and Creppy, E. E. (2002). Ochratoxin A in human plasma in Morocco: a preliminary survey. *Human and Experimental Toxicology*, *21*(5), 241–245.

Freire, L., and Sant'Ana, A. S. (2018). Modified mycotoxins: An updated review on their formation, detection, occurrence, and toxic effects. *Food and Chemical Toxicology*, *111*, 189–205.

FSA. (2015). Mycotoxins sampling guidance. Retrieved March 23, 2019, from https://www.food.gov.uk/sites/default/files/media/document/mycotoxins-sampling-guidance.pdf

Galaverna, G., Dall'Asta, C., Mangia, M., Dossena, A., and Marchelli, R. (2009). Masked mycotoxins: an emerging issue for food safety. *Czech Journal of Food Sciences*, *27*, 89–92.

Galbenu-Morvay, P. L., Trif, A., DAMIESCU, L., and Simion, G. (2011). T-2 toxin occurrence in cereals and cereal-based foods. *Bulletin UASVM Agriculture*, *68*(8), 274–280.

Gam, L., Tham, S., and Latiff, A. (2003). Immunoaffinity extraction and tandem mass spectrometric analysis of human chorionic gonadotropin in doping analysis. *Journal of Chromatography B*, *792*(2), 187–196.

Garba, M. H., Makun, H. A., Jigam, A. A., Muhammad, H. L., and Njobeh, P. B. (2017). Incidence and toxigenicity of fungi contaminating sorghum from Nigeria. *World Journal of Microbiology*, *4*(2), 105–114.

García-Marino, M., Rivas-Gonzalo, J., Ibánez, E., and García-Marino, C. (2006). Recovery of catechins and proanthocyanidins from winery by-products using subcritical water extraction. *Analytica Chimica Acta*, *563*(1–2), 44–50.

Gareis, M., Bauer, J., Thiem, J., Plank, G., Grabley, S., and Gedek, B. (1990). Cleavage of zearalenone-glycoside, a "masked" mycotoxin, during digestion in swine. *Journal of Veterinary Medicine, Series B*, *37*(1–10), 236–240.

Gbashi, S., Njobeh, P., Steenkamp, P., Tutu, H., and Madala, N. (2016). The effect of temperature and methanol–water mixture on pressurized hot water extraction (PHWE) of anti-HIV analogues from *Bidens pilosa*. *Chemistry Central Journal*, *10*(37), 1–12.

Gbashi, S., Njobeh, P., Steenkamp, P., and Madala, N. (2017a). Pressurized hot water extraction and chemometric fingerprinting of flavonoids from *Bidens pilosa* by UPLC-tandem mass spectrometry. *CYTA - Journal of Food*, *15*(2), 171–180.

Gbashi, S., Madala, N., Adebo, O. A., and Njobeh, P. B. (2017b). Development and validation of

a pressurized hot water extraction method for the extraction of aflatoxin B_1 in maize. In: *7th Euro Global Summit on Clinical Microbiology and Mycotoxins. February 27-28, 2017 Amsterdam, Netherlands* (p. Full text data not published). Amsterdam, Netherlands.

Gbashi, S., Madala, N. E., Adebo, O. A., Piater, L., Phoku, J. Z., and Njobeh, P. B. (2017c). Subcritical water extraction and its prospects for aflatoxins extraction in biological materials. In: L. B. Abdulra'uf (Ed.), *Aflatoxin-control, analysis, detection and health risks* (pp. 229–250). Rijeka, Croatia: InTech.

Gbashi, S., Adebo, O. A., Piater, L., Madala, N. E., and Njobeh, P. B. (2017d). Subcritical water extraction in biological materials. *Separation and Purification Reviews*, *46*(1), 21–34.

Gbashi, S., Madala, N. E., De Saeger, S., De Boevre, M., Adekoya, I., Adebo, O. A., and Njobeh, P. B. (2018). The Socio-economic impact of mycotoxin contamination in Africa. In: P. B. Njobeh (Ed.), *Fungi and mycotoxins - their occurrence, impact on health and the economy as well as pre- and postharvest management strategies* (pp. 1–20). Rijeka, Croatia: InTech.

Geary, P. A., Chen, G., Kimanya, M. E., Shirima, C. P., Oplatowska-Stachowiak, M., Elliott, C. T., Routledge, M. N., and Gong, Y. Y. (2016). Determination of multi-mycotoxin occurrence in maize based porridges from selected regions of Tanzania by liquid chromatography tandem mass spectrometry (LC-MS/MS), a longitudinal study. *Food Control*, *68*, 337–343.

Ghani, S. B. A., Weaver, L., Zidan, Z. H., Ali, H. M., Keevil, C. W., and Brown, R. C. D. (2008). Microwave-assisted synthesis and antimicrobial activities of flavonoid derivatives. *Bioorganic and Medicinal Chemistry Letters*, *18*, 518–522.

Giannitti, F., Diab, S. S., Pacin, A. M., Barrandeguy, M., Larrere, C., Ortega, J., and Uzal, F. A. (2011). Equine leukoencephalomalacia (ELEM) due to fumonisins B1 and B2 in Argentina. *Pesquisa Veterinária Brasileira*, *31*(5), 407–412.

Gil, L., Ruiz, P., Font, G., and Manyes, L. (2016). An overview of the applications of hazards analysis and critical control point (HACCP) system to mycotoxins. *Reviews in Toxicology*, *33*, 50–55.

Gilbert, J., and Anklam, E. (2002). Validation of analytical methods for determining mycotoxins

in foodstuffs. *TrAC Trends in Analytical Chemistry*, *21*(6–7), 468–486.

Gnonlonfin, G. J. B., Hell, K., Adjovi, Y., Fandohan, P., Koudande, D. O., Mensah, G. A., Sanni, A., and Brimer, L. (2013). A review on aflatoxin contamination and its implications in the developing world: A sub-Saharan African perspective. *Critical Reviews in Food Science and Nutrition*, *53*(4), 349–365.

Goncalves, C., Carvalho, J. J., Azenha, M. A., and Alpendurada, M. F. (2006). Optimization of supercritical fluid extraction of pesticide residues in soil by means of central composite design and analysis by gas chromatography–tandem mass spectrometry. *Journal of Chromatography A*, *1110*(1–2), 6–14.

González-Peñas, E., Leache, C., Viscarret, M., De Obanos, A. P., Araguás, C., and De Cerain, A. L. (2004). Determination of ochratoxin A in wine using liquid-phase microextraction combined with liquid chromatography with fluorescence detection. *Journal of Chromatography A*, *1025*(2), 163–168.

Gratz, S. W. (2017). Do Plant-Bound Masked Mycotoxins Contribute to Toxicity? *Toxins*, *9*(3), 85.

Grenier, B., and Oswald, I. (2011). Mycotoxin co-contamination of food and feed: Meta-analysis of publications describing toxicological interactions. *World Mycotoxin Journal*, *4*(3), 285–313.

Gromadzka, K., Waskiewicz, A., Chelkowski, J., and Golinski, P. (2008). Zearalenone and its metabolites: occurrence, detection, toxicity and guidelines. *World Mycotoxin Journal*, *1*(2), 209–220.

Groopman, J. D., and Kensler, T. W. (2005). Role of metabolism and viruses in aflatoxin-induced liver cancer. *Toxicology and Applied Pharmacology*, *206*, 131–137.

Guclu-Ustundag, O., and Mazza, G. (2009). Effects of pressurized low polarity water extraction parameters on antioxidant properties and composition of cow cockle seed extracts. *Plant Foods for Human Nutrition*, *64*(1), 32–38.

Gunst, R. F., and Mason, R. L. (2009). Fractional factorial design. *Wiley Interdisciplinary Reviews: Computational Statistics*, *1*(2), 234–244.

Gupta, A., Naraniwal, M., and Kothari, V. (2012). Modern extraction methods for preparation of bioactive plant extracts. *International Journal of Applied and Natural Sciences (IJANS)*, *1*(1), 8–26.

Haider, S. I., and Asif, E. S. (2011). *Quality control training mannual. Comprehensive training guide for API, finished pharmaceutical and biotechnologies laboratories*. Boca Raton, Florida: CRC Press.

Hartonen, K., Parshintsev, J., Sandberg, K., Bergelin, E., Nisula, L., and Riekkola, M. L. (2007). Isolation of flavonoids from aspen knotwood by pressurized hot water extraction and comparison with other extraction techniques. *Talanta*, *74*(1), 32–38.

Haschek, W. M., Voss, K. A., and Beasley, V. R. (2002). Selected mycotoxins affecting animal and human health. In: *Handbook of toxicologic pathology* (2, Vol. 1 ed., pp. 645–699). San Diego, California: Academic Press.

Haschek, W. M., Gumprecht, L. A., Smith, G., Tumbleson, M. E., and Constable, P. D. (2001). Fumonisin toxicosis in swine: an overview of porcine pulmonary edema and current perspectives. *Environmental Health Perspectives*, *109*(Suppl 2), 251–257.

Hawthorne, S. B. (2000). *Enhanced electrochemical process in sub-critical water*. *Technical Report*. Dakota. Retrieved January 12, 2019, from http://www.adktroutguide.com/files/subcritical_electrolysis_NREL.pdf.

Hawthorne, S. B., Yang, Y., and Miller, D. J. (1994). Extraction of organic pollutants from environmental solids with sub- and supercritical water. *Analytical Chemistry*, *66*, 2912–2920.

Hawthorne, S. B., Grabanski, C. B., Martin, E., and Miller, D. J. (2000). Comparisons of Soxhlet extraction, pressurized liquid extraction, supercritical fluid extraction and subcritical water extraction for environmental solids: Recovery, selectivity and effects on sample matrix. *Journal of Chromatography A*, *892*(1–2), 421–433.

Henderson, R. K., Jiménez-González, C., Constable, D. J., Alston, S. R., Inglis, G. G., Fisher, G., Sherwood, J., Binks, S. P., and Curzons, A. D. (2011). Expanding GSK's solvent selection guide–embedding sustainability into solvent selection starting at medicinal chemistry. *Green Chemistry*, *13*(4), 854–862.

Hendrickse, R. G. (1999). Of sick turkeys, kwashiorkor, malaria, perinatal mortality, heroin addicts and food poisoning: Research on the influence of aflatoxins on child health in the tropics. *Annals of Tropical Paediatrics*, *19*(3), 229–235.

Herrero, M., Ibanez, E., Senorans, J., and Cifuentes, A. (2004). Pressurized liquid extracts from Spirulina platensis microalga: Determination of their antioxidant activity and preliminary analysis by micellar electrokinetic chromatography. *Journal of Chromatography A*, *1047*, 195–203.

Hewitt, T. C., Flack, C. L., Kolodziejczyk, J. K., Chacon, A. M., and D'Ovidio, K. L. (2012). Occurrence of zearalenone in fresh corn and corn products collected from local Hispanic markets in San Diego County, CA. *Food Control*, *26*(2), 300–304.

Hickert, S., Hermes, L., Marques, L. M. M., Focke, C., Cramer, B., Lopes, N. P., Flett, B., and Humpf, H. U. (2017). Alternaria toxins in South African sunflower seeds: cooperative study. *Mycotoxin Research*, *33*(4), 309–321.

Ho, C. H. L., Cacace, J. E., and Mazza, G. (2007). Extraction of lignans, proteins and carbohydrates from flaxseed meal with pressurized low polarity water. *Lebensmittel-Wissenschaft & Technologie*, *40*, 1637–1647.

Ho, C. S., Lam, C. W. K., Chan, M. H. M., Cheung, R. C. K., Law, L. K., Lit, L. C. W., Ng, K. F., Suen, M. W. M., and Tai, H. L. (2003). Electrospray ionisation mass spectrometry: principles and clinical applications. *The Clinical Biochemist Reviews*, *24*(1), 3–12.

Horvatovich, K., Hafner, D., Bodnár, Z., Berta, G., Hancz, C., Dutton, M., and Kovács, M. (2013). Dose-related genotoxic effect of T-2 toxin measured by comet assay using peripheral blood mononuclear cells of healthy pigs. *Acta Veterinaria Hungarica*, *61*(2), 175–186.

Howard, P. C., Eppley, R. M., Stack, M. E., Warbritton, A., Voss, K. A., Lorentzen, R. J., Kovach,

R. M., and Bucci, T. J. (2001). Fumonisin B_1 carcinogenicity in a two-year feeding study using F344 rats and B6C3F1 mice. *Environmental Health Perspectives*, *109*(Suppl 2), 277–282.

Huang, L. C., Zheng, N., Zheng, B. Q., Wen, F., Cheng, J. B., Han, R. W., Xu, X. M., Li, S. L., and Wang, J. Q. (2014a). Simultaneous determination of aflatoxin M1, ochratoxin A, zearalenone and α-zearalenol in milk by UHPLC–MS/MS. *Food Chemistry*, *146*(1), 242–249.

Huang, S., Wang, J., Xing, L., Shen, H., Yan, X., Wang, J., and Zhang, X. (2014b). Impairment of cell cycle progression by sterigmatocystin in human pulmonary cells *in vitro*. *Food and Chemical Toxicology*, *66*, 89–95.

Huber, L. (2010). *Validation of analytical methods. Publication number 5990-5140EN*. Waldbronn, Germany: Agilent Technologies. Retrieved June 24, 2019, from https://www.researchgate.net/profile/Ljubomir_Jacic2/post/How_can_I_validate_a_new_analytical_method_if_there_is_no_comparable_one/attachment/59d63638c49f478072ea418a/AS%3A273671610929192%401442259805121/download/Validation+of+Analytical+Methods.pdf

Hueza, I. M., Raspantini, P. C. F., Raspantini, L. E. R., Latorre, A. O., and Górniak, S. L. (2014). Zearalenone, an estrogenic mycotoxin, is an immunotoxic compound. *Toxins*, *6*(3), 1080–1095.

Humpf, H. U., and Voss, K. A. (2004). Effects of thermal food processing on the chemical structure and toxicity of fumonisin mycotoxins. *Molecular Nutrition & Food Research*, *48*(4), 255–269.

Hussain, I. (2011). Aflatoxin measurement and analysis. In: I. Torres-Pacheco (Ed.), *Aflatoxins – Detection, measurement and control* (1st ed., pp. 129–146). Rijeka, Croatia: InTech.

Hussein, H. S., and Brasel, J. M. (2001). Toxicity, metabolism and impact of mycotoxins on humans and animals. *Toxicology*, *167*, 101–134.

IARC. (2002). *IARC monographs on the evaluation of carcinogenic risks to humans. Some*

traditional herbal medicines, some mycotoxins, naphthalene and styrene. Volume 82. Lyon, France.

Ibañez, E., Kubátová, A., Señoráns, F. J., Cavero, S., Reglero, G., and Hawthorne, S. B. (2003). Subcritical water extraction of antioxidant compounds from rosemary plants. *Journal of Agricultural and Food Chemistry*, *51*(2), 375–382.

Ibarra, I. S., Rodriguez, J. A., Galán-Vidal, C. A., Cepeda, A., and Miranda, J. M. (2015). Magnetic solid phase extraction applied to food analysis. *Journal of Chemistry*, *2015*(Article ID 919414), 1–13.

Ibrahim, A. S., Zaghloul, H., and Badria, F. A. (2013). Case report evidence of relationships between hepatocellular carcinoma and ochratoxicosis. *PloS One*, *8*(8), e71423.

Ismaiel, A., and Papenbrock, J. (2015). Mycotoxins: producing fungi and mechanisms of phytotoxicity. *Agriculture*, *5*(3), 492–537.

Ismail, O. M. S., and Hameed, R. S. A. (2013). Environmental effects of volatile organic compounds on ozone layer. *Advances in Applied Science Research*, *4*(1), 264–268.

Jackson, L. S., Hlywka, J. J., Senthil, K. R., Bullerman, L. B., and Musser, S. M. (1996). Effects of time, temperature, and pH on the stability of fumonisin B1 in an aqueous model system. *Journal of Agricultural and Food Chemistry*, *44*(3), 906–912.

Jandera, P. (2002). Comparison of reversed-phase and normal-phase column liquid chromatographic techniques for the separation of low and high molecular weight compounds. *Journal of Liquid Chromatography and Related Technologies*, *25*(19), 2901–2931.

Jolly, P. E., Jiang, Y., Ellis, W. O., Awuah, R. T., Appawu, J., Nnedu, O., and Williams, J. H. (2007). Association between aflatoxin exposure and health characteristics, liver function, hepatitis and malaria infections in Ghanaians. *Journal of Nutritional and Environmental Medicine*, *16*(3–4), 242–257.

Josephs, R. D., Krska, R., Grasserbauer, M., and Broekaert, J. C. (1998). Determination of trichothecene mycotoxins in wheat by use of supercritical fluid extraction and high-

performance liquid chromatography with diode array detection or gas chromatography with electron capture detection. *Journal of Chromatography A*, *795*(2), 297–304.

Juan, C., Oueslati, S., and Mañes, J. (2016). Evaluation of Alternaria mycotoxins in strawberries: quantification and storage condition. *Food Additives and Contaminants: Part A*, *33*(5), 861–868.

Kabak, B. (2009). The fate of mycotoxins during thermal food processing. *Journal of the Science of Food and Agriculture*, *89*(4), 549–554.

Kamala, A., Shirima, C., Jani, B., Bakari, M., Sillo, H., Rusibamayila, N., De Saeger, S., Kimanya, M., Gong, Y. Y., Simba, A., and Investigation Team. (2018). Outbreak of an acute aflatoxicosis in Tanzania during 2016. *World Mycotoxin Journal*, *11*(3), 311–320.

Katerere, D. R., Shephard, G. S., and Faber, M. (2008). Infant malnutrition and chronic aflatoxicosis in Southern Africa: is there a link? *International Journal of Food Safety, Nutrition and Public Health*, *1*(2), 127–136.

Kensler, T. W., Roebuck, B. D., Wogan, G. N., and Groopman, J. D. (2011). Aflatoxin: A 50-year odyssey of mechanistic and translational toxicology. *Toxicological Sciences*, *120*(SI), S28–S48.

Khajenoori, M., Asl, A. H., Hormozi, F., Eikani, M. H., and Bidgoli, H. N. (2009). Subcritical water extraction of essential oils from Zataria multiflora Boiss. *Journal of Food Process Engineering*, *32*(6), 804–816.

Khazaeli, E., Haddadi, H., Zargar, B., and Hatamie, A. (2016). Response surface methodology based on central composite design as a chemometric tool for optimizing dispersive liquid–liquid microextraction for determining ultra-trace amounts of zinc in oil and water samples. *Analytical Methods*, *8*(25), 5101–5110.

Khoza, B. S., Chimuka, L., Mukwevho, E., Steenkamp, P. A., and Madala, N. E. (2014). The effect of temperature on pressurised hot water extraction of pharmacologically-important metabolites as analysed by UPLC-qTOF-MS and PCA. *Evidence-Based Complementary and Alternative Medicine*, *2014*(Article ID 914759), 1–9.

Khoza, B. S., Dubery, I. A., Byth-Illing, H. A., Steenkamp, P. A., Chimuka, L., and Madala, N. E. (2015). Optimization of pressurized hot water extraction of flavonoids from *Momordica foetida* using UHPLC-qTOF-MS and multivariate chemometric approaches. *Food Analytical Methods*, *2015*, 1–10.

Khoza, B. S., Gbashi, S., Steenkamp, P. A., Njobeh, P. B., and Madala, N. E. (2016). Identification of hydroxylcinnamoyl tartaric acid esters in *Bidens pilosa* by UPLC-tandem mass spectrometry. *South African Journal of Botany*, *2016*, 96–100.

Kimanya, M. E., Tiisekwa, B., and Mpolya, E. (2012). *Country and economic assessment for aflatoxin contamination and control in Tanzania*. Retrieved June 24, 2019 from http://archives.au.int/bitstream/handle/123456789/5495/Tanzania%20Supplemental%20Aflatoxin%20Situational%20Analysis%20Report%20%2018%20July%202016.pdf?sequence=1&isAllowed=y

King, B. (1979). Outbreak of ergotism in Wollo, Ethiopia. *The Lancet*, *313*(8131), 1411.

Klinglmayr, C., Nöbauer, K., Razzazi-Fazeli, E., and Cichna-Markl, M. (2010). Determination of deoxynivalenol in organic and conventional food and feed by sol–gel immunoaffinity chromatography and HPLC–UV detection. *Journal of Chromatography B*, *878*(2), 187–193.

Kočube, Š., Varga, J., Sigeti, Đ., Baranji, N., Šuri, K., Tot, B., Toldi, E., Bartok, T., and Mešterhazi, A. (2013). Aspergillus species as mycotoxin producers in agricultural products in central Europe. *Matica Srpska Journal of Natural Sciences*, *124*, 13–25.

Kokkonen, M., Ojala, L., Parikka, P., and Jestoi, M. (2010). Mycotoxin production of selected *Fusarium* species at different culture conditions. *International Journal of Food Microbiology*, *143*, 17–25.

Kong, W. J., Liu, S. Y., Qiu, F., Xiao, X. H., and Yang, M. H. (2013). Simultaneous multi-mycotoxin determination in nutmeg by ultrasound-assisted solid-liquid extraction and immunoaffinity column clean-up coupled with liquid chromatography and on-line post-column photochemical derivatization-fluorescence detection. *Analyst*, *138*(9), 2729–2739.

Kovalsky, P., Kos, G., Nahrer, K., Schwab, C., Jenkins, T., Schatzayr, G., Sulyok, M., and Krska,

R. (2016). Co-occurrence of regulated, masked and emerging mycotoxins and secondary metabolites in finished feed and maize–An extensive survey. *Toxins*, *8*(12), 363.

Krska, R., Schubert-ullrich, P., Molinelli, A., Sulyok, M., MacDonald, S., and Crews, C. (2008). Mycotoxin analysis: An update. *Food Additives and Contaminants*, *25*(2), 152–163.

Kruse, A., and Dinjus, E. (2007). Hot compressed water as reaction medium and reactant Properties and synthesis reactions. *Journal of Supercritical Fluids*, *39*, 362–380.

Kubátová, A., Miller, D. J., and Hawthorne, S. B. (2001). Comparison of subcritical water and organic solvents for extracting kava lactones from kava root. *Journal of Chromatography A*, *923*(1–2), 187–194.

Kuiper-Goodman, T. (1998). Food safety: mycotoxins and phycotoxins in perspective. In: M. Miraglia, H. Van Edmond, C. Brera, & J. Gilbert (Eds.), *Mycotoxins and phycotoxins-developments in chemistry, toxicology and food safety* (pp. 25–48). Fort Collins, Colorado: Alaken Inc.

Kumar, S., Jyotirmayee, K., and Sarangi, M. (2013). Thin layer chromatography: a tool of biotechnology for isolation of bioactive compounds from medicinal plants. *International Journal of Pharmaceutical Sciences Review and Research*, *18*(1), 126–132.

Lamb, J. E., Mponda, O., and Kocyn, S. (2015). *Aflatoxin associated postharvest losses for selected food security crops in East Africa*. In Aflatoxin and postharvest loss (pp. 12–13). International Institute for Tropical Agriculture (IITA). Retrieved August 11, 2019, from https://d3n8a8pro7vhmx.cloudfront.net/eatradehub/pages/517/attachments/original/1429600275/Aflatoxin_-_Post_harvest_Losses_for_Selected_Crops_in_EAC.002.pdf?1429600275%0A%0A

Lattanzio, M. V. T., Solfrizzo, M., Powers, S., and Visconti, A. (2007). Simultaneous determination of aflatoxins, ochratoxin A and *Fusarium* toxins in maize by liquid chromatography/tandem mass spectrometry after multitoxin immunoaffinity cleanup. *Rapid Communications in Mass Spectrometry*, *21*, 3253–3261.

Lattanzio, V. M., Ciasca, B., Terzi, V., Ghizzoni, R., McCormick, S. P., and Pascale, M. (2015).

Study of the natural occurrence of T-2 and HT-2 toxins and their glucosyl derivatives from field barley to malt by high-resolution Orbitrap mass spectrometry. *Food Additives and Contaminants: Part A*, *32*(10), 1647–1655.

Lattanzio, V. M. T., Gatta, S. D., Godula, M., and Visconti, A. (2011). Quantitative analysis of mycotoxins in cereal foods by collision cell fragmentation-high-resolution mass spectrometry: performance and comparison with triple-stage quadrupole detection. *Food Additives and Contaminants: Part A*, *28*(10), 1424–1437.

Lewis, L., Onsongo, M., Njapau, H., Schurz-Rogers, H., Luber, G., Kieszak, S., Nyamongo, J., Backer, L., Dahiye, A. M., Misore, A., DeCock, K., and Rubin, C. (2005). Aflatoxin contamination of commercial maize products during an outbreak of acute aflatoxicosis in eastern and central Kenya. *Environmental Health Perspective*, *113*(12), 1763–1767.

Li, F. Q., Toyazaki, N., and Yoshizawa, T. (2001). Production of Alternaria mycotoxins by Alternaria alternata isolated from weather-damaged wheat. *Journal of Food Protection*, *64*(4), 567–571.

Li, M., Dai, C., Wang, F., Kong, Z., He, Y., Huang, Y. T., and Fan, B. (2017). Chemometric-assisted QuEChERS extraction method for post-harvest pesticide determination in fruits and vegetables. *Scientific Reports*, *7*, 42489.

Liang, X., and Fan, Q. (2013). Application of sub-critical water extraction in pharmaceutical industry. *Journal of Materials Science and Chemical Engineering*, *1*, 1–6.

Limsuwan, S. (2011). *Development, evaluation and application of methods for mycotoxin analysis*. PhD Thesis. Faculty of Agricultural Sciences, Georg-August-University Göttingen, Göttingen, Germany.

Liu, S., Liang, Y. Z., and Liu, H. T. (2016). Chemometrics applied to quality control and metabolomics for traditional Chinese medicines. *Journal of Chromatography B*, *1015*, 82–91.

Lizárraga-Paulín, E. G., Moreno-Martínez, E., and Miranda-Castro, S. P. (2011). Aflatoxins and their impact on human and animal health: An emerging problem. In: R. G. Guevara-Gonzalez

(Ed.), *Aflatoxins - Biochemistry and molecular biology* (pp. 255–282). Rijeka, Croatia: InTech.

Logrieco, A., Moretti, A., and Solfrizzo, M. (2009). Alternaria toxins and plant diseases: an overview of origin, occurrence and risks. *World Mycotoxin Journal*, *2*(2), 129–140.

Lopez-Garcia, R., Park, D. L., and Phillips, T. D. (1999). Integrated mycotoxin management systems. *Food Nutrition and Agriculture*, *23*, 38–48.

Maaroufi, K., Achour, A., Hammami, M., El May, M., Betbeder, A. M., Ellouz, F., Creppy, E. E., and Bacha, H. (1995). Ochratoxin A in human blood in relation to nephropathy in Tunisia. *Human and Experimental Toxicology*, *14*(7), 609–614.

Magan, N., Medina, A., and Aldred, D. (2011). Possible climate-change effects on mycotoxin contamination of food crops pre-and postharvest. *Plant Pathology*, *60*(1), 150–163.

Magoha, H., Kimanya, M., De Meulenaer, B., Roberfroid, D., Lachat, C., and Kolsteren, P. (2014). Risk of dietary exposure to aflatoxins and fumonisins in infants less than 6 months of age in Rombo, Northern Tanzania. *Maternal and Child Nutrition*, *12*(3), 516–527.

Makun, H. A., Dutton, M. F., Njobeh, P. B., Gbodi, T. A., and Ogbadu, G. H. (2012). Aflatoxin contamination in foods and feeds: A special focus on Africa. In: A. E. Ayman (Ed.), *Trends in vital food and control engineering* (pp. 187–234). Rijeka, Croatia: InTech.

Malachová, A., Sulyok, M., Beltrán, E., Berthiller, F., and Krska, R. (2014). Optimization and validation of a quantitative liquid chromatography – tandem mass spectrometric method covering 295 bacterial and fungal metabolites including all regulated mycotoxins in four model food matrices. *Journal of Chromatography A*, *1362*, 145–156.

Malir, F., Ostry, V., Pfohl-Leszkowicz, A., Malir, J., and Toman, J. (2016). Ochratoxin A: 50 years of research. *Toxins*, *8*(7), 191 (1-49).

Mally, A., and Dekant, W. (2009). Mycotoxins and the kidney: modes of action for renal tumor formation by ochratoxin A in rodents. *Molecular Nutrition and Food Research*, *53*(4), 467–478.

Maragos, C. M., Busman, M., and Plattner, R. D. (2008). Development of monoclonal antibodies for the fusarin mycotoxins. *Food Additives and Contaminants*, *25*(1), 105–114.

Maran, J. P., Manikandan, S., Thirugnanasambandham, K., Nivetha, C. V, and Dinesh, R. (2013). Box–Behnken design based statistical modeling for ultrasound-assisted extraction of corn silk polysaccharide. *Carbohydrate Polymers*, *92*(1), 604–611.

Marechera, G. (2015). Estimation of the potential adoption of Aflasafe among smallholder maize farmers in lower eastern Kenya. *African Journal of Agriculture and Resource Economics*, *10*(1), 72–85.

Marechera, G., and Ndwiga, J. (2014). Farmer perceptions of aflatoxin management strategies in lower Eastern Kenya. *Journal of Agricultural Extension and Rural Development*, *6*(12), 382–392.

Marin, S., Ramos, A. J., Cano-Sancho, G., and Sanchis, V. (2013). Mycotoxins: occurrence, toxicology, and exposure assessment. *Food and Chemical Toxicology*, *60*, 218–237.

Mata, A. T., Ferreira, J. P., Oliveira, B. R., Batoréu, M. C., Crespo, M. T. B., Pereira, V. J., and Bronze, M. R. (2015). Bottled water: Analysis of mycotoxins by LC – MS/MS. *Food Chemistry*, *176*, 455–464.

Mbundi, L., Gallar-Ayala, H., Khan, M. R., Barber, J. L., Losada, S., and Busquets, R. (2014). Advances in the analysis of challenging food contaminants: Nanoparticles, bisphenols, mycotoxins, and brominated flame retardants. *Advances in Molecular Toxicology*, *8*, 35–105.

McCormick, S. P., Stanley, A. M., Stover, N. A., and Alexander, N. J. (2011). Trichothecenes: from simple to complex mycotoxins. *Toxins*, *3*(7), 802–814.

McCormick, S. P., Kato, T., Maragos, C. M., Busman, M., Lattanzio, V. M., Galaverna, G., Dall-Asta, C., Crich, D., Price, N. P., and Kurtzman, C. P. (2015). Anomericity of T-2 toxin-glucoside: masked mycotoxin in cereal crops. *Journal of Agricultural and Food Chemistry*, *63*(2), 731–738.

Meena, M., Gupta, S. K., Swapnil, P., Zehra, A., Dubey, M. K., and Upadhyay, R. S. (2017).

Alternaria toxins: potential virulence factors and genes related to pathogenesis. *Frontiers in Microbiology*, *8*, 1451.

Meerpoel, C., Vidal, A., di Mavungu, J. D., Huybrechts, B., Tangni, E. K., Devreese, M., Croubels, S., and De Saeger, S. (2018). Development and validation of an LC–MS/MS method for the simultaneous determination of citrinin and ochratoxin a in a variety of feed and foodstuffs. *Journal of Chromatography A*, *1580*, 100–109.

Mejía-Teniente, L., Chapa-Oliver, A. M., Vazquez-Cruz, M. A., Torres-Pacheco, I., and Guevara-González, R. G. (2011). Aflatoxins biochemistry and molecular biology - Biotechnological approaches for control in crops. In: I. Torres-Pacheco (Ed.), *Aflatoxins – Detection, measurement and control* (pp. 317–354). Rijeka, Croatia: InTech.

Miller, D. J., Hawthorne, S. B., Gizir, A. M., and Clifford, A. A. (1998). Solubility of polycyclic aromatic hydrocarbons in subcritical water from 298 K to 498 K. *Journal of Chemical Engineering Data (American Chemical Society)*, *43*(6), 1043–1047.

Mircea, C., Poiata, A., Tuchilus, C., Agoroae, L., Butnaru, E., and Stanescu, U. (2008). Aflatoxigenic fungi isolated from medicinal herbs. *Toxicology Letters*, *180*, 32–246.

Mirzajani, F., Ghassempour, A., Jalali-Heravi, M., and Mirjalili, M. H. (2010). Optimisation of a microwave-assisted method for extracting withaferin A from *Withania somnifera Dunal*. using central composite design. *Phytochemical Analysis*, *21*(6), 544–549.

Misihairabgwi, J. M., Ezekiel, C. N., Sulyok, M., Shephard, G. S., and Krska, R. (2017). Mycotoxin contamination of foods in Southern Africa: A 10-year review (2007–2016). *Critical Reviews in Food Science and Nutrition*, (Published online), 1–16.

Missmer, S. A., Suarez, L., Felkner, M., Wang, E., Merrill Jr, A. H., Rothman, K. J., and Hendricks, K. A. (2005). Exposure to fumonisins and the occurrence of neural tube defects along the Texas–Mexico border. *Environmental Health Perspectives*, *114*(2), 237–241.

Mitchell, N. J., Bowers, E., Hurburgh, C., and Wu, F. (2016). Potential economic losses to the USA corn industry from aflatoxin contamination. *Food Additives and Contaminants: Part A*, *33*(3), 540–550.

Monbaliu, S., Van Poucke, C., Van Peteghem, C., Van Poucke, K., Heungens, K., and De Saeger, S. (2009). Development of a multi-mycotoxin liquid chromatography/tandem mass spectrometry method for sweet pepper analysis. *Rapid Communications in Mass Spectrometry*, *23*(1), 3–11.

Monbaliu, S., Van Poucke, C., Detavernier, C., Dumoulin, F., Van De Velde, M., Schoeters, E., Van Dyck, S., Averkieva, O., Van Peteghem, C., and De Saeger, S. (2010). Occurrence of mycotoxins in feed as analyzed by a multi-mycotoxin LC-MS/MS method. *Journal of Agricultural and Food Chemistry*, *13*(58), 66–71.

Montville, T. J., and Matthews, K. R. (2008). *Food microbiology: An introduction* (2nd ed.). Washington DC, USA: ASM Press.

Moreau, R. A., Nghiem, N. P., Rosentrater, K. A., Johnston, D. B., and Hicks, K. B. (2012). Ethanol production from starch-rich crops other than corn and the composition and value of the resulting DDGS. In: K. Liu & K. A. Rosentrater (Eds.), *Distillers grains: production, properties, and utilization* (p. 109). Boca Raton, Florida: CRC Press, Taylor and Francis Group.

Moreno, E., Reza, J., and Trejo, A. (2007). Extraction of polycyclic aromatic hydrocarbons from soil using water under subcritical conditions. *Journal of Polycyclic Aromatic Compounds*, *27*(4), 239–260.

Morshedi, A., and Akbarian, M. (2014). Application of response surface methodology: design of experiments and optimization: a mini review. *Journal of Fundamental and Applied Sciences*, *54*, 2434–2349.

Mwanza, M. (2007). *An investigation in South African domesticated animals, their products and related health issues with reference to mycotoxins and fungi*. PhD Thesis. Department of Biomedical Technology, University of Johannesburg, South Africa.

Mwanza, M., Ndou, R. V, Dzoma, B., Nyirenda, M., and Bakunzi, F. (2013). Canine aflatoxicosis outbreak in South Africa (2011): a possible multi-mycotoxins aetiology. *Journal of the South African Veterinary Association*, *84*(1), 1–5.

Nair, M. G. (1998). Fumonisins and human health. *Annals of Tropical Paediatrics*, *18*, S47–S52.

Nakagawa, H., Sakamoto, S., Sago, Y., and Nagashima, H. (2013). Detection of type A trichothecene di-glucosides produced in corn by high-resolution liquid chromatography-orbitrap mass spectrometry. *Toxins*, *5*(3), 590–604.

Narwate, B. M., Ghule, A. V, Darandale, A. S., and Wagh, J. G. (2014). Ultra-performance liquid chromatography: a new revolution in liquid chromatography. *International Journal of Pharmaceutics and Drug Analysis*, *2*(1), 25–34.

Naude, T. W., Botha, C. J., Vorster, J. H., Roux, C., Van der Linde, E. J., Van der Walt, S. I., Rottinghaus, G. E., Van Jaarsveld, L., and Lawrence, A. N. (2005). *Claviceps cyperi*, a new cause of severe ergotism in dairy cattle consuming maize silage and teff hay contaminated with ergotised *Cyperus esculentus* (nut sedge) on the Highveld of South Africa. *Onderstepoort Journal of Veterinary Research*, *72*(1), 23–37.

Nazari, F., Ebrahimi, S. N., Talebi, M., Rassouli, A., and Bijanzadeh, H. R. (2007). Multivariate optimisation of microwave-assisted extraction of capsaicin from *Capsicum frutescens* L. and quantitative analysis by 1H-NMR. *Phytochemical Analysis*, *18*(4), 333–340.

Nieto, C. H. D., Granero, A. M., Zon, M. A., and Fernández, H. (2018). Sterigmatocystin: a mycotoxin to be seriously considered. *Food and Chemical Toxicology*, *118*, 460–470.

Njobeh, B. P., Dutton, F. M., and Makun, H. A. (2010a). Mycotoxins and human health: Significance, prevention and control. In: A. K. Mishra, A. Tiwari, & S. B. Mishra (Eds.), *Smart Biomolecules in Medicine* (pp. 132–177). VBRI Press, India.

Njobeh, P. B., Dutton, M. F., Åberg, A. T., and Haggblom, P. (2012). Estimation of multi-mycotoxin contamination in South African compound feeds. *Toxins (Basel)*, *4*(10), 836–848.

Njobeh, P. B., Dutton, M. F., Koch, S. H., Chuturgoon, A. A., Stoev, S. D., and Mosonik, J. S. (2010b). Simultaneous occurrence of mycotoxins in human food commodities from Cameroon. *Mycotoxin Research*, *26*(1), 47–57.

Njobeh, P. B., and Olotu, I. (2017). The socio-economic impact of mycotoxin contamination in

Africa. In: *Proceedings of the 1st MYCOKEY International Conference. Global Mycotoxin Reduction in the Food and Feed Chain. September 11-14 2017*. Ghent, Belgium: Toxins.

Numanoglu, E., Yener, S., Gökmen, V., Uygun, U., and Koksel, H. (2013). Modelling thermal degradation of zearalenone in maize bread during baking. *Food Additives and Contaminants: Part A*, *30*(3), 528–533.

O'Mahony, J., Clarke, L., Whelan, M., O'Kennedy, R., Lehotay, S. J., and Danaher, M. (2013). The use of ultra-high pressure liquid chromatography with tandem mass spectrometric detection in the analysis of agrochemical residues and mycotoxins in food – Challenges and applications. *Journal of Chromatography A*, *1292*, 83–95.

Omurtag, G. Z. (2008). Fumonisins, trichothecenes and zearalenone in cereals. *International Journal of Molecular Sciences*, *9*(11), 2062–2090.

Ong, E. S., Cheong, J. S. H., and Goh, D. (2006). Pressurized hot water extraction of bioactive or marker compounds in botanicals and medicinal plant materials. *Journal of Chromatography A*, *1112*(1–2), 92–102.

Ostry, V. (2008). Alternaria mycotoxins: an overview of chemical characterization, producers, toxicity, analysis and occurrence in foodstuffs. *World Mycotoxin Journal*, *1*(2), 175–188.

Oyelami, O. A., Maxwell, S. M., Adelusola, K. A., Aladekoma, T. A., and Oyelese, A. O. (1997). Aflatoxins in the lungs of children with kwashiorkor and children with miscellaneous diseases in Nigeria. *Journal of Toxicology and Environmental Health*, *51*(6), 623–628.

Ozel, M. Z., Gogus, F., and Lewis, A. C. (2003). Subcritical water extraction of essential oils from Thymbra spicata. *Food Chemistry*, *82*(3), 381–386.

Pakade, V., Cukrowska, E., Lindahl, S., Turner, C., and Chimuka, L. (2013). Molecular imprinted polymer for solid-phase extraction of flavonol aglycones from *Moringa oleifera* extracts. *Journal of Separation Science*, *36*(3), 548–555.

Pallaroni, L., Von Holst, C., Eskilsson, C., and Björklund, E. (2002). Microwave-assisted extraction of zearalenone from wheat and corn. *Analytical and Bioanalytical Chemistry*,

374(1), 161–166.

Palma, N., Cinelli, S., Sapora, O., Wilson, S. H., and Dogliotti, E. (2007). Ochratoxin A-induced mutagenesis in mammalian cells is consistent with the production of oxidative stress. *Chemical Research in Toxicology*, *20*(7), 1031–1037.

Panaccione, D. G., Schardl, C. L., and Coyle, C. M. (2006). Pathways to diverse ergot alkaloid profiles in fungi. *Recent Advances in Phytochemistry*, *40*, 23–52.

Panigrahi, N. (2014). *Computing in geographic information systems*. Boca Raton, Florida: CRC Press, Taylor and Francis Group.

Paredes, J. J., Jara, R., Shaler, S. M., and Van Heiningen, A. (2008). Influence of hot water extraction on the physical and mechanical behavior of OSB. *Forest Products Journal*, *58*(12), 56–62.

Park, J. W., Kim, E. K., and Kim, Y. B. (2004a). Estimation of the daily exposure of Koreans to aflatoxin B_1 through food consumption. *Food Additives and Contaminants*, *21*, 70–75.

Park, J. W., Scott, P. M., Lau, B. Y., and Lewis, D. A. (2004b). Analysis of heat-processed corn foods for fumonisins and bound fumonisins. *Food Additives and Contaminants*, *21*(12), 1168–1178.

Pascale, M., Panzarini, G., and Visconti, A. (2012). Determination of HT-2 and T-2 toxins in oats and wheat by ultra-performance liquid chromatography with photodiode array detection. *Talanta*, *89*, 231–236.

Patel, P. (2004). Mycotoxin analysis: Current and emerging technologies. In: N. Magan & M. Olsen (Eds.), *Mycotoxins in food: Detection and control* (pp. 88–110). Cambridge, England: Woodhead Publishing Limited.

Patel, S., Hazel, C., and Shah, I. (2011). *Investigation of the loss of parent fumonisin mycotoxins during food processing. Report Number: C03061*. High Wycombe, UK. Retrieved June 24, 2019, from https://www.food.gov.uk/sites/default/files/media/document/C03061.pdf

Patterson, D. S. P., and Roberts, B. A. (1979). Mycotoxins in animal feedstuffs: Sensitive thin layer chromatographic detection of aflatoxin, ochratoxin A, sterigmatocystin, zearalenone and T-2 toxin. *Journal of the Association of Official Analytical Chemists International*, *62*, 1265–1267.

Peraica, M., Radic, B., Lucic, A., and Pavlovic, M. (1999). Toxic effects of mycotoxins in humans. *Bulletin of the World Health Organization*, *77*(9), 754–766.

Peraica, M., Richter, D., and Rašić, D. (2014). Mycotoxicoses in children. *Archives of Industrial Hygiene and Toxicology*, *65*(4), 347–363.

Pettersson, H. (2004). Controlling mycotoxins in animal feed. In: N. Magan & M. Olsen (Eds.), *Mycotoxins in food: Detection and control* (pp. 269–273). Cambridge, England: Woodhead Publishing Ltd.

Pfohl-Leszkowicz, A., and Manderville, R. A. (2007). Ochratoxin A: An overview on toxicity and carcinogenicity in animals and humans. *Molecular Nutrition and Food Research*, *51*(1), 61–99.

Pimentel, M. C. B., Araújo, A. I., Figueiredo, Z. M. B., Silva, R. A., Cavalcanti, M. T. H., Moreira, K. A., and Porto, A. L. F. (2013). Aqueous two-phase system for citrinin extraction from fermentation broth. *Separation and Purification Technology*, *110*, 158–163.

Pinton, P., Braicu, C., Nougayrede, J. P., Laffitte, J., Taranu, I., and Oswald, I. P. (2010). Deoxynivalenol impairs porcine intestinal barrier function and decreases the protein expression of claudin-4 through a mitogen-activated protein kinase dependent mechanism. *Journal of Nutrition*, *140*(11), 1956–1962.

Pitt, J. I., Taniwaki, M. H., and Cole, M. B. (2013). Mycotoxin production in major crops as influenced by growing, harvesting, storage and processing, with emphasis on the achievement of food safety objectives. *Food Control*, *32*(1), 205–215.

Pittet, A. (2005). Modern methods and trends in mycotoxin analysis. *Mitteilungen Aus Lebensmitteluntersuchung Und Hygiene*, *96*, 424–444.

Plaza, M., and Turner, C. (2015). Pressurized hot water extraction of bioactives. *TrAC Trends in Analytical Chemistry*, *71*, 39–54.

Podlech, J., and Marko, D. (2009). Alternariol acts as a topoisomerase poison, preferentially affecting the II isoform. *Molecular Nutrition and Food Research*, *53*(4), 441–451.

Prado, G., de Oliveira, M. S., Lima, A. S., and Moreira, A. P. A. (2008). Occurrence of aflatoxin M1 in parmesan cheese consumed in Minas Gerais, Brazil. *Ciência e Agrotecnologia*, *32*(6), 1906–1911.

Prat, D., Pardigon, O., Flemming, H. W., Letestu, S., Ducandas, V., Isnard, P., Guntrum, E., Senac, T., Ruisseau, S., Cruciani, P., and Hosek, P. (2013). Sanofi's solvent selection guide: a step toward more sustainable processes. *Organic Process Research and Development*, *17*(12), 1517–1525.

Rahmani, A., Jinap, S., and Soleimany, F. (2009). Qualitative and quantitative analysis of mycotoxins. *Comprehensive Reviews in Food Science and Food Safety*, *8*, 202–251.

Raistrick, H., Stickings, C. E., and Thomas, R. (1953). Studies in the biochemistry of micro-organisms. 90. Alternariol and alternariol monomethyl ether, metabolic products of Alternaria tenuis. *Biochemical Journal*, *55*(3), 421–433.

Rajani, P., Sridevi, V., and Lakshmi, M. V. V. C. (2012). A review on biological control of aflatoxin crop contamination. *International Journal of Chemical, Environmental and Pharmaceutical Research*, *3*(1), 83–86.

Rakić, T., Kasagić-Vujanović, I., Jovanović, M., Jančić-Stojanović, B., and Ivanović, D. (2014). Comparison of full factorial design, central composite design, and box-behnken design in chromatographic method development for the determination of fluconazole and its impurities. *Analytical Letters*, *47*(8), 1334–1347.

Ramni, K., Navneet, K., Ashutosh, U., Suri, O. P., and Arti, T. (2011). High performance liquid chromatography detectors – A review. *International Research Journal of Pharmacy*, *2*(5), 1–7.

Ramos, L., Kristenson, E. M., and Brinkman, U. A. T. (2002). Current use of pressurised liquid extraction and subcritical water extraction in environmental analysis. *Journal of Chromatography A*, *975*, 3–29.

Raters, M., and Matissek, R. (2008). Thermal stability of aflatoxin B 1 and ochratoxin A. *Mycotoxin Research*, *24*(3), 130–134.

Razzazi-Fazeli, E., and Reiter, E. V. (2011). Sample preparation and clean up in mycotoxin analysis: Principles, applications and recent developments. In: S. De Saeger (Ed.), *Determining mycotoxins and mycotoxigenic fungi in food and feed* (pp. 37–70). Sawston, Cambridge, UK: Woodhead Publishing Limited.

Rezaee, M., Khalilian, F., Pourjavid, M. R., Seidi, S., Chisvert, A., and Abdel-Rehim, M. (2015). Extraction and sample preparation. *International Journal of Analytical Chemistry*, *2015*(Article ID 397275), 1-2.

Richard, J. L. (2007). Some major mycotoxins and their mycotoxicoses-an overview. *International Journal of Food Microbiology*, *119*(1–2), 3–10.

Robens, J., and Cardwell, K. (2003). The costs of mycotoxin management to the USA: Management of aflatoxins in the United States. *Journal of Toxicology - Toxin Reviews*, *22*(2–3), 139–152.

Robert, C., Devillers, T., Wathelet, B., Van Herck, J. C., and Paquot, M. (2006). Use of a Plackett− Burman experimental design to examine the impact of extraction parameters on yields and compositions of pectins extracted from chicory roots (Chicorium intybus L.). *Journal of Agricultural and Food Chemistry*, *54*(19), 7167–7174.

Rodrigues, I., Handl, J., and Binder, E. M. (2011). Mycotoxin occurrence in commodities, feeds and feed ingredients sourced in the Middle East and Africa. *Food Additives and Contaminants: Part B*, *4*(3), 168–179.

Rodríguez-Carrasco, Y., Mañes, J., Berrada, H., and Juan, C. (2016). Development and validation of a LC-ESI-MS/MS method for the determination of Alternaria toxins alternariol, alternariol methyl-ether and tentoxin in tomato and tomato-based products. *Toxins*, *8*(11), 328.

Roseanu, A., Jecu, L., Badea, M., and Evans, R. W. (2010). Mycotoxins: an overview on their quantification methods. *Romanian Journal of Biochemistry*, *47*(1), 79–86.

Royer, D., Humpf, H. U., and Guy, P. A. (2004). Quantitative analysis of *Fusarium* mycotoxins in maize using accelerated solvent extraction before liquid chromatography/atmospheric pressure chemical ionization tandem mass spectrometry. *Food Additives and Contaminants*, *21*(7), 678–692.

Rychlik, M., Humpf, H. U., Marko, D., Dänicke, S., Mally, A., Berthiller, F., Klaffke, H., and Lorenz, N. (2014). Proposal of a comprehensive definition of modified and other forms of mycotoxins including "masked" mycotoxins. *Mycotoxin Research*, *34*(4), 197–205.

Saengtienchai, T., Poapolathep, S., Isariyodom, S., Ikenaka, Y., Ishizuka, M., and Poapolathep, A. (2014). Toxicokinetics and tissue depletion of Fusarenon-X and its metabolite nivalenol in piglets. *Food and Chemical Toxicology*, *66*, 307–312.

Santana, M. C., Ferrera, Z. S., Padrón, M. E. T., and Rodríguez, J. J. S. (2009). Methodologies for the extraction of phenolic compounds from environmental samples: New approaches. *Molecules*, *14*, 298–320.

Sapkale, G. N., Patil, S. M., Surwase, U. S., and Bhatbhage, P. K. (2010). Supercritical fluid extraction. *International Journal of Chemical Sciences*, *8*(2), 729–743.

Schardl, C. L., Panaccione, D. G., and Tudzynski, P. (2006). Ergot alkaloids–biology and molecular biology. *The Alkaloids: Chemistry and Biology*, *63*, 45–86.

Schmale, D. G., and Munkvold, G. P. (2009). Mycotoxins in crops: A threat to human and domestic animal health. *The Plant Health Instructor*, *3*, 340–353.

Schmidt, H. S., Becker, S., Cramer, B., and Humpf, H. U. (2017). Impact of mechanical and thermal energies on the degradation of T-2 and HT-2 toxins during extrusion cooking of oat flour. *Journal of Agricultural and Food Chemistry*, *65*(20), 4177–4183.

Schmidt, H. S., Schulz, M., Focke, C., Becker, S., Cramer, B., and Humpf, H. U. (2018). Glucosylation of T-2 and HT-2 toxins using biotransformation and chemical synthesis:

Preparation, stereochemistry, and stability. *Mycotoxin Research*, *34*(3), 159–172.

Schneider, D. J., Miles, C. O., Garthwaite, I., Van, A. H., Wessels, J. C., and Lategan, H. J. (1996). First report of field outbreaks of ergot-alkaloid toxicity in South Africa. *The Onderstepoort Journal of Veterinary Research*, *63*(2), 97–108.

Schrader, T. J., Cherry, W., Soper, K., Langlois, I., and Vijay, H. M. (2001). Examination of Alternaria alternata mutagenicity and effects of nitrosylation using the Ames Salmonella test. *Teratogenesis, Carcinogenesis, and Mutagenesis*, *21*(4), 261–274.

Scott, P. M., Zhao, W., Feng, S., and Lau, B. P. Y. (2012). Alternaria toxins alternariol and alternariol monomethyl ether in grain foods in Canada. *Mycotoxin Research*, *28*(4), 261–266.

Scott, R. P. W. (1996). *Chromatographic Detectors. Design, Function and Operation*. New York, USA: Marcel Dekker.

Scudamore, K. A. (2008). Mycotoxins. In: J. Gilbert & H. Senyuva (Eds.), *Bioactive compounds in foods* (pp. 134–162). West Sussex, United Kingdom: Blackwell Publishing Ltd.

Seefelder, W., Hartl, M., and Humpf, H. U. (2001). Determination of N-(carboxymethyl) fumonisin B1 in corn products by liquid chromatography/electrospray ionization–mass spectrometry. *Journal of Agricultural and Food Chemistry*, *49*(5), 2146–2151.

Seefelder, W., Knecht, A., and Humpf, H. U. (2003). Bound fumonisin B1: Analysis of fumonisin-B1 glyco and amino acid conjugates by liquid chromatography− electrospray ionization− tandem mass spectrometry. *Journal of Agricultural and Food Chemistry*, *51*(18), 5567–5573.

Semple, R. L., Frio, A. S., Hicks, P. A., and Lozare, J. V. (1989). *Mycotoxin prevention and control in foodgrains*. Rome, Italy. Retrieved June 24, 2019, from http://www.fao.org/docrep/x5036e/x5036E00.htm

Shabir, G. A. (2005). Step-by-step analytical methods validation and protocol in the quality system compliance industry. *Journal of Validation Technology*, *10*, 314–325.

Shaddel, R., Maskooki, A., Haddad-Khodaparast, M. H., Azadmard-Damirchi, S., Mohamadi, M., and Fathi-Achachlouei, B. (2014). Optimization of extraction process of bioactive

compounds from Bene hull using subcritical water. *Food Science and Biotechnology*, *23*(5), 1459–1468.

Shalmashi, A., Abedi, M., Golmohammad, F., and Eikani, M. H. (2010). Isolation of caffeine from tea waste using subcritical water extraction. *Journal of Food Process Engineering*, *33*(4), 701–711.

Sharma, S. R. (2015). Green chemistry, green solvents and alternative techniques in organic synthesis. *International Journal of Chemical and Physical Sciences*, *4*, 516–520.

Shephard, G. S. (2008). Determination of mycotoxins in human foods. *Chemical Society Reviews*, *37*, 2468–2477.

Shephard, G. S. (2008). Impact of mycotoxins on human health in developing countries. *Food Additives and Contaminants*, *25*(2), 146–151.

Shephard, G. S., Thiel, P. G., Sydenham, E. W., Vleggaar, R., and Alberts, J. F. (1994). Determination of the mycotoxin fumonisin B1 and identification of its partially hydrolysed metabolites in the faeces of non-human primates. *Food Chemistry and Toxicology*, *32*(1), 23–29.

Sherman, J., Chin, B., Huibers, P. D., Garcia-Valls, R., and Hatton, T. A. (1998). Solvent replacement for green processing. *Environmental Health Perspective*, *106*(Suppl 1), 253–271.

Shi, Y. Y., Guan, S. H., Tang, R. N., Tao, S. J., and Guo, D. A. (2012). Simultaneous determination of four sesquiterpenoids in Atractylodes macrocephala Rhizoma by GC-FID: Optimisation of an ultrasound-assisted extraction by central composite design. *Phytochemical Analysis*, *23*(4), 408–414.

Shine, S., Sapna, S., Anuradha, V. P., Safeena, K., Mohammed, F., Sanaa, F. M., Rifna, T. K., Zubair, P. C., and Purushoth, P. T. (2015). Review on analytical tools of supercritical fluid extraction and its application in pharmaceutical industry. *International Journal of Medical and Pharmaceutical Research*, *1*(1), 29–37.

Shitu, A., Izhar, S., and Tahir, T. M. (2015). Sub-critical water as a green solvent for production of valuable materials from agricultural waste biomass: A review of recent work. *Global Journal of Environmental Science and Management*, *1*(3), 255–264.

Shu, Y. Y., Lao, R. C., Chiu, C. H., and Turle, R. (2000). Analysis of polycyclic aromatic hydrocarbons in sediment reference materials by microwave-assisted extraction. *Chemosphere*, *41*(11), 1709–1716.

Sibiya, P., Chimuka, L., Cukrowska, E., and Tutu, H. (2013). Development and application of microwave assisted extraction (MAE) for the extraction of five polycyclic aromatic hydrocarbons in sediment samples in Johannesburg area, South Africa. *Environmental Monitoring and Assessment*, *185*(7), 5537–5550.

Siegel, D., Feist, M., Proske, M., Koch, M., and Nehls, I. (2010). Degradation of the Alternaria mycotoxins alternariol, alternariol monomethyl ether, and altenuene upon bread baking. *Journal of Agricultural and Food Chemistry*, *58*(17), 9622–9630.

Sima, I. A., Andrási, M., and Sârbu, C. (2017). Chemometric assessment of chromatographic methods for herbal medicines authentication and fingerprinting. *Journal of Chromatographic Science*, *56*(1), 49–55.

Siuzdak, G. (2004). An introduction to mass spectrometry ionization: an excerpt from The Expanding Role of Mass Spectrometry in Biotechnology; MCC Press: San Diego, 2005. *JALA: Journal of the Association for Laboratory Automation*, *9*(2), 50–63.

Snyder, L. R., Kirkland, J. J., and Dolan, J. W. (2010). *Introduction to modern liquid chroamtography*. New York, USA: John Wiley and Sons, Inc.

Sofi, F. R., Nissar, K. S., Nayak, P. C., Amin, A., Phadke, G., and Malik, M. (2013). Phenolic compounds: Successful substitute for lipid retardation. In: Y. Srivastava (Ed.), *Advances in food science and nutrition* (p. 32). Akure, Nigeria: Science and Education Development Institute.

Song, H. H., Kim, D. Y., Woo, S., Lee, H. K., and Oh, S. R. (2013). An approach for simultaneous determination for geographical origins of Korean Panax ginseng by UPLC-QTOF/MS

coupled with OPLS-DA models. *Journal of Ginseng Research*, *37*(3), 341.

Soriano, J. M., and Dragacci, S. (2004). Occurrence of fumonisins in foods. *Food Research International*, *37*, 985–1000.

Srivastava, B., Sharma, B. K., Baghel, U. S., and Sethi, N. (2010). Ultra-performance liquid chromatography (UPLC): a chromatography technique. *International Journal of Pharmaceutical Quality Assurance*, *2*(1), 19–25.

Sriveena, T., Srividya, A., Ajitha, A., and Rao, V. U. M. (2015). Time of flight mass spectrometry: review. *World Journal of Pharmacy and Pharmaceutical Sciences*, *4*(7), 614–625.

STAT503. (2018). Response surface methods and designs: Central composite designs. Retrieved July 26, 2018, from https://onlinecourses.science.psu.edu/stat503/node/59/

Stockmann-Juvala, H., and Savolainen, K. (2008). A review of the toxic effects and mechanisms of action of fumonisin B_1. *Human and Experimental Toxicology*, *27*(11), 799–809.

Stroka, J., Dasko, L., Spangenberg, B., and Anklam, E. (2004). Determination of the mycotoxin, sterigmatocystin, by thin-layer chromatography and reagent-free derivatisation. *Journal of Liquid Chromatography and Related Technologies*, *27*(13), 2101–2111.

Sulyok, M., Berthiller, F., Krska, R., and Schuhmacher, R. (2006). Development and validation of a liquid chromatography/tandem mass spectrometric method for the determination of 39 mycotoxins in wheat and maize. *Rapid Communication in Mass Spectrometry*, *20*(18), 2649–2659.

Susanti, R. F., Kurnia, K., Vania, A., and Reynaldo, I. J. (2015). Total phenol, flavanoid and antioxidant activity of *Physalis angulata* extraction, leaves extract by subcritical water. *Modern Applied Science*, *9*(7), 190–198.

Swartz, M. (2010). HPLC detectors: A review. *Journal of Chromatography and Related Technologies*, *33*(9–12), 1130–1150.

Tabaraki, R., and Nateghi, A. (2011). Optimization of ultrasonic-assisted extraction of natural

antioxidants from rice bran using response surface methodology. *Ultrasonics Sonochemistry*, *18*(6), 1279–1286.

Tambellini, N. P., Zaremberg, V., Turner, R. J., and Weljie, A. M. (2013). Evaluation of extraction protocols for simultaneous polar and non-polar yeast metabolite analysis using multivariate projection methods. *Metabolites*, *3*(3), 592–605.

Tamhane, A. C. (2014). *Staistical analysis of designed experiments: Theory and applications* (1st ed.). Moorpark, California: Content Technologies Inc.

Tan, K. C., Trengove, R. D., Maker, G. L., Oliver, R. P., and Solomon, P. S. (2009). Metabolite profiling identifies the mycotoxin alternariol in the pathogen *Stagonospora nodorum*. *Metabolomics*, *5*(3), 330–335.

Tatke, P., and Jaiswal, Y. (2011). An overview of microwave assisted extraction and its applications in herbal drug research. *Research Journal of Medicinal Plant*, *5*, 21–31.

Teo, C. C., Tan, S. N., Yong, J., Hew, C. S., and Ong, E. S. (2010). Pressurized hot water extraction (PHWE). *Journal of Chromatography A, 1217*, 2484–2494.

Toregeani-Mendes, K. A., Arroteia, C. C., Kemmelmeier, C., Dalpasquale, V. A., Bando, É., Alves, A. F., Marques, O. J., Nishiyama, P., Mossini, S. A., and Machinski, M. (2011). Application of hazard analysis critical control points system for the control of aflatoxins in the Brazilian groundnut-based food industry. *International Journal of Food Science and Technology*, *46*(12), 2611–2618.

Trombete, F. M., Santos, T. B., Direito, G. M., Fraga, M. E., and Saldanha, T. (2014). In-house validation of a method for determining aflatoxins B_1, B_2, G_1 and G_2 in wheat and wheat by-products. *Pesquisa Agropecuária Tropical*, *44*(3), 255–262.

Tugizimana, F. (2012). *Metabolite profiling of defence-related secondary metabolites in tobacco cells, in response to ergosterol, a steroid from fungal membranes*. University of Johannesburg, South Africa.

Turner, N. W., Subrahmanyam, S., and Piletsky, S. A. (2009). Analytical methods for

determination of mycotoxins: A review. *Analytica Chimica Acta*, *632*(2), 168–180.

Turner, N. W., Bramhmbhatt, H., Szabo-Vezse, M., Poma, A., Coker, R., and Piletsky, S. A. (2015). Analytical methods for determination of mycotoxins: An update (2009–2014). *Analytica Chimica Acta*, *901*, 12–33.

Turner, P. C., Collinson, A. C., Cheung, Y. B., Gong, Y., Hall, A. J., Prentice, A. M., and Wild, C. P. (2007). Aflatoxin exposure in utero causes growth faltering in Gambian infants. *International Journal of Epidemiology*, *36*, 1119–1125.

Turner, P. C., Moore, S. E., Hall, A. J., Prentice, A. M., and Wild, C. P. (2003). Modification of immune function through exposure to dietary aflatoxin in Gambian children. *Environmental Health Perspectives*, *111*(2), 217–220.

Uchigashima, M., Yamaguchi, M. Y., Narita, H., Nakajima, M., and Miyake, S. (2012). Development of an immuno-affinity column for ochratoxin analysis using an organic solvent-tolerant monoclonal antibody. *Methods (San Diego, Calif.)*, *56*(2), 180–185.

Uma, D. B., Ho, C. W., and Wan Aida, W. M. (2010). Optimization of extraction parameters of total phenolic compounds from henna (*Lawsonia inermis*) leaves. *Sains Malaysiana*, *39*(1), 119–128.

Vaghela, A., Patel, A., Patel, A., Vyas, A., and Patel, N. (2016). Sample preparation in bioanalysis: a review. *International Journal of Scientific and Technology Research*, *5*(5), 6–10.

Van de Perre, E., Jacxsens, L., Liu, C., Devlieghere, F., and De Meulenaer, B. (2015). Climate impact on Alternaria moulds and their mycotoxins in fresh produce: the case of the tomato chain. *Food Research International*, *68*, 41–46.

Van Egmond, H. P., Schothorst, R. C., and Jonker, M. A. (2007). Regulations relating to mycotoxins in food. *Analytical and Bioanalytical Chemistry*, *389*, 147–157.

Vanhoutte, I., Audenaert, K., and De Gelder, L. (2016). Biodegradation of mycotoxins: tales from known and unexplored worlds. *Frontiers in Microbiology*, *7*, 561.

Varga, E., Glauner, T., Berthiller, F., Krska, R., Schuhmacher, R., and Sulyok, M. (2013).

Development and validation of a (semi-) quantitative UHPLC-MS/MS method for the determination of 191 mycotoxins and other fungal metabolites in almonds, hazelnuts, peanuts and pistachios. *Analytical and Bioanalytical Chemistry*, *405*(15), 5087–5104.

Veggi, P. C., Martinez, J., and Meireles, M. A. A. (2013). Fundamentals of microwave extraction. In: F. Chemat & G. Cravotto (Eds.), *Microwave-assisted extraction for bioactive compounds: Theory and practice* (pp. 15–52). Springer US.

Vendruscolo, C. P., Frias, N. C., de Carvalho, C. B., de Sá, L. R. M., Belli, C. B., and Baccarin, R. Y. A. (2016). Leukoencephalomalacia outbreak in horses due to consumption of contaminated hay. *Journal of Veterinary Internal Medicine*, *3*(6), 1879–1881.

Veprikova, Z., Vaclavikova, M., Lacina, O., Dzuman, Z., Zachariasova, M., and Hajslova, J. (2012). Occurrence of mono-and di-glycosylated conjugates of T-2 and HT-2 toxins in naturally contaminated cereals. *World Mycotoxin Journal*, *5*(3), 231–240.

Vergara-Salinas, J. R., Bulnes, P., Zúñiga, M. C., Pérez-Jiménez, J., Torres, J. L., Mateos-Martín, M. L., Agosin, E., and Pérez-Correa, J. R. (2013). Effect of pressurized hot water extraction on antioxidants from grape pomace before and after enological fermentation. *Journal of Agriculture and Food Chemistry*, *61*(28), 6929–6936.

Veršilovskis, A., and De Saeger, S. (2010). Sterigmatocystin: occurrence in foodstuffs and analytical methods–an overview. *Molecular Nutrition and Food Research*, *54*(1), 136–147.

Vidal, A., Sanchis, V., Ramos, A. J., and Marín, S. (2015). Thermal stability and kinetics of degradation of deoxynivalenol, deoxynivalenol conjugates and ochratoxin A during baking of wheat bakery products. *Food Chemistry*, *178*, 276–286.

Voss, K. A., Smith, G. W., and Haschek, W. M. (2007). Fumonisins: Toxicokinetics, mechanism of action and toxicity. *Animal Feed Science and Technology*, *137*, 299–325.

Voss, K., Ryu, D., Jackson, L., Riley, R., and Gelineau-van Waes, J. (2017). Reduction of fumonisin toxicity by extrusion and nixtamalization (alkaline cooking). *Journal of Agricultural and Food Chemistry*, *65*(33), 7088–7096.

Wafa, E. W., Yahya, R. S., Sobh, M. A., Eraky, I., El-Baz, M., El-Gayar, H. A., Betbeder, A. M., and Creppy, E. E. (1998). Human ochratoxicosis and nephropathy in Egypt: A preliminary study. *Human and Experimental Toxicology*, *17*(2), 124–129.

Wagacha, J. M., and Muthomi, J. W. (2008). Mycotoxin problem in Africa: current status, implications to food safety and health and possible management strategies. *International Journal of Food Microbiology*, *124*(1), 1–12.

Wan, C., and Li, Y. (2011). Effect of hot water extraction and liquid hot water pretreatment on the fungal degradation of biomass feedstocks. *Bioresource Technology*, *102*(20), 9788–9793.

Wang, L. J. (2010). Advances in extraction of plant products in nutraceutical processing. In: Y. Pathak (Ed.), *Handbook of nutraceuticals, volume II: Scale up, processing and automation* (pp. 15–52). Boca Raton, Florida: CRC Press, Taylor and Francis Group.

Wang, M., Jiang, N., Xian, H., Wei, D., Shi, L., and Feng, X. (2016). A single-step solid phase extraction for the simultaneous determination of 8 mycotoxins in fruits by ultra-high performance liquid chromatography tandem mass spectrometry. *Journal of Chromatography A*, *1429*, 22–29.

Wang, Z. G., Feng, J. N., and Tong, Z. (1993). Human toxicosis caused by moldy rice contaminated with *Fusarium* and T-2 toxin. *Biomedical and Environmental Sciences: BES*, *6*(1), 65–70.

Weingartner, H., and Franck, E. U. (2005). Supercritical water as a solvent. *Angewandte Chemie International Edition*, *44*(18), 2672–2692.

Wen, J., Kong, W., Hu, Y., Wang, J., and Yang, M. (2014). Multi-mycotoxins analysis in ginger and related products by UHPLC-FLR detection and LC-MS/MS confirmation. *Food Control*, *43*, 82–87.

Wierucka, M., and Biziuk, M. (2014). Application of magnetic nanoparticles for magnetic solid-phase extraction in preparing biological, environmental and food samples. *TrAC Trends in Analytical Chemistry*, *59*, 50–58.

Wild, C. P., Rasheed, F. N., Jawla, M. F. B., Hall, A. J., Jansen, L. A. M., and Montesano, R. (1991). In-utero exposure to aflatoxin in West Africa. *The Lancet*, *337*(8757), 1602.

Williams, H. D., Trevaskis, N. L., Charman, S. A., Shanker, R. M., Charman, W. N., Pouton, C. W., and Porter, C. J. H. (2013). Strategies to address low drug solubility in discovery and development. *Pharmacological Reviews*, *65*, 315–449.

Wilson, D. M., and Abramson, D. (1992). Mycotoxins. In: D. B. Sauer (Ed.), *Storage of cereal grains and thier products* (pp. 341–391). Saint Paul, Minnesota: American Association of Cereal Chemists.

Wilson, I. D., Davis, P., and Ruane, R. J. (1993). Supercritical fluid chromatography and extraction of pharmaceuticals. In: J. R. Dean (Ed.), *Applications of Supercritical Fluids in Industrial Analysis* (pp. 74–103). Dordrecht, Netherlands: Springer Netherlands.

Wilson, N., McMaster, N., Gantulga, D., Soyars, C., McCormick, S., Knott, K., Senger, R., and Schmale, D. (2017). Modification of the mycotoxin deoxynivalenol using microorganisms isolated from environmental samples. *Toxins*, *9*(4), 141.

Wilson, S. J., and Otsuki, T. (2001). What price precaution? European harmonisation of aflatoxin regulations and African groundnut exports. *European Review of Agricultural Economics*, *28*(3), 263–284.

Worley, B., and Powers, R. (2013). Multivariate analysis in metabolomics. *Current Metabolomics*, *1*, 92–107.

Wu, F. (2004). Mycotoxin risk assessment for the purpose of setting international regulatory standards. *Environmental Science and Technology*, *38*(15), 4049–4055.

Wu, X., and Smith, J. S. (2007). A gas chromatography−flame ionization detection method for detection of fusaproliferin in corn. *Journal of Agricultural and Food Chemistry*, *55*(8), 3211–3216.

Wu, Y., and Engen, J. R. (2006). Ultra-performance liquid chromatography (UPLC) further improves hydrogen/deuterium exchange mass spectrometry. *Journal of the American Society*

for Mass Spectrometry, *17*, 163–167.

Yamaguchi, T. (1998). Structure of subcritical and supercritical hydrogen-bonded liquids and solutions. *Journal of Molecular Liquids*, *78*, 43–50.

Yamamoto, H., Yamaji, H., Abe, Y., Harada, K., Waluyo, D., Fukusaki, E., Kondo, A., Ohno, H., and Fukuda, H. (2009). Dimensionality reduction for metabolome data using PCA, PLS, OPLS, and RFDA with differential penalties to latent variables. *Chemometrics and Intelligent Laboratory Systems*, *98*(2), 136–142.

Yang, B., and Wyman, C. E. (2004). Effect of xylan and lignin removal by batch and flow through pretreatment on the enzymatic digestibility of corn stover Cellulose. *Biotechnology and Bioengineering*, *86*(1), 88–95.

Yang, J., Li, J., Jiang, Y., Duan, X., Qu, H., Yang, B., Chen, F., and Sivakumar, D. (2014). Natural occurrence, analysis, and prevention of mycotoxins in fruits and their processed product. *Critical Reviews in Food Science and Nutrition*, *54*(1), 64–83.

Yang, Y., Matyska, M. T., Boysen, R. I., Pesek, J. J., and Hearn, M. T. (2013). Simultaneous separation of hydrophobic and polar bases using a silica hydride stationary phase. *Journal of Separation Science*, *36*(7), 1209–1216.

Yekeler, H., Bitmiş, K., Ozçelik, N., Doymaz, M. Z., and Calta, M. E. T. I. N. (2001). Analysis of toxic effects of Alternaria toxins on esophagus of mice by light and electron microscopy. *Toxicologic Pathology*, *29*(4), 492–497.

Yuan, G., Wang, Y., Yuan, X., Zhang, T., Zhao, J., Huang, L., and Peng, S. (2014). T-2 toxin induces developmental toxicity and apoptosis in zebrafish embryos. *Journal of Environmental Sciences*, *26*(4), 917–925.

Yumbe-Guevara, B. E., Imoto, I., and Yoshizava, T. (2003). Effects of heating procedures on deoxynivalenol, nivalenol and zearalenone levels in naturally contaminated barley and wheat. *Food Additives and Contaminants*, *20*, 1132–1140.

Zabe, N., Ayalew, K., and Powers, S. (2008). Ethanol extraction method for a rapid test for

aflatoxin in corn. *Food Contaminants*, *1001*, 297–305.

Zaied, C., Bouaziz, C., Azizi, I., Bensassi, F., Chour, A., Bacha, H., and Abid, S. (2011). Presence of ochratoxin A in Tunisian blood nephropathy patients. Exposure level to OTA. *Experimental and Toxicologic Pathology*, *63*(7–8), 613–618.

Zain, M. E. (2011). Impact of mycotoxins on humans and animals. *Journal of Saudi Chemical Society*, *15*(2), 129–144.

Zhang, A., Sun, H., Wang, P., Han, Y., and Wang, X. (2012). Modern analytical techniques in metabolomics analysis. *Analyst*, *137*(2), 293–300.

Zhang, H. F., Yang, X. H., and Wang, Y. (2011). Microwave assisted extraction of secondary metabolites from plants: Current status and future directions. *Trends in Food Science and Technology*, *22*, 672–688.

Zhang, L., Dou, X. W., Zhang, C., Logrieco, A., and Yang, M. H. (2018). A review of current methods for analysis of mycotoxins in herbal medicines. *Toxins*, *10*(2), 65.

Zheng, M. Z., Richard, J. L., and Binder, J. (2006). A review of rapid methods for the analysis of mycotoxins. *Mycopathologia*, *161*(5), 261–273.

Zinedine, A., Soriano, J. M., Molto, J. C., and Manes, J. (2007). Review on the toxicity, occurrence, metabolism, detoxification, regulations and intake of zearalenone: An oestrogenic mycotoxin. *Food and Chemical Toxicology*, *45*, 1–18.

Zinedine, A., and Mañes, J. (2009). Occurrence and legislation of mycotoxins in food and feed from Morocco. *Food Control*, *20*(4), 334–344.

Zöllner, P., and Mayer-Helm, B. (2006). Trace mycotoxin analysis in complex biological and food matrices by liquid chromatography–atmospheric pressure ionisation mass spectrometry. *Journal of Chromatography A*, *1136*(2), 123–169.

Zougagh, M., and Rios, A. (2008). Supercritical fluid extraction of macrocyclic lactone mycotoxins in maize flour samples for rapid amperometric screening and alternative liquid chromatographic method for confirmation. *Journal of Chromatography A*, *1177*(1), 50–57.

CHAPTER THREE[#]

NUMERICAL OPTIMIZATION OF TEMPERATURE-TIME DEGRADATION OF MULTIPLE MYCOTOXINS

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Abstract

Mycotoxins are potent food contaminants that exert significant deleterious effects on human and animal health, yet, there is limited and often conflicting data on their thermal stability. The present study systematically investigated the thermal degradation patterns of multiple mycotoxins as a function of temperature and time, in pure form and spiked into a food matrix (maize flour), using a numerical modelling approach. Mycotoxins under investigation included aflatoxins (AFs), fumonisins (FBs), zearalenone and its analogue α and β epimers (ZEAs), ochratoxins (OTs), T-2 toxin (T-2), alternariol monomethyl ether (AME) and sterigmatocystin (STEG). A set of statistically-designed experiments were conducted, and a second-order optimization function fitted to the experimental data. The resultant models were well fit with R^2 values ranging from 0.87 to 0.99 and 0.89 to 0.99, for pure mycotoxin standards and spiked maize flour, respectively. It was also possible to statistically determine the optimum degradation conditions which were 217 °C/63.28 min and 211 °C/54.71 min for pure mycotoxins and spiked into maize flour, respectively. Our observations herein could be critical for food safety applications targeted at reducing or at best eliminating completely multi-mycotoxins in food using heat processing while limiting the destruction of food quality factors.

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Highlights

- Mycotoxins are unwanted poisonous secondary metabolites produced by fungi that contaminate a wide range of food commodities globally.
- Thermal treatment is commonly employed in food processing to ensure safety *via* degradation of toxic components present in food.
- The effectiveness of this process is proportional to the amount of thermal energy supplied and exposure time.
- Numerical modelling is useful for optimization of thermal degradation patterns of multiple mycotoxins as a function of temperature and time.

Key words: Mycotoxins; numerical modeling; optimization; thermal degradation; thermal stability

3.1 Introduction

Mycotoxins are poisonous biochemical compounds of fungal origin that contaminate various food and feed commodities on a global scale. They have been implicated as major environmental hazards due to their perpetual proliferation in food and feed products, and subsequent possible lethal effects on humans and animals (Njobeh *et al*., 2010a; Zain, 2011; Makun *et al*., 2012; Enyiukwu *et al*., 2014; Gbashi *et al*., 2017c). One of the mycotoxins, aflatoxin B_1 (AFB_1), has been classified as the most noxious naturally-occurring carcinogen known to man (FAO, 2004a). Although mycotoxins have been estimated in a wide range of food and feed commodities (Mircea *et al*., 2008; Yang *et al*., 2014; Chilaka *et al*., 2016), maize in particular is a favored food substrate for incessant contamination (often at levels above regulatory limits) due to its susceptibility to attack by mycotoxigenic fungal species (Makun *et al*., 2012; Njobeh *et al*., 2010; Njobeh *et al*., 2012). This is of great concern because maize and maize-based products are staple foods for billions of people, and as well often constitute a major component of animal feed (du Plessis, 2003; Ranum *et al*., 2014). In South Africa for instance, daily consumption of maize and maize-based meals can reach up to 328 g/person (du Plessis, 2003; Ranum *et al*., 2014). Because of that, maize is an ideal reference matrix for investigating various mitigation approaches in mycotoxicology (Brown, 1999; Lauren & Smith, 2001; Raters & Matissek, 2008).

Different approaches have been investigated for the degradation of mycotoxins, some of which include gamma irradiation (Hooshmand & Klopfenstein, 1995), UV irradiation (Murata *et al*., 2008), thermal processing (Bretz *et al*., 2006; Raters & Matissek, 2008; Kabak, 2009), microbial and enzyme degradation (Ji *et al*., 2016; Adebo *et al*., 2017), plasma-based degradation (ten Bosch *et al*., 2017), microwave-induced argon plasma degradation (Park *et al*., 2007), oxidative degradation using ozone (McKenzie *et al*., 1997), and many others (Doyle *et al*., 1982; Juodeikiene *et al*., 2012). Amongst these, heat treatment remains a cheap, simple and sustainable approach for mitigating the prevalence of mycotoxins, and has had a good track record of effectively reducing other contaminants present in food (Yazdanpanah *et al*., 2005; Kabak, 2009; Méndez-Albores *et al*., 2013; Wu *et al*., 2017). However, because of its unsophisticatedness and everyday use, a critical understanding of the goal-specific applications of thermal processing is often unintentionally neglected (Sindelar & King, 2013). This perhaps could be the reason for the limited and conflicting studies on thermal stability of many mycotoxins (Raters & Matissek, 2008; Kabak, 2009; Turner *et al*., 2009).

The efficacy of a thermal processing system is known to be in proportion to the amount of heat energy supplied and time of exposure. Higher temperatures and longer heating times are known to result in greater degradation of mycotoxins (Kabak, 2009; Gbashi *et al*., 2017e). However, due to the detrimental effects higher temperatures have on important food quality factors, it is important to optimize these parameters in order to achieve maximum degradation of these toxins at the lowest possible temperature and time conditions. Moreover, the notoriously incessant prevalence of mycotoxins even in heat-processed foods (Stoloff & Trucksess, 1981), has necessitated the systematic re-investigation of their response to heat treatment.

In this regard, numerical modelling is a useful optimization approach that has found wide applicability in thermal processes in biological systems (Sendín *et al*., 2010; Abakarov & Nuñez, 2013). Amidst various approaches, central composite design (CCD) is one of the most commonly used numerical optimization techniques in food processing. This is because it offers the advantage of a reduced number of experimental runs, and provides a function and empirical relationship between the objective function (response variable) and the various control variables, as well as provides details on the effects of different control variables on a response variable (Goncalves *et al*., 2006; Khuri & Mukhopadhyay, 2010; Uma *et al*., 2010; Gbashi *et al*., 2016). The present study

adopts a CCD numerical modelling approach to systematically investigate, and optimize the temperature-time degradation patterns of multiple mycotoxins in pure and spiked form (maize flour).

3.2 Materials and methods

3.2.1 Materials

Mycotoxin reference standards, AFB_1, aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), aflatoxin G_2 (AFG_2), ochratoxin A (OTA), ochratoxin B (OTB), fumonisin B_1 (FB_1), fumonisin B_2 (FB_2), fumonisin B_3 (FB_3), zearalenone (ZEA), α-zearalenol (α-ZEL), β-zearalenol (β-ZEL), T2-toxin (T-2), AME, and STEG were purchased from the Council for Scientific and Industrial Research (CSIR). Solvents used included LC-MS grade acetonitrile, methanol and formic acid which were purchased from Sigma, Aldrich (South Africa). Ultrapure water was obtained from a Milli-Q Gradient A10 dispensing system (Millipore, Billerica, MA, USA). Maize flour was purchased from a Shoprite grocery store in Johannesburg (South Africa).

3.2.2 Methods

3.2.2.1 Experimental design

A set of experiments was statistically designed based on the response surface methodology (RSM) using Statistica version 7 statistical software (StatSoft, USA). Specifically, the central composite design (CCD) (Hossain *et al*., 2015) approach was adopted because it permits building second-order optimization models without the need for a complete three-level factorial experimental design (Goncalves *et al*., 2006; Tamhane, 2014). Accordingly, a 2-factor, 1 block experimental design was achieved, which consisted of 10 experimental levels (Table 3.1); 2 levels of fractional factorial design for each of the factors studied, a replicated center-point to improve the precision of the model, and a set of axial points (*i.e.*, α and $-\alpha$) that permits rotatability of the model and ensures estimation of response curvature. Rotatability of the model is desirable because it allows for equal variance of prediction for all points equal-distance from the center point irrespective of the direction. The experimental region was selected based on preliminary laboratory trials (Gbashi *et al*., 2017e).

Table 3.1: Two-factor, 1 block standard order CCD experimental design for temperature-time degradation of mycotoxins

S/No	Temperature (°C)	Time (min)	RSM Codes	Comment
1	120	15.00	-1, -1	Factorial level
2	120	55.00	-1, +1	Factorial level
3	200	15.00	+1, -1	Factorial level
4	200	55.00	+1, +1	Factorial level
5	103	35.00	-α, 0	Axial point
6	217	35.00	+α, 0	Axial point
7	160	6.72	0, -α	Axial point
8	160	63.28	0, +α	Axial point
9	160	35.00	+1, +1	Center point
10	160	35.00	+1, +1	Center point

Key: S/No.: serial number. AFB_1: aflatoxin B_1. AFB_2: aflatoxin B_2. AFG_1: aflatoxin G_1. AFG_2: aflatoxin G_2. AME: alternariol monomethyl ether. FB_1: fumonisin B_1. FB_2: fumonisin B_2. FB_3: fumonisin B_3. OTA: ochratoxin A. OTB: ochratoxin B. STEG: sterigmatocystin. T-2: T-2 toxin. ZEA: zearalenone. α-ZEL: α-zearalenol. β-ZEL: β-zearalenol. RSM – response surface methodology

After conducting the experiments, a second-order optimization model described by Equation 3.1 (Adebo *et al*., 2018b) was fitted to the experimental data using the method of least squares (MLS) which generates the lowest possible residuals (Bas & Boyaci, 2007).

$$Z = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i=1}^{k} \quad \sum_{j=1}^{k} \beta_{ij} x_i \, x_j \qquad \textbf{\textit{Equation 3.1}}$$

Where z is the response variable *i.e.*, degradation (%), x_i and x_j are the factors, temperature (°C) and time (min), respectively, β_0 is the model constant, β_i, β_{ii} and β_{ij} are the coefficients for the linear, quadratic and interaction terms (Adebo *et al*., 2018b).

The model fitness and adequacy were determined by evaluating the coefficient of determination (R^2), adjusted R^2, Pearson's correlation coefficient (r), average absolute deviation (*AAD*), accuracy factor (A_f) and the bias factor (B_f). Model parameters and significance were determined at a probability level of 95% (*i.e.*, $p < 0.05$). The various mathematical functions used to compute these parameters are presented in the Equations 3.2 to 3.7 (Morshedi & Akbarian, 2014; Adebo *et al*., 2018b).

$$R^2 = \frac{\sum_{i=1}^{n} (\hat{y}_i - \bar{y})^2}{\sum_{i=1}^{n} (y_i - \bar{y})^2} \qquad \textbf{\textit{Equation 3.2}}$$

Where R^2 is the coefficient of determination, n is the sample size, $\bar{y}$ is estimated mean value, y_i and $\hat{y}$ are the experimental and predicted values, respectively.

$$R^2adj = 1 - \frac{k-1}{k-p}(1 - R^2) \qquad \textbf{Equation 3.3}$$

Where R^2adj is the adjusted coefficient of determination, R^2 is the coefficient of determination, p is number of regression coefficients and k is total number of observations.

$$r = \frac{\sum_{i=1}^{n}(x_i - \bar{x})(y_i - \bar{y})}{\sqrt{\sum_{i=1}^{n}(x_i - \bar{x})^2}\sqrt{\sum_{i=1}^{n}(y_i - \bar{y})^2}} \qquad \textbf{Equation 3.4}$$

Where r is the Pearson's correlation coefficient, n is the sample size, x_i and y_i are single measurements indexed with i for predicted and experimental values, respectively, and $\bar{x}$ and $\bar{y}$ are the means for the predicted and experimental variables, respectively.

$$AAD = \frac{\left[\sum_{i=1}^{n}\left(\frac{y_{i\,exp} - y_{i\,cal}}{y_{i\,exp}}\right)\right]}{n} \qquad \textbf{Equation 3.5}$$

Where AAD is the average absolute deviation, n is the sample size, and $y_{i\,exp}$ and $y_{i\,cal}$ are the individual experimental and predicted values indexed with i, respectively.

$$B_f = 10^{\frac{1}{n}\sum_{i=1}^{n} log\left(\frac{y_{i\,cal}}{y_{i\,exp}}\right)} \qquad \textbf{Equation 3.6}$$

Where B_f is the bias factor, n is the sample size, and $y_{i\,exp}$ and $y_{i\,cal}$ are the individual experimental and predicted values indexed with i, respectively.

$$A_f = 10^{\frac{1}{n}\sum_{i=1}^{n}\left[log\left(\frac{y_{i\,cal}}{y_{i\,exp}}\right)\right]} \qquad \textbf{Equation 3.7}$$

Where A_f is the accuracy factor, n the sample size, and $y_{i\,exp}$ and $y_{i\,cal}$ are the individual experimental and predicted values indexed with i, respectively.

After the model-fit and validation of the model adequacy, the resultant quadratic optimization models were used for optimization and computation of the globally optimal conditions for the degradation of real samples *i.e.*, pure mycotoxin standards and spiked maize flour, using the Minitab 17 global optimization function. The 3-D surface plots and Pareto charts were used to examine the degradation patterns of the analytes, with and without matrix interference.

3.2.2.2 Sample preparation

Spiking of maize matrix was performed as described by Sameni *et al*. (2014) with little modifications. To obtain a spiked maize concentration of 2 µg/g, exactly 2 mL of multi-mycotoxin standards stock solution (1 µg/mL) was added to 1 g of blank maize flour (previously tested on UHPLC-MS/MS) contained in a 16 mL glass vial (25×50 mm). The spiked sample was thoroughly mixed and left overnight in the dark in a fume hood to allow for slow evaporation of the solvent at ambient conditions and for the mycotoxins to be absorbed into the matrix. For the pure standards, 2 mL of the multi-mycotoxin stock solution (1 µg/mL) was transferred into a 16 mL glass vial and dried under similar conditions.

3.2.2.3 Thermal treatment

Thermal treatment of samples was achieved using a GC 600 Vega Series 2 oven (Carlo Erba Instruments, Italy) with an automatic temperature control unit (± 1 ^{o}C). The oven was allowed to equilibrate at a desired temperature for 5 min before samples were introduced. Samples were placed inside the oven which was maintained at the preset temperature. After heating for the desired time, samples were retrieved immediately and allowed to cool under ambient conditions. The vials containing pure mycotoxin standards were then reconstituted in 1 mL of methanol, thoroughly vortexed for 3 min, allowed to stand and vortexed again for 2 min, filtered through a 0.22 µm syringe filter into a 1.5 mL clear HPLC vial for subsequent analysis on UHPLC-MS/MS.

The thermally-treated spiked maize samples were extracted using the method of Bertuzzi *et al*. (2011) with slight modifications. To a 1 g of maize flour, 2 mL of methanol/acetonitrile (50:50, v/v) was added, and placed on a bench shaker (LABCON GmbH, Heppenheim, Germany) for 1 h. After which samples were centrifuged at 4,000 RPM, and the supernatant was filtered through a 0.22 µm syringe filter into a 1.5 mL HPLC vial for subsequent analysis on UHPLC-MS/MS.

3.2.2.4 Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS)

Chromatographic separation followed by quantification of multi-mycotoxins was accomplished using a Shimadzu UHPLC-MS/MS 8030 equipment (Shimadzu Corporation, Tokyo, Japan), which consists of an LC-30AD Nexera chromatograph connected to a SIL-30 AC Nexera autosampler and a CTO-20 AC Prominence Column Oven. Analytes were separated on a Raptor^{TM}

ARC-18 column (2.7 µm, 2.1 mm × 100 mm) (Restek Corporation, Pennsylvania USA), thermostated at 40 °C. The mobile phases used consisted of (A) 0.1% formic acid (FA) in deionized water, and (B) 0.1% FA in methanol/acetonitrile (50:50 v/v), and was delivered at a constant flow rate of 0.2 mL/min. The gradient elution program began with 10% B for 0.1 min, ramped to 95% B in 8.4 min, kept constant for 3 min, and the initial condition (10% B) was re-instated for 1 min, after which, the column was allowed to re-equilibrate for 4.5 min for the next run, bringing these to a total run time of 17 min.

Following chromatographic separation, analytes were detected and quantified on a Shimadzu triple-quadrupole MS model 8030 (Shimadzu Corporation, Kyoto, Japan) operated in positive ionization mode with an electron spray ionization (ESI^+) source. The interface nebulizing gas flow rate was 3 L/min, desolvation line (DL) temperature was 250 °C, heat block temperature was 400 °C, and drying gas flow rate was 15 L/min. Data acquisition was by means of a multiple reaction monitoring (MRM) method operated using optimized MS conditions for the analytes (Table 3.2). The Shimadzu LabSolutions software was used for subsequent processing and visualization of the data.

Table 3.2: MRM transitions, optimized MS conditions and retention times of multi-mycotoxins; Ret. time – retention time; Q1 – quadrupole 1; Q3 – quadrupole 3; CE – collision energy.

S/No	Mycotoxin	Recovery (%)*	Ret. time (min)	Precursor (*mz*)	Products (*mz*)	Q1 Pre Bias (V)	CE (eV)	Q3 Pre Bias (V)
1.	AFB_1	110	8.55	313.00	241.00*	-22.00	-41.00	-23.00
					285.10	-22.00	-24.00	-29.00
2.	AFB_2	102	8.30	315.00	259.10*	-22.00	-31.00	-25.00
					287.00	-23.00	-26.00	-30.00
3.	AFG_1	101	8.08	329.00	243.00*	-12.00	-28.00	-23.00
					311.10	-16.00	-24.00	-14.00
4.	AFG_2	96	7.83	331.00	245.10*	-12.00	-32.00	-24.00
					313.00	-12.00	-24.00	-20.00
5.	AME	100	10.32	273.00	128.10*	-10.00	-49.00	-21.00
					115.05	-18.00	-54.00	-19.00
6.	FB_1	106	8.11	722.20	352.20*	-34.00	-42.00	-11.00
					334.30	-20.00	-42.00	-11.00
7.	FB_2	109	9.17	706.10	336.30*	-20.00	-38.00	-22.00

S/No	Mycotoxin	Recovery (%)*	Ret. time (min)	Precursor (*mz*)	Products (*mz*)	Q1 Pre Bias (V)	CE (eV)	Q3 Pre Bias (V)
					318.30	-26.00	-41.00	-22.00
8.	FB_3	94	8.85	706.30	336.30*	-40.00	-39.00	-11.00
					354.40	-20.00	-35.00	-24.00
9.	OTA	119	10.31	403.80	239.00*	-15.00	-27.00	-24.00
					221.00	-12.00	-38.00	-21.00
10.	OTB	109	9.56	370.10	205.00*	-13.00	-22.00	-21.00
					324.10	-13.00	-14.00	-22.00
11.	STEG	99	10.52	324.90	310.00*	-22.00	-24.00	-30.00
					281.10	-22.00	-40.00	-27.00
12.	T-2	120	10.03	467.20	245.10*	-13.00	-11.00	-16.00
					305.20	-22.00	-11.00	-20.00
13.	ZEA	119	10.26	319.10	185.00*	-12.00	-27.00	-30.00
					187.10	-15.00	-21.00	-19.00
14.	a-ZEA	128	8.80	323.10	277.20*	-17.00	-17.00	-18.00
					305.20	-24.00	-9.00	-20.00
15.	B-ZEA	135	8.39	323.10	277.20*	-16.00	-16.00	-18.00
					305.20	-16.00	-11.00	-20.00

Key: S/No.: serial number. *Extraction recovery for mycotoxins spiked into the maize. Ret. Time: retention time. Q1 Pre bias: quadruple one pre-rod bias. Q3 Pre bias: quadruple three pre-rod bias. CE: collision energy. * Quantitative product ion. AFB_1: aflatoxin B_1. AFB_2: aflatoxin B_2. AFG_1: aflatoxin G_1. AFG_2: aflatoxin G_2. AME: alternariol monomethyl ether. FB_1: fumonisin B_1. FB_2: fumonisin B_2. FB_3: fumonisin B_3. OTA: ochratoxin A. OTB: ochratoxin B. STEG: sterigmatocystin. T-2: T-2 toxin. ZEA: zearalenone. α-ZEL: α-zearalenol. β-ZEL: β-zearalenol.

3.3 Results and discussion

3.3.1 Thermal degradation of mycotoxins

Results for the thermal stability of pure multi-mycotoxin standards as well as mycotoxins spiked into the maize matrix as a function of temperature and time are presented in Tables 3.3 and 3.4. Clearly, thermal degradation of all studied mycotoxins increased proportionately with increasing temperature and exposure time (Appendices 3.A & 3.B). For pure mycotoxin standards, OTB was the most thermally stable mycotoxin with an average degradation of 20%, followed by OTA (24%), AME (36%), AFG_2 (40%), AFB_2 (44%), T-2 (49%), α-ZEL (50%), ZEA (51%), β-ZEL (52%), AFB_1 (53%), AFG1 (55%), STEG (61%), FB_1 (63%), FB_3 and FB_2 (65% each). When

mycotoxins were spiked into the maize matrix, AME showed the highest resilience to heat treatment with an average percentage degradation of 33%, followed by AFG_2 and STEG (37% each), T-2 and α-ZEL (37% each), OTB and β-ZEL (39% each), ZEA (42%), OTA (46%), AFG_1 and AFB_1 (47% each), AFB_2 (51%), FB_2 (77%), FB_1 (78%), and FB_3 (81%). It can be observed that mycotoxins that were relatively stable to heat treatment have a compact molecular structural configuration, *i.e.*,, OTA, AME, and AFG_2, whereas the more structurally spread-out mycotoxins were more thermolabile, *i.e.*,, FB_3, FB_2 and FB_1 (Lerda, 2011; De Souza *et al*., 2013). A more compact structural configuration could possibly imply a more difficult thermal break down (Gibbs *et al*., 1998; Cremer *et al*., 2000). Generally, evaluation of the results presented in Tables 3.3 & 3.4 revealed that the temperature-time conditions that resulted in the most degradation of all classes of the mycotoxins was a temperature of 217 °C at a time of 35 min.

Although all mycotoxins showed a differential degradation pattern for pure mycotoxin standards compared with mycotoxins spiked into maize matrix, an independent-samples Student's t-test showed that only FB_1, FB_2, FB_3, and AME had statistically significant ($p \leq 0.05$) differences between pure standards and spiked maize matrix (Appendix 3.C). A temperature of 217 °C for 35 min was sufficient to completely eradicate all the aflatoxins (AFs) in the absence of matrix interference, except for AFB_2 which had over 86% degradation (Table 3.3). Whereas when spiked into maize matrix, all AFs where completely degraded at similar condition (217 °C for 35 min), except for AFG_2, which had over 97% degradation (Table 3.4). Our results agree with Yazdanpanah *et al*. (2005), who reported that roasting nuts which contained AFs at 90 to 150 °C for 30 to 120 min resulted in reduced AF levels that ranged from 17 to 63%, depending on the temperature and exposure time (Yazdanpanah *et al*., 2005). The *Fusarium* group of toxins, FBs, were the most thermolabile of all tested mycotoxins for both spiked and unspiked standards. Average degradation for FBs ranged from 79 to 82%, with FB_3 being the most susceptible analogue in this group. Scott and Lawrence (1987) made similar observations, where heating maize meal at 190 °C for 60 min degraded FBs by 60 to 80%, whereas, when the conditions were increased to 220 °C and 25 min, FBs were almost completely eliminated. Our observations also agree with reports by Castelo *et al*. (1998), who noted that roasting of artificially-contaminated maize meal at 218 °C for 15 min eliminated FB_1 from the food samples (Scott & Lawrence, 1987).

Table 3.3: Temperature-time degradation of pure multi-mycotoxin standards.

Mycotoxin	Percentage degradation of mycotoxins at different temperature-time conditions									
	120 °C/ 15 min (%)	**120 °C/ 55 min (%)**	**200 °C/ 15 min (%)**	**200 °C/ 55 min (%)**	**103.43 °C/ 35 min (%)**	**217 °C/ 35 min (%)**	**160 °C/ 6.72 min (%)**	**160 °C/ 63.28 min (%)**	**160 °C/ 35 min (%)**	**Average**
AFB_1	17^{a}±12	12^{a}±3.3	93^{d}±0.53	99^{d}±0.2	3^{a}±2.9	100^{d}±0	49^{bc}±1.3	66^{c}±4.7	41^{b}±2.4	53±3.1
AFB_2	31^{a}±17	19^{a}±7.1	62^{ab}±0.17	89^{b}±3	23^{a}±16	86^{b}±5.6	31^{a}±29	33^{a}±13.8	22^{a}±1.8	44±10
AFG_1	12^{a}±6.4	14^{a}±4	97^{e}±0.2	99^{e}±0	6^{a}±2.9	100^{e}±0	39^{b}±2.7	72^{d}±1.3	52^{c}±2.2	55±2.2
AFG_2	17^{ab}±2.1	7^{a}±0.13	68^{d}±0.06	89^{d}±0.31	6^{a}±0.25	100^{d}±0	13^{a}±8.5	30^{c}±4.1	27^{bc}±2.1	40±2
AME	7^{ab}±8.8	2^{a}±1.6	55^{d}±1.8	89^{e}±0.98	7^{ab}±4.1	94^{e}±0.4	18^{bc}±4	30^{c}±1.2	25^{c}±1.5	36±2.7
FB_1	10^{a}±13.6	6^{a}±8.4	100^{c}±0	100^{c}±0	18^{a}±13	100^{c}±0	88^{c}±0.41	93^{c}±0	52^{b}±5.8	63±4.5
FB_2	20^{b}±2.8	24^{b}±2.3	100^{e}±0	100^{e}±0	2^{a}±0	100^{e}±0	89^{d}±1.1	95^{e}±0.37	54^{c}±1	65±0.84
FB_3	20^{a}±5.6	8^{a}±4.8	99^{c}±0.24	100^{c}±0.06	15^{a}±15	100^{c}±0	84^{c}±2.2	96^{c}±0.35	60^{b}±1.7	65±3.4
OTA	10^{ab}±11	6^{ab}±3.8	39^{cd}±1.3	48^{de}±0.75	5^{a}±3.6	59^{e}±0.63	12^{ab}±8.7	24^{bc}±0.25	16^{a}±0.61	24±3.4
OTB	23^{abc}±6.6	2^{a}±0.54	29^{bc}±0.27	39^{cd}±3.9	10^{ab}±1.8	51^{d}±3	11^{ab}±5.3	11^{ab}±5.8	9^{ab}±7.2	20±3.8
STEG	17^{a}±5.8	30^{b}±2.9	94^{f}±0.44	99^{f}±0.07	18^{a}±0.51	100^{f}±0	62^{d}±2	79^{e}±0.44	48^{c}±3.2	61±1.7
T-2	13^{ab}±13	5^{a}±4	84^{de}±1.9	96^{e}±0.96	6^{a}±6.3	98^{e}±0.89	35^{bc}±4.2	66^{d}±1.9	40^{c}±7.3	49±4.5
ZEA	14^{a}±0.62	20^{a}±3.5	84^{d}±1.5	95^{e}±0.27	19^{a}±2.7	100^{e}±0	34^{b}±3	53^{c}±1	39^{b}±1.3	51±1.5
α-ZEL	12^{a}±2.9	20^{ab}±3.6	89^{e}±0.77	95^{e}±0.7	13^{a}±4.3	100^{e}±0	33^{c}±4.3	53^{d}±0.28	31^{bc}±3.5	50±2.3
β-ZEL	16^{a}±1.3	19^{a}±0.85	88^{d}±1.1	96^{e}±0.28	23^{a}±2.8	100^{e}±0	32^{b}±0.07	57^{c}±4.2	34^{b}±1.7	52±1.4

Key: Values represent means of duplicate determinations of the percentage degradations ± standard deviation of the means. Significant differences among the sample treatments as a function of time and temperature are indicated as superscripted alphabets on the means, and were compared using Tukey's pairwise multiple comparison test following a one-way ANOVA. Values in the same row followed by the same alphabet are not significantly different ($p > 0.05$). AFB_1: aflatoxin B_1. AFB_2: aflatoxin B_2. AFG_1: aflatoxin G_1. AFG_2: aflatoxin G_2. AME: alternariol monomethyl ether. FB_1: fumonisin B_1. FB_2: fumonisin B_2. FB_3: fumonisin B_3. OTA: ochratoxin A. OTB: ochratoxin B. STEG: sterigmatocystin. T-2: T-2 toxin. ZEA: zearalenone. α-ZEL: α-zearalenol. β-ZEL: β-zearalenol.

Table 3.4: Temperature-time degradation of multi-mycotoxins spiked into maize matrix.

Mycotoxin	Percentage degradation of mycotoxins at different temperature-time conditions									
	120 °C/ 15 min (%)	**120 °C/ 55 min (%)**	**200 °C/ 15 min (%)**	**200 °C/ 55 min (%)**	**103 °C/ 35 min (%)**	**217 °C/ 35 min (%)**	**160 °C/ 6.72 min (%)**	**160 °C/ 63.28 min (%)**	**160 °C/ 35 min (%)**	**Average**
AFB_1	$2.8^{a}\pm3.1$	$6^{a}\pm2.3$	$86^{e}\pm0$	$99^{f}\pm0.13$	$6^{a}\pm4.5$	$100^{f}\pm0$	$18^{b}\pm0.38$	$59^{d}\pm1.5$	$46^{c}\pm0.47$	47±1.4
AFB_2	$19^{a}\pm15$	$31^{abc}\pm11$	$73^{cd}\pm13$	$91^{d}\pm0$	$14^{a}\pm17$	$100^{d}\pm0$	$24^{ab}\pm4.7$	$62^{bcd}\pm18$	$46^{abc}\pm3.3$	51±9.1
AFG_1	$4^{a}\pm4.2$	$6^{a}\pm4$	$91^{c}\pm0$	$100^{c}\pm0$	$1^{a}\pm3.3$	$100^{c}\pm0$	$12^{a}\pm12$	$60^{b}\pm1.9$	$45^{b}\pm2.2$	47±3.1
AFG_2	$3^{a}\pm3.7$	$7^{a}\pm2.4$	$68^{c}\pm2.4$	$89^{d}\pm0$	$4^{a}\pm2.4$	$98^{d}\pm0.22$	$14^{ab}\pm6.3$	$27^{b}\pm8.5$	$22^{b}\pm0.42$	37±2.9
AME	$28^{a}\pm2.6$	$23^{a}\pm6.1$	$30^{a}\pm1.8$	$51^{b}\pm0.99$	$32^{a}\pm7.6$	$63^{b}\pm2.9$	$29^{a}\pm2.6$	$26^{a}\pm0.64$	$19^{a}\pm3.2$	33±3.2
FB_1	$53^{ab}\pm1.3$	$52^{ab}\pm11$	$100^{c}\pm0$	$100^{c}\pm0$	$42^{a}\pm5$	$100^{c}\pm0$	$65^{b}\pm4.6$	$97^{c}\pm0$	$97^{c}\pm0$	78±2.5
FB_2	$44^{ab}\pm4.8$	$53^{b}\pm5$	$100^{d}\pm0$	$100^{d}\pm0$	$42^{a}\pm5.3$	$100^{d}\pm0$	$65^{c}\pm0.84$	$97^{d}\pm0.26$	$95^{d}\pm0.16$	77±1.8
FB_3	$55^{a}\pm7$	$59^{ab}\pm3.1$	$100^{c}\pm0$	$100^{c}\pm0$	$50^{a}\pm5.7$	$100^{c}\pm0$	$70^{b}\pm2$	$98^{c}\pm0.38$	$96^{c}\pm0.49$	81±2.1
OTA	$25^{a}\pm2.4$	$34^{a}\pm6$	$56^{b}\pm1.6$	$85^{c}\pm0.65$	$26^{a}\pm2.9$	$94^{c}\pm0.18$	$33^{a}\pm6.2$	$32^{a}\pm4.2$	$26^{a}\pm1.2$	46±2.8
OTB	$17^{ab}\pm15$	$21^{ab}\pm3.1$	$46^{b}\pm2.2$	$80^{c}\pm0.78$	$16^{a}\pm13$	$92^{c}\pm0.13$	$22^{ab}\pm7.5$	$21^{ab}\pm9.4$	$30^{ab}\pm0.85$	39±5.8
STEG	$5^{a}\pm0.21$	$7^{a}\pm0.36$	$56^{c}\pm4.1$	$94^{d}\pm0.21$	$9^{a}\pm0.21$	$98^{d}\pm0.14$	$12^{a}\pm2.6$	$39^{b}\pm2.4$	$15^{a}\pm5.2$	37±1.7
T-2	$9^{a}\pm6.3$	$7^{a}\pm3.4$	$53^{b}\pm15$	$85^{c}\pm0.3$	$13^{a}\pm9.3$	$96^{c}\pm2.2$	$19^{a}\pm13$	$29^{ab}\pm6.2$	$26^{ab}\pm3$	37±6.5
ZEA	$14^{a}\pm0.12$	$17^{a}\pm11$	$56^{b}\pm1.6$	$85^{c}\pm1.1$	$21^{a}\pm4.2$	$93^{c}\pm0.3$	$22^{a}\pm2.4$	$44^{b}\pm2$	$27^{a}\pm2.2$	42±2.8
α-ZEL	$6^{ab}\pm0$	$17^{bc}\pm0.66$	$58^{e}\pm0.66$	$88^{f}\pm0.72$	$3^{a}\pm2.9$	$97^{f}\pm0.61$	$14^{abc}\pm4.1$	$35^{d}\pm7.7$	$19^{c}\pm0.18$	37±1.9
β-ZEL	$3^{b}\pm0$	$20^{b}\pm2.9$	$58^{d}\pm0.58$	$90^{e}\pm0.05$	$9^{a}\pm5.4$	$98^{e}\pm0.37$	$20^{b}\pm1$	$32^{c}\pm1.7$	$19^{b}\pm1.2$	39±1.5

Key: Values represent means of duplicate determinations ± standard deviation of the means. Significant differences among the sample treatments as a function of time and temperature are indicated as superscripted alphabets on the means, and were compared using Tukey's pairwise multiple comparison test following a one-way ANOVA. Values in the same row followed by the same alphabet are not significantly different ($P > 0.05$). AFB_1: aflatoxin B_1. AFB_2: aflatoxin B_2. AFG_1: aflatoxin G_1. AFG_2: aflatoxin G_2. AME: alternariol monomethyl ether. FB_1: fumonisin B_1. FB_2: fumonisin B_2. FB_3: fumonisin B_3. OTA: ochratoxin A. OTB: ochratoxin B. STEG: sterigmatocystin. T-2: T-2 toxin. ZEA: zearalenone. α-ZEL: α-zearalenol. β-ZEL: β-zearalenol.

In the food industry, particularly the coffee industry, the thermal stability of ochratoxins (OTs) is of particular interest, because OTs have been reported to be prevalent even in processed ready-to-eat coffee (Studer-Rohr *et al*., 1995; FAO, 2001; Kabak, 2009; Vanesa & Ana, 2013). It has not been possible to conclusively establish the thermal stability of OTs based on available literature. Tsubouchi *et al*. (1987) considers OTA as relatively heat stable, because only 0 to 12% of OTA was degraded in beans when roasted at 200 °C for 10 to 20 min (Tsubouchi *et al*., 1987). Whereas, other reports consider OTA as relatively thermolabile (Studer-Rohr *et al*., 1995; Perez de Obanos *et al*., 2005). Ultimately, our findings reveal that the thermal stability of OTs is strongly influenced by matrix interactions (Table 3.5). As pure standards, OTs were highly resilience to heat treatment, such that a temperature-time condition of 217 °C and 35 min (a condition at which almost all the other mycotoxins completely degrade) resulted in only about 50% degradation of the OTs. However, when OTs were spiked into maize matrix and subjected to similar conditions (*i.e.,* 217 °C and 35 min), there was at least 94 and 92% degradation for OTA and OTB respectively. The mechanism of thermal degradation of OTA at higher temperatures (>250 °C) has been described as partial isomerization on the molecules, which results in the formation of a less toxic diastereomer (Studer-Rohr *et al*., 1995).

Based on our results, T-2 and ZEA and its analogues (ZEAs) can be regarded as mid-thermally stable mycotoxins. The average thermal degradation of ZEAs ranged from 48 to 50% for pure standards, and 36 to 41% for spiked maize. Likewise, T-2 had an average degradation of 48 and 32% for pure standards and spiked maize respectively, which agrees with the findings by Schmidt *et al*. (2017). The thermal stability of the relatively understudied, AME and STEG, were also investigated in the present study. Alternariol monomethyl ether demonstrated a significantly strong thermal stability. In fact, it had the highest thermal stability for spiked maize (32%), second only to the OTs for pure standards with an average degradation of 35%. Just like the OTs, the thermal stability of STEG is strongly matrix-influenced. When in pure form, STEG had an average degradation of 60%, however, when spiked into maize matrix it had an average degradation of 35%. To the best of our knowledge, this is the first report on the thermal degradation of AME and STEG.

3.3.2 Matrix effect on thermal stability of mycotoxins

The effect of matrix interference on the thermal stability of mycotoxins is indisputably substantial. Depending on the mycotoxin and thermal conditions, maize matrix either accelerated or suppressed the degradation of mycotoxins (Tables 3.3 & 3.4). The mycotoxins most susceptible to matrix-enhanced degradation included FBs, OTs and AFB_2. Sterigmatocystin, T-2 and the other mycotoxins demonstrated less susceptibility, and perhaps some level of matrix-suppressed degradation (Appendix 3.C). This phenomenon can be more clearly visualized on the compound 3-D surface plots (Figure 3.1), which is discussed in greater detail in the succeeding sections of this work. Raters and Matissek (2008) also observed that the presence of matrix components (starch) led to increased degradation of AFB_1 in spiked maize matrix compared to pure-form AFB_1. In fact, they noted that when AFB_1 was spiked into a proteinous matrix (*i.e.*,, soybean), thermal degradation was much more increased compared to starch (Raters & Matissek, 2008). Other possible matrix constituents that may interfere with the thermal stability of mycotoxins are polyphenols and moisture (Boudra *et al*., 1995; Howard *et al*., 1998; Raters & Matissek, 2008).

Previous studies have shown that mycotoxin-matrix interactions during thermal degradation entails a number of physicochemical possibilities such as breakdown or modification the chemical structures of the analytes, or heat-assisted binding of the toxins to matrix components (Seefelder *et al*., 2001; Nicolás-Vázquez *et al*., 2010; Dhanasekaran *et al*., 2011), which could render the toxin undetectable during routine analysis and perhaps less toxic to humans and animals. For example, during thermal processing of maize or maize-based foods, it is known that fumonisins can bind to various components within the food matrix or react with other ingredients within the food such as reducing sugars (Seefelder *et al*., 2001; Kabak, 2009). Lu *et al*. (2002) showed that the incubation of FB_1 with D-glucose resulted in the formation of N-carboxymethyl-FB_1, a reaction product of FB_1 and reducing sugars. The four primary products of the FB_1-reducing sugars reaction have been characterized as Nmethyl-FB_1,N-carboxymethyl-FB_1,N-(3-hydroxyacetonyl)-FB_1 and N-(2-hydroxy, 2-carboxyethyl)-FB_1 using nuclear magnetic resonance and electrospray mass spectroscopy (Lu *et al*., 2002).

In another study, Seefelder and co-authors (2001) reported the formation of hydrolyzed fumonisin B_1 when samples containing FB_1 and sucrose were thermally processed. The authors also observed that N-(carboxymethyl)fumonisin B_1, which was formed via thermal processing of samples

containing FB_1 and d-glucose is less toxic compared to FB_1, the parent toxin. This heat-accentuated binding of FBs to matrix components could be the reason for the herein observed thermolabile nature of FBs.

The presence of residual moisture in sample matrices is known to enhance the thermal degradation of mycotoxins via formation of a carboxylic acid terminal on the molecule (Kabak *et al*., 2006). This terminal is formed by addition of a water molecule to the lactone ring of the molecules, which subsequently undergoes heat-induced decarboxylation (Kabak *et al*., 2006). Boudra *et al*. (1995) showed that when OTA was present in wheat matrix and dry-heated at 100 °C for 40 to 160 min there was no observable degradation, however, wet-heating at the same temperature (100 °C) for only 120 min resulted in over 50% degradation of OTA. It has been advanced that availability of moisture during heating of AFs hydrolyses its lactone ring, which makes them highly-chemically unstable, as such, subsequent mild heating (>100 °C) allows for clipping of the lactone ring of the molecule (Samarajeewa *et al*., 1990; Raters & Matissek, 2008).

3.3.3 Numerical modelling of the degradation of mycotoxins

3.3.3.1 Model fitting to experimental data

In order to describe the empirical relationship between the studied objective variables, *i.e.*, mycotoxin degradation (%) and control variables of temperature (°C) and exposure time (min), the quadratic regression function (Equation 3.1) was fitted to the experimental data. The resultant second-order model coefficients for the objective variables are presented in Tables 3.5 and 3.6 (Columns 2-6). These models provide a strong approximation to the true relationship between our objective variables and the control variables.

Table 3.5: Regression model fit coefficients and validation indices for thermal degradation for pure mycotoxins standards

Myco	$z(x,y) = C_{00} + C_{10}x + C_{20}x^2 + C_{01}y + C_{02}y^2 + C_{11}xy$												
	c_{00}	c_{10}	c_{20}	c_{01}	c_{02}	c_{11}	R^2	R^2 Adj	Residual	ρ	AAD	B_f	A_f
AFB_1	13.68	-0.22	0.00	-1.84	0.02	0.00	0.98	0.97	37.59	0.99	0.16	1.06	1.15
AFB_2	297.85	-3.41	0.01	-3.01	0.02	0.01	0.87	0.82	139.67	0.93	0.33	1.06	1.31
AFG_1	-92.07	0.79	0.00	-0.03	0.01	0.00	0.96	0.95	65.97	0.98	0.27	0.71	1.67
AFG_2	177.89	-2.51	0.01	-1.19	0.00	0.01	0.98	0.98	27.99	0.99	0.25	1.02	1.24

Myco	$z(x,y) = C_{00} + C_{10}x + C_{20}x^2 + C_{01}y + C_{02}y^2 + C_{11}xy$												
	c_{00}	c_{10}	c_{20}	c_{01}	c_{02}	c_{11}	R^2	R^2 Adj	Residual	ρ	AAD	B_f	A_f
AME	163.93	-2.25	0.01	-1.63	0.00	0.01	0.99	0.99	14.46	0.99	0.51	1.05	1.30
FB_1	-76.53	1.25	0.00	-2.64	0.04	0.00	0.95	0.84	245.39	0.94	0.47	1.17	1.26
FB_2	-124.16	1.85	0.00	-2.53	0.04	0.00	0.98	0.97	37.25	0.99	0.60	1.05	1.08
FB_3	-116.69	1.83	0.00	-2.48	0.03	0.00	0.91	0.88	170.19	0.95	0.54	1.18	1.24
OTA	97.40	-1.33	0.01	-0.81	0.00	0.00	0.96	0.95	18.86	0.98	0.38	1.10	1.32
OTB	199.85	-2.28	0.01	-1.84	0.00	0.01	0.92	0.89	28.28	0.96	0.51	0.97	1.58
STEG	-3.12	0.01	0.00	-1.14	0.03	0.00	0.98	0.97	28.41	0.99	0.09	0.96	1.10
T-2	20.96	-0.41	0.00	-1.5	0.01	0.01	0.95	0.94	80.65	0.98	0.58	1.16	1.23
ZEA	85.18	-1.36	0.01	-0.39	0.01	0.00	0.98	0.98	23.06	0.99	0.09	1.01	1.08
α-ZEL	118.92	-1.83	0.01	-0.83	0.02	0.00	0.98	0.97	30.04	0.99	0.11	1.00	1.12
β-ZEL	146.69	-2.06	0.01	-1.00	0.01	0.00	0.97	0.96	43.93	0.98	0.13	1.01	1.13

Key: Myco: mycotoxin. AFB_1: aflatoxin B_1. AFB_2: aflatoxin B_2. AFG_1: aflatoxin G_1. AFG_2: aflatoxin G_2. AME: alternariol monomethyl ether. FB_1: fumonisin B_1. FB_2: fumonisin B_2. FB_3: fumonisin B_3. OTA: ochratoxin A. OTB: ochratoxin B. STEG: sterigmatocystin. T-2: T-2 toxin. ZEA: zearalenone. α-ZEL: α-zearalenol. β-ZEL: β-zearalenol. c_{00} is a constant. c_{10} and c_{01} are the linear coefficients of *x* (temperature) and *y* (time), respectively. c_{20} and c_{02} are the quadratic coefficients of *x* and *y*, respectively. c_{11} is the interaction coefficient.

Table 3.6: Regression model fit coefficients and validation indices for thermal degradation for mycotoxin standards spiked into maize flour

Myco	$z(x,y) = C_{00} + C_{10}x + C_{20}x^2 + C_{01}y + C_{02}y^2 + C_{11}xy$												
	c_{00}	c_{10}	c_{20}	c_{01}	c_{02}	c_{11}	R^2	R^2 Adj	Residual	ρ	AAD	B_f	A_f
AFB_1	-54.76	0.08	0.00	0.53	-0.01	0.00	0.96	0.95	69.52	0.98	0.36	1.10	1.32
AFB_2	21.82	-0.63	0.00	0.34	0.00	0.00	0.92	0.89	98.83	0.96	0.49	1.09	1.33
AFG_1	-56.35	0.02	0.00	0.64	-0.01	0.00	0.94	0.92	119.28	0.97	0.83	1.28	1.37
AFG_2	154.33	-2.43	0.01	-0.72	0.00	0.01	0.99	0.98	25.17	0.99	2.52	1.19	1.48
AME	247.13	-2.70	0.01	-1.83	0.01	0.01	0.89	0.86	27.38	0.95	0.13	0.99	1.12
FB_1	-227.76	3.09	-0.01	1.59	-0.02	0.00	0.93	0.9	54.12	0.96	0.07	1.00	1.07
FB_2	-248.21	3.18	-0.01	2.08	-0.02	0.00	0.95	0.94	38.52	0.98	0.06	1.00	1.06
FB_3	-189.89	2.68	-0.01	1.57	-0.02	0.00	0.94	0.92	34.64	0.97	0.06	1.00	1.06
OTA	264.86	-3.26	0.01	-1.53	0.01	0.01	0.95	0.93	42.44	0.98	0.11	1.00	1.12
OTB	177.39	-2.36	0.01	-0.69	-0.01	0.01	0.92	0.89	76.27	0.96	0.35	1.04	1.32
STEG	261.22	-3.51	0.01	-2.28	0.01	0.01	0.99	0.99	11.36	1.00	0.13	1.01	1.13
T-2	179.08	-2.45	0.01	-1.27	0.00	0.01	0.96	0.95	48.15	0.98	0.19	1.00	1.20
ZEA	189.42	-2.49	0.01	-1.29	0.01	0.01	0.99	0.98	14.12	0.99	0.11	1.01	1.10

Myco	$z(x,y) = C_{00} + C_{10}x + C_{20}x^2 + C_{01}y + C_{02}y^2 + C_{11}xy$												
	c_{00}	c_{10}	c_{20}	c_{01}	c_{02}	c_{11}	R^2	R^2 Adj	Residual	ρ	AAD	B_f	A_f
α-ZEL	189.15	-2.76	0.01	-1.22	0.01	0.01	0.99	0.98	18.02	0.99	0.47	1.05	1.28
β-ZEL	210.15	-3.01	0.01	-1.18	0.01	0.01	0.98	0.98	23.59	0.99	0.23	1.05	1.22

Key: Myco: mycotoxin. AFB_1: aflatoxin B_1. AFB_2: aflatoxin B_2. AFG_1: aflatoxin G_1. AFG_2: aflatoxin G_2. AME: alternariol monomethyl ether. FB_1: fumonisin B_1. FB_2: fumonisin B_2. FB_3: fumonisin B_3. OTA: ochratoxin A. OTB: ochratoxin B. STEG: sterigmatocystin. T-2: T-2 toxin. ZEA: zearalenone. α-ZEL: α-zearalenol. β-ZEL: β-zearalenol. c_{00} is a constant, c_{10} and c_{01} are the linear coefficients of *x* (temperature) and *y* (time), respectively, c_{20} and c_{02} are the quadratic coefficients of *x* and *y*, respectively, and c_{11} is the interaction coefficient.

By means of these models, the compound 3-D surface plots (Figure 3.1) were generated using OriginPro 8.5 software (OriginLab, Massachusetts, US). These plots present a visual interpretation of the degradation patterns of each mycotoxin, as well allows the comparison of the degradation profile of pure mycotoxins and mycotoxins spiked into maize. Many of the degradation patterns in our data described by these plots are consistent with a first order reaction kinetics. On the plots, the blue surfaces represent the degradation profile of pure mycotoxin standards, while the green surfaces represent degradation profile of corresponding mycotoxin spiked into maize matrix. The color bands correspond to the value-ranges of the objective variable. Accordingly, lighter regions represent higher degradation, whereas, more intense (darker) regions corresponds to lower degradation.

3.3.3.2 Factor effects

In contrast to conventional optimization methods, RSM is able to make available details on the magnitude and significance of individual and pairwise factor effects on the objective variable(s) (Uma *et al*., 2010; Gbashi *et al*., 2016). Accordingly, our model fit gave the various factor effects for pure mycotoxin standards and mycotoxins spiked into maize matrix as shown in Figures 3.2a & b and Appendix 3.D. The Pareto charts of standardized factor effects (Figures 3.2a & b) graphically indicates the magnitude and importance of each effect. The red reference line indicated on the chart distinguishes between insignificant and significant effects at $\alpha = 0.05$. Any effect that is below this line is insignificant (Gbashi *et al*., 2016).

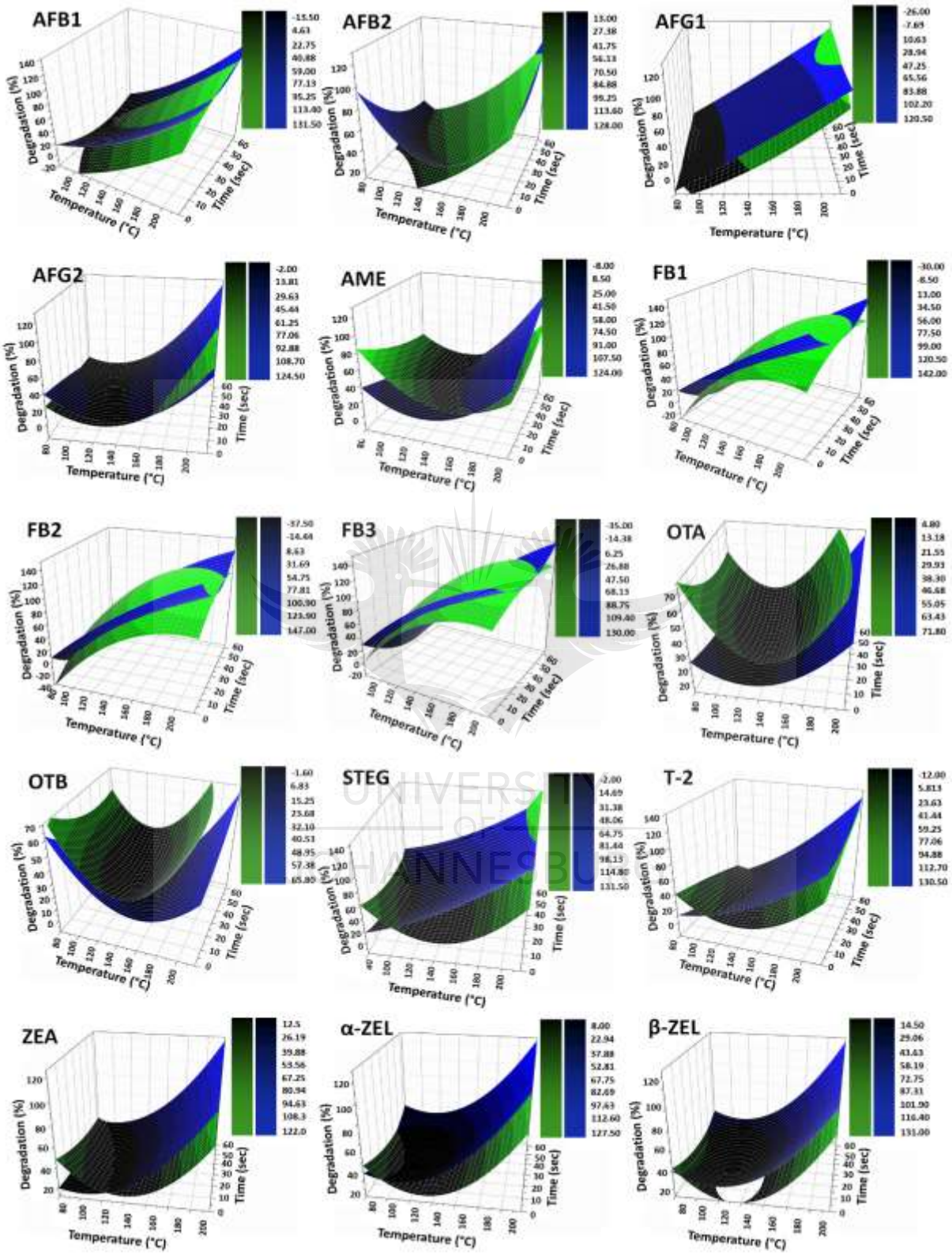

Figure 3.1: Compound response surface plots for thermal degradation for pure mycotoxins and mycotoxins spiked into maize matrix. AFB_1: aflatoxin B_1. AFB_2: aflatoxin B_2. AFG_1: aflatoxin

G_1. AFG_2: aflatoxin G_2. AME: alternariol monomethyl ether. FB_1: fumonisin B_1. FB_2: fumonisin B_2. FB_3: fumonisin B_3. OTA: ochratoxin A. OTB: ochratoxin B. STEG: sterigmatocystin. T-2: T-2 toxin. ZEA: zearalenone. α-ZEL: α-zearalenol. β-ZEL: β-zearalenol.

It can be seen that the linear effect of temperature (T1L) was significantly higher ($p<0.05$) across all response variables for pure mycotoxin standards and mycotoxins spiked into maize matrix, and ranged from 75.89 to 25.08, and 80.24 to 18.28 for pure mycotoxin standards and mycotoxins spiked into maize matrix (Appendix 3.D). This suggests that higher temperatures and shorter exposure times are more effective for mycotoxin degradation, compared to lower temperatures and longer exposure times. The other factor effects for pure mycotoxin standards and mycotoxins spiked into maize matrix, respectively, included quadratic effect of temperature (T1Q) that ranged from 35.76 to -10.49, and 39.46 to -25.5; Linear effect of time (T2L): ranged from 12.63 to -2.56, and 21.27 to 3.01; Quadratic effect of time (T2Q): ranged from 32.29 to -1.81, and 11.26 to -15.66; and interactive effect of temperature and time (T1L by T2L): ranged from 19.61 to -4.29, and 17.92 to -4.61.

3.3.3.3 Optimization of the thermal degradation of multi-mycotoxins

Using the response optimization function of the Minitab 17 statistical software (Pennsylvania, US), it was possible to derive the optimum conditions for the thermal degradation of individual mycotoxins, *i.e.*, the most efficient combination of temperature-time for the maximum degradation of individual mycotoxins (Appendix 3.E). An approximation of these solutions can also be visually extrapolated from the surface plots (Figure 3.1). For all mycotoxins, the optimum conditions varied from 178 °C/63.28 min to 217 °C/63.28 min for pure standard and 207 °C/24.43 min to 217 °C/63.28 for spiked maize matrix. Within the experimental range and model resources, it was not possible to achieve 100% degradation of pure-standard OTs due to their high thermal stability. A similar situation was observed for AME spiked into the maize matrix.

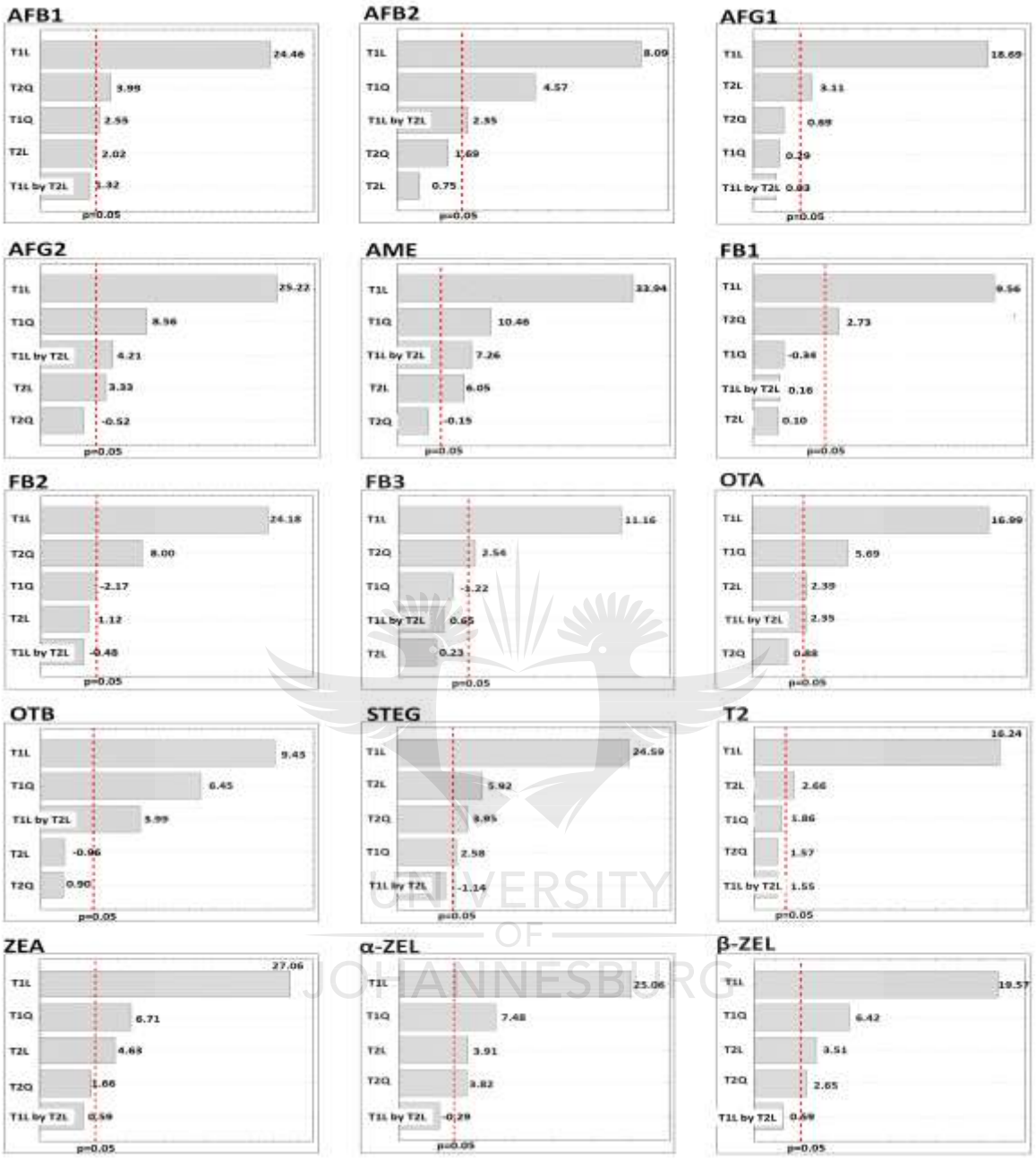

Figure 3.2a: Pareto charts of standardized factor effects for model fit of pure mycotoxin standards. T1L – Linear effect of temperature; T1Q – Quadratic effect of temperature; T2L – Linear effect of time; T2Q – Quadratic effect of time; and T1L by T2L – Interaction effect temperature and time. AFB_1: aflatoxin B_1. AFB_2: aflatoxin B_2. AFG_1: aflatoxin G_1. AFG_2: aflatoxin G_2. AME: alternariol monomethyl ether. FB_1: fumonisin B_1. FB_2: fumonisin B_2. FB_3: fumonisin B_3. OTA: ochratoxin A. OTB: ochratoxin B. STEG: sterigmatocystin. T-2: T-2 toxin. ZEA: zearalenone. α-ZEL: α-zearalenol. β-ZEL: β-zearalenol.

Figure 3.2b: Pareto charts of standardized factor effects for model fit of mycotoxins spiked into maize matrix. T1L – Linear effect of temperature; T1Q – Quadratic effect of temperature; T2L – Linear effect of time; T2Q – Quadratic effect of time; and T1L by T2L – Interaction effect temperature and time. AFB_1: aflatoxin B_1. AFB_2: aflatoxin B_2. AFG_1: aflatoxin G_1. AFG_2: aflatoxin G_2. AME: alternariol monomethyl ether. FB_1: fumonisin B_1. FB_2: fumonisin B_2. FB_3: fumonisin B_3. OTA: ochratoxin A. OTB: ochratoxin B. STEG: sterigmatocystin. T-2: T-2 toxin. ZEA: zearalenone. α-ZEL: α-zearalenol. β-ZEL: β-zearalenol.

As such, in order to at least have an idea of the region in which conditions for 100% degradation of these mycotoxins would fall, we interrogated the corresponding models beyond the available model resources, which gave values of 239 °C/63.28 for pure-standard OTs, and 230 °C/40.50 min for AME spiked into maize matrix. Although these values are not very far from the experimental range, it should be noted that because these values fall outside of the experimental range, their predictability may not be reliable, except confirmed by laboratory analysis. Essentially, the further away the solution is from the experimental range, the greater the variance and the less the precision (STAT503, 2018).

Beside optimization for individual objective variables, it is important to derive the global (synchronous) optimal solution for all the objective variables. This is critical because mycotoxins co-occur in nature in food commodities and exert synergistic effects (Serrano *et al*., 2012; Smith *et al*., 2016; Adekoya *et al*., 2018). As such, a single compromise optimum that accounts for the maximum eradication of all mycotoxins present is a more meaningful solution for food safety and health applications. In this regard, using the global optimization function of the Minitab 17 statistical software, the compromise multi-objective optimum solution for our objective variables was 217 °C/63.28 min and 211 °C/54.71 min for pure mycotoxin standards and spiked maize matrix, respectively. The desirability factor (DF) in all cases was 1.00.

3.3.3.4 Validation of model

To determine the adequacy of the models and ensure that they offer a good approximation of the true systems, we examined some important validation indices from the model fit which are presented in Tables 5 and 6 (columns 8-14). Significance was determined at a 95% probability level *i.e.,* α=0.05. Computed values of the coefficients of multiple determination (R^2) ranged from 0.87 to 0.99, and 0.89 to 0.99 for pure mycotoxin standards and mycotoxins spiked into maize matrix, respectively. This implies that our models describe between 87 to 99% of the variability in our data, reflective of its acceptability and significance (Adebo *et al*., 2018b). Also, the adjusted R^2 fell within less than ± 0.05 of the R^2 values for all the models. The closeness of the R^2 and adjusted R^2 values, and their nearness to unity indicated that our empirical models were well fitted to the actual data (Babu & Srivastava, 2007; Morshedi & Akbarian, 2014; Adebo *et al*., 2018b). The Pearson's correlation coefficient (r) ranged from 0.91 to 0.99, and 0.95 to 1.00 for pure

mycotoxin standards and mycotoxins spiked into maize matrix, respectively, indicating a strong relationship between the predicted values and the experimental values.

The degree of variability in the dataset was estimated using the average absolute deviation (*AAD*), which ranged from 0.09 to 0.60, and 0.06 to 0.83 for pure mycotoxin standards and mycotoxins spiked into maize matrix, respectively. It should be noted that *AAD* values for AFG_2 in spiked maize matrix (2.25) was not included in the above ranges because it was considered as an outlier. Outliers were determined using the Q-Q plots in SPSS by a step of 1.5×IQR (interquartile range) (Marr, 2018). Average absolute deviation values closer to zero are desirable as it indicates agreements between the predicted and experimental values (Adebo *et al*., 2018b). The bias factor (B_f) and accuracy factor (A_f) were also examined for our model fits. A_f estimates the relative deviation of predicted values from the experimental values (Dominguez & Schaffner, 2007). Our A_f values ranged from 1.08 to 1.68, and 1.06 to 1.48 for pure mycotoxin standards and mycotoxins spiked into maize matrix respectively. An A_f value of 1.12 indicates an averagely 12% variation between observed and predicted values (Oscar, 2009). Similarly, the B_f measures the relative deviation of predicted and experimental values (Dominguez & Schaffner, 2007). Our B_f values ranged from 0.71 to 1.57, and 0.99 to 1.28 for 48 for pure mycotoxin standards and mycotoxins spiked into maize matrix, respectively. The closer the values of A_f and B_f to unity, the stronger the predictability of the associated models (Desobgo *et al*., 2015; Adebo *et al*., 2018b).

3.4 Conclusion

The present study profiled temperature-time degradation patterns of multiple mycotoxins with and without the effect of a matrix. Mycotoxins such as OTs, AFB_2, ZEA and its analogues, STEG and the FBs showed a strongly enhanced degradation when spiked into maize matrix. Alternariol monomethyle ether demonstrated a very high thermal stability generally irrespective of matrix interference, while the FBs were the most thermolabile amongst the studied mycotoxins irrespective of the presence of a food matrix. Moreover, we have demonstrated for the first time the thermal stability of the mycotoxins, AME and STEG. In order to optimize the degradation of mycotoxins, the RSM optimization function was fitted to our experimental data, which yielded well-fit models that clearly define the empirical relationship between our control variables (temperature and time) and our objective variable (*i.e.,* mycotoxin degradation). The resultant

global optimum solution for multi-mycotoxin degradation was 217 °C/63.28 min and 211 °C/54.71 min for pure mycotoxin standards and spiked maize matrix, respectively. These values were the most efficient combinations of temperature and time to achieve maximum degradation of the analytes within the experimental range.

However, it was observed that these optimal conditions were not sufficient to completely eliminate OTs and AME for pure mycotoxins and in spiked maize, respectively, due to their high thermal stability, and because the optimal conditions are computed within the boundries of the experimental range. As such, in order to obtain detectable levels (100% degradation) of the two mycotoxins in question, the optimization models were interrogated beyond the experimental ranges, which gave values of 239 °C/63.28 min for pure standard OTs, and 230 °C/40.50 min for AME in spiked maize. Besides optimization, our models can be useful in estimating any desired degradation outcome within the experimental domain. This could find applicability in food processing for goal-specific thermal processing of purportedly mycotoxin contaminated foods for food safety and optimum quality.

Further research however, is required to determine the effect of thermal treatment at the obtained optimal conditions on vital food quality factors. Also, other but more efficient thermal processing techniques such as micronisation (high intensity infrared heating) could be investigated as alternatives to conventional oven heating methods. It would also be interesting to investigate the effects of some specific matrix parameters such as pH and moisture, on the thermal degradation patterns of the mycotoxins. Furthermore, there is need to establish the identities of degradation products via high resolution mass spectrometry (HRMS). We do hope that our observations reported herein would positively contribute to the debate on thermal stability of some mycotoxins such as OTs and AFs.

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References

Abakarov, A., and Nuñez, M. (2013). Thermal food processing optimization: Algorithms and software. *Journal of Food Engineering*, *115*(4), 428–442.

Adebo, O. A., Njobeh, P. B., Mulaba-Bafubiandi, A. F., Adebiyi, J. A., Desobgo, Z. S. C., and Kayitesi, E. (2018). Optimization of fermentation conditions for ting production using response surface methodology. *Journal of Food Processing and Preservation*, *42*(1), e13381.

Adebo, O. A., Njobeh, P. B., Gbashi, S., Nwinyi, O. C., and Mavumengwana, V. (2017). Review on microbial degradation of aflatoxins. *Critical Reviews in Food Science and Nutrition*, *57*(15), 3208–3217.

Adekoya, I., Obadina, A., Adaku, C. C., De Boevre, M., Okoth, S., De Saeger, S., and Njobeh, P. (2018). Mycobiota and co-occurrence of mycotoxins in South African maize-based opaque beer. *International Journal of Food Microbiology*, *270*, 22–30.

Babu, G. S., and Srivastava, A. (2007). Reliability analysis of allowable pressure on shallow foundation using response surface method. *Computers and Geotechnics*, *34*(3), 187–194.

Bas, D., and Boyaci, I. H. (2007). Modeling and optimization I: Usability of response surface methodology. *Journal of Food Engineering*, *78*, 836–845.

Bertuzzi, T., Rastelli, S., Mulazzi, A., and Pietri, A. (2011). Evaluation and improvement of extraction methods for the analysis of aflatoxins B_1, B_2, G_1 and G_2 from naturally contaminated maize. *Food Analytical Methods*, *5*(3), 512–519.

Boudra, H., Le Bars, P., and Le Bars, J. (1995). Thermostability of Ochratoxin A in wheat under two moisture conditions. *Applied and Environmental Microbiology*, *61*(3), 1156–1158.

Bretz, M., Beyer, M., Cramer, B., Knecht, A., and Humpf, H. U. (2006). Thermal degradation of the *Fusarium* mycotoxin deoxynivalenol. *Journal of Agricultural and Food Chemistry*,

54(17), 6445–6451.

Brown, J. D. (1999). *Development of a method for the simultaneous detection of mycotoxins in corn using supercritical fluid extraction (SFE), high performance liquid chromatography (HPLC), and electrospray-atmospheric pressure ionization/mass spectrometry (ES-API/MS) for extr*. The University of Iowa.

Castelo, M. M., Sumner, S. S., and Bullerman, L. B. (1998). Stability of fumonisins in thermally processed corn products. *Journal of Food Protection*, *61*, 1030–1033.

Chilaka, C. A., De Boevre, M., Atanda, O. O., and De Saeger, S. (2016). Occurrence of *Fusarium* mycotoxins in cereal crops and processed products (Ogi) from Nigeria. *Toxins*, *8*(11), 342.

Cremer, D., Wu, A., Larsson, A., and Kraka, E. (2000). Some thoughts about bond energies, bond lengths, and force constants. *Molecular Modeling Annual*, *6*(4), 396–412.

De Souza, G. D., Mithöfer, A., Daolio, C., Schneider, B., and Rodrigues-Filho, E. (2013). Identification of Alternaria alternata mycotoxins by LC-SPE-NMR and their cytotoxic effects to soybean (Glycine max) cell suspension culture. *Molecules*, *18*(3), 2528–2538.

Desobgo, Z. S., Stafford, R. A., and Metcalfe, D. J. A. (2015). Dimethyl sulfide stripping behavior during wort boiling using response surface methodology. *Journal of the ASBC*, *1*, 84–89.

Dhanasekaran, D., Shanmugapriya, S., Thajuddin, N., and Panneerselvam, A. (2011). Aflatoxins and aflatoxicosis in human and animals. In: R. G. Guevara-Gonzalez (Ed.), *Aflatoxins - Biochemistry and molecular Biology* (1st ed., pp. 221–254). Rijeka, Croatia: InTech.

Dominguez, S. A., and Schaffner, D. W. (2007). Development and validation of a mathematical model to describe the growth of *Pseudomonas* spp. in raw poultry stored under aerobic conditions. *International Journal of Food Microbiology*, *120*(3), 287–295.

Doyle, M. P., Applebaum, R. S., Brackett, R. E., and Marth, E. H. (1982). Physical, chemical and biological degradation of mycotoxins in foods and agricultural commodities. *Food Control*, *45*(10), 964–971.

du Plessis, J. (2003). *Maize production* (1st ed.). Pretoria, South Africa: Department of Agriculture, Republic of South Africa. Retrieved June 24, 2019, from http://www.arc.agric.za/arc-gci/Fact Sheets Library/Maize Production.pdf

Enyiukwu, D. N., Awurum, A. N., and Nwaneri, J. A. (2014). Mycotoxins in stored agricultural products: Implications to food safety and health and prospects of plant-derived pesticides as novel approach to their management. *Greener Journal of Microbiology and Antimicrobials*, *2*(3), 32–48.

FAO. (2001). *Guidelines for the prevention of mould formation in coffee*. Rome, Italy: Food and Agricultural Organization of the United Nations. Retrieved June 24, 2019, from http://www.fao.org/fileadmin/user_upload/agns/pdf/coffee/guidelines_final_en.pdf

FAO. (2004). Aflatoxin contamination in foods and feeds in the Philippines. *FAO/WHO Regional Conference on Food Safety for Asia and Pacific. Seremban, Malaysia, 24-27 May 2004* (pp. 1–6). Seremban, Malaysia: FAO/WHO United Nations. Retrieved June 24, 2019, from http://www.fao.org/3/ad730e/ad730e00.htm

Gbashi, S., Njobeh, P., Steenkamp, P., Tutu, H., and Madala, N. (2016). The effect of temperature and methanol–water mixture on pressurized hot water extraction (PHWE) of anti-HIV analogues from *Bidens pilosa*. *Chemistry Central Journal*, *10*(37), 1–12.

Gbashi, S., Madala, N. E., Adebo, O. A., Piater, L., Phoku, J. Z., and Njobeh, P. B. (2017a). Subcritical water extraction and its prospects for aflatoxins extraction in biological materials. In: L. B. Abdulra'uf (Ed.), *Aflatoxin-control, analysis, detection and health risks* (pp. 229–250). Rijeka, Croatia: InTech.

Gbashi, S., Madala, N., Adebo, O. A., and Njobeh, P. B. (2017b). Temperature-time numerical modelling of the thermal degradation of aflatoxin B_1 (AFB_1) using response surface methodology. In: S. De Saeger & A. Logrieco (Eds.), *Report from the 1st MYCOKEY International Conference Global Mycotoxin Reduction in the Food and Feed Chain Held in Ghent, Belgium, 11–14 September 2017* (p. 276). Ghent, Belgium: Toxins.

Gibbs, G. V, Hill, F. C., Boisen, M. B., and Downs, R. T. (1998). Power law relationships between

bond length, bond strength and electron density distributions. *Physics and Chemistry of Minerals*, *25*(8), 585–590.

Goncalves, C., Carvalho, J. J., Azenha, M. A., and Alpendurada, M. F. (2006). Optimization of supercritical fluid extraction of pesticide residues in soil by means of central composite design and analysis by gas chromatography–tandem mass spectrometry. *Journal of Chromatography A*, *1110*(1–2), 6–14.

Hooshmand, H., and Klopfenstein, C. F. (1995). Effects of gamma irradiation on mycotoxin disappearance and amino acid contents of corn, wheat, and soybeans with different moisture contents. *Plant Foods for Human Nutrition*, *47*(3), 227–238.

Hossain, M. B., Rawson, A., Aguiló-Aguayo, I., Brunton, N. P., and Rai, D. K. (2015). Recovery of steroidal alkaloids from potato peels using pressurized liquid extraction. *Molecules*, *20*, 8560–8573.

Howard, P. C., Churchwell, M. I., Couch, L. H., Marques, M. M., and Doerge, D. R. (1998). Formation of N-(carboxymethyl) fumonisin B_1, following the reaction of fumonisin B_1 with reducing sugars. *Journal of Agricultural and Food Chemistry*, *46*(9), 3546–3557.

Ji, C., Fan, Y., and Zhao, L. (2016). Review on biological degradation of mycotoxins. *Animal Nutrition*, *2*(3), 127–133.

Juodeikiene, G., Basinskiene, L., Bartkiene, E., and Matusevicius, P. (2012). Mycotoxin decontamination aspects in food, feed and renewables using fermentation processes. In: A. A. Eissa (Ed.), *Structure and function of food engineering* (pp. 171–204). Rijeka, Croatia: InTech.

Kabak, B. (2009). The fate of mycotoxins during thermal food processing. *Journal of the Science of Food and Agriculture*, *89*(4), 549–554.

Kabak, B., Dobson, A. D., and Var, I. (2006). Strategies to prevent mycotoxin contamination of food and animal feed: a review. *Critical Reviews in Food Science and Nutrition*, *46*(8), 593–619.

Khuri, A. I., and Mukhopadhyay, S. (2010). Response surface methodology. *Wiley Interdisciplinary Reviews: Computational Statistics*, *2*(2), 128–149.

Lauren, D. R., and Smith, W. A. (2001). Stability of the *Fusarium* mycotoxins nivalenol, deoxynivalenol and zearalenone in ground maize under typical cooking environments. *Food Additives and Contaminants*, *18*(11), 1011–1016.

Lerda, D. (2011). *Mycotoxins factsheet* (4th ed.). Geel, Belgium: European Commission Joint Research Centre, Institute for Reference Materials and Measurements (JRC-IRMM). Retrieved June 24, 2019, from https://ec.europa.eu/jrc/sites/jrcsh/files/Factsheet Mycotoxins_2.pdf

Lu, Y., Clifford, L., Hauck, C. C., Hendrich, S., Osweiler, G., and Murphy, P. A. (2002). Characterization of Fumonisin B_1- Glucose Reaction Kinetics and Products. *Journal of Agricultural and Food Chemistry*, *50*(16), 4726–4733.

Makun, H. A., Dutton, M. F., Njobeh, P. B., Gbodi, T. A., and Ogbadu, G. H. (2012). Aflatoxin contamination in foods and feeds: A special focus on Africa. In: A. E. Ayman (Ed.), *Trends in vital food and control engineering* (pp. 187–234). Rijeka, Croatia: InTech.

Marr, P. (2018). Quantitative methods (GEO 441). *SPSS Mini-Lab: Outliers*. Retrieved June 24, 2019, from http://webspace.ship.edu/pgmarr/Geo441/Labsheets/SPSS Mini-Lab Outline - Outliers.pdf

McKenzie, K. S., Sarr, A. B., Mayura, K., Bailey, R. H., Miller, D. R., Rogers, T. D., Norred, W. P., Voss, K. A., Plattner, R. D., Kubena, L. F., and Phillips, T. D. (1997). Oxidative degradation and detoxification of mycotoxins using a novel source of ozone. *Food and Chemical Toxicology*, *35*(8), 807–820.

Méndez-Albores, A., Campos-Agular, A. Z., Moreno-Martínez, E., and Vázquez-Durán, A. (2013). Physical and chemical degradation of B-aflatoxins during the roasting and dutching of cocoa liquor. *Journal of Agricultural Science and Technology*, *15*(3), 557–567.

Mircea, C., Poiata, A., Tuchilus, C., Agoroae, L., Butnaru, E., and Stanescu, U. (2008). Aflatoxigenic fungi isolated from medicinal herbs. *Toxicology Letters*, *180*, 32–246.

Morshedi, A., and Akbarian, M. (2014). Application of response surface methodology: design of experiments and optimization: a mini review. *Journal of Fundamental and Applied Sciences*, *54*, 2434–2349.

Murata, H., Mitsumatsu, M., and Shimada, N. (2008). Reduction of feed-contaminating mycotoxins by ultraviolet irradiation: an *in vitro* study. *Food Additives and Contaminants*, *25*(9), 1107–1110.

Nicolás-Vázquez, I., Méndez-Albores, A., Moreno-Martínez, E., Miranda, R., and Castro, M. (2010). Role of Lactone Ring in Structural, Electronic, and Reactivity Properties of Aflatoxin B_1: A Theoretical Study. *Archives of Environmental Contamination and Toxicology*, *59*(3), 393–406.

Njobeh, B. P., Dutton, F. M., and Makun, H. A. (2010a). Mycotoxins and human health: Significance, prevention and control. In: A. K. Mishra, A. Tiwari, & S. B. Mishra (Eds.), *Smart Biomolecules in Medicine* (pp. 132–177). VBRI Press, India.

Njobeh, P. B., Dutton, M. F., Åberg, A. T., and Haggblom, P. (2012). Estimation of multi-mycotoxin contamination in South African compound feeds. *Toxins (Basel)*, *4*(10), 836–848.

Njobeh, P. B., Dutton, M. F., Koch, S. H., Chuturgoon, A. A., Stoev, S. D., and Mosonik, J. S. (2010b). Simultaneous occurrence of mycotoxins in human food commodities from Cameroon. *Mycotoxin Research*, *26*(1), 47–57.

Oscar, T. P. (2009). General regression neural network and Monte Carlo simulation model for survival and growth of Salmonella on raw chicken skin as a function of serotype, temperature, and time for use in risk assessment. *Journal of Food Protection*, *72*(10), 2078–2087.

Park, B. J., Takatori, K., Sugita-Konishi, Y., Kim, I. H., Lee, M. H., Han, D. W., Chung, K. H., Hyun, S. O., and Park, J. C. (2007). Degradation of mycotoxins using microwave-induced argon plasma at atmospheric pressure. *Surface and Coatings Technology*, *201*(9–11), 5733–5737.

Perez de Obanos, A., Gonzalez-Penas, E., and Lopez de Cerain, A. (2005). Influence of roasting and brew preparation on the ochratoxin A content in coffee infusion. *Food Additives and*

Contaminants, *22*(5), 463–471.

Ranum, P., Peña-Rosas, J. P., and Garcia-Casal, M. N. (2014). Global maize production, utilization, and consumption. *Annals of the New York Academy of Sciences*, *1312*(1), 105–112.

Raters, M., and Matissek, R. (2008). Thermal stability of aflatoxin B 1 and ochratoxin A. *Mycotoxin Research*, *24*(3), 130–134.

Samarajeewa, U., Sen, A. C., Cohen, M. D., and Wei, C. I. (1990). Detoxification of aflatoxins in foods and feeds by physical and chemical methods. *Journal of Food Protection*, *53*, 489–501.

Sameni, M., Dübecke, A., and Weber, J. F. (2014). Simultaneous multi-residue determination of mycotoxins in foods using LC-MS/MS. *Journal of Environmental and Analytical Toxicology*, *5*(2), 1000259.

Schmidt, H. S., Becker, S., Cramer, B., and Humpf, H. U. (2017). Impact of mechanical and thermal energies on the degradation of T-2 and HT-2 toxins during extrusion cooking of oat flour. *Journal of Agricultural and Food Chemistry*, *65*(20), 4177–4183.

Scott, P. M., and Lawrence, G. A. (1987). Liquid chromatographic determination and stability of the *Fusarium* mycotoxin moniliformin in cereal grains. *Journal-Association of Official Analytical Chemists*, *70*(5), 850–853.

Seefelder, W., Hartl, M., and Humpf, H. U. (2001). Determination of N-(carboxymethyl) fumonisin B_1 in corn products by liquid chromatography/electrospray ionization–mass spectrometry. *Journal of Agricultural and Food Chemistry*, *49*(5), 2146–2151.

Sendín, J. O. H., Alonso, A. A., and Banga, J. R. (2010). Efficient and robust multi-objective optimization of food processing: A novel approach with application to thermal sterilization. *Journal of Food Engineering*, *98*(3), 317–324.

Serrano, A. B., Font, G., Ruiz, M. J., and Ferrer, E. (2012). Co-occurrence and risk assessment of mycotoxins in food and diet from Mediterranean area. *Food Chemistry*, *135*(2), 423–429.

Sindelar, J. J., and King, A. (2013). Thermal processing with food safety in mind. Retrieved March 16, 2018, from https://www.foodsafetymagazine.com/magazine-archive1/octobernovember-2013/thermal-processing-with-food-safety-in-mind/

Smith, M. C., Madec, S., Coton, E., and Hymery, N. (2016). Natural co-occurrence of mycotoxins in foods and feeds and their *in vitro* combined toxicological effects. *Toxins*, *8*(4), 94.

STAT503. (2018). Response surface methods and designs: Central composite designs. Retrieved July 26, 2018, from https://onlinecourses.science.psu.edu/stat503/node/59/

Stoloff, L., and Trucksess, M. W. (1981). Effect of boiling, frying, and baking on recovery of aflatoxin from naturally contaminated corn grits or cornmeal. *Journal - Association of Official Analytical Chemists*, *64*(3), 678–680.

Studer-Rohr, I., Dietrich, D. R., Schlatter, J., and Schlatter, C. (1995). The occurrence of ochratoxin A in coffee. *Food and Chemical Toxicology*, *33*(5), 341–355.

Tamhane, A. C. (2014). *Satistical analysis of designed experiments: Theory and applications* (1st ed.). Moorpark, California: Content Technologies Inc.

ten Bosch, L., Pfohl, K., Avramidis, G., Wieneke, S., Viöl, W., and Karlovsky, P. (2017). Plasma-based degradation of mycotoxins produced by *Fusarium*, *Aspergillus* and *Alternaria* species. *Toxins*, *9*(3), 97.

Tsubouchi, H., Yamamoto, K., Hisada, K., Sakabe, Y., and Udagawa, S. I. (1987). Effect of roasting on ochratoxin A level in green coffee beans inoculated with Aspergillus ochraceus. *Mycopathologia*, *97*(2), 111–115.

Turner, N. W., Subrahmanyam, S., and Piletsky, S. A. (2009). Analytical methods for determination of mycotoxins: A review. *Analytica Chimica Acta*, *632*(2), 168–180.

Uma, D. B., Ho, C. W., and Wan Aida, W. M. (2010). Optimization of extraction parameters of total phenolic compounds from henna (*Lawsonia inermis*) leaves. *Sains Malaysiana*, *39*(1), 119–128.

Vanesa, D., and Ana, P. (2013). Occurrence of Ochratoxin A in coffee beans, ground roasted coffee and soluble coffee and method validation. *Food Control*, *30*(2), 675–678.

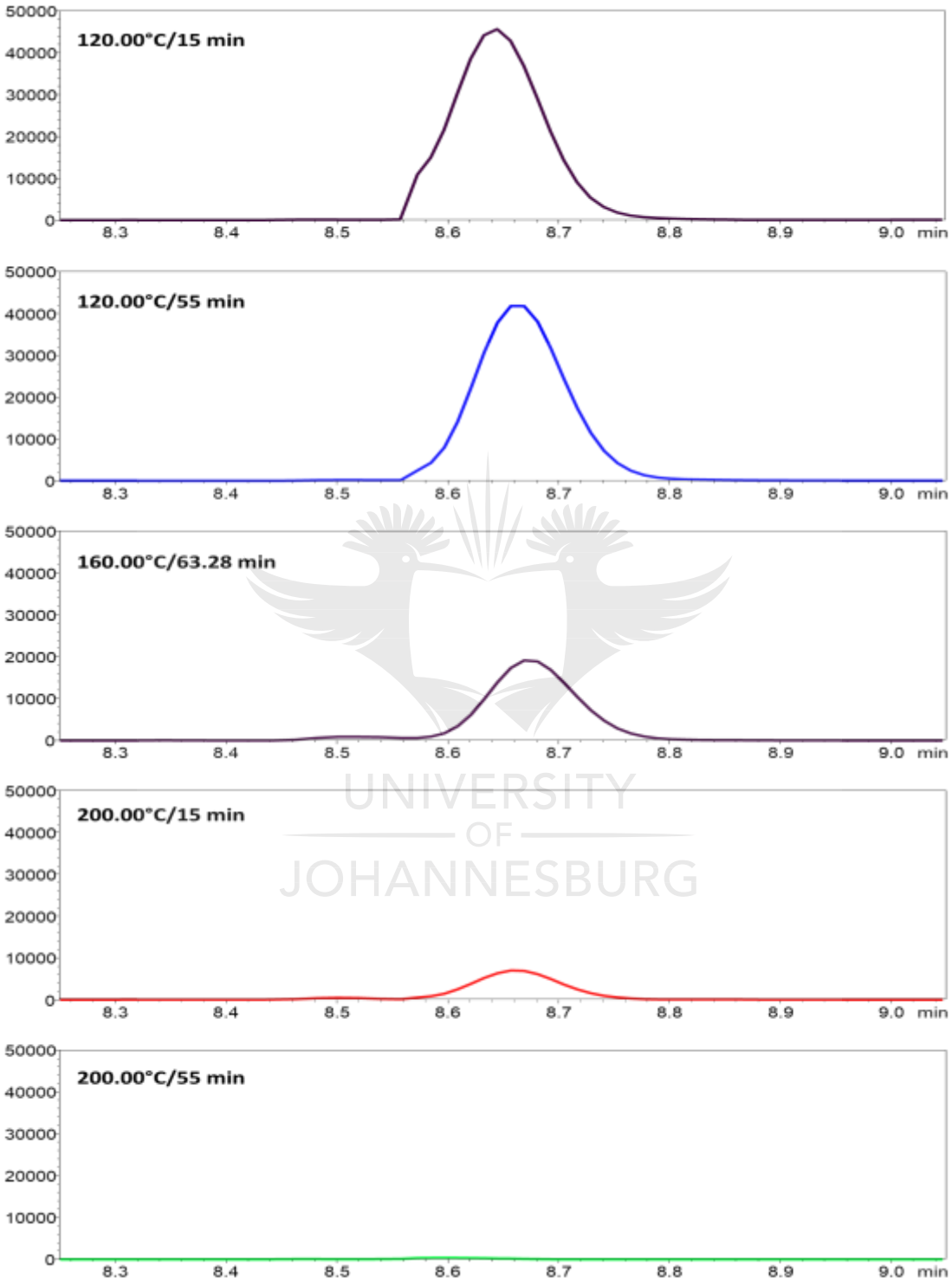
Wu, Q., Kuča, K., Humpf, H. U., Klímová, B., and Cramer, B. (2017). Fate of deoxynivalenol and deoxynivalenol-3-glucoside during cereal-based thermal food processing: a review study. *Mycotoxin Research*, *33*(1), 79–91.

Yang, J., Li, J., Jiang, Y., Duan, X., Qu, H., Yang, B., Chen, F., and Sivakumar, D. (2014). Natural occurrence, analysis, and prevention of mycotoxins in fruits and their processed product. *Critical Reviews in Food Science and Nutrition*, *54*(1), 64–83.

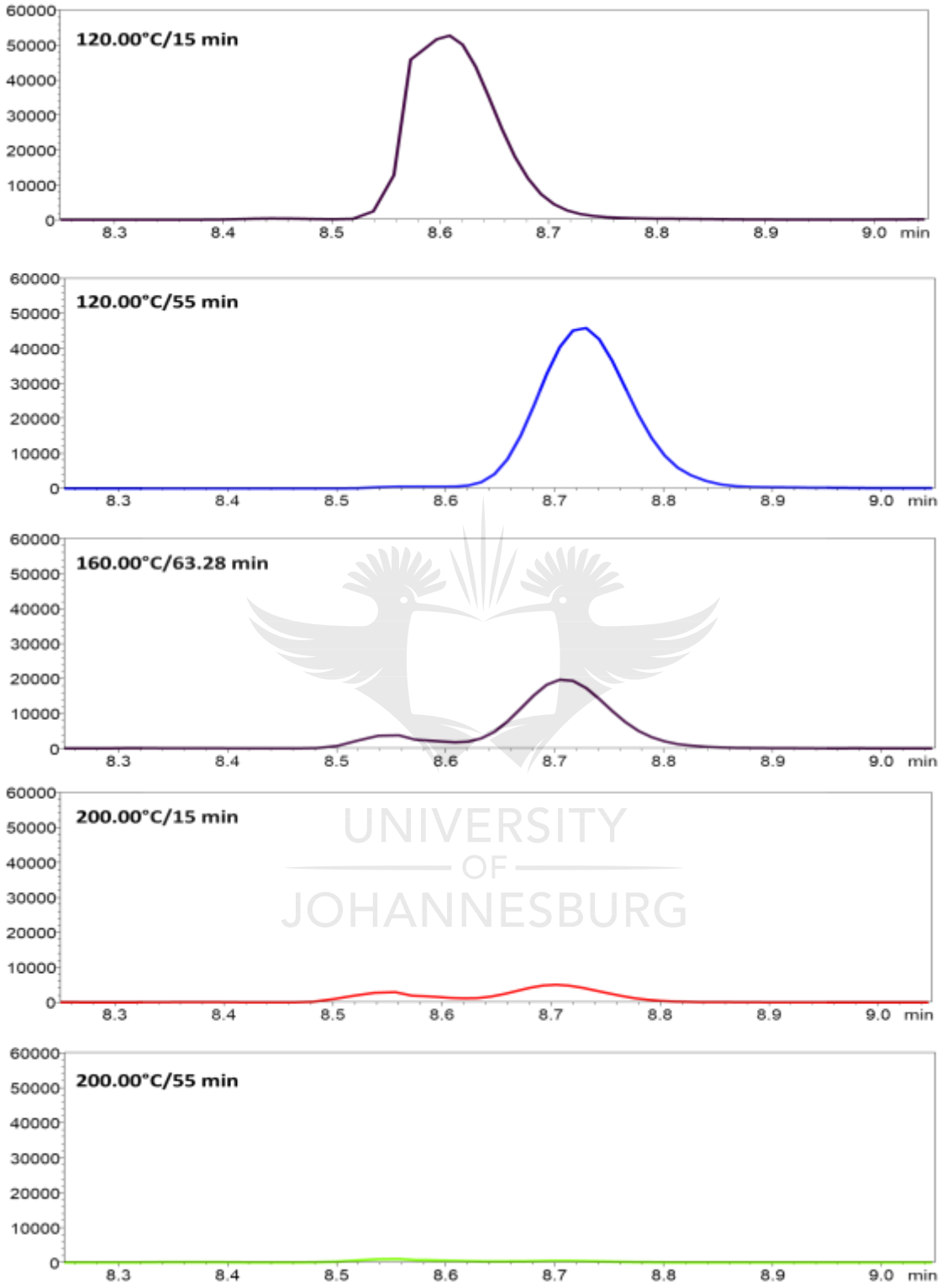
Yazdanpanah, H., Mohammadi, T., Abouhossain, G., and Cheraghali, A. M. (2005). Effect of roasting on degradation of Aflatoxins in contaminated pistachio nuts. *Food Chemistry and Toxicology*, *43*(7), 1135–1139.

Zain, M. E. (2011). Impact of mycotoxins on humans and animals. *Journal of Saudi Chemical Society*, *15*(2), 129–144.

APPENDIX 3



Appendix 3.A: Single ion chromatogram of pure AFB_1 standard at different temperature-time conditions.



Appendix 3.B: Single ion chromatogram of AFB_1 spiked into maize matrix at different temperature-time conditions.

Appendix 3.C: Independent-samples t-test for average thermal degradation of pure mycotoxin and mycotoxins spiked into maize matrix.

Mycotoxin	Mycotoxin Standards	Mycotoxin spiked into maize matrix	F-value	p-value	Significance
AFB_1	53.±3.06	47±1.37	0.10	0.75	not sig.
AFB_2	44±10.37	51±9.14	0.33	0.57	not sig.
AFG_1	55±2.2	47±3.11	0.16	0.69	not sig.
AFG_2	40±1.95	37±2.92	0.03	0.87	not sig.
AME	36±2.69	33±3.17	7.85	0.01	Sig.
FB_1	63±4.53	78±2.46	7.65	0.01	Sig.
FB_2	65±0.84	77±1.81	4.82	0.04	Sig.
FB_3	65±3.38	81±2.07	7.12	0.02	Sig.
OTA	24±3.36	46±2.81	1.04	0.32	not sig.
OTB	20±3.82	39±5.76	2.93	0.11	not sig.
STEG	61±1.71	37±1.71	0.05	0.82	not sig.
T-2	50±4.51	37±6.52	0.58	0.46	not sig.
ZEA	51±1.54	42±2.81	0.36	0.56	not sig.
α-ZEL	50±2.26	37±1.94	0.06	0.81	not sig.
β-ZEL	52±1.37	39±1.47	0.03	0.87	not sig.

Key: AFB_1: aflatoxin B_1. AFB_2: aflatoxin B_2. AFG_1: aflatoxin G_1. AFG_2: aflatoxin G_2. AME: alternariol monomethyl ether. FB_1: fumonisin B_1. FB_2: fumonisin B_2. FB_3: fumonisin B_3. OTA: ochratoxin A. OTB: ochratoxin B. STEG: sterigmatocystin. T-2: T-2 toxin. ZEA: zearalenone. α-ZEL: α-zearalenol. β-ZEL: β-zearalenol. Not sig.: not significant.

Appendix 3.D: CCD regression model fit factor effects for thermal degradation of mycotoxins

Mycotoxins	Factor effects for pure mycotoxin standards					Factor effects for mycotoxins spiked into maize matrix				
	Temp. (L)	Temp. (Q)	Time (L)	Time (Q)	1L by 2L	Temp. (L)	Temp. (Q)	Time (L)	Time (Q)	1L by 2L
AFB_1	74.99*	10.34*	6.20	16.21*	5.73	77.39*	7.80	18.96*	-6.43	5.01
AFB_2	47.80*	35.76*	4.42	13.27	19.61*	58.77*	12.94	21.27*	-1.32	3.05
AFG_1	75.89*	1.54	12.63*	3.73	0.14	80.24*	9.13	19.63*	-5.60	3.39
AFG_2	66.72*	29.94*	8.82*	-1.81	15.76*	70.14*	31.12*	11.02*	1.31	8.83*
AME	64.52*	26.32*	11.49*	-0.37	19.51*	18.28*	26.29*	3.01	6.29	13.57*
FB_1	74.85*	-3.49	0.79	28.32*	1.82	44.38*	-25.50*	11.03*	-15.66*	0.59
FB_2	73.79*	-8.77*	3.42	32.29*	-2.08	46.52*	-24.94*	13.77*	-14.57*	-4.61
FB_3	72.78*	-10.49	1.52	21.93*	5.99	39.13*	-21.45*	11.23*	-12.39*	-2.06
OTA	36.90*	16.34*	5.19*	2.53	7.23*	44.54*	35.98*	8.64*	8.62	9.90*
OTB	25.08*	22.68*	-2.56	3.18	15.01*	48.84*	26.48*	9.29	-6.24	14.68*
STEG	65.56*	9.08*	10.53*	20.88*	-4.29	66.00*	39.46*	19.73*	11.26*	17.92*
T-2	72.91*	11.04	11.93*	9.32	9.87	59.74*	28.22*	11.09*	-1.84	17.05*
ZEA	64.97*	21.32*	11.11*	5.27	2.03	53.05*	28.84*	15.64*	4.85	12.55*
α-ZEL	68.68*	27.13*	10.73*	13.87*	-1.14	63.82*	33.46*	17.83*	8.13*	9.50*
β-ZEL	64.84*	28.15*	11.64*	11.62*	2.75	62.60*	36.16*	16.78*	8.90*	8.16*

Key: *Statistically significant ($p \leq 0.05$) factors. Temp. (L) – linear effect of temperature, Temp. (Q) – quadratic effect of temperature, Time (L) – linear effect of time, Time (Q) – quadratic effect of time, 1L by 2L – interaction effect temperature and time. AFB_1: aflatoxin B_1. AFB_2: aflatoxin B_2. AFG_1: aflatoxin G_1. AFG_2: aflatoxin G_2. AME: alternariol monomethyl ether. FB_1: fumonisin B_1. FB_2: fumonisin B_2. FB_3: fumonisin B_3. OTA: ochratoxin A. OTB: ochratoxin B. STEG: sterigmatocystin. T-2: T-2 toxin. ZEA: zearalenone. α-ZEL: α-zearalenol. β-ZEL: β-zearalenol.

Appendix 3.E: Individually optimized experimental conditions for pure mycotoxins and mycotoxins spiked into maize matrix

Mycotoxin	Mycotoxin standards			Spiked maize		
	Temp (°C)	Time (sec)	Degradation (%)	Temp (°C)	Time (sec)	Degradation (%)
AFB_1	193	63.28	100	200	63.28	100
AFB_2	203	63.28	100	202	63.28	100
AFG_1	196	63.28	100	200	63.28	100
AFG_2	205	63.28	100	206	63.28	100
AME	204	63.28	100	217	63.28	80
	-	-	-	230*	63.28*	100
FB_1	206	20.10	100	209	59.78	100
FB_2	208	48.50	100	181	58.94	100
FB_3	178	63.28	100	207	24.43	100
OTA	217	63.28	72	207	63.28	100
	239*	63.28*	100	-	-	-
OTB	217	63.28	66	213	63.28	100
	239*	63.28*	100	-	-	-
STEG	206	51.14	100	197	63.28	100
T-2	195	63.28	100	207	63.28	100
ZEA	202	63.28	100	204	63.28	100
α-ZEL	200	63.28	100	203	63.28	100
β-ZEL	199	63.28	100	203	63.28	100

Key: Temp: Temperature. * Out-of-experimental range optimal conditions. AFB_1: aflatoxin B_1. AFB_2: aflatoxin B_2. AFG_1: aflatoxin G_1. AFG_2: aflatoxin G_2. AME: alternariol monomethyl ether. FB_1: fumonisin B_1. FB_2: fumonisin B_2. FB_3: fumonisin B_3. OTA: ochratoxin A. OTB: ochratoxin B. STEG: sterigmatocystin. T-2: T-2 toxin. ZEA: zearalenone. α-ZEL: α-zearalenol. β-ZEL: β-zearalenol.

CHAPTER FOUR[#]

DEVELOPMENT, CHEMOMETRIC-ASSISTED OPTIMIZATION AND IN-HOUSE VALIDATION OF A MODIFIED PRESSURIZED HOT WATER EXTRACTION METHODOLOGY FOR MULTI-MYCOTOXINS IN MAIZE

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Abstract

Effective management of mycotoxins rely on stringent regulation and routine surveillance of food/feed commodities via efficient analysis, hence the continuous need for improved methods. The present study developed, optimized and validated a modified pressurized hot water extraction (PHWE) method for the simultaneous extraction of multi-mycotoxins from maize and subsequent quantification on ultra-high performance liquid chromatography and tandem mass spectrometry (UHPLC-MS/MS). The PHWE system was modified using ethanol (EtOH) as a cosolvent, while a numerical modelling approach, the central composite design (CCD), was adopted for the optimization of the extraction conditions. The computed global optimal solution for the objective function (*i.e.,* the most efficient combination of temperature and solvent composition for maximum recovery of target mycotoxins simultaneously) was 162 °C and 45% ethanol (EtOH) for temperature and solvent composition, respectively. Using the optimized conditions, it was possible to effectively extract and quantify 15 different mycotoxins, including aflatoxins (AFs), fumonisins (FBs), ochratoxins (OTs), zearalenone and its analogues (ZEAs), T2-toxin (T-2), alternariol monomethyl ether (AME) and sterigmatocystine (STEG), from maize in a single step with satisfactory recoveries (71 to 124%) except for ochratoxin A (OTA) and α-zearalenol (α-ZEL) which had recoveries of 58 and 14%, respectively. The modified PHWE method also demonstrated adequate linearities (0.986 to 0.999), intra-day precisions (RSDr ranged from 0 to 27%) and inter-day precisions (RSD_R ranged from 3 to 34%). Further efficacious application of the method to real

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samples re-affirmed the prospects of PHWE as a suitable, cost-effective and greener alternative to traditional methods of mycotoxin extraction.

Highlights

- A modified PHWE method for mycotoxin extraction was developed and applied for the first time.
- The method validation parameters were consistent with standard guideline requirements for analytical method performance.
- In comparison to conventional methods of mycotoxin extraction, the method performed very well, and in some cases favourably.
- PHWE is promising as an alternative to traditional methods for survey-type work and exposure studies for mycotoxins.

Keywords: Mycotoxins, pressurized hot water extraction, chemometric-assisted optimization, central composite design

4.1 Introduction

Mycotoxins are poisonous secondary metabolites produced by certain fungal species that contaminate various agricultural commodities. Exposure to mycotoxin-contaminated foods results in various health-related problems, depending on the type of mycotoxin and level of exposure. Relative to their prevalence and potency, they have been identified as the most significant nutritional hazard, superior to other natural inherent plant toxins, pesticide residues, food additives and other synthetic contaminants in foods (Kuiper-Goodman, 1998; Bennett and Klich, 2003). Besides the health-related problems, mycotoxins also exert significant socio-economic effects on societies globally, particularly in the developing nations (Gbashi *et al*., 2017a; Makun *et al*., 2012). Annual losses in agricultural commodities due to mycotoxin contamination have been estimated at one billion metric tons (Schmale and Munkvold, 2009; Wilson *et al*., 2017). All these coupled with the globalization of the food supply chain, has led to more stringent regulatory limits and routine surveillance of the levels of these environmental pollutants in food and feed (Gbashi *et al*., 2017a; Rahmani *et al*., 2009). As such, analysis plays a critical tool in the control and management of mycotoxins (Gbashi *et al*., 2017a).

Development of improved methods for mycotoxin analysis, particularly extraction methods, has been an active research field (Anfossi *et al*., 2010; Arroyo-Manzanares *et al*., 2014; Karsten *et al*., 2008). Fundamental interests have been sensitivity, speed, ease, cost-effectiveness, and environmental friendliness. However, most of the conventional methods of mycotoxin extraction such as liquid-liquid solvent extraction, immuno-affinity column extraction, solid-phase extraction falls short of these qualities in one way or the other (Gbashi *et al*., 2017a). Pressurized hot water extraction (PHWE) seems very promising in this regard, as it is more environmentally friendly, efficient, cheap, fast, and relatively easy (Teo *et al*., 2010). Extraction with this method can be easily maneuvered to achieve extractability of a wide array of analytes with some degree of selectivity and efficiency by adjusting the extraction temperature and/or introduction of a co-solvent (Teo *et al*., 2010; Plaza & Turner, 2015). Moreover, preliminary studies have yielded good results for PHWE of aflatoxin B_1 (AFB_1) from maize (Gbashi *et al*., 2017b).

Because the performance of PHWE is determined by a number of factors, in particular temperature and solvent composition, optimization is essential in order to achieve maximum efficiency. This is important when developing a method for the simultaneous extraction of multiple analytes with varying physicochemical properties such as mycotoxins. For this purpose, the utilization of chemometric tools, such as the response surface methodology (RSM) is suitable (Díez *et al*., 2011; Gbashi *et al*., 2016; Li *et al*., 2017). Further driven by the need to save time, cost and physical efforts, the central composite design (CCD) (Bajer *et al*., 2015) approach of RSM is an important mathematical tool for optimizing complex multivariate and multi-analyte problems (Asghar *et al*., 2014), and has been widely used in the optimization of various extraction techniques for different analytes (Ali *et al*., 2016; Gbashi *et al*., 2016; Khazaeli *et al*., 2016).

The CCD offers the advantage of a reduced number of experimental runs for response optimization, and is useful in simultaneously studying the effects of different control variables on an objective variable (Gbashi *et al*., 2016; Zulkiply, 2012). Rakić *et al*. (2014) compared four different RSM models for the development of a chromatographic method for determining fluconazole and its impurities, and found CCD superior in terms of generation of better models and a reduced number of experimental runs (Rakić *et al*., 2014). The present study describes the development and optimization of a PHWE method for multi-mycotoxin extraction from maize

using the CCD, and subsequent validation of the optimized method against standard analytical guidelines.

4.2 Materials and methods

4.2.1 Materials

Mycotoxin reference standards used in this study included aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), aflatoxin G_2 (AFG_2), fumonisin B_1 (FB_1), fumonisin B_2 (FB_2), fumonisin B_3 (FB_3), ochratoxin A (OTA), ochratoxin B (OTB), T-2 toxin (T-2), zearalenone (ZEA), α-zearalenol (α-ZEL), β-zearalenol (β-ZEL), alternariol monomethyl ether (AME), and sterigmatocystin (STEG) were purchased from the National Metrological Institute of South Africa (NMISA). Mycotoxin standards used for the solvent extraction included all of the above listed mycotoxins purchased from Biopure (RomerLabs, Tulln, Austria), except for AME and FB_3 purchased from Sigma-Aldrich (Bornem, Belgium) and Promec unit (Tygerberg, South Africa), respectively. Deepoxy-deoxynivalenol (DOM) and zearalanone (ZAN) were used as internal standards and were purchased from Sigma-Aldrich (Bornem, Belgium) and Biopure (RomerLabs, Tulln, Austria), respectively.

Biotage® Isolute SAX cartridges were purchased from Anatech Instruments (Pty) Ltd (South Africa). C18-SPE cartridge and MultiSep®226 AflaZon+ multifunctional columns were purchased from Alltech (Lokeren, Belgium) and Romer Labs (Gernsheim, Germany), respectively. PVDF syringe filters (0.22 µm, with Luer lock) and 10 mL NORM-JECT plastic syringe (with Luer lock) were purchased from Restek (Restek Corporation, Pennsylvania USA). Whatman® glass microfiber filters (47 mm diameter, grade GF/A) was purchased from VWR International (VWR International, Leuven, Belgium), while Ultrafree®-MC PVDF centrifugal filters (0.22 µm) were purchased from Millipore (Bredford, MA, USA). Diatomaceous earth, potassium chloride, sodium sulphate, sodium bicarbonate, anhydrous sodium sulphate and dialysis tubing were purchased from Sigma (South Africa).

Solvents used for PHWE, extraction by Method 1 and extraction by Method 2 (as described in Section 4.2.4) included mass spectrometry (MS)-grade acetonitrile, MS-grade methanol and MS-grade formic acid, dichloromethane (DCM), ethanol (EtOH), iso-octane and sulphuric acid which

were also purchased from Sigma (South Africa). Ultrapure water was obtained from a Milli-Q Gradient A10 dispensing system (Millipore, Billerica, MA, USA). Solvents used for extraction by Method 3 (as described in Section 4.2.4) included HPLC grade acetonitrile (Biosolve BV, Valkenswaard, The Netherlands), LC-MS grade methanol, acetonitrile, glacial acetic acid all from (Biosolve BV, Valkenswaard, The Netherlands) and ammonium acetate from Merck (Merck KGaA, Darmstadt, Germany). N-hexane was purchased from BDH Hipersolv Chromanorm (VWR International, Leuven, Belgium) and nitrogen gas (Air Liquide, Aalter, Belgium). The water used for this method (i.e. Method 3) was obtained from a Milli-Q®SP Reagent water system (Millipore Corp., Brussels, Belgium). Maize flour was purchased from a local vendor in Johannesburg, South Africa. All other consumables were of analytical grade and purchased from accredited suppliers within South Africa.

4.2.2 Methods

4.2.2.1 Sampling

A total of 25 maize meal samples from different households in Ngwalemong village in Limpopo Province of South Africa destined for human consumption were collected. Incremental sampling was performed from different locations withing the sampling lot to achieve a combined mass of 1 kg which was thoroughly mixed and placed in sterile, airtight ziplock plastic bags. The samples were kept chilled in a cooler box and transported to the Food, Environment and Health Research Group (FEHRG) Laboratotry, University of Johannesburg, Doornfontein Campus where they were stored at -4 ° until analysis.

4.2.2.2 Experimental design

Using Statistica (version 7, StatSoft, USA), a set of experiments were designed based on the CCD RSM in order to optimize two important PHWE parameters temperature (°C) and solvent composition (% EtOH) for maximum recovery of multi-mycotoxins. By adopting the CCD approach for two factors and 1 block, 10 experimental levels were obtained (Table 4.1): 2 levels of fractional factorial design for each of the factors, a replicated center point to improve the precision of the optimization model, and a set of axial points (*i.e.*, α and -α) that allows for rotatability of the model and estimation of response curvature. Curvature estimation of the model is particularly important because one of the factors (temperature) exhibits a strong quadratic effect

on analyte recovery during PHWE (Gbashi *et al*., 2016). Rotatability of the model is desirable because it allows for equal variance of prediction for all points equal-distance from the center point irrespective of the direction. The experimental domain was selected based on preliminary laboratory trials (Gbashi *et al*., 2017b).

Table 4.1: Two-factor, 1 block standard order CCD experimental design for temperature and solvent composition optimization during PHWE of multi-mycotoxins.

S/No	Temperature (°C)	Solvent composition (% EtOH)	RSM Codes	Comment
1	50.00	0.000	-1, -1	Factorial level
2	50.00	40.00	-1, +1	Factorial level
3	150.0	0.000	+1, -1	Factorial level
4	150.0	40.00	+1, +1	Factorial level
5	29.29	20.00	-α, 0	Axial point
6	170.7	20.00	+α, 0	Axial point
7	100.0	-8.280	0, -α	Axial point
8	100.0	48.28	0, +α	Axial point
9	100.0	20.00	+1, +1	Center point
10	100.0	20.00	+1, +1	Center point

Key: S/No.: serial number. EtOH: ethanol. RSM: response surface methodology.

After conducting experiments at the above stated experimental levels, a second-order optimization model represented in Equation 4.1 (Adebo *et al*., 2018), was fitted to the experimental data using the method of least squares (MSL) which generates the lowest possible residuals (Bas & Boyaci, 2007). Adequacy of the model-fit was determined by evaluating various model-fit validation parameters described by Adebo *et al*. (2017). The parameters include coefficient of determination (R^2), adjusted coefficient of determination (R^2 adjusted), MS residual (*MSR*), average absolute deviation (*AAD*), bias factor (B_f), accuracy factor (A_f) and Pearson's correlation coefficient (r). Model parameters and significance were determined at a probability level of 95% (*i.e.*, $p < 0.05$).

$$z = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i=1}^{k} \quad \sum_{j=1}^{k} \beta_{ij} x_i x_j \qquad \textbf{Equation 4.1}$$

Where z is the response variable *i.e.*, recovery (%), x_i and x_j are the factors *i.e.*, temperature (°C) and solvent composition (% EtOH) respectively, β_0 is the model constant, β_i, β_{ii} and β_{ij} are the coefficients for the linear, quadratic and interaction terms.

4.2.2.3 Pressurized hot water extraction (PHWE)

Pressurized hot water extraction was performed using a make-shift laboratory setup described by Gbashi *et al*. (2016). The system consisted of an HPLC pump (Waters 6000 fluid controller, Waters Corporation, Manchester, UK), a stainless steel extraction cell (70 x 30 mm and approximately 20 mL), a refurbished GC 600 Vega Series 2 oven (Carlo Erba Instruments, Italy) with a digital temperature controllable unit (±1 ^{o}C), stainless tubing (1.58 mm in outer dimension (OD) and 0.18 mm inner dimension (ID), a back-pressure valve (Swagelok, Johannesburg, South Africa), and a collection flask. For the extraction, 4 g of spiked maize flour was thoroughly mixed with 3 g of diatomaceous earth and placed inside the extraction cell. Extraction was performed at the different pre-designed experimental conditions of temperature (°C) and solvent composition (% EtOH) described previously in Table 4.1. The solvent was made to flow through the extraction cell at a steady flow rate of 5 mL/min with pressure maintained at 1000 ± 200 psi using the back-pressure valve. The extract was collected into a 50 mL centrifugal tube up to the 50 mL mark. Each extraction experiment was performed in duplicate. The extracts were filtered using a 0.22 µm nylon syringe filter into a 1.5 mL HPLC vial, and subsequently analyzed on the UHPLC-MS/MS.

4.2.2.4 Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS)

Mycotoxins were quantified using a Shimadzu UHPLC-MS/MS 8030 equipment (Shimadzu Corporation, Tokyo, Japan), which consisted of an LC-30AD Nexera chromatograph connected to an autosampler (SIL-30 AC Nexera) and a column oven (CTO-20 AC Prominence) maintained at a constant temperature of 40 ^{o}C. Chromatographic separation of multi-mycotoxins was achieved on a Raptor^{TM} ARC-18 column (2.7 µm, 2.1 mm × 100 mm) (Restek Corporation, Pennsylvania USA). The sample injection volume was 2 µL, and the mobile phases were composed of an aqueous phase *i.e.*, solvent A: 0.1% formic acid (FA) in deionized water, and an organic phase *i.e.*, solvent B: 0.1% FA in methanol/acetonitrile (50/50 v/v).

The gradient elution program began with 10% B for 0.1 min, ramped to 95% B in 8.4 min, maintained at this condition for 3 min, and the initial condition *i.e.*, 10% B re-established for 1 min, after which the column was allowed to re-equilibrate for 4.5 min for the next run. The mobile phase was delivered at a constant flow rate of 0.2 mL/min, and the total run time was 17 min. The LC was connected to a Shimadzu triple-quadrupole MS model 8030 (Shimadzu Corporation,

Kyoto, Japan) detector with an electron spray ionization source where mycotoxins were detected and quantified in positive ionization mode (ESI^+). The MS method consisted of a multiple reaction monitoring (MRM) method operated at optimized MS conditions for the analytes (Table 4.2). The heat block temperature was 400 °C, desolvation line (DL) temperature was 250 °C, interface nebulizing gas flow rate was 3 L/min and drying gas flow rate was 15 L/min. The Shimadzu LabSolutions software was used for subsequent processing and visualization of the data.

Table 4.2: MRM transitions, optimized MS conditions and retention times of multi-mycotoxins

S/No	Mycotoxin	Ret. time (min)	Precursor	Products	Q1 Pre bias (V)	CE (eV)	Q3 Pre bias (V)
1.	AFB_1	8.25	313.00	241.00*	-22.00	-41.00	-23.00
				285.10	-22.00	-24.00	-29.00
2.	AFB_2	8.01	315.00	259.10*	-22.00	-31.00	-25.00
				287.00	-23.00	-26.00	-30.00
3.	AFG_1	7.77	329.00	243.00*	-12.00	-28.00	-23.00
				311.10	-16.00	-24.00	-14.00
4.	AFG_2	7.51	331.00	245.10*	-12.00	-32.00	-24.00
				313.00	-12.00	-24.00	-20.00
5.	AME	10.13	273.00	128.10*	-10.00	-49.00	-21.00
				115.10	-18.00	-54.00	-19.00
6.	FB_1	7.97	722.20	352.20*	-34.00	-42.00	-11.00
				334.30	-20.00	-42.00	-11.00
7.	FB_2	8.95	706.10	336.30*	-20.00	-38.00	-22.00
				318.30	-26.00	-41.00	-22.00
8.	FB_3	8.75	706.30	336.30*	-40.00	-39.00	-11.00
				354.40	-20.00	-35.00	-24.00
9.	OTA	10.13	403.80	239.00*	-15.00	-27.00	-24.00
				221.00	-12.00	-38.00	-21.00
10.	OTB	9.33	370.10	205.00*	-13.00	-22.00	-21.00
				324.10	-13.00	-14.00	-22.00
11.	STEG	10.45	324.90	310.00*	-22.00	-24.00	-30.00
				281.10	-22.00	-40.00	-27.00
12.	T-2	9.67	467.20	245.10*	-13.00	-11.00	-16.00
				305.20	-22.00	-11.00	-20.00
13.	ZEA	10.06	319.10	185.00*	-12.00	-27.00	-30.00
				187.10	-15.00	-21.00	-19.00
14.	a-ZEA	9.42	323.10	277.20*	-17.00	-17.00	-18.00
				305.20	-24.00	-9.000	-20.00
15.	B-ZEA	8.95	323.10	277.20*	-16.00	-16.00	-18.00
				305.20	-16.00	-11.00	-20.00

Key: S/No.: serial number. Ret. Time: retention time. Q1 Pre bias: quadruple one pre-rod bias. Q3 Pre bias: quadruple three pre-rod bias. CE: collision energy. * Quantitative product ion. AFB_1: aflatoxin B_1. AFB_2: aflatoxin B_2. AFG_1: aflatoxin G_1. AFG_2: aflatoxin G_2. AME: alternariol monomethyl ether. FB_1: fumonisin B_1. FB_2: fumonisin B_2. FB_3: fumonisin B_3. OTA: ochratoxin A. OTB: ochratoxin B. STEG: sterigmatocystin. T-2: T-2 toxin. ZEA: zearalenone. α-ZEL: α-zearalenol. β-ZEL: β-zearalenol.

4.2.3 Validation of the modified PHWE method for multi-mycotoxins extraction

Matrix effects (ME) for the different mycotoxins were determined using the signal enhancement or suppression approaches of Arroyo-Manzanares *et al*. (2018) and Sulyok *et al*. (2006). For this purpose, calibration curves were plotted for standards prepared in extracts obtained from blank samples, as well as, for standards prepared in neat solvents. Matrix effect was calculated using the difference in the slopes of the calibration curves as described in Equation 4.2. The linearity of the PHWE method was determined by evaluating the coefficient of determination (R^2) between the instrumental responses and the analyte concentrations at 5 concentration levels within the range of 160 to 5,000 µg/mL. The limits of detection (LOD) and limits of quantification (LOQ) (Equation 4.3) of the analytes were determined using the signal to noise ratio of the matrix-matched standards as described by Kim *et al*. (2017).

$$ME = \frac{Slope_m - Slope_n}{Slope_n} \times 100 \qquad \textbf{\textit{Equation 4.2}}$$

Where *ME* is the matrix effect, $Slope_m$ is the slope of calibration curve of standards prepared in sample extracts and $Slope_n$ is the slope of standards prepared in neat solvent.

$$Limit_{DQ} = F \times \left[\frac{C}{\left(\frac{S}{N}\right)}\right] \qquad \textbf{\textit{Equation 4.3}}$$

Where $Limit_{DQ}$ is the *LOD* or *LOQ* depending on the value of the multiplication factor *F*, which is 3.33 for *LOD* and 10 for *LOQ*. *C* is the concentration, while S is the signal at concentration *C*, and *N* is the noise level at similar concentration.

For accuracy (recovery) measurement, spiked maize samples (previously confirmed to be free of any of the analytes on UHPLC-MS/MS) were extracted using PHWE and the results presented as the percentage ratio of the recovered concentration to that of the originally spiked concentration (Equation 4.4) (Arroyo-Manzanares *et al*., 2018). The spiking concentration was determined in accordance with the maximum theoretical extraction for the PHWE setup (described in Section 4.2.2.3) using a 20 mL extraction cell. Accordingly, sample spiking was achieved by adding 4 mL

of multi-mycotoxin mix standard solution (1 µg/mL) to 4 g of blank maize flour, mixing thoroughly and storing for 24 h in the dark at ambient conditions. Intra-day precision or repeatability (RSDr) of the method was determined by evaluating the coefficient of variation of recoveries of three PHWE procedures performed under the same working conditions within the period of 24 h at two different spiking levels, 250 and 1,000 µg/kg, levels 1 and levels 2, respectively. Inter-day precision or intermediate precision (RSD_R), was determined in a similar manner to RSDr, however, 3 PHWE operations at the two spiking levesl were performed over three consecutive days, and the coefficient of variation of the recoveries computed. Where E_r is the recovered concentration after spiking and E_i is the spiked concentration.

$$Recovery\ (\%) = \frac{E_r}{E_i} \times 100 \qquad \textbf{\textit{Equation 4.4}}$$

4.2.4 Comparative assessment of the recovery of PHWE with other conventional methods of mycotoxin extraction

The extraction efficiency of the modified PHWE method was compared with those of some well-defined and validated methods for multi-mycotoxin extraction reported in literature. Method 1 is the method of Patterson and Roberts (1979) as described by Chilaka *et al*. (2012). Briefly, extraction was executed with acetonitrile/4% potassium chloride (KCl) (90/10, v/v), followed by defatting with iso-octane, phase separation by addition of DCM and saturated sodium bicarbonate. After separation, the aqueous phase was acidified with sulphuric acid (H_2SO_4), and further extracted with DCM. The organic phase was dialysed against 30% acetone overnight, and re-extracted into DCM. Both fractions were quantified for appropriate mycotoxins using the UHPLC-MS/MS. Extraction time was 48 h.

Method 2 is a solvent extraction method adapted from Sulyok *et al*. (2007). Extraction involved adding acetonitrile/water/acetic acid (79/20/1, v/v/v) to the sample in the ratio of 1/4. The samples were agitated for 90 min, centrifuged for 10 min at 4,000 g. To 350 µL of aliquot 350 µL of extraction solvent was added, filtered through a 0.22 µm PVDF syringe filter into a 1.5 mL HPLC vial without further sample clean-up, and injected into the UHPLC-MS/MS.

Method 3 is a multi-analyte solvent partitioning method comprising of an extraction phase, extensive sample clean-up steps using solid phase extraction (SPE) cartridges, and a thorough quality control procedure via addition of internal standards described by Majeed *et al*. (2018). To

5 g of spiked sample, 20 mL of extraction solvent acetonitrile/water/acetic acid (79/20/1, v/v/v) was added, vortexed for 5 min, agitated for 60 min using an end-over-end shaker (Exacta, Delhi, India), and centrifuged at 4,000 g for 15 min. The supernatant was filtered through a pre-conditioned C18-SPE cartridge under ambient pressure, and the extracts defatted with 20 mL of hexane. The defatted extract was divided into two portions. The first portion (10 mL) was made up to 30 mL using acetonitrile/acetic acid (99/1, v/v), and passed through a MultiSep®226 AflaZon^{+} multifunctional column. The second portion (10 mL) was filtered using a glass microfilter (0.22 µm), and 2 mL of the filtrate added to the eluent of the first portion. The combined portions were evaporated to complete dryness over a stream of nitrogen gas at 40 °C, and reconstituted with 150 µL of mobile phase [5 mM ammonium acetate in water/methanol/acetic acid (94/5/1, v/v/v) and 5 mM ammonium acetate in acetonitrile/acetic acid (99/1, v/v), in the ratio of 3/2, v/v]. The reconstituted extract was subsequently thoroughly agitated for 4 min on a vortex machine (Labinco, Breda, The Netherlands), and filtered using a Ultrafree® PVDF centrifuge filter prior to injection on the LC-MS/MS.

4.3 Results and discussion

4.3.1 Method development and optimization

A modified PHWE method was developed for the simultaneous extraction of 15 different mycotoxins from maize. The mechanism and dynamics behind PHWE had been previously described in detail (Gbashi *et al*., 2017a). Maize was selected based on the fact that it is a widely-consumed staple food also in South Africa and a favored substrate for fungal contamination and subsequent mycotoxin production (Chilaka *et al*., 2012; Ranum *et al*., 2014). Preliminary attempts at adopting a conventional PHWE system (which uses only hot water as the extraction solvent) for multi-mycotoxin extraction was unsuccessful due to low recoveries for many of the mycotoxins. As such, in order to improve the extractability multi-mycotoxin using PHWE, ethanol was incorporated as a co-solvent. Ethanol is an environmentally friendly, cheap and readily available solvent that has been proven to be effective in improving the efficiency of PHWE (Herrero *et al*., 2011; Shang *et al*., 2014; Shang *et al*., 2017).

The extraction conditions of temperature and solvent composition were thus optimized using the CCD model in order to ensure an optimum performance of the modified PHWE method. In this

regard, experiments were conducted at a set of statistically pre-designed experimental conditions (Table 4.1) and a quadratic optimization function (Equation 4.1) fitted to the experimental data (Appendix 4.A). The resultant regression models [Appendix 4.B (columns 2-7)] were validated using standard model-fit validation parameters of R^2, B_f and A_f [Appendix 4.B (columns 8-14)] as described by Adebo *et al*. (2018). The R^2 values ranged from 0.56 to 0.97 indicating that the models were well-fit, whereas, the B_f and A_f values ranged from 0.98 to 1.22 and 1.10 to 1.59, respectively, indicating that the models presented a good estimation of the true relationship between the dependent variable (i.e. recovery) and the control variables (i.e. temperature and solvent composition). This functional relationship can be clearly visualized on the generated 3-D surface plots (Figure 4.1).

Following the model-fit, the multi-objective optimum solution for the dependent variables (*i.e.*, the most efficient combination of temperature and solvent composition for the maximum recovery of all mycotoxins simultaneously) was computed using the global optimization function of the Minitab 17 statistical software (Minitab Inc., Pennsylvania, US) as described by Adebo *et al*. (2018). This optimum solution was 162 °C and 45% EtOH for temperature and solvent composition, respectively. Subsequent validation of this optimized conditions by laboratory experiments showed a clear enhancement in recovery of the analytes with recoveries ranging from 14 to 124%, with 13 out of 15 of the analytes having recoveries above 70% in one single extraction (Figure 4.2). Even T-2 which had a pre-optimization recovery of 0% using conventional PHWE, had a post-optimization recovery of over 82% using the optimized PHWE method. Alternariol monomethyl ether had the highest recovery rate (129%), followed in descending order by AFB_1 (127%), AFG_1 (125%), FB2 (118%), ZEA (114%), AFB_2 (110%), β-ZEL (108%), OTB (98%), AFG_2 (94%), FB_3 (90%), T-2 (83%), STEG (73%), FB_1 (71%), OTA (58%) and α-ZEL (14%).

In addition to obtaining the optimal extraction condition, the model-fit also provided details on how the two extraction variables individually and interactively affected the recovery of each mycotoxin (Figure 4.3). These Pareto charts summarize the relative importance of the linear, quadratic and interaction effects of temperature and solvent composition on the recovery of individual mycotoxins. The effects are displayed on the y-axis and their corresponding magnitudes are plotted as horizontal bars on the x-axis. Bars that extended beyond the red line on the chart are significant at a 95% probability.

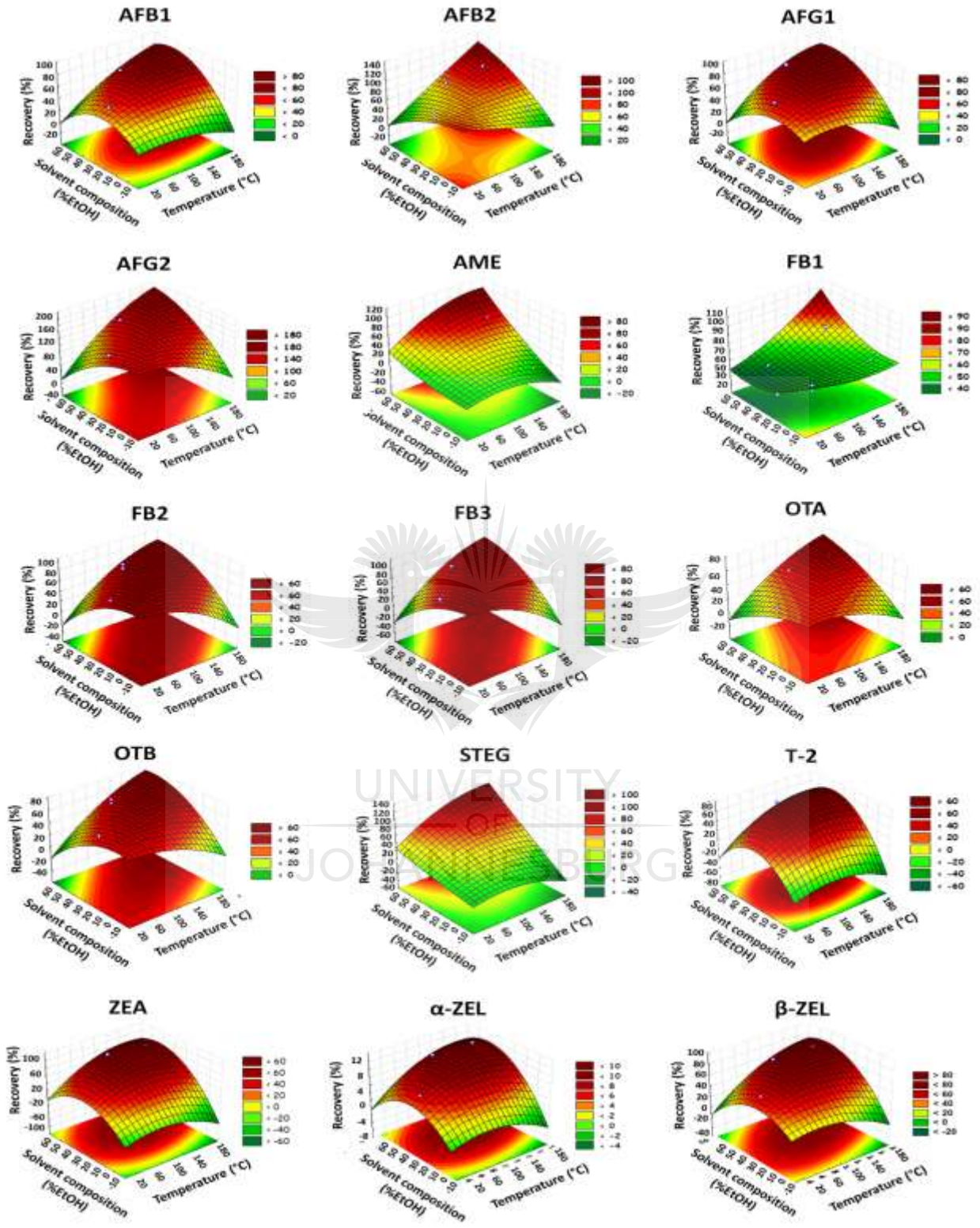

Figure 4.1: Response surface plots showing the relationship between mycotoxin recovery and extraction parameters (temperature and solvent composition). EtOH: ethanol. AFB_1: aflatoxin B_1.

AFB_2: aflatoxin B_2. AFG_1: aflatoxin G_1. AFG_2: aflatoxin G_2. AME: alternariol monomethyl ether. FB_1: fumonisin B1. FB_2: fumonisin B_2. FB_3: fumonisin B_3. OTA: ochratoxin A. OTB: ochratoxin B. STEG: sterigmatocystin. T-2: T-2 toxin. ZEA: zearalenone. α-ZEL: α-zearalenol. β-ZEL: β-zearalenol.

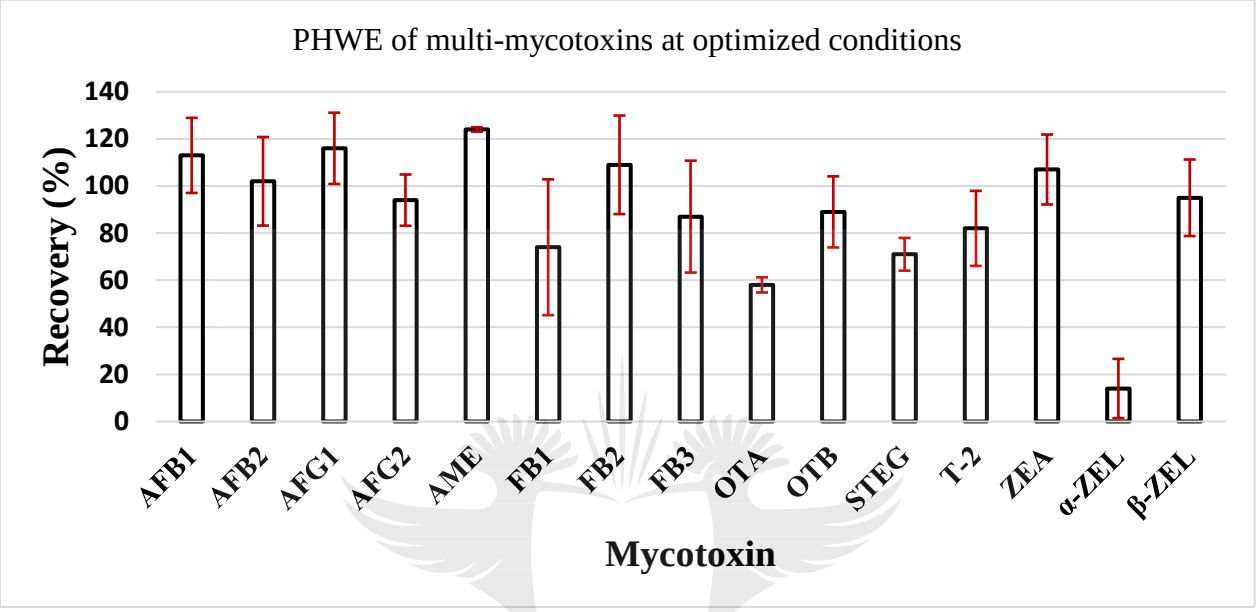

Figure 4.2: PHWE of multi-mycotoxins using CCD-optimized extraction conditions of 162 °C for temperature and 45% for solvent composition. AFB_1: aflatoxin B_1. AFB_2: aflatoxin B_2. AFG_1: aflatoxin G_1. AFG_2: aflatoxin G_2. AME: alternariol monomethyl ether. FB_1: fumonisin B1. FB_2: fumonisin B_2. FB_3: fumonisin B_3. OTA: ochratoxin A. OTB: ochratoxin B. STEG: sterigmatocystin. T-2: T-2 toxin. ZEA: zearalenone. α-ZEL: α-zearalenol. β-ZEL: β-zearalenol.

Eight (8) out of 15 of the mycotoxins were most strongly influenced by the linear effect of solvent composition (S1L) indicating that an increase the solvent composition results in a corresponding increase in recoveries of these mycotoxins. This observed strong positive linear effect of solvent composition on the extractability of mycotoxins could be due to the polarity interactions, lower surface tension and higher diffusivity of the water-ethanol mixture as compared to water only. These features allow for greater permeability of the extraction solvent into matrix cellular structures, as well as, higher solubility of some of the analytes in the extraction solvent (Gbashi *et al*., 2016; Teo *et al*., 2010). Six (6) of the mycotoxins (i.e. OTs, FB_2, FB_3, AFB_2 and AFG_2) were

most strongly influenced by the interaction effect of temperature and solvent composition (S1byT1) while only FB_1 was most strongly influenced by the linear effect of temperature (T1L).

4.3.3 Validation of the optimized PHWE method performance characteristics

Subsequent to optimization, the performance of the modified PHWE method was validated against benchmark standards of legislation and criteria. This was necessary for further authentication of the extraction process, and to compare the results obtained to established specifications of mycotoxin extraction and analysis.

4.3.3.1 Matrix effect (ME)

The possibility of co-extracted matrix components interfering with the signals of target analytes in the MS could not be ruled out, as such we investigated the ME of the modified PHWE method to determine whether a matrix-matched calibration was needed for quantitation of the analytes. Matrix effect can occur by way of signal suppression or amplification in the MS depending on the nature of the analyte and type of matrix (Kim *et al*., 2017). From Figure 4.5, it can be seen that AFs, OTA, and ZEAs demonstrated matrix-induced signal suppression with AFG_2 having the highest suppression of 48%, followed in descending order by AFB_1 (45%), AFG_1 (44%), AFB_2 (15%), ZEA and β-ZEL (9%), OTA (5%) and α-ZEL (3%). In contrast, OTB, T-2, AME and STEG demonstrated signal enhancement, with T-2 being the most enhanced at 10% (Figure 4.5). Apart from the AFs, all signal interferences were negligible (≤10%). The main components in maize which are likely to cause matrix effects during LC-MS/MS analysis are lysophospholipids (Xia & Jemal, 2009; Panuwet *et al*., 2016). The mechanism behind this phenomenon is not yet well understood, however a commonly acknowledged explanation for ion suppression is that matrix components compete with analytes for available charges in the liquid phase hence limiting ionization (Cappiello *et al*., 2010; Panuwet *et al*., 2016). Also, at higher concentrations of co-eluting components, there may be an increase in surface tension and viscosity of the eluents in the ESI interface, leading to an inefficient transfer of the analyte to the gas phase (Panuwet *et al*., 2016).

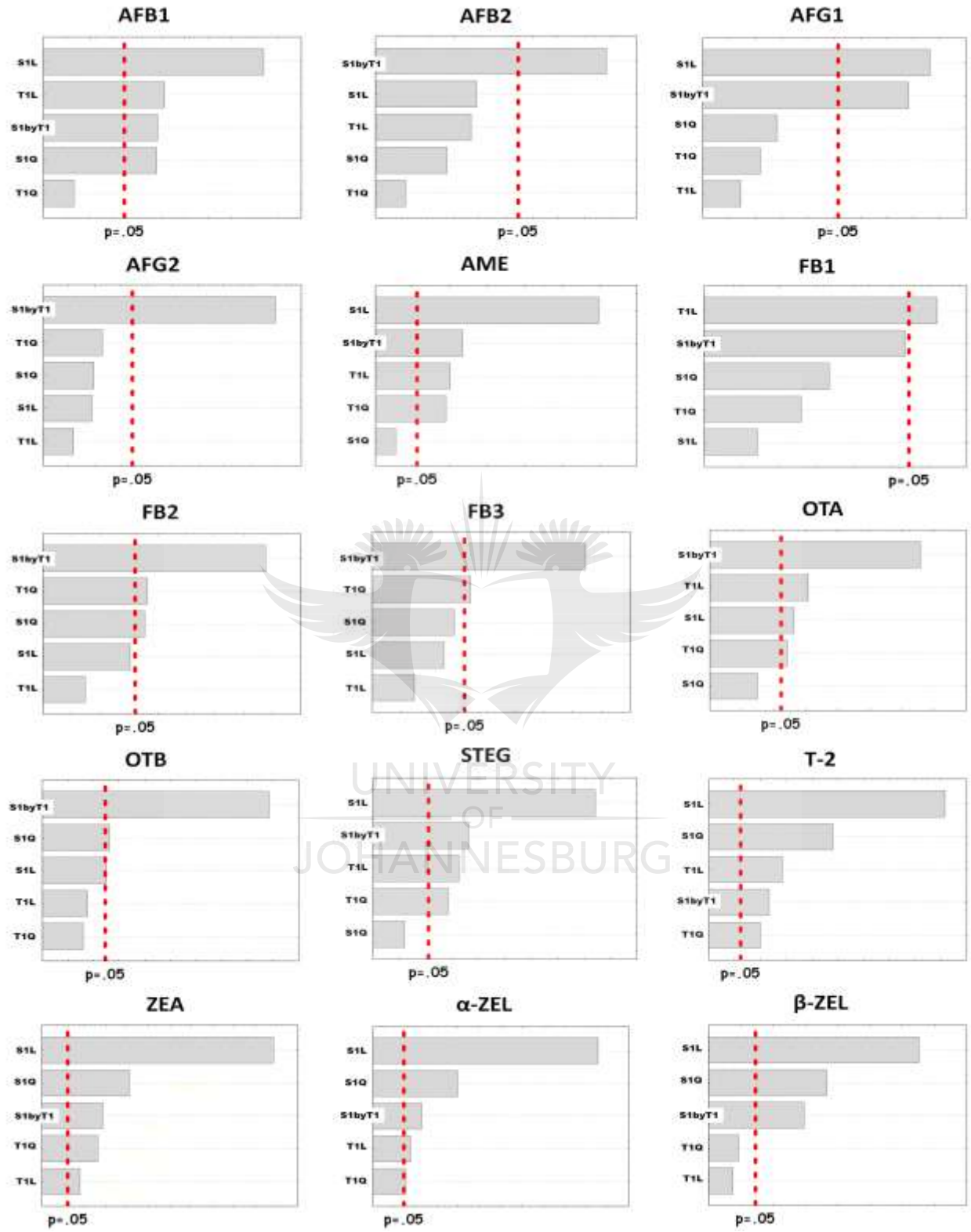

Figure 4.3: Pareto charts of standardized factor effects for pressurized hot water extraction (PHWE) of multi-mycotoxins. S1L: linear effect of solvent composition. S1Q: quadratic effect of

solvent composition. T1L: linear effect of temperature. T1Q: quadratic effect of temperature. S1byT1: interaction effect of solvent composition and temperature. AFB_1: aflatoxin B_1. AFB_2: aflatoxin B_2. AFG_1: aflatoxin G_1. AFG_2: aflatoxin G_2. AME: alternariol monomethyl ether. FB_1: fumonisin B_1. FB_2: fumonisin B_2. FB_3: fumonisin B_3. OTA: ochratoxin A. OTB: ochratoxin B. STEG: sterigmatocystin. T-2: T-2 toxin. ZEA: zearalenone. α-ZEL: α-zearalenol. β-ZEL: β-zearalenol.

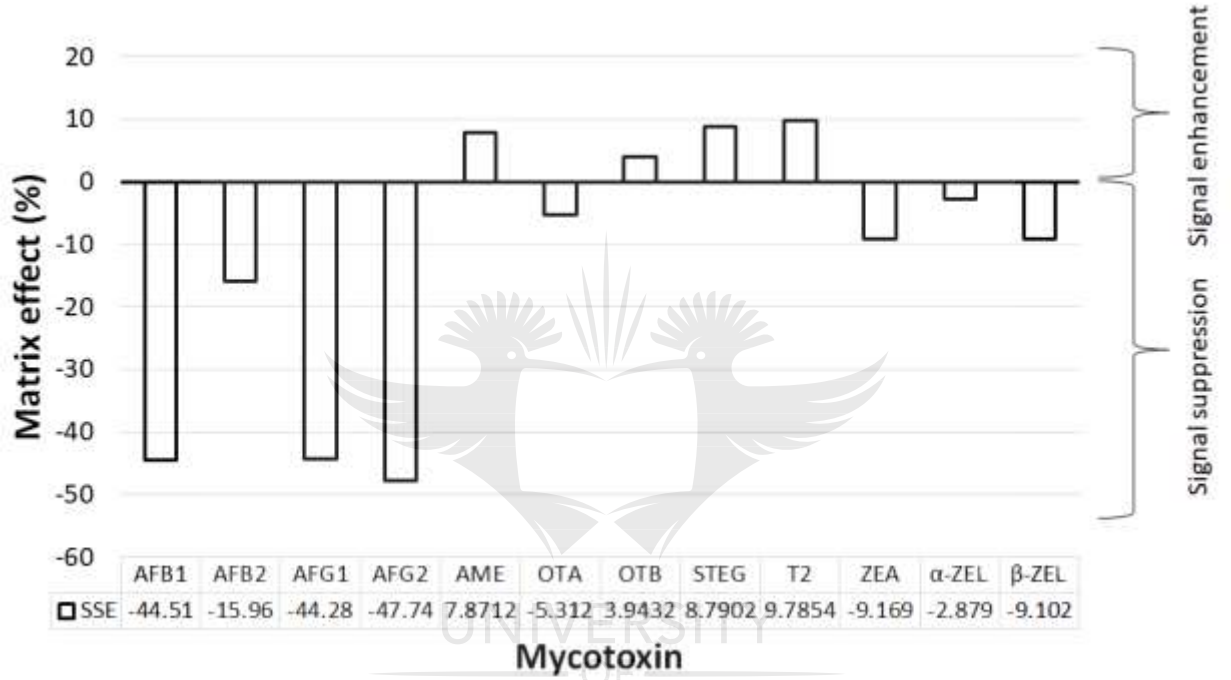

Figure 4.4: Matrix-induced signal suppression/enhancement for mycotoxins using pressurized hot water extraction (PHWE). AFB_1: aflatoxin B_1. AFB_2: aflatoxin B_2. AFG_1: aflatoxin G_1. AFG_2: aflatoxin G_2. AME: alternariol monomethyl ether. FB_1: fumonisin B1. FB_2: fumonisin B_2. FB_3: fumonisin B_3. OTA: ochratoxin A. OTB: ochratoxin B. STEG: sterigmatocystin. T-2: T-2 toxin. ZEA: zearalenone. α-ZEL: α-zearalenol. β-ZEL: β-zearalenol.

The AOAC recommends the use of matrix-matched external standard calibration to compensate for quantitation bias in the absence of isotopically-labeled standards (Lehotay, 2007; Liu *et al*., 2015; Zrostlıkova *et al*., 2002). To this effect, quantitation of analytes was achieved using matrix-matched calibration curves, except for FBs, which for reasons unclear, did not yield linear calibration curves using matrix-matched solutions, as such, were quantified using calibration curves of standards prepared in neat solutions. Elsewhere, under different circumstances, a similar

phenomenon was observed for AFGs when using a solvent-based extraction method (Sameni *et al*., 2014).

4.3.3.2 Linearity, accuracy, LOD and LOQ

The UHPLC-MS/MS method demonstrated an adequate linearity with R^2 values ranging from 0.986 to 0.999 (Table 4.3). The International Conference on Harmonization (ICH) recommends that coefficients of determination for biologics should be ≥ 0.950 (Shabir, 2005). In all cases, the y-intercept was zero which did not violate the maximum of 2% (of the target concentration response) recommended by the ICH (Shabir, 2005). Computed values for LOD and LOQ ranged from 0.06 to 40.85 µg/kg and 0.18 to 122.60 µg/kg, respectively (Table 4.3).

Table 4.3: Accuracy or recovery efficiency (%), linear calibration function, coefficient of linearity (R^2), LOD and LOQ (µg/kg).

Mycotoxin	Accuracy (%)	Equation	R^2	LOD (µg/kg)	LOQ (µg/kg)	Intra-day precision (RSDr)		Inter-day precision (RSD_R)	
						Level 1	Level 2	Level 1	Level 2
AFB_1	113	y=963239.00x+0	0.986	0.94	2.8	5	3	16	18
AFB_2	102	y=11376.000x+0	0.997	0.08	0.3	10	21	19	23
AFG_1	116	y=1133280.0x+0	0.996	0.43	1.3	20	11	15	16
AFG_2	94	y=603053.00x+0	0.998	1.7	5.1	ND	ND	ND	ND
AME	124	y=159974.00x+0	0.996	2.4	7.2	ND	ND	ND	ND
FB_1	74	y=17830.000x+0	0.998	2.3	6.8	10	10	29	20
FB_2	109	y=9478.7900x+0	0.999	4.3	13	9	7	21	34
FB_3	87	y=28677.900x+0	0.999	0.1	0.2	19	25	24	34
OTA	58	y=2539650.0x+0	0.998	0.4	1.3	22	20	3	30
OTB	89	y=22408600x+0	0.987	0.1	0.3	25	23	15	28
STEG	71	y=2407810.0x+0	0.998	0.5	1.5	5	19	7	20
T-2	82	y=18644.300x+0	0.999	16	47.3	27	23	16	31
ZEA	107	y=370444.00x+0	0.996	1.6	4.9	16	24	15	26
α-ZEL	14	y=35358.600x+0	0.999	41	123	0.00	6	13	30
β-ZEL	95	y=218599.00x+0	0.999	12	36	23	17	16	30

Key: LOD: limit of detection. LOQ: limit of quantification. ND: not determined. AFB_1: aflatoxin B_1. AFB_2: aflatoxin B_2. AFG_1: aflatoxin G_1. AFG_2: aflatoxin G_2. AME: alternariol monomethyl ether. FB_1: fumonisin B_1. FB_2: fumonisin B_2. FB_3: fumonisin

B_3. OTA: ochratoxin A. OTB: ochratoxin B. STEG: sterigmatocystin. T-2: T-2 toxin. ZEA: zearalenone. α-ZEL: α-zearalenol. β-ZEL: β-zearalenol.

Results of accuracy of the method revealed that analytes were determined with reasonable accuracy ranging from 71 to 124%, with the exception of OTA and α-ZEL which had recoveries of 58 and 14%, respectively (Table 4.3). The FDA recommends values between 80-110% for concentrations ranging from 1000 to 10,000 µg/kg (FDA, 2015). The EC recommends recovery values between 60 and 130% (Commission Regulation, 2006a), the AOAC recommends 60 to 125% (AOAC, 2009) in foodstuffs contaminated with 10 µg/kg of mycotoxins, whereas, the CODEX recommends 80 to 110% (CODEX, 2015) for accuracy in agricultural commodities contaminated with mycotoxins depending on the type of mycotoxins and level of contamination.

4.3.3.3 Intra-day precision (RSDr) and inter-day precision (RSD_R)

The results for the method precision i.e. RSDr and RSD_R are presented in Table 4.3 (Columns 7-10). The method demonstrated good intra-day precision with all RSDr values ≤ 27%. These values did not vary wide from the requirements by the EC and AOAC. The AOAC recommends a repeatability variation coefficient of < 25% for each mycotoxin (AOAC, 2009) and the EC recommends ≤ 20% for OTA, ≤ 25 for ZEA, ≤ 20% for FBs, ≤ 30 for T-2 (Commission Regulation, 2006a). The RSD_R values for inter-day precision ranged from 3 to 34%. The AOAC recommends RSD_R values less than 40% for each mycotoxin (AOAC, 2009) whereas the EC recommends RSD_R values of ≤ 30 for OTA (at a concentration > 1 µg/kg), ≤ 30 for FB_1 and FB_3 (at a concentration > 500 µg/kg), ≤ 40 for ZEA (at a concentration > 50 µg/kg), and ≤ 40 for T-2 (at a concentration > 250 µg/kg) (Commission Regulation, 2014).

4.3.4 Comparison with other conventional mycotoxin extraction methods

The recovery efficiency of our method for the extraction of multi-mycotoxins in maize was compared to those of other validated and widely-referenced methods reported in literature. The results showed significantly different (α=0.05) recovery patterns amongst the different extraction methods for some of the analytes. Relative to the performance of the conventional methods in terms of recovery, Method 1 (0 to 124%), Method 2 (0.80 to 184%), and Method 3 (99 to 100%), our method performed sufficiently (71 to 124%) with the exception of OTA (58%) and α-ZEL (14%), and in some cases favorably (Table 4.4). Particularly, the recovery of FBs was poor using

the tested conventional methods with maximum recoveries <10%, whereas, the recovery of FBs with PHWE ranged from 74 to 109%. Chilaka *et al*. (2012) noted the difficulty of extracting FBs from maize using Method 1, and rather adopted a separate extraction procedure for this purpose using SAX SPE. Method 3 (Majeed *et al*., 2018) involved the use of a strong organic eluent (acetonitrile/water) and 1% acetic acid for extraction, followed by defatting and an extensive clean-up using two different SPE clean-up. Further to this, two internal standards deepoxy-deoxynivalenol (DOM) and zearalanone (ZAN) were used for quality control to correct for unintended losses of analytes during the extraction and clean-up processes, as well as, during sample injection into the UHPLC-MS/MS as described in Section 4.2.4.

Table 4.4: Comparison of method performance (recovery) between PHWE and conventional extraction methods

Mycotoxin	Recovery (%)			
	PHWE	**Method 1 (MME)**	**Method 2 (SOLV-DS)**	**Method 3 (SOLV-SPE)**
AFB_1	113^{a}±16	118^{a}±0.64	159^{b}±3.4	100^{a}±1.6
AFB_2	102^{a}±19	69^{a}±2.2	98^{a}±2.7	100^{a}±0
AFG_1	116^{a}±15	124^{a}±4.5	184^{b}±1.7	100^{a}±0.83
AFG_2	94^{b}±11	54^{a}±1.3	73^{ab}±2.3	100^{b}±0.85
AME	124^{d}±0.93	51^{a}±1.2	77^{b}±2.8	100^{c}±3
FB_1	74^{b}±29	0.1^{a}±0.1	3^{a}±4.6	100^{b}±2.7
FB_2	109^{b}±21	0^{a}±0.	0.8^{a}±0	100^{b}±1.8
FB_3	87^{b}±24	0.3^{a}±0.4	5^{a}±0.45	100^{b}±0.77
OTA	58^{b}±3.2	41^{a}±2.6	101^{c}±0.79	100^{c}±1.4
OTB	89^{b}±15	51^{a}±1.6	124^{c}±9.6	NI
STEG	71^{a}±6.9	108^{b}±0.98	133^{c}±0.62	100^{b}±1.6
T-2	82^{a}±16	78^{ab}±0.13	112^{b}±0.51	100^{ab}±1.1
ZEA	107^{a}±15	95^{a}±4.9	115^{a}±1.6	99^{a}±4.6
α-ZEL	14^{b}±13	17^{a}±0.54	22^{a}±0.57	NI
β-ZEL	95^{a}±16	107^{a}±1.2	105^{a}±2.6	NI

Key: Values represent the mean recoveries of duplicate extractions ± standard deviations. Significant differences amongst the recovery values are indicated by superscripted alphabets on the means, and were compared using Tukey's pairwise multiple comparison test following a one-way ANOVA. Values in the same row followed by the same alphabet are not significantly different ($p > 0.05$). MME: multi-mycotoxin extraction using a combination of solvents and organic salts (Chilaka *et al*., 2012). SOLV-DS: solvent extraction followed by dilute and shoot injection (Sulyok *et al*., 2007). SOLV-SPE: solvent extraction followed by clean-up using different SPE cartridges (Majeed *et al*., 2018). NI: not investigated. AFB_1: aflatoxin B_1. AFB_2: aflatoxin B_2. AFG_1: aflatoxin G_1. AFG_2: aflatoxin G_2. AME: alternariol monomethyl ether. FB_1: fumonisin B_1. FB_2: fumonisin B_2. FB_3: fumonisin B_3. OTA: ochratoxin A. OTB: ochratoxin B. STEG: sterigmatocystin. T-2: T-2 toxin. ZEA: zearalenone. α-ZEL: α-zearalenol. β-ZEL: β-zearalenol.

In terms of cost, effort and time, the PHWE method was more desirable. Despite foregoing clean-up, the observed recoveries were in the range of other single-laboratory validated methods widely reported in literature which required extensive clean-up procedures (Di Mavungu *et al*., 2009; Monbaliu *et al*., 2009, 2010; Bardsley & Oliver, 2015; Liu *et al*., 2015; Solfrizzo *et al*., 2018). For example, the recovery of the modified PHWE (which ranged from 58 to 124%, with the exception of α-ZEL) is in line with those of Lattanzio *et al*. (2014) and Kim *et al*. (2017) who reported recoveries ranging from 63 to 93% and 74 to 133%, respectively, for multi-mycotoxin extraction in cereals (maize inclusive) followed by immuno-affinity column cleanup prior to LC-MS/MS analysis.

4.3.6 Sample analysis

Sequel to adequate performance, the developed PHWE method was applied for the extraction of the understudied mycotoxins in 25 maize meal samples intended for human consumption obtained from rural households in Ngwalemong village in Limpopo Province (South Africa). The results revealed that all samples were free from AFs, OTS and STEG. Ninety-six percent of the samples (n=25) were positive for FB_1, 52% positive for FB_3, and 48% positive for T-2 (Table 4.7). Fumonisin B_1 was the highest contaminant of the food products with a mean contamination levels of 119 µg/kg, followed in descending order by α-ZEL (24 µg/kg), and FB_3 (5.1 µg/kg). For all samples, the contamination levels for the tested mycotoxins were all below the maximum levels stipulated by the South African government, EC, CODEX, and other regulatory bodies (Arroyo-Manzanares *et al*., 2018; CODEX, 1995; Commission Regulation, 2006b, 2013; FAO, 2004). It can thus be inferred that household maize meal in the aforementioned village in South Africa was safe for human consumption in terms of mycotoxins. Our observations further reiterate the adequacy of our developed PHWE method for the analysis of multi-mycotoxins in food using UHPLC-MS/MS.

Table 4.5: Mycotoxin contamination of maize meal from Ngwalemong village in Limpopo Province of South Africa.

Mycotoxin	Number of positive samples (n=25)	Mean* (µg/kg) ± STD	Contamination range (µg/kg)
AFB_1	<LOQ	<LOQ	NA
AFB_2	<LOQ	<LOQ	NA

Mycotoxin	Number of positive samples (n=25)	Mean* (µg/kg) ± STD	Contamination range (µg/kg)
AFG_1	0	<LOQ	NA
AFG_2	0	<LOQ	NA
AME	9	4.1±7.2	<LOQ-32
FB_1	24	119±84	<LOQ-297
FB_2	0	<LOQ	NA
FB_3	13	5.1±5.8	<LOQ-16
OTA	0	<LOQ	NA
OTB	0	<LOQ	NA
STEG	0	<LOQ	NA
T-2	12	4.3±5.2	<LOQ-18
ZEA	3	0.99±2.7	<LOQ-8.8
α-ZEL	6	24±46	<LOQ-160
β-ZEL	8	4.4±6.9	<LOQ-18

Key: Mean*: average contamination of the samples including positive (i.e. >LOD) and negative (i.e. <LOD) samples. STD: standard deviation. <LOD: below limit of detection. NA: not applicable. AFB_1: aflatoxin B_1. AFB_2: aflatoxin B_2. AFG_1: aflatoxin G_1. AFG_2: aflatoxin G_2. AME: alternariol monomethyl ether. FB_1: fumonisin B_1. FB_2: fumonisin B_2. FB_3: fumonisin B_3. OTA: ochratoxin A. OTB: ochratoxin B. STEG: sterigmatocystin. T-2: T-2 toxin. ZEA: zearalenone. α-ZEL: α-zearalenol. β-ZEL: β-zearalenol.

4.4 Conclusion

The occurrence of mycotoxins in food and feed and their consequent severe effects in animals and humans have made it essential to routinely monitor the levels of these contaminants in various agricultural products. This has positioned analysis as a critical element in the management and control of mycotoxins. Extraction is an important step during mycotoxin analysis, hence improvement in extraction methods has been a recurrent interest in mycotoxicology. Shortcomings of other conventional methods have led to the development of new applications. Herein is reported for the first time the use of PHWE for the extraction of multi-mycotoxins and their subsequent quantitation on UHPLC-MS/MS. This method was developed as an efficient, safer, greener and faster alternative to other conventional methods for multi-mycotoxin extraction. All these were conveniently achieved in one single step extraction process with good values obtained for the accuracy and linearity and other associated validation parameters. The adoption of a chemometric approach for method optimization not only resulted in the optimal performance of the method, but also allows for the possibility of future optimizations geared towards analyte-specific applications. In any case, there is need to improve on some important aspects of the method, such as the working range of the method and the recovery values of OTA and α-ZEL. Notwithstanding, the overall

performance of the method is highly promising for mycotoxin research and survey work. While the method is promising, further studies in the future can focus on the cost-benefit analysis of using PHWE for extraction, as compared to other conventional mycotoxin extraction techniques.

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References

Adebo, O. A., Njobeh, P. B., Adebiyi, J. A., and Kayitesi, E. (2018). Co-influence of fermentation time and temperature on physicochemical properties, bioactive components and microstructure of ting (a Southern African food) from whole grain sorghum. *Food Bioscience, 25*, 118–127.

Adebo, O. A., Njobeh, P. B., Mulaba-Bafubiandi, A. F., Adebiyi, J. A., Desobgo, Z. S. C., & Kayitesi, E. (2018). Optimization of fermentation conditions for ting production using response surface methodology. *Journal of Food Processing and Preservation, 42*(1), e13381.

Ali, S., Noor, S., Siddiqua, U. H., Jabeen, S., and Hussain, T. (2016). Central composite design approach for optimization of extraction and dyeing conditions of marigold colorant. *Natural Products Chemistry and Research, 4*(224), 1–5.

Anfossi, L., Baggiani, C., Giovannoli, C., and Giraudi, G. (2010). Mycotoxins in food and feed: Extraction, analysis and emerging technologies for rapid and on-field detection. *Recent Patents on Food, Nutrition and Agriculture, 2*(2), 140–153.

Lehotay, S. J. (2007). Determination of pesticide residues in foods by acetonitrile extraction and partitioning with magnesium sulfate: collaborative study. *Journal of AOAC International, 90*(2), 485-520.

AOAC. (2009). A method for the determination of multiple mycotoxins in feeds and raw grains intended for feeds. *AOAC Agricultural Materials Task Force. Sub-Committee on Feed Additives and Contaminants* (pp. 2–3). Rockville, MD, USA: Association of Official Agricultural Chemists (AOAC).

Arroyo-Manzanares, N., De Ruyck, K., Uka, V., Gámiz-Gracia, L., García-Campaña, A. M., De Saeger, S., and Di Mavungu, J. D. (2018). In-house validation of a rapid and efficient procedure for simultaneous determination of ergot alkaloids and other mycotoxins in wheat and maize. *Analytical and Bioanalytical Chemistry*, 410(22), 5567–5581.

Arroyo-Manzanares, N., Huertas-Pérez, J. F., García-Campaña, A. M., and Gámiz-Gracia, L. (2014). Mycotoxin analysis: new proposals for sample treatment. *Advances in Chemistry, 2014*, 1–12.

Asghar, A., Raman, A., Aziz, A., and Daud, W. M. A. W. (2014). A comparison of central composite design and Taguchi method for optimizing Fenton process. *The Scientific World Journal, 2014*(Article ID 869120), 1–14.

Bajer, T., Bajerova, P., Kremr, D., Eisner, A., and Ventura, K. (2015). Central composite design of pressurised hot water extraction process for extracting capsaicinoids from chili peppers. *Journal of Food Composition and Analysis, 40*(2015), 32–38.

Bardsley, J., and Oliver, M. (2015). Determination of multiple mycotoxins in grain using a QuEChERS sample preparation approach and LC-MS/MS detection. *Application Note 21121* (pp. 1–7). Runcorn, UK: Thermo Fisher Scientific. Retrieved June 28, 2019, from http://tools.thermofisher.com/content/sfs/brochures/AN-21121-SP-Mycotoxins-Grain-AN21121-EN.pdf

Bas, D., and Boyaci, I. H. (2007). Modeling and optimization I: Usability of response surface methodology. *Journal of Food Engineering, 78*, 836–845.

Bennett, J., and Klich, M. (2003). Mycotoxins. *Clinical Microbiology Reviews, 16*(3), 497–516.

Cappiello, A., Famiglini, G., Palma, P., and Trufelli, H. (2010). Matrix effects in liquid chromatography-mass spectrometry. *Journal of Liquid Chromatography and Related Technologies, 33*(9–12), 1067–1081.

Chilaka, C. A., Kock, S. De, Phoku, J. Z., Mwanza, M., Augustina, M., and Dutton, M. F. (2012). Fungal and mycotoxin contamination of South African commercial maize. *Journal of Food, Agriculture and Environment*, *10*(2), 296–303.

CODEX. (1995). Codex general standard for contaminants and toxins in food and feed (Codex Stan 193–1995). *Codex Standard 193-1995*, 1–39.

CODEX. (2015). General standard for contaminants and toxins in food and feed (CODEX STAN 193-1995). *CODEX Alimentarius. International Food Standards, CODEX STAN*, 25.

Commission Regulation (2006a). Commission Regulation (EC) No. 401/2006 of laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs. *Official Journal of the European Communities, L70*, 20–23.

Commission Regulation (2006b). Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. *Official Journal of the European Communities*, *2006R1881*(016.001), 5–24.

Commission Regulation (2013). Commission Recommendation of 27 March 2013 on the presence of T-2 and HT-2 toxin in cereals and cereal products Text with EEA relevance. *Official Journal of the European Communities*, *L 91/12*, 12–15.

Commission Regulation (2014). Commission Regulation (EU) No 519/2014 of 16 May 2014 - amending Regulation (EC) No 401/2006 as regards methods of sampling of large lots, spices and food supplements, performance criteria for T-2, HT-2 toxin and citrinin and screening methods of analysis. *Official Journal of the European Union*.

Di Mavungu, J. D., Monbaliu, S., Scippo, M. L., Maghuin-Rogister, G., Schneider, Y. J., Larondelle, Y., Callebaut, A., Robbens, J., Van Peteghem, C., and De Saeger, S. (2009). LC-MS/MS multi-analyte method for mycotoxin determination in food supplements. *Food Additives and Contaminants: Part A*, *26*(6), 885–895.

Díez, C., Barrado, E., and Rodríguez, J. A. (2011). Chemometric strategies for the extraction and analysis optimization of herbicide residues in soil samples. In: M. Larramendy (Ed.), *Herbicides, theory and applications* (pp. 345–368). Rijeka, Croatia: InTech.

FAO. (2004). *Worldwide regulations for mycotoxins in food and feed in 2003* (Food Quality and

Standards Service (ESNS) No. 81). *FAO Food and Nutrition Paper 81*. Rome, Italy.

FDA. (2015). Guidelines for the validation of chemical methods for the FDA FVM program. In P. A. Orlandi, W. T. Flynn, D. L. Zink, P. E. Norris, V. K. Bunning, T. Mcgrath, and M. E. Torrence (Eds.), *US Food and Drug Administration. Office of Foods and Veterinary Medicine* (2nd ed.). Silver Spring, Maryland.

Gbashi, S., Madala, N., Adebo, O. A., and Njobeh, P. B. (2017). Development and validation of a pressurized hot water extraction method for the extraction of aflatoxin B1 in maize. In: *7th Euro Global Summit on Clinical Microbiology and Mycotoxins. February 27-28, 2017 Amsterdam, Netherlands* (Full text data not published). Amsterdam, Netherlands.

Gbashi, S., Madala, N. E., Adebo, O. A., Piater, L., Phoku, J. Z., and Njobeh, P. B. (2017). Subcritical water extraction and its prospects for aflatoxins extraction in biological materials. In: L. B. Abdulra'uf (Ed.), *Aflatoxin-control, analysis, detection and health risks* (pp. 229–250). Rijeka, Croatia: InTech.

Gbashi, S., Njobeh, P., Steenkamp, P., Tutu, H., and Madala, N. (2016). The effect of temperature and methanol–water mixture on pressurized hot water extraction (PHWE) of anti-HIV analogues from *Bidens pilosa*. *Chemistry Central Journal*, *10*(37), 1–12.

Herrero, M., Temirzoda, T. N., Segura-Carretero, A., Quirantes, R., Plaza, M., and Ibañez, E. (2011). New possibilities for the valorization of olive oil by-products. *Journal of Chromatography A*, *1218*(42), 7511–7520.

Karsten, M., Swart, R., Mcleod, F., Murphy, B., Henderson, S., and Richter, B. (2008). Fast and effective determination of aflatoxins in grains or food using accelerated solvent extraction followed by HPLC. *Dionex Corporation*, 1–5.

Khazaeli, E., Haddadi, H., Zargar, B., and Hatamie, A. (2016). Response surface methodology based on central composite design as a chemometric tool for optimizing dispersive liquid–liquid microextraction for determining ultra-trace amounts of zinc in oil and water samples. *Analytical Methods*, *8*(25), 5101–5110.

Kim, D. H., Hong, S. Y., Kang, J. W., Cho, S. M., Lee, K. R., An, T. K., Lee, C., Chung, S. H. (2017). Simultaneous determination of multi-mycotoxins in cereal grains collected from

South Korea by LC/MS/MS. *Toxins*, *9*(3), 106.

Kuiper-Goodman, T. (1998). Food safety: mycotoxins and phycotoxins in perspective. In: M. Miraglia, H. Van Edmond, C. Brera, and J. Gilbert (Eds.), *Mycotoxins and phycotoxins-developments in chemistry, toxicology and food safety* (pp. 25–48). Fort Collins, Colorado: Alaken Inc.

Lattanzio, V. M. T., Ciasca, B., Powers, S., and Visconti, A. (2014). Improved method for the simultaneous determination of aflatoxins, ochratoxin A and *Fusarium* toxins in cereals and derived products by liquid chromatography–tandem mass spectrometry after multi-toxin immunoaffinity clean up. *Journal of Chromatography A*, *1354*, 139–143.

Lehotay, S. J. (2007). Determination of pesticide residues in foods by acetonitrile extraction and partitioning with magnesium sulfate: collaborative study. *Journal of AOA*, *90*(2), 485–520.

Li, M., Dai, C., Wang, F., Kong, Z., He, Y., Huang, Y. T., and Fan, B. (2017). Chemometric-assisted QuEChERS extraction method for post-harvest pesticide determination in fruits and vegetables. *Scientific Reports*, *7*, 42489.

Liu, Y., Han, S., Wang, P. Y., Han, J., Wang, J. H., Lu, M., and Zhou, S. (2015). Simultaneous determination of 26 mycotoxins in sesame butter using modified QuEChERS coupled with UHPLC-ESI/triple quadrupole mass spectrometry. *Application Note - Agilent Technologies*, *5991–5347E*, 1–10.

Majeed, S., De Boevre, M., De Saeger, S., Rauf, W., Tawab, A., Rahman, M., and Iqbal, M. (2018). Multiple mycotoxins in rice: occurrence and health risk assessment in children and adults of Punjab, Pakistan. *Toxins*, *10*(2), 77.

Makun, H. A., Dutton, M. F., Njobeh, P. B., Gbodi, T. A., and Ogbadu, G. H. (2012). Aflatoxin contamination in foods and feeds: A special focus on Africa. In: A. E. Ayman (Ed.), *Trends in vital food and control engineering* (pp. 187–234). Rijeka, Croatia: InTech.

Monbaliu, S., Van Poucke, C., Detavernier, C., Dumoulin, F., Van De Velde, M., Schoeters, E., Van Dyck, S., Averkieva, O., Van Peteghem, C., De Saeger, S. (2010). Occurrence of mycotoxins in feed as analyzed by a multi-mycotoxin LC-MS/MS method. *Journal of Agricultural and Food Chemistry*, *13*(58), 66–71.

Monbaliu, S., Van Poucke, C., Van Peteghem, C., Van Poucke, K., Heungens, K., and De Saeger,

S. (2009). Development of a multi-mycotoxin liquid chromatography/tandem mass spectrometry method for sweet pepper analysis. *Rapid Communications in Mass Spectrometry*, *23*(1), 3–11.

Panuwet, P., Hunter Jr, R. E., D'Souza, P. E., Chen, X., Radford, S. A., Cohen, J. R., Marder, M. E., Kartavenka, K., Ryan, P. B. and Barr, D. B. (2016). Biological matrix effects in quantitative tandem mass spectrometry-based analytical methods: Advancing biomonitoring. *Critical Reviews in Analytical Chemistry*, *46*(2), 93–105.

Patterson, D. S. P., and Roberts, B. A. (1979). Mycotoxins in animal feedstuffs: Sensitive thin layer chromatographic detection of aflatoxin, ochratoxin A, sterigmatocystin, zearalenone and T-2 toxin. *Journal of the Association of Official Analytical Chemists International*, *62*, 1265–1267.

Plaza, M., and Turner, C. (2015). Pressurized hot water extraction of bioactives. *TrAC Trends in Analytical Chemistry*, *71*, 39–54.

Rahmani, A., Jinap, S., and Soleimany, F. (2009). Qualitative and quantitative analysis of mycotoxins. *Comprehensive Reviews in Food Science and Food Safety*, *8*, 202–251.

Rakić, T., Kasagić-Vujanović, I., Jovanović, M., Jančić-Stojanović, B., and Ivanović, D. (2014). Comparison of full factorial design, central composite design, and box-behnken design in chromatographic method development for the determination of fluconazole and its impurities. *Analytical Letters*, *47*(8), 1334–1347.

Ranum, P., Peña-Rosas, J. P., and Garcia-Casal, M. N. (2014). Global maize production, utilization, and consumption. *Annals of the New York Academy of Sciences*, *1312*(1), 105–112.

Sameni, M., Dübecke, A., and Weber, J. F. F. (2014). Simultaneous multi-residue determination of mycotoxins in foods using LC-MS/MS. *Journal of Environmental and Analytical Toxicology*, *5*(2), 1–6.

Schmale, D. G., and Munkvold, G. P. (2009). Mycotoxins in crops: A threat to human and domestic animal health. *The Plant Health Instructor*, *3*, 340–353.

Shabir, G. A. (2005). Step-by-step analytical methods validation and protocol in the quality system

compliance industry. *Journal of Validation Technology*, *10*, 314–325.

Shang, Y. F., Kim, S. M., and Um, B. H. (2014). Optimization of pressurized liquid extraction of antioxidants from black bamboo leaves. *Food Chemistry*, *154*, 164–170.

Shang, Y. F., Xu, J. L., Lee, W. J., and Um, B. H. (2017). Antioxidative polyphenolics obtained from spent coffee grounds by pressurized liquid extraction. *South African Journal of Botany*, *109*, 75–80.

Solfrizzo, M., Gambacorta, L., Bibi, R., Ciriaci, M., Paoloni, A., and Pecorelli, I. (2018). Multimycotoxin analysis by LC-MS/MS in cereal food and feed: Comparison of different approaches for extraction, purification, and calibration. *Journal of AOAC International*, *101*(3), 647–657.

Sulyok, M., Berthiller, F., Krska, R., and Schuhmacher, R. (2006). Development and validation of a liquid chromatography/tandem mass spectrometric method for the determination of 39 mycotoxins in wheat and maize. *Rapid Communication in Mass Spectrometry*, *20*(18), 2649–2659.

Sulyok, M., Krska, R., and Schuhmacher, R. (2007). Application of a liquid chromatography–tandem mass spectrometric method to multi-mycotoxin determination in raw cereals and evaluation of matrix effects. *Food Additives and Contaminants*, *24*(10), 1184–1195.

Teo, C. C., Tan, S. N., Yong, J., Hew, C. S., and Ong, E. S. (2010). Pressurized hot water extraction (PHWE). *Journal of Chromatography A*, *1217*, 2484–2494.

Uma, D. B., Ho, C. W., & Wan Aida, W. M. (2010). Optimization of extraction parameters of total phenolic compounds from henna (Lawsonia inermis) leaves. *Sains Malaysiana*, *39*(1), 119–128.

Wilson, N., McMaster, N., Gantulga, D., Soyars, C., McCormick, S., Knott, K., Senger, R., and Schmale, D. (2017). Modification of the mycotoxin deoxynivalenol using microorganisms isolated from environmental samples. *Toxins*, *9*(4), 141.

Xia, Y. Q., and Jemal, M. (2009). Phospholipids in liquid chromatography/mass spectrometry bioanalysis: comparison of three tandem mass spectrometric techniques for monitoring plasma phospholipids, the effect of mobile phase composition on phospholipids elution and

the association of phospholipids with matrix effects. *Rapid Communication in Mass Spectrometry*, *23*(14), 2125–2138.

Zrostlıkova, J., Hajšlová, J., Poustka, J., and Begany, P. (2002). Alternative calibration approaches to compensate the effect of co-extracted matrix components in liquid chromatography–electrospray ionisation tandem mass spectrometry analysis of pesticide residues in plant materials. *Journal of Chromatography A*, *973*(1–2), 13–26.

APPENDIX 4

Appendix 4.A: Pressurized hot water extraction (PHWE) of multi-mycotoxins at different levels of temperature and solvent composition based on the central composite design (CCD)

Mycotoxin	Recovery of mycotoxins (%) at different solvent composition and temperature conditions							
	50 °C/ 0% EtOH	50 °C/ 40% EtOH	150 °C/ 0% EtOH	150 °C/ 40% EtOH	29 °C/ 20% EtOH	170 °C/ 20% EtOH	100 °C/ 48.28% EtOH	100 °C/ 20% EtOH
AFB_1	31±0.93	38±1.48	38±0.75	84±0.93	61±1.12	64±2.42	72±0.56	68±0.56
AFB_2	66±2.05	38±10.05	51±8.19	89±15.07	74±3.54	72±15.26	76±2.42	70±3.54
AFG_1	45±1.31	38±0.37	45±1.12	82±0.74	67±0.93	60±1.49	80±1.68	74±4.09
AFG_2	128±0.93	76±0.37	98±1.68	159±3.16	136±0.56	123±1.67	147±0.37	145±0.74
AME	1±0.00	27±2.52	1±0.00	64±2.17	5±1.08	11±0.72	60±3.61	27±1.08
FB_1	53±4.65	45±8.56	52±10.98	70±2.05	38±3.54	52±7.81	44±0.18	43±1.86
FB_2	64±0.56	33±1.86	32±3.16	71±0.75	63±0.56	54±1.12	68±5.21	71±4.84
FB_3	61±4.09	26±6.70	28±2.23	70±6.14	59±2.98	46±2.05	65±0.00	66±5.21
OTA	38±0.19	23±1.12	31±0.37	59±0.74	38±1.12	38±1.68	47±0.93	46±0.93
OTB	48±1.12	28±0.37	36±0.74	67±1.48	53±0.75	53±0.37	59±4.28	60±1.68
STEG	0±0.00	34±1.31	0±0.00	76±0.56	13±0.00	19±0.19	72±2.23	34±1.12
T-2	0±0.00	25±0.93	0±0.00	61±1.48	34±1.68	49±3.16	59±0.00	56±4.29
ZEA	0±0.00	35±0.56	1±0.00	79±2.42	39±0.18	39±1.12	69±1.87	61±0.37
α-ZEL	2±0.00	5±0.00	3±0.00	11±0.37	7±0.00	6±0.00	9±0.00	9±0.19
β-ZEL	35±0.19	38±0.93	35±0.74	83±1.48	61±0.93	55±0.93	70±1.12	71±1.12

Key: Sol. comp.: solvent composition. Temp.: Temperature. AFB_1: aflatoxin B_1. AFB_2: aflatoxin B_2. AFG_1: aflatoxin G_1. AFG_2: aflatoxin G_2. AME: alternariol monomethyl ether. FB_1: fumonisin B_1. FB_2: fumonisin B_2. FB_3: fumonisin B_3. OTA: ochratoxin A. OTB: ochratoxin B. STEG: sterigmatocystin. T-2: T-2 toxin. ZEA: zearalenone. α-ZEL: α-zearalenol. β-ZEL: β-zearalenol.

Appendix 4.B: Model-fit regression coefficients and validation parameters

$$z(x,y) = C_{00} + C_{10}x + C_{20}x^2 + C_{01}y + C_{02}y^2 + C_{11}xy$$

Mycotoxin	c_{00}	c_{10}	c_{20}	c_{01}	c_{02}	c_{11}	R^2	R^2 Adj	Residual	ρ	AAD	B_f	A_f
AFB_1	27.40	0.310	0.000	1.070	-0.030	0.010	0.830	0.740	84.36	0.910	0.130	1.010	1.130
AFB_2	83.02	-0.220	0.000	-0.880	-0.010	0.020	0.660	0.490	139.9	0.810	0.140	1.010	1.140
AFG_1	37.08	0.480	0.000	0.360	-0.020	0.010	0.690	0.540	125.5	0.830	0.140	1.010	1.150
AFG_2	129.56	0.400	0.000	-1.330	-0.030	0.030	0.760	0.640	253.0	0.870	0.090	1.010	1.100
AME	-19.11	0.630	0.000	-0.130	0.010	0.010	0.970	0.950	30.47	0.980	1.960	1.200	1.590
FB_1	61.88	-0.250	0.000	-1.140	0.010	0.010	0.560	0.350	71.09	0.750	0.110	1.010	1.110
FB_2	61.88	-0.250	0.000	-1.140	0.010	0.010	0.860	0.780	52.23	0.920	0.090	1.010	1.100
FB_3	60.39	0.290	0.000	-0.980	-0.020	0.020	0.840	0.750	71.03	0.910	0.130	1.010	1.130
OTA	34.67	0.240	0.000	-0.550	-0.010	0.010	0.870	0.810	21.41	0.930	0.090	1.010	1.100
OTB	46.92	0.220	0.000	-0.320	-0.020	0.010	0.810	0.720	45.71	0.900	0.100	1.010	1.100
STEG	-18.18	0.620	0.000	0.190	0.000	0.010	0.970	0.960	31.09	0.990	2.270	0.980	1.140
T-2	-23.82	0.730	0.000	2.390	-0.050	0.010	0.940	0.900	56.69	0.970	0.120	1.000	1.120
ZEA	-23.29	0.840	0.000	2.260	-0.050	0.010	0.950	0.920	59.51	0.970	2.150	1.220	1.340
α-ZEL	-0.450	0.090	0.000	0.240	-0.010	0.000	0.870	0.810	1.780	0.930	0.220	1.020	1.230
β-ZEL	25.32	0.490	0.000	0.930	-0.030	0.010	0.810	0.720	89.98	0.900	0.140	1.010	1.150

Key: c_{00} is a constant. c_{10} and c_{01} are the linear coefficients of x (temperature) and y (solvent composition), respectively. c_{20} and c_{02} are the quadratic coefficients of x and y, respectively. c_{11} is the interaction coefficient. AFB_1: aflatoxin B_1. AFB_2: aflatoxin B_2. AFG_1: aflatoxin G_1. AFG_2: aflatoxin G_2. AME: alternariol monomethyl ether. FB_1: fumonisin B_1. FB_2: fumonisin B_2. FB_3: fumonisin B_3. OTA: ochratoxin A. OTB: ochratoxin B. STEG: sterigmatocystin. T-2: T-2 toxin. ZEA: zearalenone. α-ZEL: α-zearalenol. β-ZEL: β-zearalenol.

CHAPTER FIVE[#]

COMPARISON OF PRESSURIZED HOT WATER EXTRACTION (PHWE) AND A SOLVENT-BASED EXTRACTION METHOD FOR THE ESTIMATION OF MULTI-MYCOTOXIN LEVELS IN CEREALS

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Abstract

Mycotoxin contamination of crops has contributed significantly to the elusive sustainable development in Africa. Routine analysis of food commodities is critical for control and management of these toxins in foods. In the present study, 15 different mycotoxins were estimated in three staple cereals from selected agro-ecological regions in Nigeria using a 'novel' green extraction method, pressurized hot water extraction (PHWE) in comparison to a conventional solvent extraction method. Tested mycotoxins included zearalenone and its analogues (ZEAs), ochratoxins (OTs), T-2 toxin (T-2), fumonisins (FBs), aflatoxins (AFs), alternariol monomethyl ether (AME) and sterigmatocystin (STEG). Further discrimination of the results of PHWE and solvent extraction using principal component analysis (PCA) and orthogonal projection to latent structures discriminate analysis (OPLS-DA) did not yield any differential clustering patterns, indicating the relative similarities of the results obtained from the two extraction methods. All the maize samples (n=16) and 32% (n=38) of sorghum and 35% (n=37) of millet samples were positive for at least one of the 15 tested mycotoxins. The FBs, in particular FB_1 had the highest prevalence in terms of rate of occurrence and levels of contamination in all three cereals. Maize samples generally had higher mycotoxin contamination levels for FB_1 (17 to 7,947 µg/kg), as compared to sorghum (188 to 248 µg/kg) and millet which was negative for the toxin. Contamination levels

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for the cereals were higher in the warm humid rain forest region and gradually decreased towards the hot and arid region in the north of the country. Co-occurrence of multiple mycotoxins was observed in many of the analyzed samples. A total of 14 different combinations were observed from the 5 major groups of regulated mycotoxins (*i.e.*, AFs, FBs, OTs, T-2 and ZEAs). The highest co-occurrence was AFs+FBs that occurred in 38% (n=16) of the maize samples, while AFs+OTs+ZEAs occurred in 3% (n=38) of the sorghum samples. These results indicate the relative risk of exposure of the Nigerian populace to mycotoxins, hence the need to prioritize food safety interventions, including establishing appropriate legislation to safeguard the public, particularly those from the southern part of the country where climatic conditions are warm and humid, and mycotoxins are prevalent. The results also demonstrate the applicability of PHWE as a possible alternative extraction method to conventional methods of extraction which are solvent based, hence promoting analytical techniques that are greener and more environmentally sustainable.

Key words: Mycotoxins, pressurized hot water extraction (PHWE), agroecological zones, co-occurrence, LC-MS/MS, green extraction, principal component analysis (PCA), orthogonal projection to latent structures discriminate analysis (OPLS-DA).

5.1 Introduction

Mycotoxins are well-known food and feed contaminants that are produced by ubiquitous toxigenic fungal species belonging mainly to the *Aspergillus*, *Penicillium*, *Fusarium*, *Claviceps* and *Alternaria* genera (Njobeh *et al*., 2010a; Chilaka *et al*., 2012; Atanda *et al*., 2013). It has been estimated that approximately 4.5 billion people in the world, of which a majority from sub-Saharan Africa are chronically exposed to uncontrolled amounts of these toxins *via* exposure to contaminated foods (Turner *et al*., 2007). Although about 300 to 400 different mycotoxins have been clearly identified in nature, only a few have received significant research attention due to their economic and health significance (Gbashi *et al*., 2018), some of which include fumonisins (FBs), aflatoxins (AFs), zearalenone (ZEA) and its analogues, ochratoxins (OTs), T-2 toxin (T-2), and some emerging ones such as alternariol monomethyl ether (AME) and sterigmatocystin (STEG).

Relative to their incessant prevalence in the sub-Saharan African food supply chain, mycotoxins have been implicated in a number of adverse socio-economic effects, ranging from human and animal health, impact on food security, impact on livelihood, damage to the African agricultural export market brand, and impact on Africa's self-sustainability and increased dependence on foreign aid (Wagacha & Muthomi, 2008; Darwish *et al*., 2014; Gbashi *et al*., 2018). In the global food market, annual losses associated with mycotoxins have been estimated at approximately one billion metric tons of agricultural produce and food commodities (Schmale and Munkvold, 2009; Wilson *et al*., 2017). Nigeria, a sub-Saharan African nation, and the most populous country on the continent, is one of the countries that have been severely plagued by the mycotoxin menace in Africa (Makun *et al*., 2012; Atanda *et al*., 2013; Ezekiel *et al*., 2014).

Amidst several factors, favorable environmental conditions for colonization of crops by toxigenic fungi species, coupled with susceptibility of endemic staple crops such as maize, sorghum and millet to mycotoxin proliferation have been identified as a critical precursor to the pervasive impact of the toxins in Nigeria (Oyelami *et al*., 1997; Atanda *et al*., 2015; Apeh *et al*., 2016; Chilaka *et al*., 2016). Majority of the Nigerian populace (78 to >85%) rely on these cereal grains for dietary supply of energy, household incomes, as well as food and feed ingredients for their livestock (Awotide, 2012; Nicely *et al*., 2012; Chilaka *et al*., 2016). The farmers produce these crops under varying agronomic practices and different climatic conditions across the country. Nigeria has a humid tropical climate in its southern region which is close to the equator, and a hot arid climate gradually unfolds towards the northern part of the country, resulting in definable floral and agroecological patterns across the country.

Since these cereal crops are mostly produced by peasant farmers and marketed in local markets within the country and to other neighboring countries *via* unofficial sales channels (Bandyopadhyay *et al*., 2007; Akhidenor & Nzeka, 2018), surveillance of mycotoxin contamination levels seldom occurs (Adetunji *et al*., 2014). In this regard, despite the compelling evidence of risk exposure to mycotoxins on the Nigerian populace and their effects thereof (Liu & Wu, 2010; Atanda *et al*., 2013), there is limited data on mycotoxins in Nigeria. It is thus imperative to routinely monitor the prevalence and levels of these toxins in food/feed in order to adopt appropriate control measures and policies. While it is important to adopt sensitive testing methods for mycotoxins, it is equally expedient to use methods that are fast, effective, more sustainable and

environmentally friendly. The present study describes the screening of samples of three staple cereals (maize, sorghum and millet) obtained from selected regions within Nigeria for possible contamination with mycotoxins using a novel green and effective method, pressurized hot water extraction (PHWE) in contrast to a conventional solvent-based extraction followed by UHPLC and tandem MS analysis.

5.2 Materials and methods

5.2.1 Materials

Mycotoxin reference standards used in this study included aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), aflatoxin G_2 (AFG_2), fumonisin B_1 (FB_1), fumonisin B_2 (FB_2), fumonisin B_3 (FB_3), ochratoxin A (OTA), ochratoxin B (OTB), T-2 toxin (T-2), zearalenone (ZEA), α-zearalenol (α-ZEL), β-zearalenol (β-ZEL), alternariol monomethyl ether (AME), and sterigmatocystin (STEG) were purchased from the National Metrological Institute of South Africa (NMISA). Mycotoxin standards used for the solvent extraction included all of the above purchased from Biopure (RomerLabs, Tulln, Austria), except for AME and FB_3 purchased from Sigma-Aldrich (Bornem, Belgium) and Promec unit (Tygerberg, South Africa), respectively. Deepoxy-deoxynivalenol (DOM) and zearalanone (ZAN) were used as internal standards and were purchased from Sigma-Aldrich (Bornem, Belgium) and Biopure (RomerLabs, Tulln, Austria), respectively.

Biotage® Isolute SAX cartridges were purchased from Anatech Instruments (Pty) Ltd (South Africa). C18 solid phase extraction (SPE) columns and MultiSep®226 AflaZon+ multifunctional columns were purchased from Alltech (Lokeren, Belgium) and Romer Labs (Gernsheim, Germany), respectively. PVDF syringe filters (0.22 µm, with Luer lock) and 10 mL NORM-JECT plastic syringe (with Luer lock) from Restek (Restek Corporation, Pennsylvania USA). Whatman® glass microfiber filters (47 mm diameter, grade GF/A) was purchased from VWR International (VWR International, Leuven, Belgium), while Ultrafree®-MC PVDF centrifugal filters (0.22 µm) were purchased from Millipore (Bredford, MA, USA). Diatomaceous earth, potassium chloride, sodium sulphate, sodium bicarbonate, anhydrous sodium sulphate and dialysis tubing were purchased from Sigma (South Africa).

Solvents used for PHWE included MS-grade acetonitrile, MS-grade methanol and MS-grade formic acid, dichloromethane (DCM), ethanol (EtOH), iso-octane and sulphuric acid which were also purchased from Sigma (South Africa). Ultrapure water was obtained from a Milli-Q Gradient A10 dispensing system (Millipore, Billerica, MA, USA). Solvents used for the solvent extraction method included HPLC grade acetonitrile (Biosolve BV, Valkenswaard, The Netherlands), LC-MS grade methanol, acetonitrile, glacial acetic acid all from (Biosolve BV, Valkenswaard, The Netherlands) and ammonium acetate from Merck (Merck KGaA, Darmstadt, Germany). N-hexane was purchased from BDH Hipersolv Chromanorm (VWR International, Leuven, Belgium) and nitrogen gas (Air Liquide, Aalter, Belgium). The water used for this method (i.e. solvent extraction) was obtained from a Milli-Q®SP Reagent water system (Millipore Corp., Brussels, Belgium).

5.2.2 Methods

5.2.2.1 Sampling and sampling preparations

Sampling

Sampling was done as described by Atehnkeng *et al*. (2008). Sixteen maize (*Zea mays*) samples, 38 sorghum (*Sorghum bicolor*) samples and 37 millet (*Pennisetum glaucum*) samples were obtained from farmers/traders from five different agroecological zones in Nigeria (Adetuniji *et al*., 2014; Ezekiel *et al*., 2014) as described in Table 5.1. Choice of grains was based on consumption patterns of the West African diet, which is characterized by increased consumption of the selected cereals.

The Humid Rain Forest zone (HRF) falls within latitudes 6°4′ & 7°5′ N and longitudes 3°5′ & 8°8′ E, with maximum temperatures ranging from 26 to 28 °C, and average rainfall between 1,300 and 2,000 mm from two raining seasons in a year. Lying between latitudes 6°8′ & 9°30′ N and longitudes 2°40′ & 12°15′ E is the Derived Savannah zone (DRS), with maximum temperatures in a year averaging 25 to 35 °C and a bimodal rainfall averaging between 1,300 mm and 1,500 mm annually. The Southern Guinea Savannah (SGS) is characterized by a mean bimodal rainfall distribution between 1000 mm to 1300 mm per annum, maximum temperatures averaging 26 to 38 °C, and lies within latitudes 8°4′ & 11°3′ N and longitudes 2°41′ & 13° 33′ E, while the Northern

Guinea Savannah (NGS) is characterized by a single raining season per annum averaging between 900 mm to 1000 mm, and maximum temperatures ranging between 28 and 40 °C, and lies within latitudes 9°10′ & 11°59′ N and longitudes 3°19′ & 13°37′ E. The Sahel Savannah (SHS) lies within latitudes 12°2′ & 13°8′ N and longitudes 3°9′ & 13°9′ E, and is characterized by a Saharan climate, with maximum temperatures averaging between 30 to 40 °C and a single raining season per annum with rainfall distribution averaging between 650 and 1,000 mm (Atehnkeng *et al*., 2008; Adetuniji *et al*., 2014).

Table 5.1: Selected sampling locations for maize, sorghum and millet in Nigeria

Crop	Number of samples	Agroecological zone	Sub regions	Cultural/Geopolitical jurisdiction
Maize	16	Humid Rain Forest and Derived Savannah (HRF/DRS)	Lagos, Ondo, Ogun, Osun and Ekiti	Southern Nigeria
Sorghum	12	Humid Rain Forest and Derived Savannah (HRF/DRS)	Ondo, Ogun, Osun and Ekiti	Southern Nigeria
	26	Sahel Savannah (SHS)	Sokoto, Katsina, Kebbi, Jigawa and Kano	Northern Nigeria
Millet	20	Humid Rain Forest and Derived Savannah (HRF/DRS)	Ondo, Ogun, Osun and Ekiti	Southern Nigeria
	9	Northern and Southern Guinea Savannah (NGS/SGS)	Niger, Bauchi, Plateau, Kogi, Benue and Kwara,	North-Central Nigeria
	8	Sahel Savannah	Kebbi and Sokoto	Northern Nigeria

Pressurized hot water extraction (PHWE)

The cereal samples (maize, sorghum and millet) were milled to sieve size of < 0.6 to 1 mm using a mechanical blender. Extraction was performed using a laboratory-scale PHWE equipment (Addendum A) (Gbashi *et al*., 2017) operated at previously optimized extraction conditions of 45% EtOH solvent composition and 162 °C temperature (Gbashi *et al*., 2019a). For the extraction, 4 g of grounded cereal sample was thoroughly mixed with 3 g of diatomaceous earth and transferred into the extraction cell (70 x 30 mm and approximately 20 mL) which was contained in a GC oven (Carlo Erba Instruments, Italy) maintained at a temperature of 162 °C using a digital

temperature controllable unit (±1 °C). The extraction solvent (45% EtOH) was pumped at a constant flow rate of 5 mL/min through the extraction cell *via* a stainless-steel tubbing (1.58 mm in outer dimension and 0.18 mm inner dimension), and the pressure maintained at 1000 ± 200 psi by means of a back-pressure valve (Swagelok, Johannesburg, South Africa). The extract was made to pass through a cooling coil and collected into a 50 mL centrifuge tube up to the 50 mL mark. Two (2) mL of the extracts were filtered through a 0.22 µm PVDF syringe filter into a 2 mL HPLC vial for subsequent analysis on UHPLC-MS/MS.

Solvent extraction

Solvent extraction was performed using the method of Majeed *et al*. (2018). Briefly, five (5) grams of samples were spiked with internal standards, DOM (1 µg/kg) and ZAN (1 µg/kg), prior to extraction. For the extraction, 20 mL of extraction solvent, acetonitrile/water/acetic acid (79/20/1, v/v/v), was added to the spiked samples, agitated for 1 h on an overhead shaker (AG 6A, Exacta, Mery sour Oise, France) and centrifuged for 15 min at 3,300 g using an IEC Central (type MP4) centrifuge (VWR International, Leuven Belgium). The supernatant was passed through a pre-conditioned (10 mL of the extraction solvent) octadecyl (C18) solid phase extraction (SPE) column (Grace octadecyl C18, Lokeren, Belgium) under gravity. A second extraction was performed on the samples by adding 5 mL of extraction solvent, agitating, centrifuging and passing through the SPE column as described above. The total volume of the eluate was adjusted to 25 mL and defatted with 10 mL n-hexane. The defatted extract was split into two equal parts for further clean-up using different approaches. The first portion (10 mL defatted extract) was diluted with 20 mL acetonitrile/acetic acid (99/1, v/v) and was subjected to clean-up by a Multisep226, Afla-ZON+ multifunctional column, under gravity, followed by further column washing using 5 mL of acetonitrile/acetic acid (99/1, v/v). The second portion (10 mL defatted extract) was filtered using a Whatman glass microfilter (VWR International, Zaventem, Belgium). The first extract was combined with 2 mL of the second extract, evaporated to dryness under a stream of nitrogen gas at 40 °C. The residue was reconstituted in 150 µL of mobile phase, methanol/water/acetic acid (57.20/41.80/1, v/v/v) and 5 mM ammonium acetate, and filtered through a 0.22 µm Ultrafree®-MC centrifugal filter (Bedford, MA, USA) at 14,000 g for 5 min.

5.2.2.2 Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS)

Chromatographic separation and UHPLC-MS/MS for PHWE

Chromatographic separation, detection and quantitation of mycotoxin levels was achieved using Shimadzu UHPLC-MS/MS 8030 equipment (Shimadzu Corporation, Tokyo, Japan). The system consisted of a chromatograph (LC-30AD Nexera) linked to an autosampler (SIL-30 AC Nexera). Two (2) microlitres of sample were injected and pumped through a Raptor^{TM} ARC-18 column (2.7 µm, 2.1 mm × 100 mm) (Restek Corporation, Pennsylvania USA) maintained at 40 ^{o}C in a column oven (CTO-20 AC Prominence). A binary pump (LC-20AD) connected to the system was used to pump the mobile phases A (aqueous phase) and B (organic phase) through the column at a constant flow rate of 0.2 mL/min. Mobile phase A consisted of 0.1% formic acid (FA) in deionized water, and mobile phase B consisted of 0.1% FA in methanol/acetonitrile (50/50 v/v). The elution gradient program started with pumping 10% B for 0.1 min, which was steadily increased to 95% B within 8.4 min, held constant at 95% B for 3 min, initial gradient condition of 10% B re-established within 1 min, and then the column was allowed to re-equilibrate at this condition for 4.5 min prior to the next injection, making a total run time of 17 min.

Separated analytes were delivered to a Shimadzu triple-quadrupole MS model 8030 (Shimadzu Corporation, Kyoto, Japan) detector equipped with an electron spray ionization operated in positive mode (ESI^{+}). A time-scheduled ultrafast multiple reaction monitoring (MRM) MS method was used for the quantification and identification of the analytes of interest. To improve specificity and confidence in analytical detection, two MRM-transitions were monitored per analyte. The desolvation line (DL) temperature was 250 °C, heat block temperature was 400 °C, drying gas flow rate was 15 L/min, and interface nebulizing gas flow rate was 3 L/min. The Shimadzu LabSolutions software was used for subsequent data visualization and analysis. The optimized chromatographic and MS method parameters of the 15 mycotoxins under investigation are presented in Table 5.2.

Table 5.2: MRM transitions, optimized MS conditions and retention times of mycotoxins.

S/No	Mycotoxin	Ret. time (min)	Precursor (*mz*)	Products (*mz*)	Q1 Pre bias (V)	CE (eV)	Q3 Pre bias (V)
1.	AFB_1	8.25	313.00	241.00*	-22.00	-41.00	-23.00
				285.10	-22.00	-24.00	-29.00
2.	AFB_2	8.01	315.00	259.10*	-22.00	-31.00	-25.00

S/No	Mycotoxin	Ret. time (min)	Precursor (*mz*)	Products (*mz*)	Q1 Pre bias (V)	CE (eV)	Q3 Pre bias (V)
				287.00	-23.00	-26.00	-30.00
3.	AFG_1	7.77	329.00	243.00*	-12.00	-28.00	-23.00
				311.10	-16.00	-24.00	-14.00
4.	AFG_2	7.17	331.00	245.10*	-12.00	-32.00	-24.00
				313.00	-12.00	-24.00	-20.00
5.	AME	10.13	273.00	128.10*	-10.00	-49.00	-21.00
				115.05	-18.00	-54.00	-19.00
6.	FB_1	7.97	722.20	352.20*	-34.00	-42.00	-11.00
				334.30	-20.00	-42.00	-11.00
7.	FB_2	8.95	706.10	336.30*	-20.00	-38.00	-22.00
				318.30	-26.00	-41.00	-22.00
8.	FB_3	8.75	706.30	336.30*	-40.00	-39.00	-11.00
				354.40	-20.00	-35.00	-24.00
9.	OTA	10.13	403.80	239.00*	-15.00	-27.00	-24.00
				221.00	-12.00	-38.00	-21.00
10.	OTB	9.33	370.10	205.00*	-13.00	-22.00	-21.00
				324.10	-13.00	-14.00	-22.00
11.	STEG	10.45	324.90	310.00*	-22.00	-24.00	-30.00
				281.10	-22.00	-40.00	-27.00
12.	T-2	9.67	467.20	245.10*	-13.00	-11.00	-16.00
				305.20	-22.00	-11.00	-20.00
13.	ZEA	10.06	319.10	185.00*	-12.00	-27.00	-30.00
				187.10	-15.00	-21.00	-19.00
14.	α-ZEL	9.42	323.10	277.20*	-17.00	-17.00	-18.00
				305.20	-24.00	-9.000	-20.00
15.	β-ZEL	8.95	323.10	277.20*	-16.00	-16.00	-18.00
				305.20	-16.00	-11.00	-20.00

Key: S/No: serial number. Ret. Time: retention time. Q1 Pre bias: quadruple one pre-rod bias. Q3 Pre bias: quadruple three pre-rod bias. CE: collision energy. * Quantitative product ion. AFB_1: aflatoxin B_1, AFB_2: aflatoxin B_2. AFG_1: aflatoxin G_1. AFG_2: aflatoxin G_2. AME: alternariol monomethyl ether. FB_1: fumonisin B_1. FB_2: fumonisin B_2. FB_3: fumonisin B_3. OTA: ochratoxin A. OTB: ochratoxin B. STEG: sterigmatocystin. T-2: T-2 toxin. ZEA: zearalenone. α-ZEL: α-zearalenol. β-ZEL: β-zearalenol.

Chromatographic separation and LC-MS/MS for solvent extraction

A Waters Acquity UPLC system (Waters, Milford, MA, USA) linked to a Waters Micromass Quattro Micro triple-quadrupole mass spectrometer (Waters, Milford, MA, USA), was used for chromatographic separation, detection and quantification of the extracts obtained from the solvent extraction. The above described LC-MS/MS system was equipped with a Waters Symmetry C18 analytical column (5 µm, 2.1 × 150 mm) and a Waters Sentry guard column (3.5 µm 2.1 × 10 mm)

purchased from the same vendor. The column oven was kept at room temperature (25 °C), and 20 µL of sample was injected into the column. The aqueous part of the mobile phase (*i.e.*, mobile phase A) consisted of water/methanol/acetic acid (94/5/1, v/v/v) and 5 mM ammonium acetate, and the organic part of the mobile phase (*i.e.*, mobile phase B) consisted of methanol/water/acetic acid (97/2/1, v/v/v) and 5 mM, ammonium acetate. The gradient elution program and MS parameters are as described by Monbaliu *et al*. (2009).

5.2.2.3 Validation of the modified PHWE method for multi-mycotoxin extraction

The effects of the cereal-matrix components on the analytical signals of the different mycotoxins in the MS were determined using the signal suppression/enhancement method described by Arroyo-Manzanares *et al*. (2018) and Sulyok *et al*. (2006). Calibration curves were plotted for standards prepared in analyte-free sample extracts, as well as for standards prepared in neat organic solvents (100% methanol). Matrix-effect (ME) was determined as the percentage ratio of the difference between the slope of matrix-matched calibration curve and neat standards calibration curve divided by the slope of neat standards calibration curve (Equation 5.1).

$$ME = \frac{Slope_m - Slope_n}{Slope_n} \times 100 \qquad \textbf{\textit{Equation 5.1}}$$

Where *ME* is the matrix effect, $Slope_m$ is the slope of calibration curve of standards prepared in sample extracts and $Slope_n$ is the slope of standards prepared in neat solvent.

The limits of detection (LOD) and limits of quantification (LOQ) (Equation 5.2) of the mycotoxins were determined using the signal-to-noise ratio of the matrix-matched standards as described by Kim *et al*. (2017). Linearity was determined by least-square regression of a 6-point matrix-matched calibration curve within the ranges of 9 to 5,000 µg/kg depending on the mycotoxin. Recovery efficiency of the method was determined by spiking analyte-free samples with known concentrations of mycotoxins, extracting the analytes the same day using PHWE as described above. The percentage ratio of post-extraction concentration (recovered concentration) to that of pre-extraction concentration (initial concentration) was taken as the recovery value (Equation 5.3) (Arroyo-Manzanares *et al*., 2018).

$$Limit_{DQ} = F \times \left[\frac{C}{\left(\frac{S}{N}\right)}\right] \qquad \textbf{\textit{Equation 5.2}}$$

$$Recovery\ (\%) = \frac{E_r}{E_i} \times 100 \qquad \textbf{\textit{Equation 5.3}}$$

Where $Limit_{DQ}$ is the *LOD* or *LOQ* depending on the value of the multiplication factor *F*, which is 3.33 for *LOD* and 10 for *LOQ*. *C* is the concentration, while *S* is the signal at concentration *C*, and *N* is the noise level at similar concentration. E_r is the recovered concentration after spiking, and E_i is the spiked concentration.

5.2.2.4 Multivariate discriminant analysis

The pre-processed data set was subjected to multivariate discriminant analysis in order to scrutinize for discriminatory patterns between the two extraction methods. Using the SIMCA-P$^+$ 14.0 chemometrics software (Umetrics, MKS Instruments Inc., Sweden), the data was mean-centered, Pareto-scaled (Aliferis *et al*., 2010) and subjected to PCA and OPLSD-DA analysis in order to extract maximum information from the data set. The adopted models, PCA and OPLS-DA, are advanced dimensionality reduction tools which highlights contrasts or similarities between data groups *via* construction of few interpretable linearly uncorrelated variables called latent variable or principal components from the dataset (Worley & Powers, 2013). PCA does not supervise the construction of latent variables from the dataset while the OPLS-DA supervises the construction of latent variables from the dataset (Aliferis *et al*., 2010; Worley & Powers, 2013).

For the OPLS-DA model, the data variables were classified into two major groups depending on the adopted method of analysis, either PHWE or solvent extraction. This was critical because, OPLS-DA is a supervised model, as such, information regarding variable class member is a prerequisite for location of the principal components. Usually, OPLS-DA is best applicable when there are only two classification groups in the data set, such as a control group and a dependent group. As such, classification of the data set into two groups permitted the extraction of a between-class variation referred to as the Y-predictive block, and a within-class variation referred to as the Y-orthogonal block or uncorrelated variation (Song *et al*., 2013). By doing so, OPLS-DA maximizes the discrimination of the two groups of variables and provides an improved model interpretability without modifying its predictive power (Trygg & Wold, 2002).

For the evaluation of model performances, the quantitative *goodness-of-fit* parameters *i.e.*, *R2*X(cum) and *Q2X*(cum) values, and the *goodness-of-prediction* parameters *i.e.*, the *Q2*(cum)

values, were calculated. The *R2*X(cum) and *Q2X*(cum) values for the PCA model were used to measure the degree to which the latent structures (*i.e.*, principal components) describe the variations and patterns in the data set (Worley & Powers, 2013). Whereas for the OPLS-DA model, the *R2*X(cum) was used to estimate the cumulative fraction of the variation of the *X* variables explained by the model, *R2*Y(cum) was used to measure the cumulative ratio of the variation of the *Y* variables explained by the model, and the *Q2*(cum) estimated the cumulative predictive capacity of the full model (Aliferis *et al*., 2010; Matthews *et al*., 2012). For internal validation of the OPLS-DA models to assess the statistical significance of the model, the goodness-of-fit and goodness-of-prediction of the OPLS-DA model was compared with those of 100 random Y-permutated models, which generates a distribution of *Q2* values that are suitable for testing the null hypothesis for a model's *Q2* (Worley & Powers, 2013; Zhang *et al*., 2015b).

5.3 Results and discussion

Pressurized hot water extraction, a 'novel' green extraction technique was adopted for the analysis of multi-mycotoxin in 91 samples of maize, sorghum and millet intended for human consumption obtained from different agroecological zones in Nigeria as described in Section 5.2.2.1. In order to authenticate the PHWE method, a method validation was performed, as well as, a comparison with a solvent-based extraction method.

5.3.1 Method validation and comparative evaluation of PHWE and solvent extraction

The results of PHWE method validation in comparison with solvent extraction is presented in Appendix 5.A and Table 5.3. In order to compensate for matrix effects, matrix-matched calibrations were adopted for quantification of the mycotoxin concentrations in the samples for the two methods.

5.3.1.1 Validation of PHWE and solvent extraction

The performances of both methods showed good consistency with EC, AOAC, and ICH guidelines (EC, 2002; Shabir, 2005; Commission Regulation, 2006a; AOAC, 2009; Arroyo-Manzanares *et al*., 2018). The linearity correlation (R^2) of PHWE ranged from 0.98 to 1.00 for the 15 mycotoxins in all 3 sample matrices within the linear ranges of 60 to 2,000 µg/kg for FB_1, 16 to 500 µg/kg for FB_2, 10 to 300 µg/kg for AFB_1 and 30 to 1,000 µg/kg for the other analytes (Appendix 5.A). The

benchmark for the acceptance of linearity of R^2 equal or higher than 0.95 by the International Conference on Harmonization (ICH) was fulfilled for all understudied analytes (Shabir, 2005), though there existed a significant difference ($p \leq 0.05$) in the linearities of the two methods. The sensitivity of the methods was determined by assessing the LOD's and LOQ's of the method for each of the mycotoxins. For PHWE, the LOD's and LOQ's ranged respectively from 0.06 to 41 µg/kg and 0.32 to 123 µg/kg for maize, 0.07 to 98 µg/kg and 0.21 to 295 µg/kg for sorghum, and 0 to 26 µg/kg and 0.01 to 79 µg/kg for millet (Table 5.3). These values were sufficiently low for detection and quantitation of small amounts of the analytes under investigation in cereal grains (Chilaka *et al*., 2016; Kim *et al*., 2017), as such, trace amounts of the analytes in the sample extracts can be quantitatively reported with a high degree of confidence. There was no statistically significant difference ($p \leq 0.05$) between the LOD's and LOQ's of PHWE and the solvent extraction method.

The recovery rates of the 15 mycotoxins varied from 74 to 126% in maize, 73 to 115% in sorghum and 67 to 133% in millet for PHWE, which is not far from the AOAC recommendations of between 60% to 125% for foodstuff contaminated with 10 µg/kg of mycotoxins, and the EC recommendations of 60 and 130% (Commission Regulation, 2006a; Arroyo-Manzanares *et al*., 2018). The recovery rates for the solvent extraction method varied from 99 to 100% for maize, 100 to 101% in sorghum, and 100 to 102% for millet. Though the recoveries of the solvent extraction method were more consistent and closer to 100%, when compared to those of PHWE using the Independent Sample's T-test at a 95% probability, there existed no statistically significant differences in the mean recoveries of the two methods. The RSDr values for all the analytes in maize, sorghum and millet matrices ranged from 2 to 19% for PHWE and satisfied the guideline criterion of <25% by CODEX and the EC (Commission Regulation, 2006a; AOAC, 2009; Arroyo-Manzanares *et al*., 2018). In comparison with the RSD values for the solvent extraction, those of maize ranged from 0 to 5% and 5 to 20% for sorghum. These values were significantly different ($p \leq 0.05$) from the corresponding RSD values for PHWE while those of millet (3 to 15%) were not significantly different ($p > 0.05$).

Table 5.3: Method validation parameters for PHWE and solvent extraction[#] of multi-mycotoxin in maize, sorghum and millet matrices.

Mycotoxin	Maize				Sorghum				Millet			
	LOD (µg/kg)	LOQ (µg/kg)	Recovery (%)	RSDr (%)	LOD (µg/kg)	LOQ (µg/kg)	Recovery (%)	RSDr (%)	LOD (µg/kg)	LOQ (µg/kg)	Recovery (%)	RSDr (%)
AFB_1	0.94	2.8	108	3.1	0.37	1.1	104	1.7	2.2	6.6	97	10
	0.28[#]	0.9[#]	100[#]	1.6[#]	0.89[#]	2.7[#]	101[#]	6.4[#]	2.0[#]	6.0[#]	101[#]	5.3[#]
AFB_2	0.08	0.25	98	12	0.07	0.20	108	3.2	1.0	3.1	72	9.0
	0.04[#]	0.13[#]	100[#]	0[#]	0.76[#]	2.3[#]	101[#]	11[#]	3.4[#]	10[#]	101[#]	8.0[#]
AFG_1	0.43	1.3	126	13	1.2	3.7	125	4.3	2.7	8.2	96	8.3
	0.13[#]	0.38[#]	100[#]	0.83[#]	0.27[#]	0.80[#]	101[#]	9.6[#]	1.0[#]	2.9[#]	100[#]	4.1[#]
AFG_2	1.2	3.6	107	8.2	1.2	3.7	115	2.6	2.7	8.1	85	6.5
	4.2[#]	13[#]	100[#]	0.85[#]	1.7[#]	4.9[#]	102[#]	11[#]	7.3[#]	22[#]	100[#]	6.4[#]
AME	2.4	7.2	77	11	5.7	17	79	11	26	78	72	14
	0.86[#]	2.6[#]	100[#]	2.9[#]	3.9[#]	12[#]	101[#]	5.3[#]	6.8[#]	21[#]	101[#]	7.8[#]
FB_1	2.3	6.8	111	9.6	1.1	3.2	81	2.9	2.2	6.5	133	6.8
	1.3[#]	3.8[#]	100[#]	2.7[#]	1.3[#]	3.9[#]	101[#]	8.6[#]	5.1[#]	15[#]	102[#]	9.4[#]
FB_2	4.3	13	89	1.7	7.3	22	78	5.2	0	0.01	119	11
	3.3[#]	9.8[#]	100[#]	1.8[#]	4.7[#]	14[#]	101[#]	7.0[#]	5.6[#]	17[#]	102[#]	11[#]
FB_3	0.06	0.18	95	5.5	0.07	0.20	84	6.3	0.0	0.01	72	8.3
	2.7[#]	8.2[#]	100[#]	0.77[#]	0.63[#]	1.9[#]	101[#]	8.0[#]	1.3[#]	4.0[#]	102[#]	14[#]
OTA	0.44	1.3	75	8.8	0.17	0.50	72	8.9	0.40	1.2	67	12
	0.32[#]	0.95[#]	100[#]	1.4[#]	1.1[#]	3.3[#]	101[#]	7.8[#]	0.54[#]	1.6[#]	100[#]	8.3[#]
OTB	0.11	0.32	77	6.3	0.07	0.21	79	4.9	0.18	0.55	72	7.9
	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]
STEG	0.50	1.5	95	8.7	0.33	0.99	90	3.2	1.6	4.7	92	13
	0.07[#]	0.21[#]	100[#]	1.6[#]	0.68[#]	2.1[#]	101[#]	5.4[#]	1.4[#]	4.3[#]	100[#]	3.1[#]
T-2	0.04	0.13	87	16	0.07	0.20	84	6.2	1.1	3.4	69	12

Mycotoxin	Maize				Sorghum				Millet			
	LOD (µg/kg)	**LOQ (µg/kg)**	**Recovery (%)**	**RSDr (%)**	**LOD (µg/kg)**	**LOQ (µg/kg)**	**Recovery (%)**	**RSDr (%)**	**LOD (µg/kg)**	**LOQ (µg/kg)**	**Recovery (%)**	**RSDr (%)**
	0.86[#]	2.6[#]	100[#]	1.1[#]	2.5[#]	7.4[#]	101[#]	6.6[#]	2.6[#]	7.7[#]	100[#]	5.8[#]
ZEA	1.6	4.9	80	9.8	0.88	2.6	93	3.4	20	59	83	9.5
	4.2[#]	13[#]	99[#]	4.7[#]	15[#]	44[#]	102[#]	20[#]	16[#]	47[#]	102[#]	15[#]
α-ZEL	41	123	84	19	60	180	73	5.3	26	79	100	13
	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]
β-ZEL	12	36	74	7.4	17	50	83	4.1	14	42	73	12
	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]
Mean	1.2	3.6	96	8.9	1.5	4.6	93	4.9	5.0	15	88	10
	1.5[#]	4.6[#]	100[#]	1.7[#]	2.7[#]	8.2[#]	101[#]	8.8[#]	4.4[#]	13[#]	101[#]	8.1[#]
Sig. (2-tailed)	0.59	0.58	0.34	0	0.38	0.38	0.12	0.01	0.83	0.83	0.06	0.15

Key: [#] Parameter values for solvent extraction. ND: not determined. Recovery values are presented mean of duplicate determinations ± standard deviation of mean. LOD: Limit of detection; LOQ: Limit of quantification. AFB_1: aflatoxin B_1. AFB_2: aflatoxin B_2. AFG_1: aflatoxin G_1. AFG_2: aflatoxin G_2. AME: alternariol monomethyl ether. FB_1: fumonisin B_1. FB_2: fumonisin B_2. FB_3: fumonisin B_3. OTA: ochratoxin A. OTB: ochratoxin B. STEG: sterigmatocystin. T-2: T-2 toxin. ZEA: zearalenone. α-ZEL: α-zearalenol. β-ZEL: β-zearalenol.

In general, there was less variability in the validation parameters (*i.e.*, recovery, linearity and RSD) of the solvent extraction method as compared to those of PHWE. This could be due to the fact that the solvent extraction method involved multiple clean-up steps (defatting and two SPE purification procedures using different SPE cartridges). This was in addition to the use of two internal standards to correct for loss of analytes during the sample preparation or injection steps in the analytical process. On the other hand, PHWE was designed with the aim of reduction of cost and the amount of harmful organic solvents used during extraction and increased speed of the analytical process. Forfeiture of a clean-up step facilitated the achievement of these objectives. This however contributed to the higher variations observed in the PHWE analytical results, which was not unexpected. Such variations are negligible provided they were within the acceptable limits stipulated by regulatory bodies (Anklam *et al*., 2002; Commission Regulation, 2006a; AOAC, 2009; CODEX, 2015; FDA, 2015; Arroyo-Manzanares *et al*., 2018). As would be discussed in the succeeding sections of this chapter, the overall variations between the two analytical procedures have been shown to be statistically insignificant.

5.3.1.2 Comparative evaluation of PHWE and solvent extraction

Principal component analysis (PCA) and orthogonal projection to latent structures discriminate analysis (OPLS-DA) approaches were adopted to scrutinize the entire data set (*i.e.*, combined data from method validation and samples analysis) for inherent global discriminatory patterns such as multiple pairwise correlations and/or co-variances between the data obtained by the two extraction methods, which may not be readily observed by using conventional statistical analysis.

5.3.1.2.1 Discriminatory analysis of PHWE and solvent extraction methods using PCA and OPLS-DA

The results of the PCA and OPLS-DA analysis of the data and their corresponding model-fit quality parameters are shown in Table 5.4 and Figure 5.1. PCA was used for an initial screening of the overall structure of the data set for discriminatory patterns and detection of outliers. The results revealed a single latent variable, which indicated there were no differential patterns between the variables pertaining to PWHE and corresponding variables pertaining to the solvent extraction. Notwithstanding, the PCA model accounted for 97% of the variations in the data set [*i.e.*,

*R2*X(cum)=0.97], with a predictive ability of 77% [*i.e., Q2X*(cum)=0.77]. This observation was in agreement with the results of the Independent Samples T-test performed on each of the variables from the two extraction methods (Tables 5.3 & 5.5, and Appendix 5.A), where it was observed that the majority of the variables were not significantly different (p>0.05).

Table 5.4: Summary of PCA-X model and OPLS-DA models with the corresponding model validation parameters

Model	Data classification	Number of observations	Number of components	*R2*X(cum)	*R2*Y(cum)	Q2(cum)
PCA	None	84 (*X*=14, *Y*=2)	1	0.97	-	0.77
OPLS-DA	PHWE & SOLV-EXT	84 (*X*=14, *Y*=2)	1 predictive and 2 orthogonal	0.99	-0.11	0.05

Key: PCA-principal component analysis. OPLS-DA-orthogonal projection to latent structures discriminate analysis. PHWE-pressurized hot water extraction. SOLV-EXT-solvent extraction

Since PCA does not take into account the classification label associated with the data set, discrimination of the data groups is not maximized. This problem is solved by the OPLS-DA model which supervises the construction of the latent variables, hence, yielding a more class-specific discrimination of the data. Results of the OPLS-DA analysis showed the construction of 2 explanatory and 1 predictive principal component with a 99% total explained variation in *X* [*i.e.*, *R2*X(cum)=0.99]. However, the model could not account for the cumulative variations in Y [*R2*Y(cum)=-0.11], and the total amount of predicted variability in the full model was low [*i.e.*, *Q2*(cum)=0.05]. As such, despite the OPLS-DA models making reference to the pre-defined sample class membership in order to maximize separation of the data, there was no clear separation of the data groups. A pattern which can be more clearly visualized on the OPLS-DA scores plot (Figure 5.1). On this score plot, the green circular dots represent variables of the PHWE class, while the red triangular dots represent variables of the solvent extraction (*i.e.*, SOLV-EXT) class. Variables from the two data groups can be seen clustered together towards the middle of the plot and while some arbitrarily distributed across the graph. This is an indication of intrinsic similarities and randomness in the patterns within and between the groups.

This observation is in strong agreement to the results of PCA analysis as well as the Independent Sample's T-test. Overall, PHWE compared well with the solvent-based extraction method, and other validated methods for multi-mycotoxin extraction reported in literature (Sulyok *et al*., 2006; Lattanzio *et al*., 2014; Kim *et al*., 2017; Arroyo-Manzanares *et al*., 2018). Hence, considering its good validation performance, it was deemed adequate for investigating the natural occurrence of mycotoxins in food commodities.

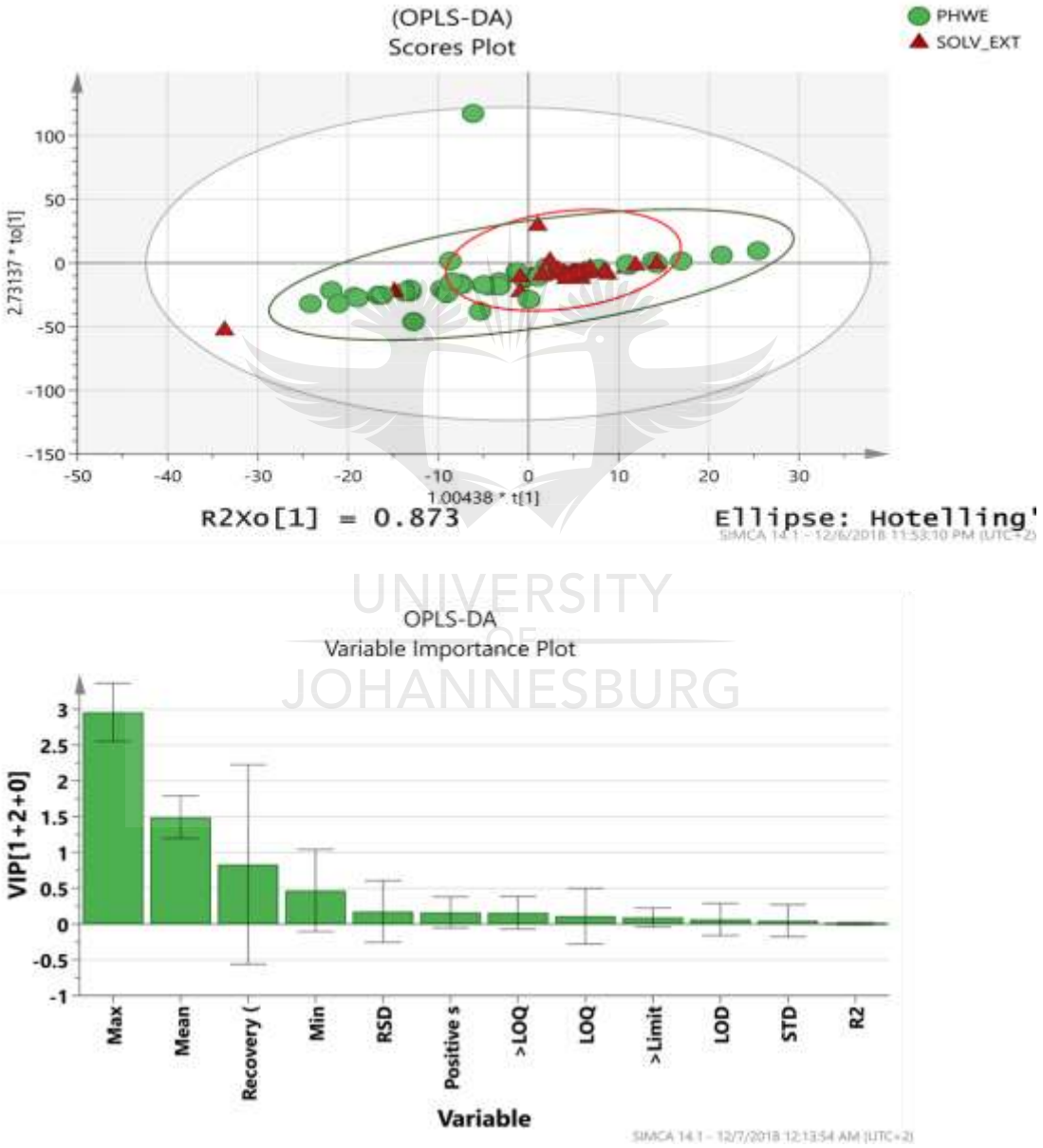

Figure 5.1: OPLS-DA scores plot and variable importance plot (VIP) plot for discriminate analysis of PHWE and solvent extraction.

5.3.2 Mycotoxin contamination of staple cereals

The above validated PHWE method together with the solvent extraction method was used for the screening of 15 different mycotoxin in samples of maize (n=16), sorghum (n=38) and millet (n=37) obtained from six agroecological zones from Nigeria (Table 5.5). Only samples contaminated with mycotoxins at levels above the respective LODs were considered positive. The minor differences between the results from the two extraction methods, are possibly due to the differences in the LOD's and LOQ's, as well as the recovery rates of the two methods. For the sake of discussing the results, in the remaining sections of this paper, reference is made only to the results of the PHWE.

5.3.2.1 Mycotoxin levels and incidence rate in maize, sorghum and millet from Nigeria

The incidence rates and levels of mycotoxin contamination in maize, sorghum and millet samples are presented in Table 5.5. All of the maize samples (n=16), 32% (n=38) of sorghum and 35% (n=37) of millet samples were non-complaint, *i.e.,* were positive for at least one of the 15 tested mycotoxins. Fumonisins, in particular FB_1 had the highest prevalence in terms of rate of occurrence and levels of contamination in all three cereals. All the maize samples (n=16) were positive for FBs with contamination levels ranging from 17 to 7,947 µg/kg for FB_1 and 30 to 14,603 µg/kg for $\Sigma FB_1\&FB_2$. Out of the 16 analyzed maize samples, 7 samples (*i.e.,* 44%) contained $\Sigma FB_1\&FB_2$ at levels above the maximum levels of 4,000 µg/kg in unprocessed maize stipulated by the European Commission (EC) and CODEX (EC, 2007; WHO, 2017; Arroyo-Manzanares *et al*., 2018). Previous studies have equally reported high incidence rates and levels of FBs contamination in maize from Nigeria (Afolabi *et al*., 2006; Adetunji *et al*., 2014). Bankole and Mabekoje (2004) found that FB_1 was the predominant mycotoxin, occurring in 79% of samples of maize obtained from a similar region from Nigeria as we sampled (Southern Nigeria).

Such high incidence rates and even higher levels of FBs contamination have likewise been reported in other West African countries. Ngoko *et al*. (2001) found FB_1 in 16 out of 18 maize samples from Cameroon at levels within the range 300 to 26,000 µg/kg. Fumonisins occurred in sorghum samples at relatively lower levels, with a mean value of 17 µg/kg and maximum value of 248 µg/kg for $\Sigma FB_1\&FB_2$, while, no FBs contamination was recorded in millet samples. Chilaka *et al*. (2016) reported a mean value of 83 µg/kg and a maximum value of 180 µg/kg for $\Sigma FB_1\&FB_2$ in

sorghum. The International Agency for Research on Cancer (IARC) has classified FB_1 as a group 2B carcinogen (possibly carcinogenic to humans) (IARC, 2002). Consumption of foods contaminated with FBs have been directly linked with upper gastro-intestinal tract cancer (Soriano and Dragacci, 2004). Moreover, FBs are also nephrotoxic, hepatotoxic, immunosuppressive, atherogenic and embryotoxic in experimental animal systems (Nair, 1998).

AFs contamination was also relatively high in the cereal samples. In maize, levels for AFB_1 ranged from 6.5 to 315 µg/kg, with a mean of 54 µg/kg, while ΣAFs ranged from 16 to 323 µg/kg. Sixty-nine percent (n=16) of the maize samples were contaminated above the maximum level of 2 and 4 µg/kg for AFB_1 and ΣAFs, respectively, stipulated by the European Commission (EC) (Commission Regulation, 2006b; Arroyo-Manzanares *et al*., 2018). These levels are similar to those reported by Bandyopadhyay *et al*. (2007), who reported ΣAFs contamination ranging from 1.1 to 480 µg/kg and a mean of 36 µg/kg in freshly harvested maize in Nigeria. In sorghum, 8% of samples were above the EC limits for AFB_1 (2 µg/kg) and ΣAFs (4 µg/kg), respectively, whereas, 5% of millet samples exceeded similar limits for AFB_1 and ΣAFs. Aflatoxin B_2, AFG_1 and AFG_2 were not detected in any of the millet samples, however, the observed levels for AFB_1 (5.5 to 50 µg/kg) were in agreement to those reported by Apeh *et al*. (2016) (1.1 to 15 µg/kg) in millet grain from Nigeria. Observed levels for ΣAFs in sorghum (15 to 116 µg/kg) were less than those reported by Makun *et al*. (2009) (<LOQ to 1,164 µg/kg) in stored sorghum samples. This could be due to variations in fungi colonization of crops over different years which could be stimulated by annual variations in temperature and rainfall, in addition to other climatic conditions (Streit *et al*., 2013; Guerre, 2016). Generally, AFs were more prevalent in maize, followed by sorghum and then millet. A similar trend was observed by Bandyopadhyay *et al*. (2007), in their study on the relative severity of AFs contamination of cereal crops in West Africa. In fact, they observed that Nigerians consume 138 kg cereals annually, and if the main cereal is sorghum instead of maize, associated AF problems will be diminished 4-fold, whereas, if it is millet, then the AF-related risks will be reduced at least 8-fold (Bandyopadhyay *et al*., 2007). Diversification of diets, instead of diets that are heavily dependent on maize could greatly reduce exposure to AFs and their consequent health-related problems. Aflatoxins are highly carcinogenic and are equally recognized as being immunosuppressive. Among the AFs group, AFB_1 is considered the most toxic, and has been identified as the most potent naturally occurring carcinogen known to man (IARC, 2002; D'Mello, 2003).

Table 5.5: Mycotoxin contamination of maize, sorghum and millet from Nigeria using PHWE and solvent extraction[#].

Mycotoxin	Maize (n=16)					Sorghum (n=38)					Millet (n=37)				
	Positive samples (%)	**Mean*** (µg/kg)	**Range** (µg/kg)	**Samples >LOQ (%)**	**Samples >Limit (%)**	**Positive samples (%)**	**Mean*** (µg/kg)	**Range** (µg/kg)	**Samples >LOQ (%)**	**Samples >Limit (%)**	**Positive samples (%)**	**Mean*** (µg/kg)	**Range** (µg/kg)	**Samples >LOQ (%)**	**Samples >Limit (%)**
*AFB_1	69	54	6.5-315	69	69	8	3.6	7.3-116	8	8	5	2.4	38-50	5	5
	44[#]	18[#]	15-74[#]	44[#]	44[#]	0[#]	0[#]	<LOQ[#]	0[#]	0[#]	3[#]	0.33[#]	<LOQ-9.4[#]	3[#]	3[#]
AFB_2	69	1.3	0.31-7.8	63	0	0	0[#]	<LOQ	0	0	0	0	<LOQ	0	0
	44[#]	7.0[#]	3.3-13[#]	44[#]	0[#]	0[#]	0[#]	<LOQ[#]	0[#]	0[#]	0[#]	0[#]	<LOQ[#]	0[#]	0[#]
AFG_1	0	0	<LOQ	0	0	3	0.19	<LOQ-7.3	3	0	0	0	<LOQ	0	0
	0[#]	0[#]	<LOQ[#]	0[#]	0[#]	0[#]	0[#]	<LOQ[#]	0[#]	0[#]	0[#]	0[#]	<LOQ[#]	0[#]	0[#]
AFG_2	0	0	<LOQ	0	0	0	0	<LOQ	0	0	0	0	<LOQ	0	0
	0[#]	0[#]	<LOQ[#]	0[#]	0[#]	0[#]	0[#]	<LOQ[#]	0[#]	0[#]	0[#]	0[#]	<LOQ[#]	0[#]	0[#]
*ΣAFs	69	56	16-323	50	69	8	3.8	15-116	8	8	5	2.4	38-50	5	5
	44[#]	21[#]	18-83[#]	44[#]	44[#]	0[#]	0[#]	<LOQ[#]	0[#]	0[#]	3[#]	0.67[#]	<LOQ[#]	0[#]	3[#]
AME	0	0	<LOQ	0	0	3	0.99	<LOQ-38	3	0	8	21	128-343	8	0
	0[#]	0[#]	<LOQ[#]	0[#]	0[#]	3[#]	0.89[#]	<LOQ-34[#]	3[#]	0[#]	16[#]	8.5[#]	24-117[#]	16[#]	0[#]
*FB_1	100	2,033	17-7,947	100	0	8	17	188-248	8	0	0	0	<LOQ	0	0
	94[#]	1,644[#]	68-7,105[#]	94[#]	0[#]	5[#]	6.8[#]	97-159[#]	5[#]	0[#]	0[#]	0[#]	<LOQ[#]	0[#]	0[#]
FB_2	100	1,525	37-5,961	94	6	0	0	<LOQ	0	0	0	0	<LOQ	0	0
	88[#]	527[#]	57-2,074[#]	88[#]	0[#]	0[#]	0[#]	<LOQ[#]	0[#]	0[#]	0[#]	0[#]	<LOQ[#]	0[#]	0[#]
FB_3	94	292	7.5-767	94	0	3	0.41	<LOQ -16	3	0	0	0	<LOQ	0	0
	81[#]	186[#]	33-689[#]	81[#]	0[#]	0[#]	0[#]	<LOQ[#]	0[#]	0[#]	0[#]	0[#]	<LOQ[#]	0[#]	0[#]
*$\Sigma FB_1\&B_2$	100	3,851	30-1,460	100	44	8	17	188-248	8	0	0	0	<LOQ	0	0
	94[#]	2,171[#]	68-9,179[#]	94[#]	19[#]	5[#]	6.8[#]	97-159[#]	5[#]	0[#]	0[#]	0[#]	<LOQ[#]	0[#]	0[#]
*OTA	31	8.3	6.5-54	31	31	5	0.59	7.5-15	5	5	5	0.73	6.9-20	5	5
	13[#]	4.0[#]	4.7-54[#]	13[#]	6[#]	0[#]	0[#]	<LOQ[#]	0[#]	0[#]	0[#]	0[#]	<LOQ[#]	0[#]	0[#]
OTB	13	1.5	7.8-17	13	0	3	0.20	<LOQ -7.5	3	0	5	0.37	6.8-6.9	5	0
	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]
ΣOTs	31	9.8	6.5-58	31	0	5	0.79	7.5-23	5	0	5	1.1	14-27	5	0
	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]

Mycotoxin	Maize (n=16)					Sorghum (n=38)					Millet (n=37)				
	Positive samples (%)	**Mean*** (µg/kg)	**Range** (µg/kg)	**Samples >LOQ (%)**	**Samples >Limit (%)**	**Positive samples (%)**	**Mean*** (µg/kg)	**Range** (µg/kg)	**Samples >LOQ (%)**	**Samples >Limit (%)**	**Positive samples (%)**	**Mean*** (µg/kg)	**Range** (µg/kg)	**Samples >LOQ (%)**	**Samples >Limit (%)**
STEG	13	0.86	6.5-7.3	13	0	13	15	1.9-330	13	0	24	7.4	5.0-208	14	0
	0[#]	0[#]	<LOQ[#]	0[#]	0[#]	13[#]	18[#]	9.9-272[#]	13[#]	0[#]	5[#]	6.9[#]	5.0-188[#]	5[#]	0[#]
*T-2	0	0	<LOQ	0	0	3	0.20	<LOQ -7.5	3	0	3	0.65	<LOQ-23	3	0
	0[#]	0[#]	<LOQ[#]	0[#]	0[#]	0[#]	0[#]	<LOQ[#]	0[#]	0[#]	0[#]	0[#]	<LOQ[#]	0[#]	0[#]
*ZEA	19	3.5	7.4-33	19	0	11	1.1	4.8-22	11	0	14	12	80-94	8	0
	0[#]	0[#]	<LOQ[#]	0[#]	0[#]	0[#]	0[#]	<LOQ[#]	0[#]	0[#]	5[#]	1.5[#]	<LOQ[#]	0[#]	0[#]
α-ZEL	31	31	<LOQ-140	6	0	5	6.5	<LOQ	0	0	0	0	<LOQ	0	0
	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]
β-ZEL	19	4.7	<LOQ	0	0	8	2.9	<LOQ-63	3	0	0	0	<LOQ	0	0
	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]
ΣZEAs	44	39	72-155	31	0	11	10	<LOQ	0	0	14	12	80-94	8	0
	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]	ND[#]

Key: [#] Parameter values for solvent extraction. % + ve Samples: percentage of positive samples (*i.e.* samples >LOD). Mean*: average contamination of the samples including positive (*i.e.* >LOD) and negative (*i.e.* <LOD) samples. Samples >Limit: number of samples with contamination levels above the maximum limit set by the EC. N.D.: not detected. AFB_1: aflatoxin B_1. AFB_2: aflatoxin B_2. AFG_1: aflatoxin G_1. AFG_2: aflatoxin G_2. AME: alternariol monomethyl ether. FB_1: fumonisin B_1. FB_2: fumonisin B_2. FB_3: fumonisin B_3. OTA: ochratoxin A. OTB: ochratoxin B. STEG: sterigmatocystin. T-2: T-2 toxin. ZEA: zearalenone. α-ZEL: α-zearalenol. β-ZEL: β-zearalenol. ΣZEAs: sum of ZEAs. ΣOTs: sum of OTs. $\Sigma FB_1\&FB_2$: sum of FB_1 and FB_2. ΣAFs: sum of AFs. *Indicate the mycotoxins that are regulated by the EC (Arroyo-Manzanares *et al.*, 2018. Commission Recommendation, 2013, 2006b), with their respective regulatory limits as follows: AFB_1 in all cereals and their derived products 2 µg/kg and 4 µg/kg for ΣAFs (Commission Regulation, 2006b), $\Sigma FB_1\&FB_2$ in unprocessed maize and maize flour/meal – 4,000 µg/kg (Arroyo-Manzanares *et al.*, 2018. EC, 2007. WHO, 2017), OTA in unprocessed cereals – 5 µg/kg (Commission Regulation, 2006b), Sum of T-2 and HT-2 in unprocessed cereals – 100 µg/kg (Commission Recommendation, 2013), ZEA in unprocessed cereals other than maize – 100 µg/kg (EC, 2007) and ZEA in unprocessed maize – 350 µg/kg (EC, 2007).

Ochratoxin A was present in 31% (n=16) of the maize samples at levels ranging from 6.5 to 54 µg/kg. All positive maize samples for OTA were above the maximum level of 5 µg/kg in unprocessed cereals stipulated by the EC (Commission Regulation, 2006b). In sorghum, the levels of OTs varied from 7.5 to 15 µg/kg, while those for millet ranged from 0.75 to 20 µg/kg. Sangare-Tigori *et al*. (2006) reported higher OTA levels (17 to 204 µg/kg) in millet from the West African country of Côte d'Ivoire sampled between 1998 to 2002, which may be due to yearly variations in mycotoxin contamination patterns across the continent (Streit *et al*., 2013; Guerre, 2016; Van der Fels-Klerx *et al*., 2016). Exposure to OTA has been linked with nephropathy (Maaroufi *et al*., 1995), urinary tract tumors (Gazinska *et al*., 2012) and oxidative DNA damage leading to mutagenesis and eventually cancer (Zepnik *et al*., 2001). Based on its carcinogenicity in animal studies, OTA has also been classified as a group 2B possible human carcinogen by the International Agency for Research on Cancer (IARC) (IARC, 1993).

The incidence rate and contamination range of ZEA in maize and sorghum and millet was 19% (7.4 to 33 µg/kg) and 11% (4.8 to 22 µg/kg), respectively. These levels are negligible when compared with the EC maximum limit of 350 µg/kg for unprocessed maize and 100 µg/kg for unprocessed cereals other than maize, respectively (EC, 2007). On the other hand, 5 out of 37 millet samples were positive for ZEA within the range 80 to 94 µg/kg, none of which exceeded the EC limits of 100 µg/kg in unprocessed cereals other than maize (EC, 2007). The ZEA analogues, α-ZEL and β-ZEL, were also detected in at least one of maize and sorghum samples each, while none of the analogues was detected in millet samples. Chilaka *et al*. (2016) and Adetunji *et al* (2014) reported maize and millet contamination with α-ZEL and β-ZEL (Adetunji *et al*., 2014; Chilaka *et al*., 2016).

Cereal contamination by ZEAs could be a major health concern as this toxin is known to be chemically stable both during various food processing operations such as cooking, heating, fermentation, milling etc., and has been quantified in a number of processed cereal-based products from Africa (Abia *et al*., 2013; Hueza *et al*., 2014; Adekoya *et al*., 2017). Zearalenone has been implicated in the manifestations of gynecomastia with testicular atrophy in rural males in Southern Africa (Shephard, 2008b). Among human populations, children are the most vulnerable to ZEA exposure and the toxin has been implicated in several incidents of precocious pubertal changes

(Zain, 2011) and other fertility problems (Sherif *et al*., 2009). The potency of ZEA's estrogenic activity is reportedly greater than that of many naturally occurring non-steroidal estrogens (Bennett and Klich, 2003). Exposure to high concentrations of ZEA in cattle feed has been linked with enlargement of the mammary gland, infertility, reduced milk production, vaginal secretions and vaginitis particularly in young dairy heifers (Zinedine *et al*., 2007). Whereas in swine, effects of ZEA include enlargement of the uterus, vaginal prolapse, swelling of the vulva, infertility, reduced litre size and embryonic death (Agag, 2004).

The Alternaria toxin, AME, and the trichothecene toxin, T-2, were not detected in any of the maize samples. Bankole *et al*. (2010) also reported the absence of T-2 contamination in maize from Nigeria. In sorghum (n=38) and millet (n=37) samples, T-2 occurred in 3% each, whereas, the average AME contamination was 0.99 and 21 µg/kg for sorghum and millet, respectively. Sterigmatocystin occurred in 13% of both maize (n=16) and sorghum (n=38) samples within the ranges of 6.5 to 7.3 µg/kg and 1.9 to 330 µg/kg, respectively, whereas 9 out of 37 millet samples were positive for STEG at concentrations ranging from 5 to 208 µg/kg. Elsewhere, STEG was reported as a contaminant of Nigerian maize (Adetunji *et al*., 2014). While the toxicity of T-2 has been established in literature (Li *et al*., 2011; Adhikari *et al*., 2017), the toxic effects of STEG and AME to humans have remained largely limited. Nonetheless, it is known that STEG is a precursor for the biosynthesis of AFB_1 and both have similar structural configurations, as such, STEG is considered as a potent mutagen, carcinogen, and teratogen (Davis, 1981; Chu, 2003). The IARC classifies STEG as a group 2B carcinogen (IARC, 1976; Chu, 2003).

A number of studies have demonstrated the stability of AME during extreme food processing conditions such as during wet baking of bread (Siegel *et al*., 2010; Abia *et al*., 2013), as such, there is a risk of secondary exposure to AME through processed cereal-based foods. Adekoya *et al*. (2017) reported AME contamination of gruels derived directly from maize and sorghum in Nigeria. Some studies have demonstrated the possible carcinogenicity and mutagenicity of AME (Liu *et al*., 1992; Brugger *et al*., 2006; Ostry, 2008). For example, NIH/3T3 cells mutated by AME caused subcutaneous tumors in mice (Liu *et al*., 1991). It has also been shown to induce DNA strand breaks in cell cultures (Podlech & Marko, 2009). In general, mycotoxin contamination was higher in maize, followed by sorghum and then millet (Table 5.5). A similar trend was observed by Makun *et al*. (2013) and Gwary *et al*. (2012). It has been shown that tannin-rich varieties of

sorghum and millet are less susceptible to fungal colonization (Apeh *et al*., 2016; Dykes & Rooney, 2006), indicating that phytochemicals present in these cereals could exert antimycological properties as such resulting in less mycotoxin contamination.

5.3.2.2 Mycotoxin distribution patterns in maize, sorghum and millet from different agroecological zones of Nigeria

Geoclimatic conditions under which crops are cultivated are critical for fungi proliferation and attendant mycotoxin production (Leema *et al*., 2011; Medina *et al*., 2014; Apeh *et al*., 2016). Table 5.6 presents the distribution patterns of mycotoxins in maize, sorghum and millet across 5 agroecological zones in Nigeria. Maize samples generally had higher incidence rate and mycotoxin contamination levels. This could be because all the maize samples were obtained from the HRF/DRS agroecological zones. Also, as stated earlier in Section 5.3.2.1, maize is generally more susceptible to contamination by mycotoxigenic fungal species and consequent mycotoxin production as compared to sorghum and millet (Chilaka *et al*., 2016; Sirma *et al*., 2016). Likewise, in millet samples contamination levels increased from Southern to Northern Nigeria, with samples from Northern having the lowest levels of contamination and incidence rates. A similar trend was observed by Chilaka *et al*. (2016), who reported contamination/incidence rate of multi-mycotoxin contamination in DRS>NGS>SHS.

The observed high incidence rate and contamination levels of mycotoxins in maize, sorghum and millet samples from the HRF/DRS agroecological zones could be partly due to the climatic conditions in these regions that favor the proliferation of mycotoxigenic fungi species and subsequent mycotoxin production (Atehnkeng *et al*., 2008). As already described in Section 5.2.2.1, the HRS is characterized by abundant rainfall (1,500 to 2,000 mm/yr), high humidity (78 to 100%) and average temperatures between 25 to 28 °C (Afolabi *et al*., 2013; Amajama *et al*., 2016), while the DRS vegetation represents a transition between the humid rainforest and guinea savannah zones. Annual rainfall in this zone ranges from 1,200 mm to 1,700 mm, while average humidity and temperature are 66 to 78% and 26 to 27 °C, respectively (Adejumo & Adejoro, 2014). Adejumo and Adejoro (2014), noted that the important agroecological zones in terms of mycotoxin research in Nigeria are the HRF, DRS and SGS/NGS zones.

Table 5.6: Distribution of mycotoxins in maize, sorghum and millet across different agroecological zones in Nigeria as analyzed using PHWE followed by UHPLC-MS/MS.

Mycotoxin	Maize		Sorghum				Millet					
	HRF/DRS (n=16)		HRF/DRS (n=12)		SHS (n=26)		HRF/DRS (n=20)		NGS/SGS (n=9)		SHS (n=8)	
	Mean* (µg/kg)	Range (µg/kg)	Mean* (µg/kg)	Range (µg/kg)	Mean* (µg/kg)	Range (µg/kg)	Mean* (µg/kg)	Range (µg/kg)	Mean* (µg/kg)	Range (µg/kg)	Mean* (µg/kg)	Range (µg/kg)
AFB_1	54.41	6.50-315.00	10.92	15.00-116.00	0.28	<LOQ-7.25	2.55	<LOQ-49.50	4.17	<LOQ-37.50	0.00	<LOQ
AFB_2	1.30	0.31-7.80	0.00	<LOQ	0.00	<LOQ	0.00	<LOQ	0.00	<LOQ	0.00	<LOQ
AFG_1	0.00	<LOQ	0.00	<LOQ	0.28	<LOQ-7.25	0.00	<LOQ	0.00	<LOQ	0.00	<LOQ
AFG_2	0.00	<LOQ	0.00	<LOQ	0.00	<LOQ	0.00	<LOQ	0.00	<LOQ	0.00	<LOQ
ΣAFs	55.71	15.60-322.80	10.92	15.00-116.00	0.56	<LOQ-14.50	2.55	<LOQ-49.50	4.17	<LOQ-37.50	0.00	<LOQ
AME	0.00	<LOQ	0.00	<LOQ	1.44	<LOQ-37.50	24.30	127.50-343.00	30.50	<LOQ-274.50	0.00	<LOQ
FB_1	2,033.00	16.92-7,947.00	0.00	<LOQ	24.25	187.50-248.00	0.00	<LOQ	0.00	<LOQ	0.00	<LOQ
FB_2	1,525.00	36.56-5,961.00	0.00	<LOQ	0.00	<LOQ	0.00	<LOQ	0.00	<LOQ	0.00	<LOQ
FB_3	292.10	7.50-767.30	0.00	<LOQ	0.60	15.50-15.50	0.00	<LOQ	0.00	<LOQ	0.00	<LOQ
$\Sigma FB_1\&FB_2$	3,851.00	29.60-14,603.00	0.00	<LOQ	24.25	187.50-248.00	0.00	<LOQ	0.00	<LOQ	0.00	<LOQ
OTA	8.28	6.50-54.25	1.88	7.50-15.00	0.00	<LOQ	1.36	6.88-20.25	0.00	<LOQ	0.00	<LOQ
OTB	1.52	7.75-16.50	0.63	<LOQ-7.50	0.00	<LOQ	0.68	6.75-6.88	0.00	<LOQ	0.00	<LOQ
ΣOTs	9.80	6.50-57.75	2.50	7.50-22.50	0.00	<LOQ	2.04	13.75-27.00	0.00	<LOQ	0.00	<LOQ
STEG	0.86	6.50-7.25	27.81	3.75-330.00	9.64	1.94-204.80	12.81	11.00-207.70	1.74	<LOQ -5.00	0.00	<LOQ
T-2	0.00	<LOQ	0.00	<LOQ	0.29	<LOQ-7.50	0.04	<LOQ	2.58	<LOQ-23.25	0.00	<LOQ
ZEA	3.49	7.38-33.00	3.06	7.50-21.75	0.18	4.75-4.75	9.99	90.00-90.00	26.94	80.00-93.50	0.94	<LOQ
α-ZEL	30.53	139.50-139.50	0.00	<LOQ	9.19	<LOQ	0.00	<LOQ	0.00	<LOQ	0.00	<LOQ
β-ZEL	4.66	<LOQ	5.25	63.00-63.00	1.80	<LOQ	0.00	<LOQ	0.89	<LOQ	0.00	<LOQ
ΣZEAs	38.68	71.50-155.00	8.31	<LOQ	11.18	<LOQ	9.99	<LOQ-90.00	27.83	80.00-93.50	0.94	<LOQ

Key: HRF: Humid rain forest. DRS: Derived savannah. NGS: Northern guinea savannah. SGS: Southern guinea savannah. SHS: Sahel savannah. Mean*: average contamination of the samples including positive (i.e. >LOD) and negative (i.e. <LOD) samples. AFB_1: aflatoxin B_1. AFB_2: aflatoxin B_2. AFG_1: aflatoxin G_1. AFG_2: aflatoxin G_2. AME: alternariol monomethyl ether. FB_1: fumonisin B_1. FB_2: fumonisin B_2. FB_3: fumonisin B_3. OTA: ochratoxin A. OTB: ochratoxin B. STEG: sterigmatocystin. T-2: T-2 toxin. ZEA: zearalenone. α-ZEL: α-zearalenol. β-ZEL: β-zearalenol. ΣZEAs: sum of ZEAs. ΣOTs: sum of OTs. $\Sigma FB_1\&FB_2$: sum of FB_1 and FB_2. ΣAFs: sum of AFs.

Interestingly, this HRF zone spans across many of the West African countries, as such, similar patterns of mycotoxin contamination have been recorded in some of these countries (Hell *et al*., 2000; Ngoko *et al*., 2001). Hell *et al*. (2000), Udoh *et al*. (2000) and Gong *et al*. (2003) observed significant AFs contamination of maize in lowland areas in Cameroon, Nigeria, Benin and Togo, respectively. A similar trend was reported for FBs (10 to 16,040 μg/kg) in maize from Burkina Faso (Nikiema *et al*., 2004) and FB_1 (300 to 26,000 μg/kg) in maize from Cameroon (Ngoko *et al*., 2001). On the other hand, the lower levels of mycotoxin contamination and incidence rates observed in the Middle-Belt and Northern parts of Nigeria *i.e.*, the SGS/NGS and SHS, respectively, could be due to dryer and more arid climatic conditions in these regions. Particularly the SHS has a much lower annual rain fall and humidity as compared to DRS and HRF. Temperatures can reach as high as 40 °C (Atehnkeng *et al*., 2008; Benson *et al*., 2017). Such conditions may not favor the proliferation of mycotoxigenic fungi species.

5.3.2.3 Simultaneous occurrence of multiple mycotoxins in maize, sorghum and millet from Nigeria as analyzed using PHWE followed by UHPLC-MS/MS

Based on the results of this study, exposure of humans and animals to multiple mycotoxins is highly likely in the selected regions in Nigeria, as we observed co-occurrence of different groups of mycotoxins in many of the analyzed samples. This observation is in line with previous literature reports (Chilaka *et al*., 2016; Somorin *et al*., 2016; Adekoya *et al*., 2018). Mycotoxin co-contamination of crops is a complex phenomenon, and a number of interrelating factors, such as fungi specie, crop genotype, and climatic conditions may be responsible for the co-occurrence of mycotoxins in foods. It is known that a single fungal specie may be able to produce more than one mycotoxin (Bayman & Baker, 2006; McCormick *et al*., 2011), while one mycotoxin can be produced by different fungi species (Alasiri *et al*., 2013; Ismaiel & Papenbrock, 2015).

Table 5.7: Co-occurrence of different groups of regulated mycotoxins in maize, sorghum and millet samples from Nigeria.

Co-contaminants	Number of co-occurrences		
	Maize (n=16)	Sorghum (n=38)	Millet (n=37)
AFs+FBs	6	0	0
AFs+FBs+OTs	3	0	0

Co-contaminants	Number of co-occurrences		
	Maize (n=16)	Sorghum (n=38)	Millet (n=37)
AFs+FBs+OTs+ZEAs	1	0	0
AFs+FBs+ZEAs	1	0	0
AFs+OTs+ZEAs	0	1	0
AFs+ZEAs	0	1	0
FBs+OTs+ZEAs	1	0	0
FBs+T-2	0	1	0
OTs+ZEAs	0	0	1

Key: AFs: aflatoxins. FBs: fumonisins. OTs: ochratoxins. T-2: T-2 toxin. ZEAs: zearalenone and its analogues α- and β-zearalenol

Table 5.7 and Appendix 5.B describe all the possible co-occurrence patterns of the five groups of regulated mycotoxins detected in the samples, which are AFs, FBs, OTs, T-2 and ZEAs. This was achieved using the Venn diagram web application (Bioinformatics and Evolutionary Genomics, Ghent University, Belgium) (BEG Group, 2018). The 5 groups of mycotoxins subjected to this analysis yielded 9 different unique intersections as summarized in Table 5.7. The highest co-occurrence of mycotoxins that appeared in the maize samples was the co-contamination of AFs and FBs (*i.e.* AFs+FBs) which occurred in 6 (n=16) of the maize samples, while 3 of the samples simultaneously contained AFs, FBs and OTs. A similar pattern was observed in 5,000 samples submitted to the GEMS/Food contaminants database between 2011 and 2016, where reiterations of FBs+AFs combinations occurred in approximately 6% of the maize samples, 1% of sorghum (JECFA, 2016). A combination of AFs+FBs+OTs+ZEAs also occurred in one (n=16) of the maize samples, whereas, a combination of AFs+OTs+ZEAs occurred in one (n=38) of the sorghum samples. The co-occurrence of OTs+ZEAs was observed in a single millet sample.

Such co-contamination patterns have previously been reported in cereals from Nigeria (Bankole & Mabekoje, 2004; Makun *et al*., 2011; Adetunji *et al*., 2014; Chilaka *et al*., 2016). Bankole and Mabekoje (2004) reported that 15 samples (n=103) of pre-harvest maize from Southern Nigeria were contaminated with both FBs and AFs simultaneously. A prevalence rate of 10% and mean contamination level of 111 µg/kg have been reported for OTA, concurrently with OTB 7% (7.5 µg/kg), STEG 37% (3 µg/kg), ZEA 17% (174 µg/kg), α-ZEL 1% (17 µg/kg), and β-ZEL 1% (13 µg/kg) in stored maize from five different agroecological zones in Nigeria (Adetunji *et al*., 2014). A review of over a hundred papers between 1987 to 2016, revealed 127 mycotoxin combinations, of which AFs+FBs, AFs+OTA, DON+ZEA, and FBs+ZEA were amongst the most frequently co-

occurring combinations in cereal crops (Smith *et al*., 2016). In Tanzania, co-exposure to FBs+AFs has been confirmed by means of plasma or urinary biomarkers of AF_1 and FB_1 (JECFA, 2016). Co-occurrence can be caused by substrate colonization with a single fungus that produces more than one mycotoxin, or due to colonization by different fungi species that produce different mycotoxins. It has been reported that ZEA usually co-occurs with one or more of the THs, because of the ability of its producing fungi to synthesize more than one mycotoxin (Grenier & Oswald, 2011).

Since the individual toxins that make these combinations are all amongst the most potent mycotoxins, their co-existence must not be neglected (Smith *et al*., 2016). The combined effects of different mycotoxins have been extensively reviewed in literature (De Ruyck *et al*., 2015; JECFA, 2016; Smith *et al*., 2016), and could manifest as additive, synergistic or antagonistic (JECFA, 2016). For example, exposure of F344 rats to FB_1+AFB_1 combinations increased liver preneoplastic changes suggestive of a synergistic interaction (Qian *et al*., 2016), whereas, health concerns in humans include possible childhood stunting (Chen *et al*., 2018). All cytotoxic effects of the binary combinations of OTA, FB_1 and AFB_1 in low concentrations at their EU regulatory limits to MDBK cell lines were additive, and in the order OTA+FB_1 > AFB_1+FB_1 > AFB_1+OTA (Clarke *et al*., 2014), while FB_1+α-ZEL combination significantly diminished interferon γ mRNA expression as compared to α-ZEL alone (Luongo *et al*., 2006).

5.3.3 Significance of mycotoxin contamination of Nigerian staple cereals

We reiterate that mycotoxin contamination of maize, sorghum and millet crops in Nigeria represents a major food safety concern because these crops are staples. Atanda *et al*. (2015) asserted that AF contamination in maize and peanuts in Nigeria contributed to at least 7,761 cases of liver cancer, resulting in a total burden of 100,965 Disability-Adjusted Life Years (DALYs) (Atanda *et al*., 2015). Derived food products from these cereals such as *masa, tuwo*, *ogi*, *ogi-baba*, *kunu*, *burukutu* and others are also at risk of mycotoxin contamination (Kpodo *et al*., 1996; Adejumo & Adejoro, 2014; Ezekiel *et al*., 2015; Chilaka *et al*., 2016, 2018; Adekoya *et al*., 2017; Njobeh & Olotu, 2017). Some of these processed food products (e.g. *ogi* and *ogi-baba*) are used as weaning foods for children. Sadly, a number of studies have reported mycotoxin contamination in these weaning foods (Oluwafemi & Ibeh, 2011; Adekoya *et al*., 2017; Adetunji *et al*., 2017). In

fact, a study (Oluwafemi & Ibeh, 2011), reported AFB_1 contamination levels up to 4,806 µg/kg in home-made weaning food made from maize and soybeans (Oluwafemi & Ibeh, 2011). Moreover, a posthumous autopsy of infants who suffered from kwashiorkor showed a significant level of AFs in their brains, because of consumption of contaminated maize-derived gruels (Oyelami *et al*., 1997).

Aside the health effects on humans and animals, mycotoxin prevalence in Nigeria and Africa as a whole has other significant socio-economic impacts, ranging from food security, decreased market value of crops, regulatory rejections of goods mainly at ports of exit, damage to the African agricultural export brand amongst others (Gbashi *et al*., 2018). For example, in 2010, the monetized burden of AFs contamination in Nigeria was estimated to be between 112 million U$D and 942 million U$D, which accounts for roughly 0.5% of the nation's GDP (Atanda *et al*., 2015). These enormous impacts of mycotoxin could significantly jeopardize prospects of attaining the UN's sustainable development goal number 2, of achieving food security, improved nutrition, and a healthy agroeconomic growth by 2030 (Gbashi *et al*., 2018).

5.4 Conclusion

Mycotoxin contamination of food and feed continues to remain a major challenge to public health in Nigeria. In this study, an environmentally friendly extraction method (PHWE), coupled with UHPLC-MS/MS analysis in comparison to a solvent-based method of extraction was used to determine the natural occurrence of multi-mycotoxins in maize, sorghum and millet, collected from different agroecological regions in Nigeria. Aside the adequacy of the method validation parameters, both univariate and multivariate (PCA and OPLS-DA) statistical models indicated the relative similarities of the results obtained from the two methods, which further authenticated the applicability of PHWE as an effective method for a research-related survey of multi-mycotoxin screening in cereals.

The survey results revealed the presence of all fifteen analyzed mycotoxins, including FBs, AFs, OTs, ZEAs, T-2, AME and STEG, in at least one sample of each cereal. Fumonisins, AFs and ZEAs were the predominant contaminants of the cereals, and maize was the most susceptible substrate, followed by sorghum and millet. Some of the samples were contaminated with mycotoxins at levels significantly higher than the maximum limit by the EC and CODEX. Also,

elaborate combinations of the different groups of regulated mycotoxins were observed. This stimulates curiosity about the possibility of their synergistic effects on the local populace that consume these crops. We also observed some fairly discrete mycotoxin contamination patterns across the different agroecological zones in the country depending on the substrate.

Considering the economic importance of these cereals in Nigeria, it is thus imperative to prioritize the adoption of functional mitigation strategies that are both cost-effective, crop-specific and locally adapted to the climatic conditions and agronomic practices of the region, in order to adequately combat the prevalence of these toxins in Nigeria. Further to this, more research should be done to determine the toxicological effects of different mycotoxin combinations in order to better understand their associated public health risks. If this is done, then future establishment of regulatory limits or as the case may be, reassessment of already existing limits could take into account the effects of co-existing mycotoxins. The successful adoption of PHWE in this study further represents a step closer to sustainability in green-solvent extraction in the field of mycotoxicology.

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References

Abia, W. A., Warth, B., Sulyok, M., Krska, R., Tchana, A. N., Njobeh, P. B., Dutton, M. F. and Moundipa, P. F. (2013). Determination of multi-mycotoxin occurrence in cereals, nuts and their products in Cameroon by liquid chromatography tandem mass spectrometry (LC-MS/MS). *Food Control*, *31*(2), 438–453.

Adejumo, T. O., and Adejoro, D. O. (2014). Incidence of aflatoxins, fumonisins, trichothecenes and ochratoxins in Nigerian foods and possible intervention strategies. *Food Science and*

Quality Management, *31*, 127–146.

Adekoya, I., Njobeh, P., Obadina, A., Chilaka, C., Okoth, S., De Boevre, M., and De Saeger, S. (2017). Awareness and prevalence of mycotoxin contamination in selected Nigerian fermented foods. *Toxins*, *9*(11), 363.

Adekoya, I., Obadina, A., Adaku, C. C., De Boevre, M., Okoth, S., De Saeger, S., and Njobeh, P. (2018). Mycobiota and co-occurrence of mycotoxins in South African maize-based opaque beer. *International Journal of Food Microbiology*, *270*, 22–30.

Adetuniji, M. C., Atanda, O. O., Ezekiel, C. N., Dipeolu, A. O., Uzochukwu, S. V. A., Oyedepo, J., and Chilaka, C. A. (2014). Distribution of mycotoxins and risk assessment of maize consumers in five agro-ecological zones of Nigeria. *European Food Research and Technology*, *239*(2), 287–296.

Adetunji, M., Atanda, O., Ezekiel, C. N., Sulyok, M., Warth, B., Beltrán, E., and Chilaka, C. A. (2014). Fungal and bacterial metabolites of stored maize (*Zea mays*, L.) from five agro-ecological zones of Nigeria. *Mycotoxin Research*, *30*(9), 89–102.

Adetunji, M. C., Atanda, O. O., and Ezekiel, C. N. (2017). Risk assessment of mycotoxins in stored maize grains consumed by infants and young children in Nigeria. *Children*, *4*(7), 58.

Adhikari, M., Negi, B., Kaushik, N., Adhikari, A., Al-Khedhairy, A. A., Kaushik, N. K., and Choi, E. H. (2017). T-2 mycotoxin: toxicological effects and decontamination strategies. *Oncotarget*, *8*(20), 33933–33952.

Afolabi, C. G., Bandyopadhyay, R., Leslie, J. F., and Ekpo, E. J. (2006). Effect of sorting on incidence and occurrence of fumonisins and *Fusarium verticillioides* on maize from Nigeria. *Journal of Food Protection*, *69*(8), 2019–2023.

Afolabi, C. G., Ephraim, J., and Bandyopadhyay, R. (2013). Maize contamination by zearalenone and T-2 toxin and human exposure in Nigeria. *Mycotoxins*, *63*(2), 143–149.

Agag, B. I. (2004). Mycotoxins in foods and feeds 3-zearalenone. *Assiut University Bulletin for Environmental Researches*, *7*(2), 169–176.

Akhidenor, J., and Nzeka, U. (2018). *Nigeria: grain and feed annual report 2018*. Washington,

DC.

Alasiri, H. S., Bahkali, A. A., Abd-Elsalam, K. A., El-Zeaagi, K. S., Elgorban, A. M., and Al-Sum, B. A. (2013). Detection of *Aspergillus* and *Penicillium* species producing aflatoxin in rice grains imported into Saudi Arabia. *Wulfenia*, *20*(10), 313–323.

Aliferis, K. A., Tarantilis, P. A., Harizanis, P. C., & Alissandrakis, E. (2010). Botanical discrimination and classification of honey samples applying gas chromatography/mass spectrometry fingerprinting of headspace volatile compounds. *Food Chemistry*, *121*(3), 856–862.

Amajama, J., Fina, F., and Mopta, S. E. (2016). Variations of tropospheric radio wave refractivities across Southern Nigeria – the rainforest to mangrove. *International Journal of Science, Engineering and Technology Research (IJSETR)*, *5*(6), 1914–1918.

Anklam, E., Stroka, J., and Boenke, A. (2002). Acceptance of analytical methods for implementation of EU legislation with a focus on mycotoxins. *Food Control*, 13(3), 173–183.

AOAC. (2009). A method for the determination of multiple mycotoxins in feeds and raw grains intended for feeds. *AOAC Agricultural Materials Task Force. Sub-Committee on Feed Additives and Contaminants* (pp. 2–3). Rockville, MD, USA: Association of Official Agricultural Chemists (AOAC).

Apeh, D. O., Ochai, D. O., Adejumo, A., Muhammad, H. L., Saidu, A. N., Atehnkeng, J., Adeyemi, R. H., Mailafiya, S. C., and Makun, H. A. (2016). Mycotoxicological concerns with sorghum, millet and sesame in Northern Nigeria. *Journal of Analytical and Bioanalytical Techniques*, *7*(5), 336.

Arroyo-Manzanares, N., De Ruyck, K., Uka, V., Gámiz-Gracia, L., García-Campaña, A. M., De Saeger, S., and Di Mavungu, J. D. (2018). In-house validation of a rapid and efficient procedure for simultaneous determination of ergot alkaloids and other mycotoxins in wheat and maize. *Analytical and Bioanalytical Chemistry*, *410*(22), 5567–5581.

Atanda, O., Makun, H. A., Ogara, I. M., Edema, M., Idahor, K. O., Eshiett, M. E., and Oluwabamiwo, B. F. (2013). Fungal and mycotoxin contamination of Nigerian foods and feeds. In: H. A. Makun (Ed.), *Mycotoxin and food safety in developing countries* (pp. 1–38).

Rijeka, Croatia: InTech.

Atanda, O., Ndenn, J., and Diedhiou, P. (2015). The economic impact of aflatoxins in West Africa: The case of Nigeria, Gambia and Senegal. Retrieved February 5, 2018, from http://aflatoxinpartnership.org/uploads/2.3 - Economic impact of aflatoxin in West Africa.pdf

Atehnkeng, J., Ojiambo, P. S., Donner, M., Ikotun, T., Sikora, R. A., Cotty, P. J., and Bandyopadhyay, R. (2008). Distribution and toxigenicity of Aspergillus species isolated from maize kernels from three agro-ecological zones in Nigeria. *International Journal of Food Microbiology*, *122*(1–2), 74–84.

Awotide, D. O. (2012). Supply response of maize, millet and sorghum in Nigeria: an error correction model approach. *Nigerian Journal of Agricultural Economics*, *3*(1), 19–27.

Bandyopadhyay, R., Kumar, M., and Leslie, J. F. (2007). Relative severity of aflatoxin contamination of cereal crops in West Africa. *Food Additives and Contaminants*, *24*(10), 1109–1114.

Bankole, S. A., and Mabekoje, O. O. (2004). Occurrence of aflatoxins and fumonisins in preharvest maize from south-western Nigeria. *Food Additives and Contaminants*, *21*(3), 251–255.

Bankole, S. A., Schollenberger, M., and Drochner, W. (2010). Survey of ergosterol, zearalenone and trichothecene contamination in maize from Nigeria. *Journal of Food Composition and Analysis*, *23*(8), 837–842.

Bayman, P., and Baker, J. L. (2006). Ochratoxins: A global perspective. *Mycopathologia*, *162*(3), 215–223.

BEG Group. (2018). Venn diagrams online bioinformatics software. Retrieved October 9, 2018, from http://bioinformatics.psb.ugent.be/webtools/Venn/

Bennett, J., and Klich, M. (2003). Mycotoxins. *Clinical Microbiology Reviews*, *16*(3), 497–516.

Benson, N., Nwokike, C., Williams, A., Adedapo, A., and Fred-Ahmadu, O. (2017). Changes in diurnal temperature and precipitation extremes in North Central Nigeria. In: *The 2nd International Electronic Conference on Atmospheric Sciences (ECAS 2017), 16–31 July*

2017; Sciforum Electronic Conference Series, Volume 2, 2017 (pp. 1–12). Basel, Switzerland: Sciforum.

Brugger, E. M., Wagner, J., Schumacher, D. M., Koch, K., Podlech, J., Metzler, M., and Lehmann, L. (2006). Mutagenicity of the mycotoxin alternariol in cultured mammalian cells. *Toxicology Letters*, *164*(3), 221–230.

Chen, C., Mitchell, N. J., Gratz, J., Houpt, E. R., Gong, Y., Egner, P. A., Groopman, J. D., Riley, R. T., Showker, J. L., Svensen, E., and Mduma, E. R. (2018). Exposure to aflatoxin and fumonisin in children at risk for growth impairment in rural Tanzania. *Environment International*, *115*, 29–37.

Chilaka, C. A., De Boevre, M., Atanda, O. O., and De Saeger, S. (2016). Occurrence of *Fusarium* mycotoxins in cereal crops and processed products (*Ogi*) from Nigeria. *Toxins*, *8*(11), 342.

Chilaka, C. A., De Boevre, M., Atanda, O. O., and De Saeger, S. (2018). Fate of *Fusarium* mycotoxins during processing of Nigerian traditional infant foods (*ogi* and soybean powder). *Food Research International, Accepted m*, Available online.

Chilaka, C. A., Kock, S. De, Phoku, J. Z., Mwanza, M., Augustina, M., and Dutton, M. F. (2012). Fungal and mycotoxin contamination of South African commercial maize. *Journal of Food, Agriculture and Environment*, *10*(2), 296–303.

Chu, F. S. (2003). Mycotoxins | Toxicology. In: B. Caballero, L. C. Trugo, and P. M. Finglas (Eds.), *Encyclopedia of food sciences and nutrition* (2nd ed., pp. 4096–4108). Academic Press (Elsevier Science Ltd).

Clarke, R., Connolly, L., Frizzell, C., and Elliott, C. T. (2014). Cytotoxic assessment of the regulated, co-existing mycotoxins aflatoxin B1, fumonisin B1 and ochratoxin, in single, binary and tertiary mixtures. *Toxicon*, *90*, 70–81.

CODEX (2015). Codex general standard for contaminants and toxins in food and feed (Codex Stan 193–1995). *CODEX Alimentarius. International Food Standards*, *CODEX STAN*, 25.

Commission Regulation (2006a). Commission Regulation (EC) No. 401/2006 of laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs. *Official Journal of the European Communities, L70*, 20–23.

Commission Regulation (2006b). Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. *Official Journal of the European Communities*, *2006R1881*(016.001), 5–24.

Commission Regulation (2013). Commission Recommendation of 27 March 2013 on the presence of T-2 and HT-2 toxin in cereals and cereal products Text with EEA relevance. *Official Journal of the European Communities, L 91/12*, 12–15.

D'Mello, J. (2003). *Mycotoxins in cereal grains, nuts and other plant products: Food safety: Contaminants and toxins*. Wallingford, England: CAB International.

Darwish, W. S., Ikenaka, Y., Nakayama, S. M., and Ishizuka, M. (2014). An overview on mycotoxin contamination of foods in Africa. *Journal of Veterinary Medical Science*, *76*(6), 789–797.

Davis, N. D. (1981). Sterigmatocystin and other mycotoxins produced by Aspergillus species. *Journal of Food Protection*, *44*(9), 711–714.

De Ruyck, K., De Boevre, M., Huybrechts, I., and De Saeger, S. (2015). Dietary mycotoxins, co-exposure, and carcinogenesis in humans: short review. *Mutation Research/Reviews in Mutation Research*, *766*, 32–41.

European Commission (2002). The Commission of the European Communities. Commission Decision 2002/657/EC of 12 August 2002. Implementing Council Directive 96/23/EC, Concerning the Performance of Analytical Methods and the Interpretation of Results. Brussels. *Official Journal of the European Communities*.

European Commission (2007). Commission Regulation (EC) No 1126/2007 of 28 September 2007 amending Regulation (EC) No 1881/2006 setting maximum levels for certain contaminants in foodstuffs as regards *Fusarium* toxins in maize and maize products. *Official Journal of the European Union, L255*, vol. L255, pp. 14–17.

Ezekiel, C. N., Abia, W. A., Ogara, I. M., Sulyok, M., Warth, B., and Krska, R. (2015). Fate of mycotoxins in two popular traditional cereal-based beverages (*kunu-zaki* and *pito*) from rural Nigeria. *LWT-Food Science and Technology*, *60*(1), 137–141.

Ezekiel, C. N., Warth, B., Ogara, I. M., Abia, W. A., Ezekiel, V. C., Atehnkeng, J., Sulyok, M

Turner, P. C., Tayo, G. O., Krska, R., and Bandyopadhyay, R. (2014). Mycotoxin exposure in rural residents in northern Nigeria: A pilot study using multi-urinary biomarkers. *Environment International*, *66*, 138–145.

Food Drug Administratio (2015). Guidelines for the validation of chemical methods for the FDA FVM program. In: P. A. Orlandi, W. T. Flynn, D. L. Zink, P. E. Norris, V. K. Bunning, T. Mcgrath, and M. E. Torrence (Eds.), *US Food and Drug Administration. Office of Foods and Veterinary Medicine* (2nd ed.). Silver Spring, Maryland.

Gazinska, P., Herman, D., Gillett, C., Pinder, S., and Mantle, P. (2012). Comparative immunohistochemical analysis of ochratoxin A tumourigenesis in rats and urinary tract carcinoma in humans; mechanistic significance of p-S6 ribosomal protein expression. *Toxins*, *4*(9), 643–662.

Gbashi, S., Madala, N. E., De Saeger, S., De Boevre, M., Adekoya, I., Adebo, O. A., and Njobeh, P. B. (2018). The Socio-economic impact of mycotoxin contamination in Africa. In P. B. Njobeh (Ed.), *Fungi and mycotoxins - their occurrence, impact on health and the economy as well as pre- and postharvest management strategies* (pp. 1-20). Rijeka, Croatia: InTech.

Gbashi, S., Madala, N. E., Adebo, O. A., Piater, L., Phoku, J. Z., and Njobeh, P. B. (2017). Subcritical water extraction and its prospects for aflatoxins extraction in biological materials. In: L. B. Abdulra'uf (Ed.), *Aflatoxin-control, analysis, detection and health risks* (pp. 229–250). Rijeka, Croatia: InTech.

Gbashi, S., Madala, N. E., De Saeger, S., De Boevre, M., and Njobeh, P. B. (2019). Development, chemometric-assisted optimization and in-house validation of a modified pressurized hot water extraction methodology for multi-mycotoxins in maize. *Food Chemistry* (Manuscript to be submitted).

Gbashi, S., Njobeh, P., Steenkamp, P., and Madala, N. (2017). Pressurized hot water extraction and chemometric fingerprinting of flavonoids from *Bidens pilosa* by UPLC-tandem mass spectrometry. *CYTA - Journal of Food*, *15*(2), 171–180.

Gong, Y. Y., Egal, S., Hounsa, A., Turner, P. C., Hall, A. J., Cardwell, K., and Wild, C. P. (2003). Determinants of aflatoxin exposure in young children from Benin and Togo, West Africa: the

critical role of weaning. *International Journal of Epidemiology*, *32*, 556–562.

Grenier, B., and Oswald, I. (2011). Mycotoxin co-contamination of food and feed: Meta-analysis of publications describing toxicological interactions. *World Mycotoxin Journal*, *4*(3), 285–313.

Guerre, P. (2016). Worldwide mycotoxins exposure in pig and poultry feed formulations. *Toxins*, *8*(12), 350.

Gwary, O. M., Hati, S. S., Dimari, G. A., and Ameh, J. A. (2012). Assessment of mycotoxins (total aflatoxins and ochratoxin-A) contamination of staple cereals. *International Journal of Chemical and Biochemical Sciences*, *2*, 1–6.

Hell, K., Cardwell, K. F., Setamou, M., and Poehling, H. M. (2000). The influence of storage practices on aflatoxin contamination in maize in four agroecological zones of Benin, West Africa. *Journal of Stored Products Research*, *36*(4), 365–382.

Hueza, I. M., Raspantini, P. C. F., Raspantini, L. E. R., Latorre, A. O., and Górniak, S. L. (2014). Zearalenone, an estrogenic mycotoxin, is an immunotoxic compound. *Toxins*, *6*(3), 1080–1095.

IARC. (1976). Sterigmatocystin. IARC monographs on the evaluation of the carcinogenic risk of chemicals to humans. *World Health Organization, International Agency for Research on Cancer*, *10*, 245.

IARC. (1993). Ochratoxin A. Some naturally occurring substances: food items and constituents, heterocyclic aromatic amines and mycotoxins. *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans*, *56*, 489–521.

IARC. (2002). *IARC monographs on the evaluation of carcinogenic risks to humans. Some traditional herbal medicines, some mycotoxins, naphthalene and styrene. Volume 82*. Lyon, France.

Ismaiel, A., and Papenbrock, J. (2015). Mycotoxins: producing fungi and mechanisms of phytotoxicity. *Agriculture*, *5*(3), 492–537.

JECFA. (2016). *Co-exposure of fumonisins with aflatoxins*. Joint FAO/WHO Expert Committee

on Food Additives. Eighty-third meeting. JECFA/83/SC. Rome, Italy.

Kim, D. H., Hong, S. Y., Kang, J. W., Cho, S. M., Lee, K. R., An, T. K., Lee, C., and Chung, S. H. (2017). Simultaneous determination of multi-mycotoxins in cereal grains collected from South Korea by LC/MS/MS. *Toxins*, *9*(3), 106.

Kpodo, K., Sørensen, A. K., and Jakobsen, M. (1996). The occurrence of mycotoxins in fermented maize products. *Food Chemistry*, *56*(2), 147–153.

Lattanzio, V. M. T., Ciasca, B., Powers, S., and Visconti, A. (2014). Improved method for the simultaneous determination of aflatoxins, ochratoxin A and *Fusarium* toxins in cereals and derived products by liquid chromatography–tandem mass spectrometry after multi-toxin immunoaffinity clean up. *Journal of Chromatography A*, *1354*, 139–143.

Leema, G., Chou, D. S., Jesudasan, C. A. N., Geraldine, P., and Thomas, P. A. (2011). Expression of genes of the aflatoxin biosynthetic pathway in *Aspergillus flavus* isolates from keratitis. *Molecular Vision*, *17*, 2889.

Li, Y., Wang, Z., Beier, R. C., Shen, J., Smet, D. D., De Saeger, S., and Zhang, S. (2011). T-2 toxin, a trichothecene mycotoxin: review of toxicity, metabolism, and analytical methods. *Journal of Agricultural and Food Chemistry*, *59*(8), 3441–3453.

Liu, G. T., Qian, Y. Z., Zhang, P., Dong, W. H., Qi, Y. M., and Guo, H. T. (1992). Etiological role of Alternaria alternata in human esophageal cancer. *Chinese Medical Journal*, *105*(5), 394–400.

Liu, G. T., Qian, Y. Z., Zhang, P., Dong, Z. M., Shi, Z. Y., Zhen, Y. Z., Miao, J., and Xu, Y. M. (1991). Relationships between *Alternaria alternata* and oesophageal cancer. In: I. K. O'Neill, J. Chen, and H. Bartsch (Eds.), *Relevance to human cancer of N-nitroso compounds, tobacco smoke and mycotoxins* (pp. 258–262). Lyon, France: International Agency for Research on Cancer.

Liu, Y., and Wu, F. (2010). Global burden of aflatoxin-induced hepatocellular carcinoma: a risk assessment. *Environmental Health Perspectives*, *118*(6), 818.

Luongo, D., Severino, L., Bergamo, P., De Luna, R., Lucisano, A., and Rossi, M. (2006). Interactive effects of fumonisin B1 and α-zearalenol on proliferation and cytokine expression

in Jurkat T cells. *Toxicology In-Vitron Vitro*, *20*(8), 1403–1410.

Maaroufi, K., Achour, A., Betbeder, A. M., Hammami, M., Ellouz, F., Creppy, E. E., and Bacha, H. (1995). Foodstuffs and human blood contamination by the mycotoxin ochratoxin A: correlation with chronic interstitial nephropathy in Tunisia. *Archives of Toxicology*, *69*(8), 552–558.

Majeed, S., De Boevre, M., De Saeger, S., Rauf, W., Tawab, A., Rahman, M., and Iqbal, M. (2018). Multiple mycotoxins in rice: occurrence and health risk assessment in children and adults of Punjab, Pakistan. *Toxins*, *10*(2), 77.

Makun, H. A., Adeniran, A. L., Mailafiya, S. C., Ayanda, I. S., Mudashiru, A. T., Ojukwu, U. J., Jagaba, A. S., Usman, Z., and Salihu, D. A. (2013). Natural occurrence of ochratoxin A in some marketed Nigerian foods. *Food Control*, *31*(2), 566–571.

Makun, H. A., Dutton, M. F., Njobeh, P. B., Mwanza, M., and Kabiru, A. Y. (2011). Natural multi-occurrence of mycotoxins in rice from Niger State, Nigeria. *Mycotoxin Research*, *27*(2), 97–104.

Makun, H. A., Dutton, M. F., Njobeh, P. B., Gbodi, T. A., and Ogbadu, G. H. (2012). Aflatoxin contamination in foods and feeds: A special focus on Africa. In: A. E. Ayman (Ed.), *Trends in vital food and control engineering* (pp. 187–234). Rijeka, Croatia: InTech.

Makun, H. A., Timothy, A. G., Olufunmilayo, H. A., Ezekiel, A. S., and Godwin, H. O. (2009). Fungi and some mycotoxins found in mouldy sorghum in Niger State, Nigeria. *World Journal of Agricultural Sciences*, *5*(1), 5–17.

Matthews, S. B., Santra, M., Mensack, M. M., Wolfe, P., Byrne, P. F., & Thompson, H. J. (2012). Metabolite profiling of a diverse collection of wheat lines using ultraperformance liquid chromatography coupled with time-of-flight mass spectrometry. *PloS One*, *7*(8), e44179.

McCormick, S. P., Stanley, A. M., Stover, N. A., and Alexander, N. J. (2011). Trichothecenes: from simple to complex mycotoxins. *Toxins*, *3*(7), 802–814.

Medina, A., Rodriguez, A., and Magan, N. (2014). Effect of climate change on *Aspergillus flavus* and aflatoxin B_1 production. *Frontiers in Microbiology*, *5*, 348.

Monbaliu, S., Van Poucke, C., Van Peteghem, C., Van Poucke, K., Heungens, K., and De Saeger, S. (2009). Development of a multi-mycotoxin liquid chromatography/tandem mass spectrometry method for sweet pepper analysis. *Rapid Communications in Mass Spectrometry*, *23*(1), 3–11.

Nair, M. G. (1998). Fumonisins and human health. *Annals of Tropical Paediatrics*, *18*, S47–S52.

Ngoko, Z., Marasas, W. F. O., Rheeder, J. P., Shephard, G. S., Wingfield, M. J., and Cardwell, K. F. (2001). Fungal infection and mycotoxin contamination of maize in the humid forest and the western highlands of Cameroon. *Phytoparasitica*, *29*(4), 352–360.

Nicely, R., David, M., and Nzeka, U. (2012). *Nigerian grain and feed annual report. Global agricultural information network (GAIN) report number NI1204*. Washington, DC.

Nikiema, P. N., Worrillow, L., Traore, A. S., Wild, C. P., and Turner, P. C. (2004). Fumonisin contamination of maize in Burkina Faso, West Africa. *Food Additives and Contaminants*, *21*(9), 865–870.

Njobeh, B. P., Dutton, F. M., and Makun, H. A. (2010). Mycotoxins and human health: Significance, prevention and control. In: A. K. Mishra, A. Tiwari, and S. B. Mishra (Eds.), *Smart Biomolecules in Medicine* (pp. 132–177). VBRI Press, India.

Njobeh, P. B., and Olotu, I. (2017). The socio-economic impact of mycotoxin contamination in Africa. In: *Proceedings of the 1st MYCOKEY International Conference. Global Mycotoxin Reduction in the Food and Feed Chain.* September 11-14, 2017. Ghent, Belgium: Toxins.

Oluwafemi, F., and Ibeh, I. N. (2011). Microbial contamination of seven major weaning foods in Nigeria. *Journal of Health, Population and Nutrition*, *29*(4), 415.

Ostry, V. (2008). Alternaria mycotoxins: an overview of chemical characterization, producers, toxicity, analysis and occurrence in foodstuffs. *World Mycotoxin Journal*, *1*(2), 175–188.

Oyelami, O. A., Maxwell, S. M., Adelusola, K. A., Aladekoma, T. A., and Oyelese, A. O. (1997). Aflatoxins in the lungs of children with kwashiorkor and children with miscellaneous diseases in Nigeria. *Journal of Toxicology and Environmental Health*, *51*(6), 623–628.

Podlech, J., and Marko, D. (2009). Alternariol acts as a topoisomerase poison, preferentially

affecting the II isoform. *Molecular Nutrition and Food Research*, *53*(4), 441–451.

Qian, G., Tang, L., Lin, S., Xue, K. S., Mitchell, N. J., Su, J., Gelderblom, W. C., Riley, R. T., Phillips, T. D., and Wang, J. S. (2016). Sequential dietary exposure to aflatoxin B_1 and fumonisin B_1 in F344 rats increases liver preneoplastic changes indicative of a synergistic interaction. *Food and Chemical Toxicology*, *95*, 188–195.

Sangare-Tigori, B., Dem, A. A., Kouadio, H. J., Betbeder, A. M., Dano, D. S., Moukha, S., and Creppy, E. E. (2006). Preliminary survey of ochratoxin A in millet, maize, rice and peanuts in Cote d'Ivoire from 1998 to 2002. *Human and Experimental Toxicology*, *25*(4), 211–216.

Schmale, D. G., and Munkvold, G. P. (2009). Mycotoxins in crops: A threat to human and domestic animal health. *The Plant Health Instructor*, *3*, 340–353.

Shabir, G. A. (2005). Step-by-step analytical methods validation and protocol in the quality system compliance industry. *Journal of Validation Technology*, *10*, 314–325.

Shephard, G. S. (2008). Impact of mycotoxins on human health in developing countries. *Food Additives and Contaminants*, *25*(2), 146–151.

Sherif, S. O., Salama, E. E., and Abdel-Wahhab, M. A. (2009). Mycotoxins and child health: The need for health risk assessment. *International Journal of Hygiene and Environmental Health*, *212*(4), 347–368.

Siegel, D., Feist, M., Proske, M., Koch, M., and Nehls, I. (2010). Degradation of the Alternaria mycotoxins alternariol, alternariol monomethyl ether, and altenuene upon bread baking. *Journal of Agricultural and Food Chemistry*, *58*(17), 9622–9630.

Sirma, A. J., Senerwa, D. M., Grace, D., Makita, K., Mtimet, N., Kang'ethe, E. K., & Lindahl, J. F. (2016). Aflatoxin B1 occurrence in millet, sorghum and maize from four agro-ecological zones in Kenya. *African Journal of Food, Agriculture, Nutrition and Development*, *16*(3), 10991–11003.

Smith, M. C., Madec, S., Coton, E., and Hymery, N. (2016). Natural co-occurrence of mycotoxins in foods and feeds and their *in vitro* combined toxicological effects. *Toxins*, *8*(4), 94.

Somorin, Y., Akinyemi, A., Bertuzzi, T., and Pietri, A. (2016). Co-occurrence of aflatoxins,

ochratoxin A and citrinin in "*egusi*" melon (Colocynthis citrullus L.) seeds consumed in Ireland and the United Kingdom. *Food Additives and Contaminants*, *9*(3), 230–235.

Song, H. H., Kim, D. Y., Woo, S., Lee, H. K., and Oh, S. R. (2013). An approach for simultaneous determination for geographical origins of Korean Panax ginseng by UPLC-QTOF/MS coupled with OPLS-DA models. *Journal of Ginseng Research*, *37*(3), 341.

Soriano, J. M., and Dragacci, S. (2004). Occurrence of fumonisins in foods. *Food Research International*, *37*, 985–1000.

Streit, E., Naehrer, K., Rodrigues, I., and Schatzmayr, G. (2013). Mycotoxin occurrence in feed and feed raw materials worldwide: long-term analysis with special focus on Europe and Asia. *Journal of the Science of Food and Agriculture*, *93*(12), 2892–2899.

Sulyok, M., Berthiller, F., Krska, R., and Schuhmacher, R. (2006). Development and validation of a liquid chromatography/tandem mass spectrometric method for the determination of 39 mycotoxins in wheat and maize. *Rapid Communication in Mass Spectrometry*, *20*(18), 2649–2659.

Trygg, J., and Wold, S. (2002). Orthogonal projections to latent structures (O-PLS). *Journal of Chemometrics*, *16*(3), 119–128.

Turner, P. C., Collinson, A. C., Cheung, Y. B., Gong, Y., Hall, A. J., Prentice, A. M., and Wild, C. P. (2007). Aflatoxin exposure in utero causes growth faltering in Gambian infants. *International Journal of Epidemiology*, *36*, 1119–1125.

Udoh, J. M., Cardwell, K. F., and Ikotun, T. (2000). Storage structures and aflatoxin content of maize in five agroecological zones of Nigeria. *Journal of Stored Products Research*, *36*(2), 187–201.

Van der Fels-Klerx, H. J., Liu, C., and Battilani, P. (2016). Modelling climate change impacts on mycotoxin contamination. *World Mycotoxin Journal*, *9*(5), 717–726.

Wagacha, J. M., and Muthomi, J. W. (2008). Mycotoxin problem in Africa: current status, implications to food safety and health and possible management strategies. *International Journal of Food Microbiology*, *124*(1), 1–12.

Wilson, N., McMaster, N., Gantulga, D., Soyars, C., McCormick, S., Knott, K., Senger, R., and Schmale, D. (2017). Modification of the mycotoxin deoxynivalenol using microorganisms isolated from environmental samples. *Toxins*, *9*(4), 141.

World Health Organization (2017). Evaluation of certain contaminants in food (Eighty-third report of the Joint FAO/WHO Expert Committee on Food Additives). *World Health Organization (WHO) Technical Report Series: WHO Technical Report Series, No.1002*, 67–68.

Worley, B., and Powers, R. (2013). Multivariate analysis in metabolomics. *Current Metabolomics*, *1*, 92–107.

Zain, M. E. (2011). Impact of mycotoxins on humans and animals. *Journal of Saudi Chemical Society*, *15*(2), 129–144.

Zepnik, H., Pähler, A., Schauer, U., and Dekant, W. (2001). Ochratoxin A-induced tumor formation: Is there a role of reactive Ochratoxin A metabolites? *Toxicological Sciences*, *59*(1), 59–67.

Zhang, L., Li, M., Zhan, L., Lu, X., Liang, L., Su, B., Sui, H., Gao, Z., Li, Y., Liu, Y., and Wu, B. (2015). Plasma metabolomic profiling of patients with diabetes-associated cognitive decline. *PloS One*, *10*(5), e0126952.

Zinedine, A., Soriano, J. M., Molto, J. C., and Manes, J. (2007). Review on the toxicity, occurrence, metabolism, detoxification, regulations and intake of zearalenone: An oestrogenic mycotoxin. *Food and Chemical Toxicology*, *45*, 1–18.

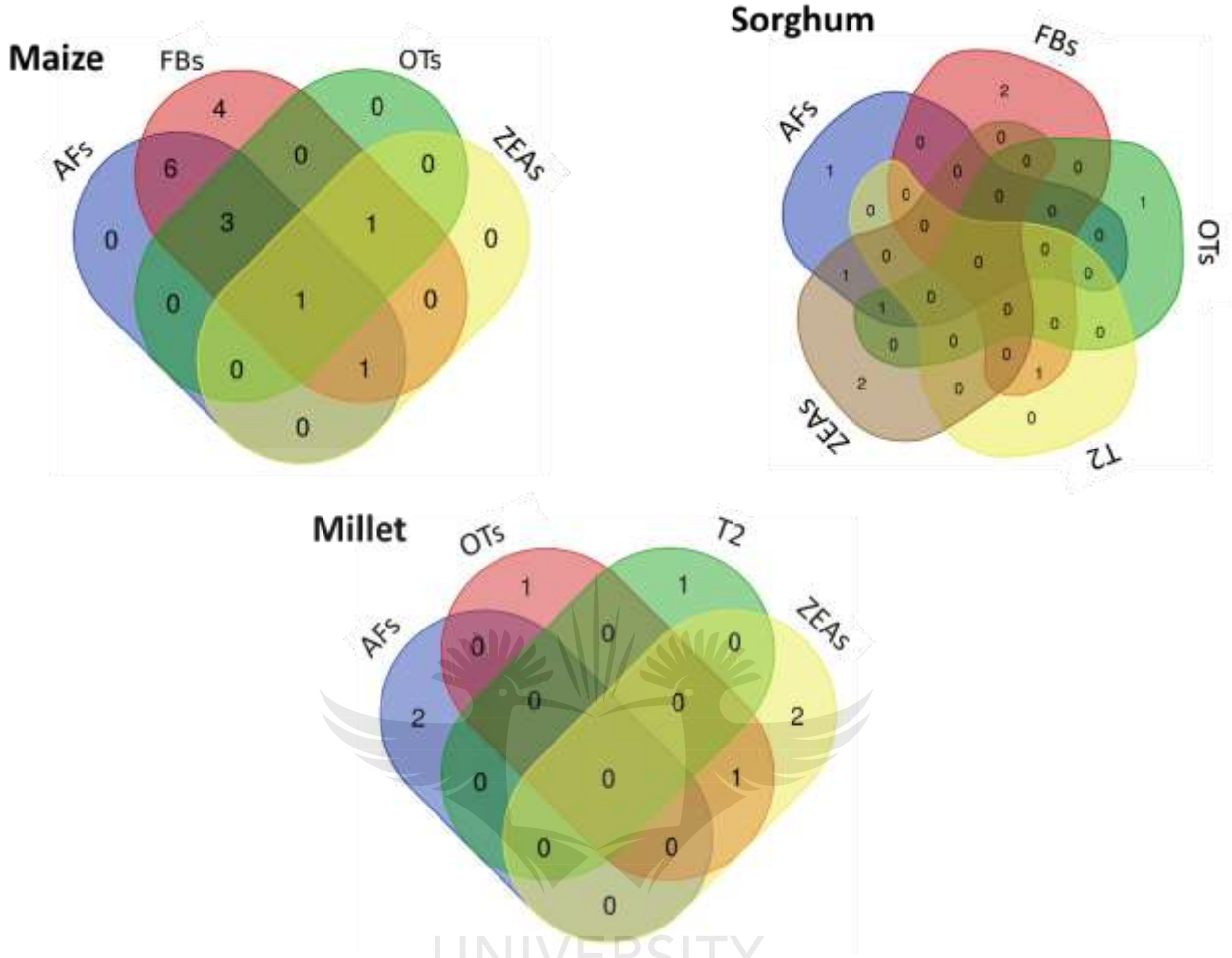
APPENDIX 5

Appendix 5.A: Linearity parameters and matrix effect of PHWE of multi-mycotoxins in maize, sorghum and millet.

Myco	Linear Range (µg/kg)	Maize			Sorghum			Millet		
		Equation	R^2	ME (%)	Equation	R^2	ME (%)	Equation	R^2	ME (%)
AFB_1	30-1,000	y=726418.00x+0.0000000	0.998	43	y=758813.00x+0.0000000	0.998	45	y=394509.00x+0.0000000	0.998	-5
	10-40#	y=0.0388236x-0.0297569#	0.999#	ND#	y=0.02246.00x-0.0551951#	0.992#	ND#	y= 0.0205976x-0.0481812#	0.994#	ND#
AFB_2	10-300	y=29922.300x+0.0000000	0.990	ND	y=30003.100x+0.0000000	0.996	ND	y=1017810.0x+0.0000000	0.999	-14
	10-40#	y= 0.0389391x-0.0724287#	0.999#	ND#	y=0.0217079x-0.0288360#	0.972#	ND#	y= 0.0182912x-0.0454976#	0.988#	ND#
AFG_1	30-1,000	y=733883.00x+0.0000000	0.997	49	y=656177.00x+0.0000000	0.984	43	y=364241.00x+0.0000000	0.991	-3
	10-40#	y= 0.0282378x-0.0290143#	0.999#	ND#	y= 0.0190237x-0.0468256#	0.988#	ND#	y= 0.0155844x-0.0182782#	0.993#	ND#
AFG_2	30-1,000	y=602178.00x+0.0000000	0.999	44	y=596522.00x+0.0000000	0.995	44	y=358975.00x+0.0000000	0.998	6
	10-40#	y=0.0137443x -0.0346290#	0.999#	ND#	y= 0.0197771x-0.0440019#	0.984#	ND#	y= 0.0117548x-0.0147432#	0.982#	ND#
AME	30-1,000	y=282992.00x+0.0000000	0.998	57	y=269502.00x+0.0000000	0.994	55	y=134284.00x+0.0000000	1.000	9
	10-40#	y=0.0012392x-0.0053106#	0.999#	ND#	y=0.0006475x-0.0170763#	0.996#	ND#	y=0.0005798x-0.0085391#	0.989#	ND#
FB_1	60-2,000	y=108498.00x+0.0000000	0.992	63	y=109.64500x+0.0000000	0.998	64	y=56694.100x+0.0000000	0.998	30
	20-80#	y=0.0031071x-0.0163114#	0.998#	ND#	y=0.0021193x-0.0772540#	0.989#	ND#	y= 0.0014962x-0.0995423#	0.984#	ND#
FB_2	16-500	y=15175200x+0.0000000	0.997	57	y=16315600x+0.0000000	0.996	60	y=9292170.0x+0.0000000	0.995	30
	20-80#	y=0.0036764x+0.0021624#	0.999#	ND#	y=0.00188148x-0.0115376#	0.989#	ND#	y=0.0015081x-0.1082910#	0.977#	ND#
FB_3	30-1,000	y=467233.00x+0.0000000	0.997	61	y=494279.00x+0.0000000	0.999	63	y=242114.00x+0.0000000	0.998	24
	20-80#	y= 0.0051078x-0.0189341#	0.999#	ND#	y= 0.0036043x-0.0766144#	0.987#	ND#	y=0.0023971x-0.1025880#	0.974#	ND#
OTA	30-1,000	y=2569440.0x+0.0000000	0.999	60	y=2462030.0x+0.0000000	0.998	59	y=1242000.0x+0.0000000	0.999	18
	25-100#	y= 0.0059043x-0.0187238#	0.999#	ND#	y=0.0028299x-0.0278753#	0.991#	ND#	y= 0.0042255x-0.0060401#	0.968#	ND#
OTB	30-1,000	y=25504200x+0.0000000	0.996	62	y=21534600x+0.0000000	0.997	55	y=1165260.0x+0.0000000	1.000	18
	N.D.#	N.D.#	N.D.#	N.D.#	N.D.#	N.D.#	N.D.#	N.D.#	N.D.#	N.D.#
STEG	30-1,000	y=1697490.0x+0.0000000	0.995	60	y=1766930.0x+0.0000000	0.997	61	y=794631.00x+0.0000000	0.999	14
	25-100#	y=0.0189498x-0.0007388#	0.999#	ND#	y= 0.0087249x-0.0812727#	0.988#	ND#	y=0.0097481x-0.0207013#	0.999#	ND#
T-2	30-1,000	y=15881.200x+0.0000000	0.996	61	y=62117.600x+0.0000000	0.998	90	y=33360.200x+0.0000000	0.999	82

Myco	Linear Range (µg/kg)	Maize			Sorghum			Millet		
		Equation	R^2	**ME (%)**	**Equation**	R^2	**ME (%)**	**Equation**	R^2	**ME (%)**
	50-200[#]	y=0.00739964x-0.0429551[#]	0.999[#]	ND[#]	y=0.0033890x-0.0488755[#]	0.993[#]	ND[#]	y= 0.0038011x+0.0089581[#]	0.987[#]	ND[#]
ZEA	30-1,000	y=571027.00x+0.0000000	0.997	60	y=494014.00x+0.0000000	0.997	54	y=240378.00x+0.0000000	0.993	6
	50-200[#]	y=0.0010048x-0.0007694[#]	0.996[#]	ND[#]	y=0.0002811x-0.0015622[#]	0.917[#]	ND[#]	y=0.0002970x-0.0043136[#]	0.969[#]	ND[#]
α-ZEL	30-1,000	y=48991.100x+0.0000000	0.999	61	y=45737.600x+0.0000000	0.999	58	y=20146.000x+0.0000000	0.998	5
	N.D.[#]	N.D.[#]	N.D.[#]	N.D.[#]	N.D.[#]	N.D.[#]	N.D.[#]	N.D.[#]	N.D.[#]	N.D.[#]
β-ZEL	30-1,000	y=128721.00x+0.0000000	0.999	60	y=257624.00x+0.0000000	0.997	56	y=128721.00x+0.0000000	0.999	13
	N.D.[#]	N.D.[#]	N.D.[#]	N.D.[#]	N.D.[#]	N.D.[#]	N.D.[#]	N.D.[#]	N.D.[#]	N.D.[#]
Mean[#]			0.996±0.00			0.996±0.00			0.996±0.00	
			0.999±0.00[#]			0.982±0.01[#]			0.999±0.00[#]	
Sign (2-tailed)			0.01			0.04			0.01	

Key: [#] Parameter values for solvent extraction. Myco: mycotoxin. ME: Matrix effect. N.D.: not detected. R^2: coefficient of determination. AFB_1: aflatoxin B_1. AFB_2: aflatoxin B_2. AFG_1: aflatoxin G_1. AFG_2: aflatoxin G_2. AME: alternariol monomethyl ether. FB_1: fumonisin B_1. FB_2: fumonisin B_2. FB_3: fumonisin B_3. OTA: ochratoxin A. OTB: ochratoxin B. STEG: sterigmatocystin. T-2: T-2 toxin. ZEA: zearalenone. α-ZEL: α-zearalenol. β-ZEL: β-zearalenol.



Appendix 5.B: Incidences of co-contamination of different classes of regulated mycotoxins in maize, sorghum and millet from Nigeria as analyzed using PHWE followed by UHPLC-MS/MS. AFs: aflatoxins. FBs: fumonisins. OTs: ochratoxins. T-2: T-2 toxin. ZEAs: zearalenone and its analogues α- and β-zearalenol.

CHAPTER SIX

6.0 GENERAL DISCUSSION AND CONCLUSION

6.1 GENERAL DISCUSSION

Mycotoxins are one of the most notorious food contaminants due to their severe toxicity and perpetual proliferation in various agricultural commodities globally (Njobeh *et al*., 2010a; Reddy *et al*., 2010). Despite much efforts to control mycotoxin contamination, these contaminants remain incessantly prevalent in food and feed, to the detriment of consumers of such products (Aldred *et al*., 2004; Zinedine *et al*., 2007; Njobeh *et al*., 2010a). Their presence in food/feed is highly unwanted and results in huge economic losses to the agricultural industry and international trade due to stringent trade policies to ensure public health and safety (Kimanya *et al*., 2012; Mitchell *et al*., 2016; Gbashi *et al*., 2018). Reliable analytical data is critical for adequate risk assessment, legislative actions and provides a strong scientific basis for adoption of relevant mitigation strategies along the supply chain (Anklam *et al*., 2002; Aldred *et al*., 2004). Such data is based on efficient and validated methods of analysis (Gbashi *et al*., 2018). It is therefore obligatory that such methods conform to certain performance criteria with respect to reliability, accuracy and sensitivity (Gilbert & Anklam, 2002; Commission Regulation, 2006a; Arroyo-Manzanares *et al*., 2018). Due to the urgency to meet up with ever increasing demand for more healthy and safe foods, it is expedient that methods employed for quality control and surveillance are equally fast in addition to safety and cos-effectiveness, an aspect which many of the conventional methods of mycotoxin analysis are grossly lacking (Schenck & Hobbs, 2004; Karsten *et al*., 2008; Frenich *et al*., 2009; Arroyo-manzanares *et al*., 2014).

In this research work, a fast, efficient and environmentally-friendly method called pressurized hot water extraction (PHWE), was developed, validated and applied for the extraction of multiple mycotoxins in a single step. This method depends on the heat- and pressure-induced dissolution, partitioning and solvation characteristics of water, which makes it act in a manner similar to organic solvents in terms of extractability (Teo *et al*., 2010; Gbashi *et al*., 2017c). Since PHWE is heat-dependent, there are concerns, which have been shown to be genuine (Khoza *et al*., 2014, 2015; Sani *et al*., 2014), that some analytes might experience thermal degradation resulting in lower recoveries during PHWE. It was thus imperative to first investigate the thermal degradation

patterns of the mycotoxins, in order to ascertain the feasibility of this extraction approach. This constituted the first objective of this research work which was successfully achieved (Gbashi *et al*., 2019b) as described in Chapter Three of this thesis.

In this experiment, the thermal stability of 15 different mycotoxins was determined as a function of temperature and time for pure standards and maize matrix spiked with mycotoxins. Maize was chosen as the reference matrix because it is a widely-consumed staple food around the world and a favored substrate for fungal contamination and subsequent mycotoxin production. In order to optimize the degradation of the multi-mycotoxins, a set of experiments were statistically designed based on the response surface methodology (RSM). By adopting the central composite design (CCD) approach of the RSM, 10 experimental levels were obtained, with temperature values ranging from 50 to 150 °C and time values ranging from 10 to 50 min. After heat treatment, the residues were then quantified on an UHPLC-MS/MS. Inference from the obtained results indicated that depending on the type of mycotoxins and conditions of exposure, the test mycotoxins were stable during thermal treatment. This observation is in line with previously published reports on the thermal stability of mycotoxins (Castelo *et al*., 1998; Raters & Matissek, 2008; Karlovsky *et al*., 2016; Kim *et al*., 2017). This implies that with a carefully optimized combination of temperature and time, the challenge of thermal degradation of mycotoxins may easily be overcomed during PHWE.

After confirming the stability of the mycotoxins, the design, optimization and validation of the PHWE method was performed in fulfillment of Objective 2 of this research as described in Chapter Four of this thesis. For the method development and optimization, a similar chemometric approach to that of the previously performed experiment, *i.e.*, the CCD, was adopted. Two extraction parameters (temperature and solvent composition) were optimized to determine the best conditions for the simultaneous extraction of the 15 different mycotoxins spiked into maize. The computed results yielded a condition of 162 °C and 45% ethanol (EtOH) for temperature and solvent composition, respectively. Using this optimized condition, with the exception of OTA and α-ZEL which had a recoveries of 58 and 14%, respectively, it was possible to achieve satisfactory recoveries (71 to 124%) for all analytes, which were consistent with regulatory requirements of

the EC, AOAC, CODEX, FDA and ICH (Shabir, 2005; Commission Regulation, 2006a; AOAC, 2009; CODEX, 2015; FDA, 2015).

Other method validation parameters such as linearity [0.986 to 0.999 (Addendums B-E)], limit of detection (LOD) (range: 0.06 to 41 µg/kg) and limit of quantification (LOQ) (range: 0.18 to 123 µg/kg), intra-day precision or repeatability (0 to 27%) and inter-day precision or reproducibility (3 to 34%) were also in line with analytical method performance criteria by same regulatory bodies (Shabir, 2005; Commission Regulation, 2006a; AOAC, 2009; CODEX, 2015; FDA, 2015). When compared with other conventional mycotoxin extraction methods, the extraction efficiency of the modified PHWE method was akin, and in some cases better. A small-scale pilot study was subsequently performed to test the applicability of the PHWE method for the analysis of mycotoxins in real samples. Twenty-five maize meal samples intended for human consumption obtained from rural households in Ngwalemong village in Limpopo Province (South Africa) were subjected to PHWE followed by UHPLC-MS/MS analysis to determine the levels of 15 different naturally occurring mycotoxins in the samples. The results revealed that though many of the samples were positive for the tested mycotoxins, none of the detected levels exceeded the regulatory limits set by the EC.

The pilot study was thus followed by a much larger scale study, to estimate multi-mycotoxin contamination levels in 91 samples of staple cereals, maize, sorghum and millet, collected from 5 different agroecological zones in Nigeria. This was in achieving Objective 3 of this research work as described in Chapter Five of this thesis. During this experiment for a larger scale application of the PHWE method, the 91 samples that were extracted using the modified PHWE method were also analyzed using a solvent extraction method which served as a control for cross-validation and authentication of the validity of the PHWE method. Discrimination of the results obtained by the two methods using the PCA and OPLS-DA multivariate discriminatory approach indicated that the variations in the results were not significantly different ($p>0.05$). The results of the PHWE analysis revealed that all the 15-tested mycotoxins were detected in at least one of the samples, some being contaminated with mycotoxins at levels significantly higher than the corresponding EC maximum levels in the food commodities. For example, 100% of the maize samples were positive for FB_1 and $\Sigma FB_1\&FB_2$ at levels ranging from 17 to 7,950 µg/kg and 30 to 14,600 µg/kg, respectively, with 44% of the samples exceeding the maximum levels of 4,000 µg/kg in

unprocessed maize as stipulated by EC and CODEX (EC, 2007; WHO, 2017; Arroyo-Manzanares *et al*., 2018).

Selected single ion chromatograms (SIM) showing the peaks of the different mycotoxins for the mycotoxin standards and contaminated maize, sorghum and millet samples are shown in Addendums F-G. Much lower levels of FBs were however, observed in sorghum samples (mean value of 17 µg/kg and maximum value of 248 µg/kg for $\Sigma FB_1 \& FB_2$) while none of the millet samples were contaminated with FBs. A similar trend was observed for AFs contamination in the cereals, with maize registering the highest prevalence, 54 µg/kg for AFB_1 and 56 µg/kg for ΣAFs, while sorghum and millet had lower levels of AFB_1 (3.6 and 2.4 µg/kg, respectively) and ΣAFs (3.8 and 2.4 µg/kg, respectively). This trend and the contamination levels thereof are in agreement with a number of previously published literature on mycotoxin contamination of maize, sorghum and millet in Nigeria (Bankole & Mabekoje, 2004; Afolabi *et al*., 2006; Adetunji *et al*., 2014; Chilaka *et al*., 2016). It is evident from these results that mycotoxins, particularly AFs and FBs, still constitute a significant concern to food safety in Nigeria, and perhaps other West African countries.

6.2 CONCLUSION

In this research work, it was noted that risks of mycotoxins present in agricultural commodities are assessed by adequate analytical procedures. At present, mycotoxins are extracted using solvent-based extraction methods involving multiple steps, which are expensive, labor-intensive and time-consuming. We developed, validated and used a fast, low-cost and environmentally friendly extraction method, PHWE, for the extraction of multi-mycotoxins in food samples in comparison to the conventional methods routinely used. To the best of our knowledge, this constitutes the first report on the application of this method for the extraction mycotoxins in food. In the experiments that preceded the actual method development, the thermal degradation patterns of some of the understudied mycotoxins (*i.e.*, STEG and AME) were also reported for the first time. The use of multivariate chemometric models was fundamental in the overall experimental design, data analysis and interpretations of the results.

The efficacious application of the validated method to real samples and the results thereof not only re-affirm the adequacy of the modified PHWE method, but also offer insights on the risk of

exposure of the populace, as well as, contribute to existing knowledge on the prevalence of mycotoxins in commercially available cereals (maize, maize meal, sorghum and millet) in Nigeria and South Africa, where applicable. This further draws attention to the mycotoxin issue in Africa, and the need for more proactive approaches in tackling this problem such as routine surveillance of these toxins in the food and feed supply chain using efficient, fast and cheap analytical approaches. This is not undermining the need for preventive and other mitigation approaches. Furthermore, inference from the data obtained are expected to stimulate policy makers and stakeholders along the food supply chain to identify critical areas of collaboration and strengthen alliances in order to ameliorate the effects of these toxicants on the continent of Africa, and the world at large. In this regard, research objectives should be prioritized to ensure a positive impact for public health, food safety and security and economic development. Critical areas to concentrate efforts include development of efficient and cost-effective analytical and intervention strategies, public awareness, strengthening research and human capacity development as well as harmonizing and enforcing regulations.

In light of the focus of this thesis, possible future works include improvements in some aspects of the PHWE method, such as the recovery of OTA and α-ZEL, as well as, the linear range of the method. In future developments, a sample concentration could improve the performance of the method. A collaborative inter-laboratory study to assess the performance characteristics of the PHWE could be organized between the Mycotoxin Laboratory, University of Johannesburg, Doornfontein Campus, South Africa and the Molecular Sciences Institute, School of Chemistry, University of Witwatersrand, South Africa, as both laboratories have a similar PHWE set up. Another future work includes extending the applicability of the PHWE method for the extraction of a wider range of other fungal metabolites and environmental pollutants that contaminate different food and feed substrates. Also, imminent developments could enable complete elimination of organic solvents from the PHWE method, as well as, the possibility to combine the method with other advanced chromatographic and mass spectrometric intruments such as the Pegasus GC-HRTOF-MS for the analysis of mycotoxins and other fungal metabolites where applicable. The choice of the Pegasus GC-HRTOF-MS can be explained in terms of its rich analytical capabilities (ultra-high mass resolution, accuracy and sensitivity) as well as its availability in our department. A further goal will be an accredited method for routine analysis for

the implementation of regulatory limits and trade specifications, as well as, obtaining a patent for the PHWE method by the end of 2020.

In general, based on the results of this study, the adoption of PHWE in combination with UHPLC and tandem MS techniques is very promising as an alternative to conventional methods for survey-type work and exposure studies for multi-mycotoxin levels in food products. Though several other benefits can be derived from reduced chemical usage, this study emphasizes the significance of greener methods of analysis in the field of mycotoxicology and bioanalysis, hence stimulating interest and contributing to green chemistry, environmental sustainability and public health in many ways.

References

Adetunji, M., Atanda, O., Ezekiel, C. N., Sulyok, M., Warth, B., Beltrán, E., and Chilaka, C. A. (2014). Fungal and bacterial metabolites of stored maize (*Zea mays*, L.) from five agro-ecological zones of Nigeria. *Mycotoxin Research*, *30*(9), 89–102.

Afolabi, C. G., Bandyopadhyay, R., Leslie, J. F., and Ekpo, E. J. (2006). Effect of sorting on incidence and occurrence of fumonisins and *Fusarium verticillioides* on maize from Nigeria. *Journal of Food Protection*, *69*(8), 2019–2023.

Aldred, D., Magan, N., and Olsen, M. (2004). The use of HACCP in the control of mycotoxins: the case of cereals. In: N. Magan & M. Olsen (Eds.), *Mycotoxins in food: Detection and control* (pp. 139–173). Cambridge, England: Woodhead Publishing Ltd.

Anklam, E., Stroka, J., and Boenke, A. (2002). Acceptance of analytical methods for implementation of EU legislation with a focus on mycotoxins. *Food Control*, *13*(3), 173–183.

AOAC. (2009). A method for the determination of multiple mycotoxins in feeds and raw grains intended for feeds. *AOAC Agricultural Materials Task Force. Sub-Committee on Feed Additives and Contaminants* (pp. 2–3). Rockville, MD, USA: Association of Official Agricultural Chemists (AOAC). Retrieved June 24, 2019, from https://www.aafco.org /Portals/0/SiteContent/Regulatory/Committees/Lab-Methods-and-Services/Methods/cfia_ac ia_multi_mycotoxin_needs_staement.pdf

Arroyo-Manzanares, N., De Ruyck, K., Uka, V., Gámiz-Gracia, L., García-Campaña, A. M., De

Saeger, S., and Di Mavungu, J. D. (2018). In-house validation of a rapid and efficient procedure for simultaneous determination of ergot alkaloids and other mycotoxins in wheat and maize. *Analytical and Bioanalytical Chemistry*, *410*(22), 5567–5581.

Arroyo-manzanares, N., Huertas-pérez, J. F., García-campaña, A. M., and Gámiz-gracia, L. (2014). Simple methodology for the determination of mycotoxins in pseudocereals , spelt and rice. *Food Control*, *36*(1), 94–101.

Bankole, S. A., and Mabekoje, O. O. (2004). Occurrence of aflatoxins and fumonisins in preharvest maize from south-western Nigeria. *Food Additives and Contaminants*, *21*(3), 251–255.

Castelo, M. M., Sumner, S. S., and Bullerman, L. B. (1998). Stability of fumonisins in thermally processed corn products. *Journal of Food Protection*, *61*, 1030–1033.

Chilaka, C. A., De Boevre, M., Atanda, O. O., and De Saeger, S. (2016). Occurrence of *Fusarium* mycotoxins in cereal crops and processed products (*Ogi*) from Nigeria. *Toxins*, *8*(11), 342.

CODEX. (2015). General standard for contaminants and toxins in food and feed (CODEX STAN 193-1995). *CODEX Alimentarius. International Food Standards, CODEX STAN*, 25. Retrieved June 24, 2019 from http://www.fao.org/fileadmin/user_upload/livestockgov/documents/1_CXS_193e.pdf

Commission Regulation, E. (2006). Commission Regulation (EC) No. 401/2006 of laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs. *Official Journal of the European Communities, L70*, 20–23.

EC. (2007). Commission Regulation (EC) No 1126/2007 of 28 September 2007 amending Regulation (EC) No 1881/2006 setting maximum levels for certain contaminants in foodstuffs as regards *Fusarium* toxins in maize and maize products. *Official Journal of the European Union*, *L255*, vol. L255, pp. 14–17.

FDA. (2015). Guidelines for the validation of chemical methods for the FDA FVM program. In: P. A. Orlandi, W. T. Flynn, D. L. Zink, P. E. Norris, V. K. Bunning, T. Mcgrath, & M. E. Torrence (Eds.), *US Food and Drug Administration. Office of Foods and Veterinary Medicine* (2nd ed.). Silver Spring, Maryland. Retrieved June 24, 2019, from

https://www.fda.gov/downloads/scienceresearch/fieldscience/ucm273418.pdf

Frenich, A. G., Luis, J., Vidal, M., Romero-gonzález, R., and Aguilera-luiz, M. M. (2009). Simple and high-throughput method for the multimycotoxin analysis in cereals and related foods by ultra-high performance liquid chromatography / tandem mass spectrometry. *Food Chemistry*, *117*(4), 705–712.

Gbashi, S., Madala, N. E., Adebo, O. A., Piater, L., Phoku, J. Z., and Njobeh, P. B. (2017). Subcritical water extraction and its prospects for aflatoxins extraction in biological materials. In: L. B. Abdulra'uf (Ed.), *Aflatoxin-control, analysis, detection and health risks* (pp. 229–250). Rijeka, Croatia: InTech.

Gbashi, S., Madala, N. E., De Saeger, S., De Boevre, M., Adekoya, I., Adebo, O. A., and Njobeh, P. B. (2018). The Socio-economic impact of mycotoxin contamination in Africa. In: P. B. Njobeh (Ed.), *Fungi and mycotoxins - their occurrence, impact on health and the economy as well as pre- and postharvest management strategies* (pp. 1–20). Rijeka, Croatia: InTech.

Gbashi, S., Madala, N. E., De Saeger, S., De Boevre, M., and Njobeh, P. B. (2019). Numerical optimization of temperature-time degradation of multiple mycotoxins. *Food and Chemical Toxicology*.

Gilbert, J., and Anklam, E. (2002). Validation of analytical methods for determining mycotoxins in foodstuffs. *TrAC Trends in Analytical Chemistry*, *21*(6–7), 468–486.

Karlovsky, P., Suman, M., Berthiller, F., De Meester, J., Eisenbrand, G., Perrin, I., Oswald, I. P., Speijers, G., Chiodini, A., Recker, T., and Dussort, P. (2016). Impact of food processing and detoxification treatments on mycotoxin contamination. *Mycotoxin Research*, *32*(4), 179–205.

Karsten, M., Swart, R., Mcleod, F., Murphy, B., Henderson, S., and Richter, B. (2008). Fast and effective determination of aflatoxins in grains or food using accelerated solvent extraction followed by HPLC. *Dionex Corporation*, 1–5.

Khoza, B. S., Chimuka, L., Mukwevho, E., Steenkamp, P. A., and Madala, N. E. (2014). The effect of temperature on pressurised hot water extraction of pharmacologically-important metabolites as analysed by UPLC-qTOF-MS and PCA. *Evidence-Based Complementary and Alternative Medicine*, *2014*(Article ID 914759), 1–9.

Khoza, B. S., Dubery, I. A., Byth-Illing, H. A., Steenkamp, P. A., Chimuka, L., and Madala, N. E. (2015). Optimization of pressurized hot water extraction of flavonoids from *Momordica foetida* using UHPLC-qTOF-MS and multivariate chemometric approaches. *Food Analytical Methods*, *2015*, 1–10.

Kim, D. H., Hong, S. Y., Kang, J. W., Cho, S. M., Lee, K. R., An, T. K., Lee, C., and Chung, S. H. (2017). Simultaneous determination of multi-mycotoxins in cereal grains collected from South Korea by LC/MS/MS. *Toxins*, *9*(3), 106.

Kimanya, M. E., Tiisekwa, B., and Mpolya, E. (2012). *Country and economic assessment for aflatoxin contamination and control in Tanzania; A supplement to the 2012 Report*. Arusha, Tanzania. Retrieved June 24, 2019 from http://archives.au.int/bitstream/handle/123456789/5495/Tanzania%20Supplemental%20Aflatoxin%20Situational%20Analysis%20Report%20%2018%20July%202016.pdf?sequence=1&isAllowed=y

Mitchell, N. J., Bowers, E., Hurburgh, C., and Wu, F. (2016). Potential economic losses to the USA corn industry from aflatoxin contamination. *Food Additives and Contaminants*: *Part A*, *33*(3), 540–550.

Njobeh, B. P., Dutton, F. M., and Makun, H. A. (2010). Mycotoxins and human health: Significance, prevention and control. In: A. K. Mishra, A. Tiwari, & S. B. Mishra (Eds.), *Smart Biomolecules in Medicine* (pp. 132–177). VBRI Press, India.

Raters, M., and Matissek, R. (2008). Thermal stability of aflatoxin B 1 and ochratoxin A. *Mycotoxin Research*, *24*(3), 130–134.

Reddy, K. R. N., Salleh, B., Saad, B., Abba, H. K., Abel, C. A., and Shier, W. T. (2010). An overview of mycotoxin contamination in foods and its implications for human health. *Toxin Reviews*, *29*(1), 3–26.

Sani, T., Golmakani, E., Mohammadi, A., Feyzi, P., and Kamali, H. (2014). Optimization of pressurized hot water extraction on the extract yield and antioxidant activity from Biebersteinia multifida DC using a modified supercritical fluid extractor. *Journal of Supercritical Fluids*, *94*, 130–137.

Schenck, F. J., and Hobbs, J. E. (2004). Evaluation of the Quick, Easy, Cheap, Effective, Rugged,

and Safe (QuEChERS) approach to pesticide residue analysis. *Bulletin of Environmental Contamination and Toxicology*, *73*(1), 23–30.

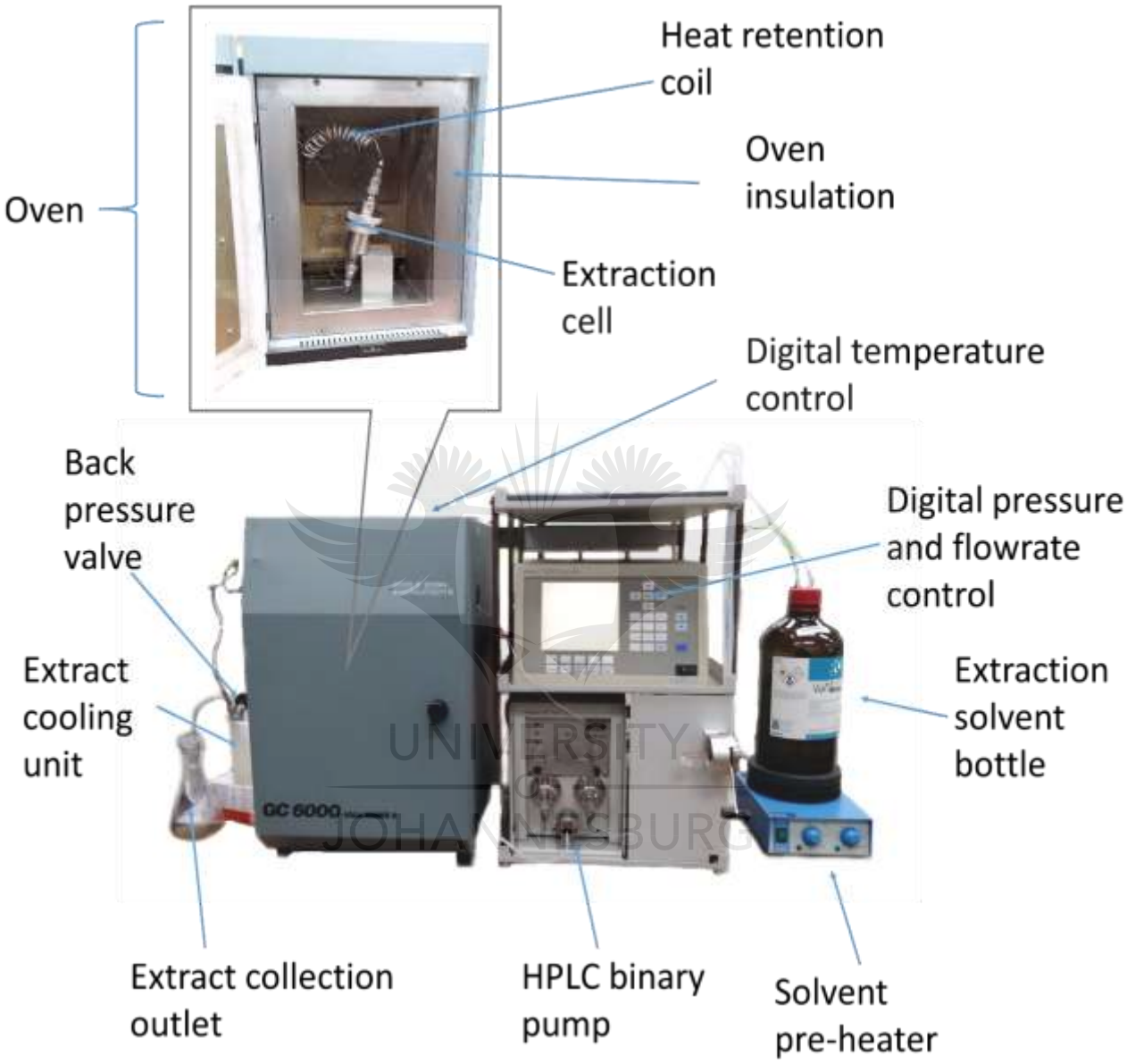
Shabir, G. A. (2005). Step-by-step analytical methods validation and protocol in the quality system compliance industry. *Journal of Validation Technology*, *10*, 314–325.

Teo, C. C., Tan, S. N., Yong, J., Hew, C. S., and Ong, E. S. (2010). Pressurized hot water extraction (PHWE). *Journal of Chromatography A*, *1217*, 2484–2494.

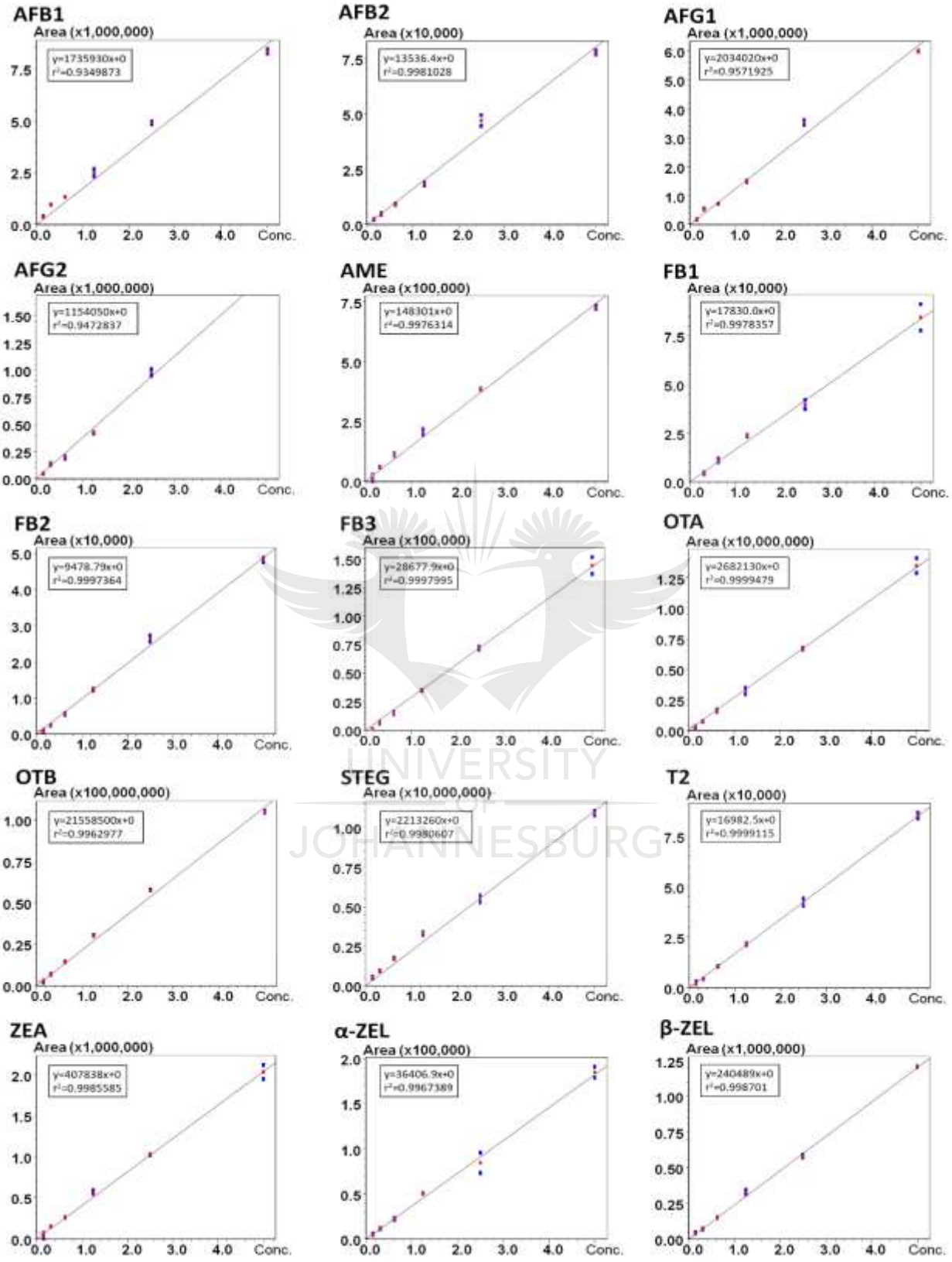
WHO. (2017). Evaluation of certain contaminants in food (Eighty-third report of the Joint FAO/WHO Expert Committee on Food Additives). *World Health Organization (WHO) Technical Report Series: WHO Technical Report Series, No.1002*, 67–68.

Zinedine, A., Soriano, J. M., Molto, J. C., and Manes, J. (2007). Review on the toxicity, occurrence, metabolism, detoxification, regulations and intake of zearalenone: An oestrogenic mycotoxin. *Food and Chemical Toxicology*, *45*, 1–18.

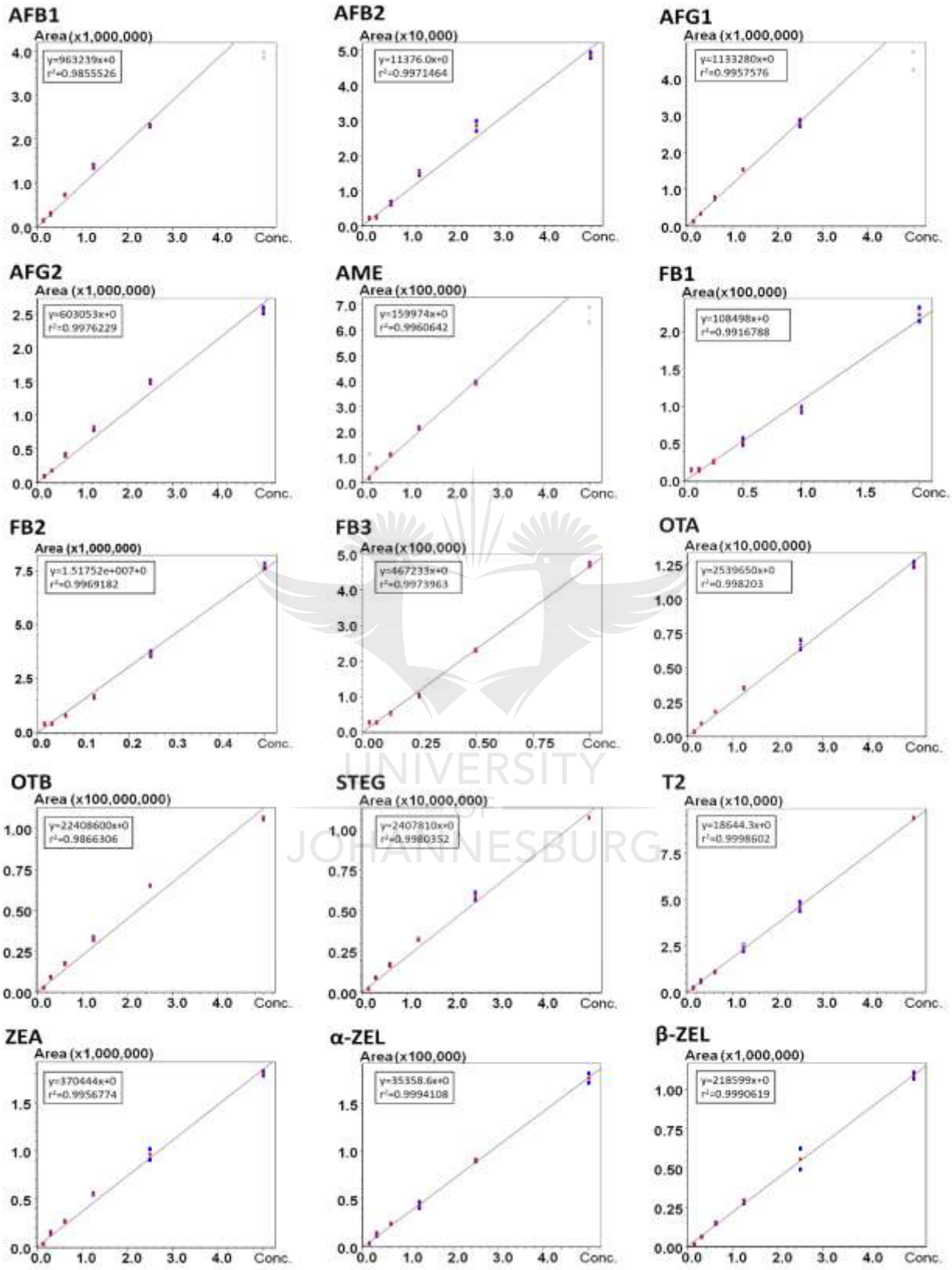
ADDENDUM



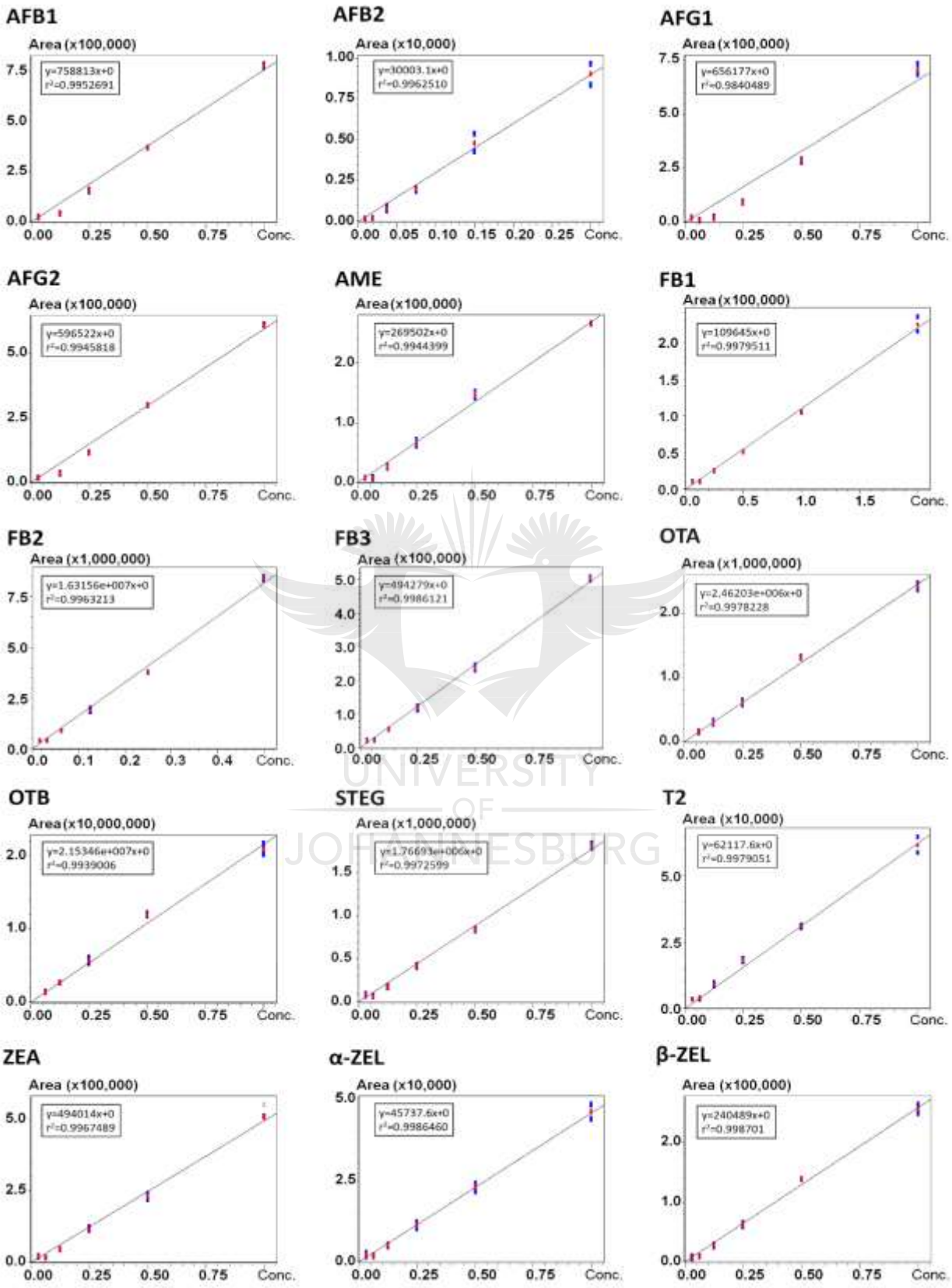
Addendum A: Picture of the homemade laboratory scale PHWE unit.



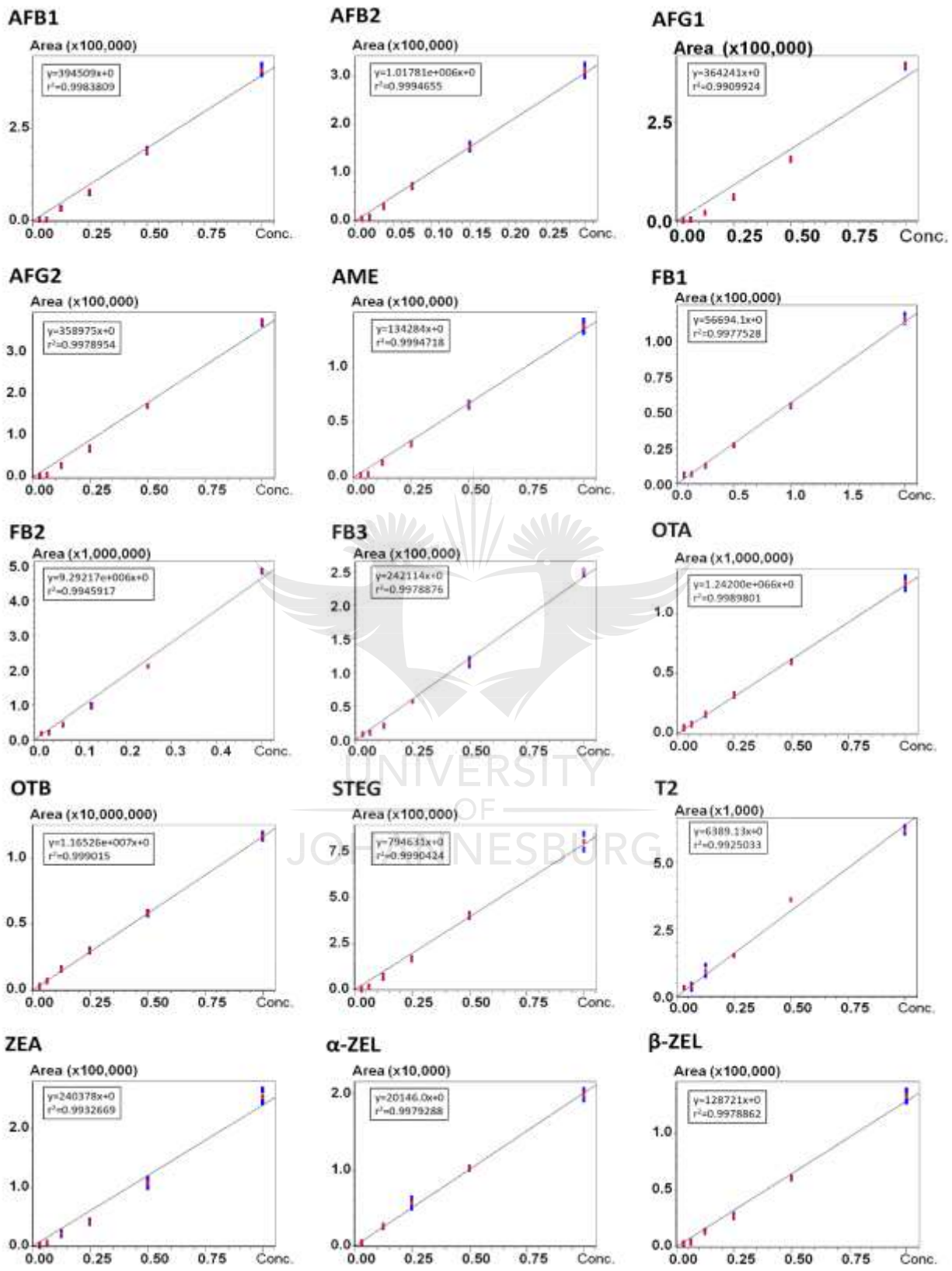
Addendum B: Calibration curves for mycotoxins prepared in neat solvent.



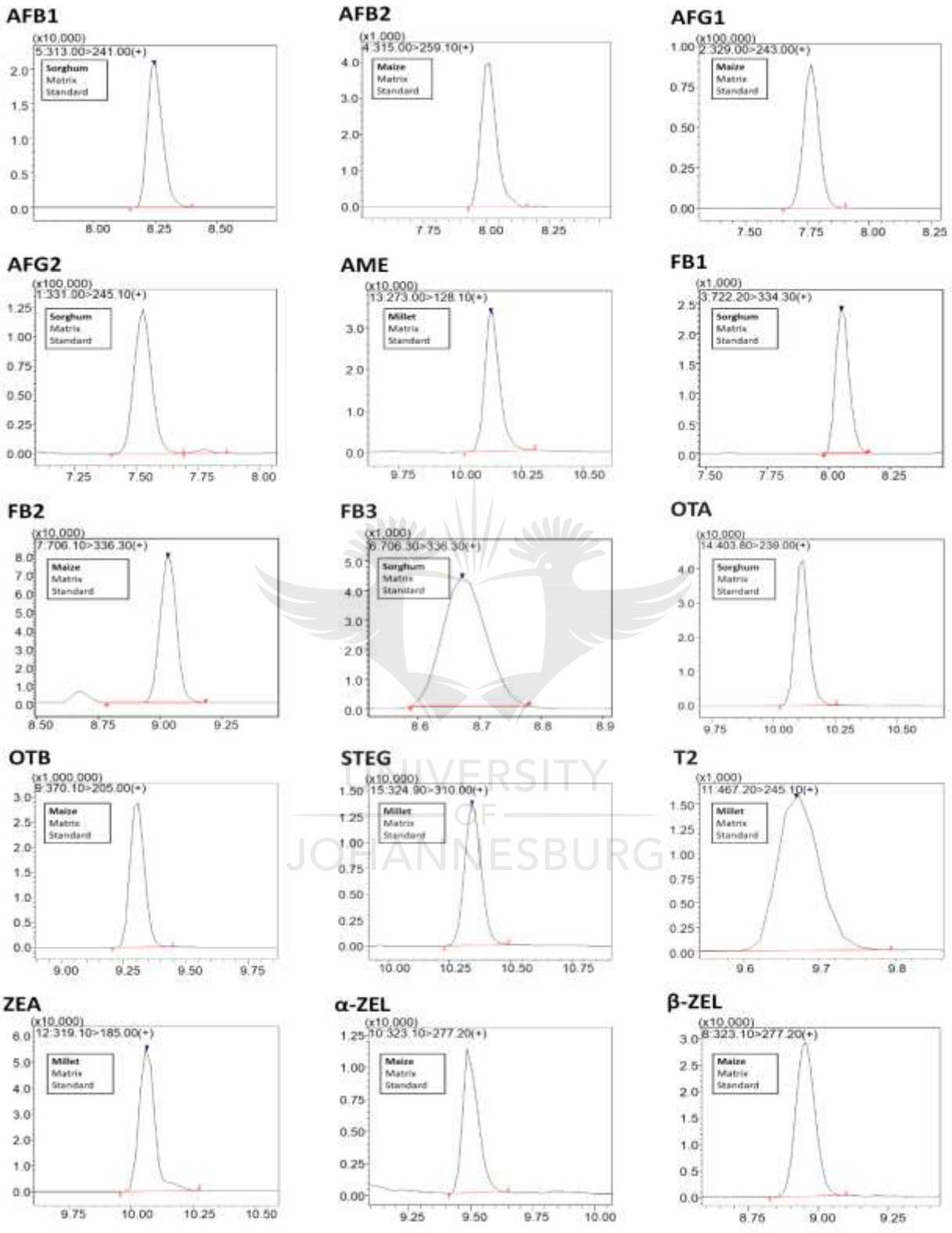
Addendum C: Maize matrix-matched calibration curves.



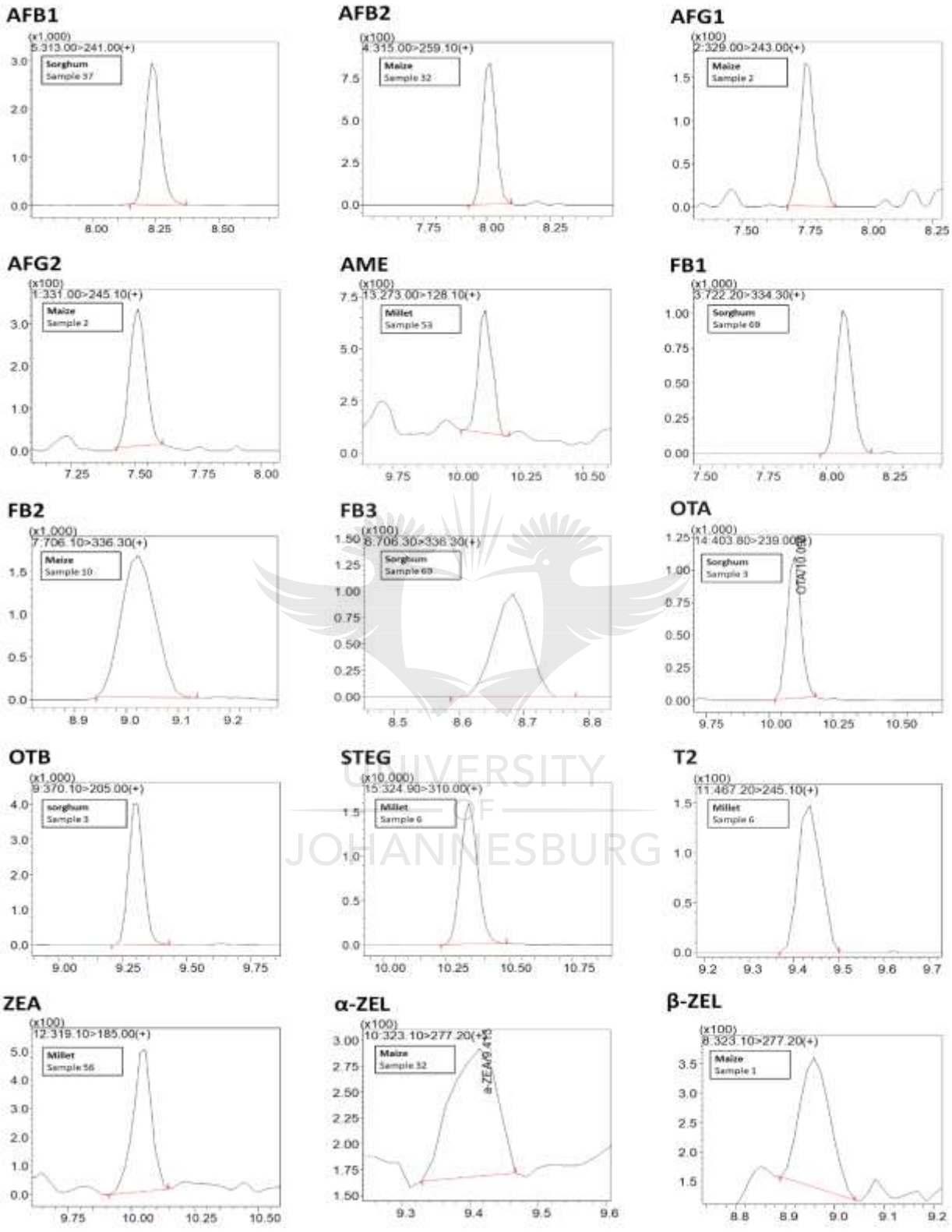
Addendum D: Sorghum matrix-matched calibration curves.



Addendum E: Millet matrix-matched calibration curves.



Addendum F: Selected single ion chromatograms (SIM) showing the peaks of the different mycotoxins prepared in neat solvent, maize-matrix, sorghum-matrix and millet-matrix.



Addendum G: Selected single ion chromatograms (SIM) showing the peaks of the different mycotoxins for contaminated maize, sorghum and millet samples.