Development of LC-HRMS Assay for the Measurement of 12 Mycotoxins in Urine

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ABSTRACT

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Melika Mirabi

Mycotoxins are the secondary metabolites of certain molds. These toxic compounds naturally contaminate food products and beverages and can cause severe health effects in humans and animals. Environmental changes and industrialization are currently promoting the spread of these mycotoxins worldwide. Health Canada regularly monitors the levels of specific mycotoxins in various food products. Complementarily to food monitoring, periodic biomonitoring is especially important to determine the exposure to mycotoxins in the Canadian population considering the variability of individual diets and metabolism. Urine biomonitoring is a non-invasive approach and sample collection is easy. The purpose of this study is to develop a sensitive and accurate LC-HRMS method for the detection in urine of 12 mycotoxins that impact human health. These mycotoxins are enniatin A (ENNA), enniatin A1 (ENNA1), enniatin B (ENNB), enniatin B1 (ENNB1), alternariol (AOH), alternariol monomethyl ether (AME), beauvericin (BEA), citrinin (CIT), fumonisin B1 (FB1), fumonisin B2 (FB2), ochratoxin A (OTA) and ochratoxin alpha (OTα). The final 24-min LC-HRMS method used CORTECS T3 reversed-phase separation and employed time-segmented polarity switching to cover the 12 analytes of interest in a single analysis. To allow high-throughput for large-scale monitoring, two sample preparation procedures were evaluated: "dilute-and-shoot" and solid-phase extraction with hydrophilic and lipophilic sorbent (HLB SPE). Evaluation of solubility and non-specific adsorption with the dilute-and-shoot method revealed that enniatins (ENNs) and BEA have low solubility in a highly aqueous solvent (H_2O/ACN/FA 94/5/1 v/v) with a 70-98% decrease in signal intensity compared to a highly organic solvent (MeOH/ H_2O/ FA 60/39/1 v/v). This issue also caused loss of ENNs and BEA during storage of urine samples in plastic containers. Rinsing the containers with MeOH allowed the recovery of 17, 30, 57, 44 and 67% of ENNB, ENNB1, ENNA, ENNA1, and BEA, respectively. Use of 20x dilution in the dilute-and-shoot method resulted in LOQs > 2 ng/mL for almost all mycotoxins, which are present at < 1 ng/mL in real samples. Thus, dilute-and-shoot is not sensitive enough for the intended application, so HLB SPE was used for sample clean-up and enrichment. This sample preparation method recovered 68 - 88% of all the

mycotoxins tested with 10x enrichment, which led to significant ionization suppression of CIT, OTα, OTA, AOH, AME and FB1. Reducing their enrichment decreased the matrix effects for all the mycotoxins (77% - 150%) except for AOH (22%) and AME (66%), which was compensated for by the addition of an internal standard ($AMEd_3$). An optimized HLB SPE LC-HRMS method is proposed for validation and further application to real samples in the biomonitoring of mycotoxins in urine.

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Table of Contents

List of Figures ix

Supplementary Figures in Appendix xii

List of Tables xiv

List of Abbreviations xv

List of Equations xviii

Chapter 1. Introduction 1

1.1. Classification and occurrence of mycotoxins 1

1.2. Toxicity of mycotoxins 5

1.3. Biomonitoring studies 8

1.4. Urine biomonitoring 8

1.5. Existing methods for the detection of mycotoxins in urine 10

1.6. Results of biomonitoring studies for mycotoxins in urine 16

1.7. LC-MS methods for biomonitoring of mycotoxins in urine 22

1.7.1. Liquid chromatography 22

1.7.2. Mass spectrometry 23

1.7.2.1. Electrospray ionization 23

1.7.2.2. Quadrupole-time-of-flight high resolution mass spectrometer 26

1.8. Solid-phase extraction 30

1.9. Objectives 31

Chapter 2. Materials and methods 33

2.1. Chemicals 33

2.2. Mycotoxin standards 33

2.3. Urine creatinine measurement 33

2.4. Development and evaluation of dilute-and-shoot method 34
2.4.1. Comparison of mycotoxin solubility and non-specific adsorption in two solvents using dilute-and-shoot method 34
2.4.1.1. Comparison of two dilute-and-shoot methods 34
2.4.1.2. Dilute-and-shoot method using 60% methanol (v/v) with 1% formic acid 35
2.4.1.3. Dilute-and-shoot method using 5% acetonitrile with 1% formic acid (v/v) 35
2.4.2. Evaluation of the effect of dilution factor on matrix effects in dilute-and-shoot method 35
2.4.3. Evaluation of recovery of dilute-and-shoot method in plastic and glass inserts 36
2.4.4. Calibration curve using dilute-and-shoot method 36
2.5. Development and evaluation of SPE method 37
2.5.1.1. Evaluation of the effect of urine pH on Oasis HLB SPE 37
2.5.1.2. Comparison of different washing solvents in SPE method 38
2.5.1.3. Evaluation of process efficiency, recovery, and matrix effects during method development of SPE 38
2.5.1.4. Comparison of HLB vs. C18 SPE 39
2.5.1.5. Comparison of MCX and MAX SPE 39
2.5.1.6. Optimization of evaporation-reconstitution step 40
2.5.1.7. Modification of HLB SPE loading and evaporation step 41
2.6. Non-specific adsorption during urine collection and storage 42
2.6.1. Testing MeOH in the loading step in HLB SPE 43
2.7. Final HLB SPE sample preparation 44
2.8. LC-MS analysis 44
2.9. Data analysis 46
Chapter 3. Results and discussion 48
3.1. Development of LC-HRMS method 48

3.1.1. Selection of internal standards 48

3.1.2. Modification of LC-MS time-segmented polarity switching method for urine biomonitoring 49

3.2. Development of sample preparation method for biomonitoring of 12 mycotoxins in urine 53

3.2.1. Dilute-and-shoot 53

3.2.1.1. Evaluation of solubility and non-specific adsorption of mycotoxins 53

3.2.1.2. Evaluation of matrix effects in different lots of urine 58

3.2.1.3. Evaluation of dilution factor 60

3.2.1.4. Evaluation of recovery in dilute-and-shoot method 60

3.2.2. Determination of LOQ in dilute-and-shoot method 63

3.2.2.1. Non-specific adsorption of ENNs and BEA to polypropylene plastic tubes 64

3.2.3. Development of solid-phase extraction method for 12 mycotoxins 67

3.2.3.1. Effect of urine pH on process efficiency (PE) 68

3.2.4. Effect of washing solvent and volume on process efficiency (PE) 69

3.2.5. Comparison of HLB, C18, MCX and MAX SPE 71

3.2.5.1. Recovery 71

3.2.5.2. Matrix effects 73

3.2.6. Effect of creatinine on matrix effects using HLB SPE 75

3.2.7. Optimization of evaporation-reconstitution step in SPE 77

3.2.8. HLB SPE finalized method 87

Chapter 4. Conclusions and future work 94

References 97

Appendix 112

List of Figures

Figure 1.1. Structure of the selected mycotoxins....8

Figure 1.2. Urine composition....10

Figure 1.3 Illustration of electrochemical reaction and creation of Taylor cone in ESI....24

Figure 1.4 Schematic of quadrupole-time-of-flight mass spectrometer....28

Figure 1.5 Schematic of solid-phase extraction procedure....30

Figure 2.1 Experiment design for the comparison of dilute-and-shoot methods....35

Figure 2.2 Calibration curve in dilute-and-shoot method....37

Figure 2.3. Comparison of two different elution solvents in MAX SPE....40

Figure 2.4 Schematic of experimental plan for testing the effect of SPE lot on recovery....42

Figure 2.5 Summary of experimental design to evaluate mycotoxin recovery from polypropylene containers....43

Figure 2.6 HLB SPE final protocol for measurment of 12 mycotoxins in urine....44

Figure 3.1. Chromatographic separation of 12 mycotoxins with CORTECS T3 C_{18}....51

Figure 3.2 Determination of fragmentor voltage for AOH and CIT in ESI (-)....52

Figure 3.3 Determination of fragmentor voltage for AME and $AMEd_3$ in ESI (-)....52

Figure 3.4. Solubility and non-specific adsorption effect in standards....55

Figure 3.5. Solubility and non-specific adsorption effect in spiked urine diluted 10x....57

Figure 3.6. Overlay of non-spiked urine TIC with EIC of mycotoxins in spiked diluted urine58

Figure 3.7. Effect of creatinine on matrix effects....59

Figure 3.8. Effect of 10x, 15x and 20x dilution on matrix effects....60

Figure 3.9. Schematic of mycotoxin recovery evaluation in dilute-and-shoot method using polypropylene and glass inserts....61

Figure 3.10. Evaluation of recovery in dilute-and-shoot method using plastic....62

Figure 3.11 Evaluation of recovery in dilute-and-shoot method using glass 63

Figure 3.12 Workflowof recovery evaluation for ENNs and BEA from Eppendorf tube.....65

Figure 3.13 Evaluation of recovery of mycotoxins from plastic Eppendorf tube 66

Figure 3.14. Effect of pH on process efficiency of HLB SPE.....68

Figure 3.15. Effect of washing solvent composition on C18 and HLB SPE.....71

Figure 3.16. Comparison of recoveries in HLB, C18, MCX and MAX SPE.....73

Figure 3.17. Comaprison of matrix effects in HLB, C18, MCX and MAX SPE 74

Figure 3.18 Matrix effects in blank HLB SPE.....75

Figure 3.19 Effect of creatinine on the matrix effects in HLB SPE. 76

Figure 3.20 Recovery of standards during evaporation/reconstitution step with and without 6 µl glycerol. 77

Figure 3.21 Effect of increase contact time during loading on recovery of mycotoxins in HLB SPE.....80

Figure 3.22 Recovery of mycotoxins using HLB SPE in spiked urine with 10x enrichment. .81

Figure 3.24 Matrix effects of mycotoxins in spiked urine using HLB SPE with evaporation and no enrichment.....83

Figure 3.25 Matrix effects of mycotoxins in spiked urine using HLB SPE with evaporation and no enrichment.....84

Figure 3.26 Comparison of recovery and matrix effects of mycotoxins with different lots of HLB SPE.....86

Figure 3.27 Effect of MeOH during sample loading on recovery (A), and matrix effects (B) of mycotoxins using HLB SPE 89

Figure 3.28 Effect of 5% MeOH during sample loading on recovery of mycotoxins using HLB SPE.....90

Figure 3.29 Matrix effects of mycotoxins using 5% MeOH during sample loading of HLB SPE.....92

Figure 4.1 Proposed experimental approach for reducing mycotoxin LOQs 96

Supplementary Figures in Appendix

Supplementary Figure 1 Co-elution of fumonisins with alternariols in positive mode.....112

Supplementary Figure 2 Comparison of negative and positive ESI modes for AOH and AME standards 113

Supplementary Figure 3 EIC of CIT and OTα in 10x diluted non-spiked urine versus standard114

Supplementary Figure 4 EIC of AOH and AME in 10x diluted non-spiked urine versus standard.....114

Supplementary Figure 5 EIC of $OTAd_5$ and OTA in 10x diluted non-spiked urine versus standard.....115

Supplementary Figure 6 EIC of ENNB and ENNB1 in 10x diluted non-spiked urine versus standard.....115

Supplementary Figure 7 EIC of ENNA1 and ENNA in 10x diluted non-spiked urine versus standard.....116

Supplementary Figure 8 EIC of FB1 and $AMEd_3$ in 10x diluted non-spiked urine versus standard.....116

Supplementary Figure 9 EIC of FB3 and FB2 and BEA in 10x diluted non-spiked urine versus standard.....117

Supplementary Figure 10 EIC of creatinine standard.....117

Supplementary Figure 11 Intensity of OTA in ESI (+) and ESI (-) 118

Supplementary Figure 12 Elution of AOH and AME after adding extra mL of MeOH 119

Supplementary Figure 13 Flowchart for collection of washing solvent from HLB SPE 119

Supplementary Figure 14 The OTα losses using 20% MeOH as washing step in HLB SPE 120

Supplementary Figure 15 Losses of polar to mid-polar mycotoxins in MCX SPE using 10% MeOH as the washing step 121

Supplementary Figure 16 Elution of ENNs by adding extra MeOH in MCX SPE.....122

Supplementary Figure 17 EIC of mycotoxins in urine spiked at 10 ng/mL using HLB SPE with 20x enrichment. 123

Supplementary Figure 18 Detection of ENNA and ENNA1 in non-spiked pooled urine HLB SPE.....124

List of Tables

Table 1.1 Mycotoxins produced by fungi 2

Table 1.2 Summary of chemical properties of mycotoxins 8

Table 1.3 Summary of LC-MS methods for detection of mycotoxins in urine samples 13

Table 1.4 Summary of mycotoxin occurrence and concentration in urine in different countries 18

Table 1.5 Summary of LC methods for separation of common mycotoxin of interest in urine samples 23

Table 2.1 Gender, pH and creatinine level of different lots of urine used during method development. 34

Table 2.2 LC gradient using CORTECS T3 column 45

Table 2.3 Time segments, ionization mode and fragmentor voltage 46

Table 2.4 Summary of mycotoxin retention time. 47

Table 3.1 LOQs and R^2 obtained for dilute-and-shoot method in solvent and urine 64

Table 3.2 Concentration of ENNs and BEA found in 10x diluted urine and in MeOH used for rinsing Eppendorf tube 66

Table 3.3 Concentration of ENNs and BEA extracted from the second-time rinsing from the Eppendorf tube 67

Table 3.4 Retention time of mycotoxins using evaporation-reconstitution step with different enrichment factor 79

Table 3.5 The p-values of recovery of mycotoxins with and without 10% MeOH in the loading using two-tailed t-test. 87

Table 3.6 The p-values of recovery of mycotoxins with and without 5% MeOH in the loading using two-tailed t-test 90

List of Abbreviations

Acetic acid	AA
Alternariol	AOH
Alternariol monomethyl ether	AME
Alternariol monomethyl ether-d_3	AMEd_3
Beauvericin	BEA
Charged-residue model	CRM
Creatinine	CRE
Dispersive liquid-liquid microextraction	DLLME
Dispersive magnetic solid-phase extraction	DMSPE
Enzyme-linked immunosorbent assay	ELISA
Enniatin A	ENNA
Enniatin A1	ENNA1
Enniatin B	ENNB
Enniatin B1	ENNB1
Extracted-ion chromatogram	EIC
Fluorometric detector	FD
Food and Drug Administration	FDA
Formic acid	FA
Full width at half maximum	FWHM
Fumonisin B1	FB1
Fumonisin B2	FB2
Fumonisin B3	FB3
High-performance liquid chromatography	HPLC
High resolution mass spectrometry	HRMS
Hybrid triple quadrupole linear ion trap mass spectrometer	QTRAP
Waters OASIS hydrophilic-lipophilic balanced sorbent	HLB
Immunoaffinity column	IAC

Ion-desorption model	IDM
Isopropanol	ISP
Limit of detection	LOD
Liquid chromatography − mass spectrometry	LC-MS
Liquid-liquid extraction	LLE
Limit of quantification	LOQ
Matrix effect	ME
Methanol	MeOH
Mixed-mode strong anion exchange	MAX
Mixed-mode strong cation exchange	MCX
Magnetic multiwalled carbon nano tube	MWCNT
Negative electrospray ionization	ESI (-)
Ochratoxin A	OTA
Ochratoxin A-d_5	OTAd_5
Ochratoxin alpha	OTα
Positive electrospray ionization	ESI (+)
Process efficiency, %	PE%
Quadrupole-time-of-flight mass spectrometer	QTOF
Recovery	RE
Relative standard deviation	RSD
Reversed-phase (Liquid chromatography)	RP (LC)
Salting-out assisted liquid-liquid extraction	SALLE
Strong anion exchange solid-phase extraction	SAX SPE
Signal-to-noise ratio	S/N
Solid-phase extraction	SPE
Tetramethylbenzidine	TMB
Total ion chromatogram	TIC
Triple quadrupole mass spectrometer	QqQ
Ultra-high performance liquid chromatography	UHPLC

List of Equations

Equation 1.1 Rayleigh limit equation 25

Equation 1.2 Kinetic energy of ion 26

Equation 1.3 Velocity of ion 27

Equation 1.4 Mass of ion 27

Equation 1.5 Calibration equation in TOF 27

Equation 2.1 Calculation of ME% 36

Equation 2.2 Calculation of RE% 36

Equation 2.3 Calculation of PE% 38

Chapter 1. Introduction

1.1. Classification and occurrence of mycotoxins

Mycotoxins are low molecular weight toxins that are produced as a secondary metabolites by different filamentous fungi.[1-2] The term "mycotoxin" was first introduced when 100,000 turkey poults died in 1962, and the reason for their death was linked to peanuts tainted with secondary metabolites from *Aspergillus flavus* (aflatoxin).[1, 3] Mycotoxins can be produced by different genera of fungi such as *Aspergillus, Penicillium, Fusarium, Claviceps* and *Alternaria, Stachybotrys,* though the first three are attributed to most of mycotoxin production.[3-4] The most prevalent genera of fungi that produce mycotoxins are shown in Table 1.1. However, this is not an exhaustive list. For instance, beauvericin can be produced by different species of *Fusarium* fungi such as *F. concentricum, F. oxysporum, F. proliferatum, F. fujikuroi,* and *F. poae* strains.[5] Sometimes different mycotoxins can be produced by the same genera and species of fungi. For example, *Fusarium beauveria, F. halosarpheia, F. paecilomyces, F. polyporus, F. verticillium* can produce both enniatins (ENNs) and beauvericin (BEA).[4] Hence, the classification of mycotoxins according to the fungi that produce them is challenging.[6] In addition, mycotoxins can be classified according to their chemical structures and biological effects. For instance, clinicians frequently group them according to the organs they affect, organic chemists according to their chemical structure and biochemists according to the biosynthesis pathways. However, none of these categorizations are satisfactory.[3]

To date, more than 400 mycotoxins have been discovered, but only some types pose a threat to food safety and human health.[4, 7] Long-term chronic human exposure to low levels of mycotoxins can cause severe health effects such as cancer, hepatic diseases, hemorrhagic syndrome, neurological disorders, fetal toxicity, teratogenicity, and immunosuppression.[4, 7-9] The exposure can occur by inhalation (presence of mold) or by consumption of different types of grains, nuts, vegetables, beverages like wine, juice, coffee and so on. Consumption of animal products such as meat, eggs and milk can also lead to indirect exposure to mycotoxins.[2, 4, 10-12]

The mycotoxins commonly found in different food products are fumonisin B1 (FB1), fumonisin B2 (FB2), ochratoxins A (OTA) and ochratoxin alpha (OTα).[13-14] Emerging mycotoxins detected in cereal-based infant foods, various grains, fruits, and food products include citrinin (CIT),

alternariol (AOH), alternariol monomethyl ether (AME), beauvericin (BEA) and enniatins (ENNs) including enniatin A (ENNA), enniatin A1 (ENNA1), enniatin B (ENNB) and enniatin B1(ENNB1).[13-14]

Table 1.1. Mycotoxins produced by fungi

Mycotoxin	Genera of fungi	Species	References
Ochratoxins	*Aspergillus*	*A. ochraceus*	15,4
		A. niger	
		A. corbonarius	
	Penicillium	*P. verrucosum*	
		P. viridicatum	
Enniatins	*Fusarium*	*F. avenaceum*	16,17
Beauvericin		*F. oxysporum*	18
		F. subglutinans	
		F. proliferatum	
Fumonisins		*F. verticilloides*	19-20
		F. proliferatum	21
Alternariol group	*Alternaria*	*A. alternata*	22
Citrinin	*Penicillium*	*P. citrinum*	15
		P. expansum	
		P. radicicola	
		P. verrucosum	
	Monascus	-	

Molds can contaminate cereal grains both in the field and during storage. Although the moldy part can be removed through different manufacturing processes, mycotoxins are stable and can remain in food products, dried nuts, coffee, fruits or beverages (e.g. beer and wine) after processing.[23-24] Mycotoxin concentrations can be reduced by a variable degree by using high-temperature procedures. For instance, a 50% decrease in CIT concentration can be achieved by boiling and heating it for 20 minutes. Also, CIT will degrade at a temperature of around 175°C. Therefore, it is absent in beer after the pressing process.

Due to global warming, there is a lot of variations in the growth of fungi and the spread of their secondary metabolites across the world. Humidity and temperature have a direct effect on the

production of fungi in different grains such as corn, wheat, rice and oats. A 3-year long study recently reported high levels of fumonisins (FBs) found in corn, rice and sorghum in Africa.[11] The average levels of FB1 found in corn in Spain, Algeria, Serbia and Italy were about 1559, 14812, 4140 and 4868 μg/kg, respectively. The highest content of FBs in corn was reported to be about 43297 μg/kg in Italy.[11] The maximum recommended allowable level of FBs in corn products set by the Food and Drug Administration (FDA),[25] and European Commission are 5000 and 2000 μg/kg, respectively.[26] Additionally, FBs have been also detected in rice samples in Somalia as high as 20596 μg/kg.[27] High levels of FBs were also detected in sorghum (8400 μg/kg) in Nigeria, exceeding the limits recommended by European Union (1000 μg/kg).[26, 28]

OTA is another mycotoxin found in various quantities in grains from different countries. In Vietnam, significant levels of OTA (1662 μg/kg) have been detected in corn.[29] This level exceeds the limit set by Health Canada and the EU, which is 5 μg/kg for raw cereal grains.[26, 28] OTA and FBs short half-lives in blood are 39 days and 18 minutes, respectively. Therefore, the regulation limits are lower for OTA than for FBs.[30-31] OTA has been detected in wheat at the highest level of 45 μg/kg in Qatar and the lowest level of 0.1 μg/kg in Turkey.[32-33] Furthermore, OTA has been detected at 15, 24, 2.2 μg/kg in rice samples from West Africa, Pakistan and Greece, respectively, from which the first two exceed the limit proposed by Health Canada (3 μg/kg).[28] In a recent study on barley samples in Canada, OTA was found at a mean level of 64 μg/kg in 10% of samples.[34] In Tunisia, Somalia and Nigeria, the maximum levels of OTA were determined to be 27.8, 2.44 and 5.6 μg/kg in sorghum samples, respectively.[11] Ochratoxins have also been detected in wine, coffee, juice, cheese and dried fruits.[8]

Many foodstuffs have presented detectable levels of citrinin (CIT). Citrinin has been detected in grains such as rice in Japan, China, Taiwan, Malaysia, Canada, Egypt, Vietnam, Spain, Iran and India. The highest quantities of CIT detected in different types of rice were, the red fermented rice in China with the highest level of 44240 μg/kg, red rice in Malaysia (20650 μg/kg), red mold rice in Taiwan (27,000 μg/kg) and 2 samples of rice in Canada (1130 μg/kg)[35] The limits of CIT in rice fermented with red yeast set by European Union No. 212/2014, China and Japan are 200, 50 and 200 μg/kg, respectively. In addition, the incidence of CIT in wheat samples in Canada and Tunisia was about 67.6% and 50%, respectively. In maize samples from Burkina Faso and Mozambique, only 3 out of 26 had detectable CIT, whereas 23 out of 51 samples from

Serbia contained CIT.[36] In different samples from spices in India, CIT has been also detected. The highest level was detected in dry ginger (85.1 ng/g).[37] CIT was also found in apples (320-920 µg/kg) in Portugal, figs (60 µg/kg) in Germany, and black olives (75-350 µg/kg) in Turkey.[35] CIT in cheese in France and moldy cheese in Egypt was found at 600 µg/kg and 50 µg/kg, respectively.[35, 38]

Enniatins (ENNs) are emerging mycotoxins that can contaminate different grains such as corn, rice, barley, oat or other foodstuffs (potatoes, coffee, apples and sugar beets). This is partially because they can be produced by various species of *Fusarium* in different ecological conditions around the world. ENNs have been mostly detected in European crops. The greatest levels of contamination were seen in wheat at maximum level of (635000 µg/kg) and maize (813000 µg/kg) for ENNA1 grown in Spain.[39] They have been also detected in wheat grown in Canada, the Shandong province of China, Tunisia and Morocco.[40]

Beauvericin (BEA) is one of the mycotoxins most frequently found in rice samples in Iran with a maximum concentration of 0.47 µg/kg. BEA has also been detected in wheat, barley and corn and cereal-based foods. The percentages of samples contaminated with BEA (>2.1 µg/kg) from corn products in Poland, Italy, South Africa, Brazil and Spain were found to be 93%, 62%, 100%, 96%, and 31%, respectively. All samples derived from Finland were positive for BEA and contained a maximum of 3500 µg/kg.[41] Since acute effects of ENNs and BEA have not been detected in humans, there are no specific regulations for the maximum acceptable limits of these mycotoxins in food products.[7, 40]

Species of *Alternaria* wreak havoc on a wide range of crops in part due to their propensity to grow at low temperature. Thus, storage at low temperatures cannot prevent *Alternaria* growth in vegetables and fruits. The secondary metabolites produced by *Alternaria* species, AOH and AME, were detected in different types of juice, tomato and its by-products, olives, peppers, edible oils, red wine, lentils, and so forth. AOH (maximum level of 5.02 µg/kg) and AME (maximum level of 0.23 µg/kg) have been detected in Canadian wine.[22] The highest levels of AOH and AME were determined in tomato puree in Argentina at 8756 and 1734 µg/kg, respectively. Furthermore, these mycotoxins have contaminated edible oils in Germany, wheat products in Sweden, tomato products, pea, and wheat in the Czech republic.[22]

In general, climate change is promoting the growth of fungi and therefore enhancing the occurrence of mycotoxins in a variety of environments worldwide. The increase of these secondary metabolites may endanger what once were safe food supplies considering the expansion of imports and storage of food products in different countries.

1.2. Toxicity of mycotoxins

As mentioned earlier, mycotoxins are widespread in different food products worldwide, and some mycotoxins can be relatively stable during food processing and storage.[23] Therefore, for those that are highly toxic, their presence in food products can be hazardous to humans and animals causing severe chronic or acute health effects.[8] Mycotoxicosis is a mycotoxin-induced illness in animals and humans.[2] OTA has shown to have carcinogenic, teratogenic, immunotoxic, nephrotoxic and neurotoxic effects.[8, 42] Renal failure due to inhalation of OTA was detected in a case where a farmer and his wife suffered from temporary respiratory stress and tubulonecrosis after they spent 8 hours in a barn that was closed for months.[42-43] Wheat in the granary contained a strain of *A. ochraceus* which produced OTA.[42-43] A study of the activity of two renal tubule enzymes in pigs demonstrated a 40% reduction in enzyme activity after they were subjected to OTA for one week.[42] Furthermore, OTA's half-life is about 840h (35 days) in blood after ingestion. Reabsorption during enterohepatic circulation, reabsorption from the urine following tubular release, or significant protein binding, may all contribute to the toxin's delayed excretion in humans and its accumulation in tissues.[30] Consumption of more than 70 μg/kg of bodyweight of OTA per day may lead to renal tumors and DNA adducts in renal tissues and tumors.[42, 44]

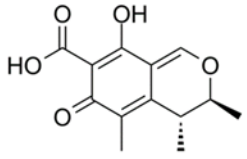
Co-occurrence of OTA with other nephrotoxic mycotoxins such as CIT or FBs can lead to synergistic effects and augment their genotoxicity.[30, 45] In addition, OTA and FBs (FB1 and FB2) are classified as potential carcinogens to human (group 2B).[9] The main source of FBs production is moldy maize. In one case of acute food poisoning in India, FBs were linked to borborygmi, abdominal pain, and diarrhea.[46] Moreover, leukoencephalomalacia in horses and pulmonary edema in pigs have both been associated with FBs.[47] FBs are similar in their structure to sphingoid bases, sphinganine (Sa) and sphingosine (So), and thus they disrupt sphingolipid metabolism.[47] In a study on rats, Shephard *et al.* showed that the elimination half-life of FB1 in plasma is about 18 min.[31] In other studies, the elimination half-life of FB1 in liver and kidney was found to be about 4h and 7h, respectively. Thus, the nephrotoxic effect of FB1 is due to its

aggregation in the kidney in rats, which is 10-fold higher than in liver in rats.[47-48] CIT has similar nephrotoxic effect and its major target organ is also the kidney.[49] CIT was first isolated from *P. citrinum*, but it can be produced from the same fungi that produce OTs. CIT co-occurrence with OTA can escalate its toxicity due to additive or synergistic effect.[49-50] CIT is categorized as a group 3 carcinogen since there is limited evidence to prove its carcinogenic effect.[9] It can lead to embryocidal and fetotoxic effects in mice, and it was demonstrated to induce apoptosis in promyelocytic leukemia cells in humans and porcine kidney PK15 cells.[49]

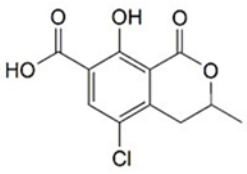
Beauvericin can cause apoptosis and DNA fragmentation by changing the concentration of calcium due to its ionophoric properties inside the cell.[7, 41] When BEA and ENNs were combined, the myelotoxic effects were amplified.[41] ENNs can lead to inhibition of many enzymes such as the acyl-CoA:cholesterol acyl transferase.[5] AOH and AME, two emerging mycotoxins can be genotoxic to bacteria, human and animal cells.[22] *A. alternata*, which produces AOH and AME is involved in the origin of oesophageal cancer in humans.[22, 51]

In summary, mycotoxins can have adverse health effects on humans and animals leading potentially to acute or chronic conditions. Thus, it is generally prudent to reduce exposure to mycotoxins and to monitor their level in food supplies and biological samples.

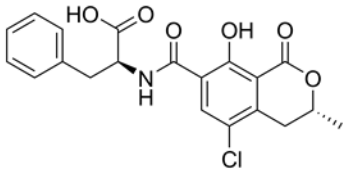
(a) Citrinin and ochratoxins



Citrinin (CIT)

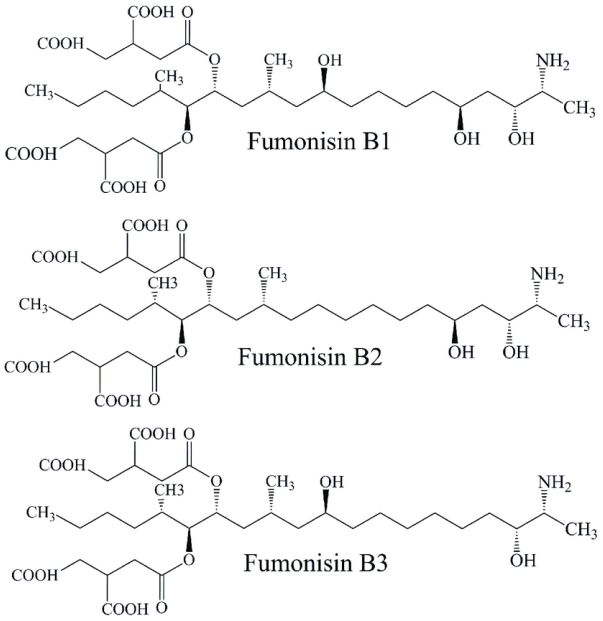


Ochratoxin alpha (OTα)

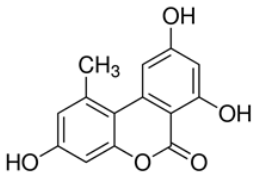


Ochratoxin A (OTA)

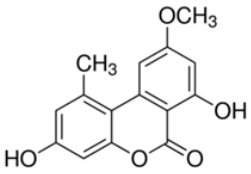
(b) Fumonisins



(c) Alternariols

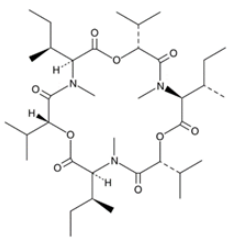


Alternariol (AOH)

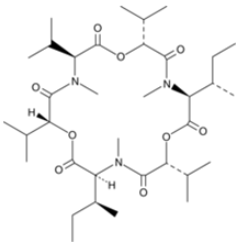


Alternariol monomethyl ether (AME)

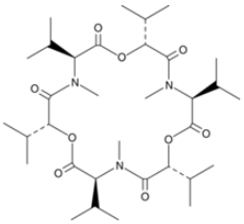
(d) Enniatins and beauvericin



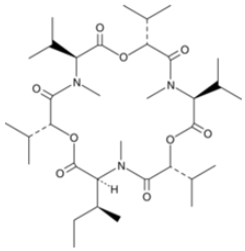
Enniatin A (ENNA)



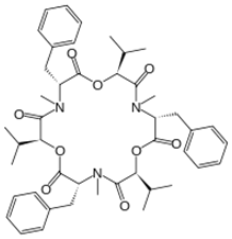
Enniatin A1 (ENNA1)



Enniatin B (ENNB)



Enniatin B1 (ENNB1)



Beauvericin (BEA)

Figure 1.1. Structures of mycotoxins, (a) Citrinin[52-53] and ochratoxins[53], (b) fumonisins,[54] (c) alternariols[52-53], (d) enniatins and beauvericin[55] Enniatins (https://www.caymanchem.com/), Citrinin, ochratoxins, alternariol, alternariol monomethyl ether (https://www.lookchem.com/Chempedia/Chemical-Resource/Chemical-DataBase/13857.html). Fumonisins figure reprinted with permission from Ren, Yiping, *et al.* "Simultaneous

determination of fumonisins B1, B2 and B3 contaminants in maize by ultra high-performance liquid chromatography tandem mass spectrometry." Analytica chimica acta 692.1-2 (2011): 138-145. Copyright 2011 Elsevier.

Table 1.2. Chemical properties of mycotoxins.

Mycotoxins	Chemical formula[56]	Log P[a]	pKa[a]
Citrinin (CIT)	$C_{13}H_{14}O_5$	0.81	3.55
Fumonisin B1(FB1)	$C_{34}H_{59}NO_{15}$	-0.67	3.16
Ochratoxin A (OTA)	$C_{20}H_{18}ClNO_6$	5.43	3.26
Fumonisin B2 (FB2)	$C_{34}H_{59}NO_{15}$	0.72	3.16
Enniatin A (ENNA)	$C_{36}H_{63}N_3O_9$	6.46	18.8
Enniatin A1 (ENNA1)	$C_{35}H_{61}N_3O_9$	5.93	18.8
Beauvericin (BEA)	$C_{45}H_{57}N_3O_9$	7.27	18.8
Enniatin B (ENNB)	$C_{33}H_{57}N_3O_9$	4.96	18.8
Enniatin B1(ENNB1)	$C_{34}H_{59}N_3O_9$	5.41	18.8
Ochratoxin alpha (OTα)	$C_{11}H_9ClO_5$	3.4	2.36
Alternariol (AOH)	$C_{14}H_{10}O_5$	3.18	7.63
Alternariol monomethyl ether (AME)	$C_{15}H_{12}O_5$	3.32	7.71

a. http://www.t3db.ca/.

1.3. Biomonitoring studies

Mycotoxins are widely monitored in different food products to ensure they meet regulatory guidelines. However, the exposure effects can be hard to predict in human beings. The reason is that each person has specific diet and numerous sources of grains, beverages, nuts, meats, etc. that can be consumed.[57] Therefore, to better understand the extent of exposure, the presence of mycotoxins needs to be studied or monitored periodically in biological fluids such as urine and plasma. Human biomonitoring can assist to detect trends of these mycotoxins in different populations and help verify if current guidelines for foodstuffs are sufficient to protect the consumer health.

1.4. Urine biomonitoring

Urine samples consist of 95% water and different percentages of various salts and minerals.[58] The typical composition of human urine is shown in Figure 1.2. Urine sample collection is non-invasive and easier to collect compared to plasma and other biological fluids. However, urine samples vary greatly in terms of pH and composition across different lots. Therefore, urine

normalization is essential when comparing results across different urine samples. The concentration of urine can be adjusted by various normalization approaches, for example specific gravity or osmolality for normalization.[59-60] In addition, different biomarkers can also be used. Historically, creatinine is the most frequently used biomarker for urine normalization. Basically, creatinine (CRE) is a by-product of creatine which is mostly produced in skeletal muscle and is considered as a good biomarker for diseases related to kidney excretion.[61-62,63] Also, creatinine is the best available marker in healthy humans to normalize the excretion rates across different lots of urine, and to compare across people, different days, different times of collection and so forth. For instance, Adedeji *et al.*, compared urinary creatinine to urinary Cystatin C and urine volume, and determined creatinine as the best approach for urine normalization.[64] Mycotoxin concentration can be reported per volume or per volume and mg creatinine.

Urine normalization can be performed either before or after analysis. For example, for methods such as LC-fluorescence detection and ELISA, urine normalization is frequently reported after analysis and the mycotoxin concentration can be later reported according to creatinine concentration. However, in LC-MS analysis, differences in urine composition and pH can cause different matrix effects across different lots of urine and thus affect the quality of analysis, especially when isotopically-labelled internal standards are not available for every mycotoxin of interest. Thus, urine normalization and pH adjustment should be performed before LC-MS analysis to ensure the best quality of the measurement. By normalizing before LC-MS analysis, both ionization and sample preparation matrix effects can be minimized, and retention time shifts of early eluting analytes by overloading the column can be controlled. In addition, although creatinine can be measured using LC-MS, it is present in high concentrations (>10 mg/dL) and would typically exceed linear range of LC-MS/MS measurement especially during simultaneous trace analysis measurement of mycotoxins. In this thesis, creatinine normalization as determined by rapid CLINITEK Status® method will be used to classify urine samples used during the method development as low, normal or high in creatinine. For more accurate creatinine measurements in real samples for biomonitoring, other methods such as Jaffe can be used.

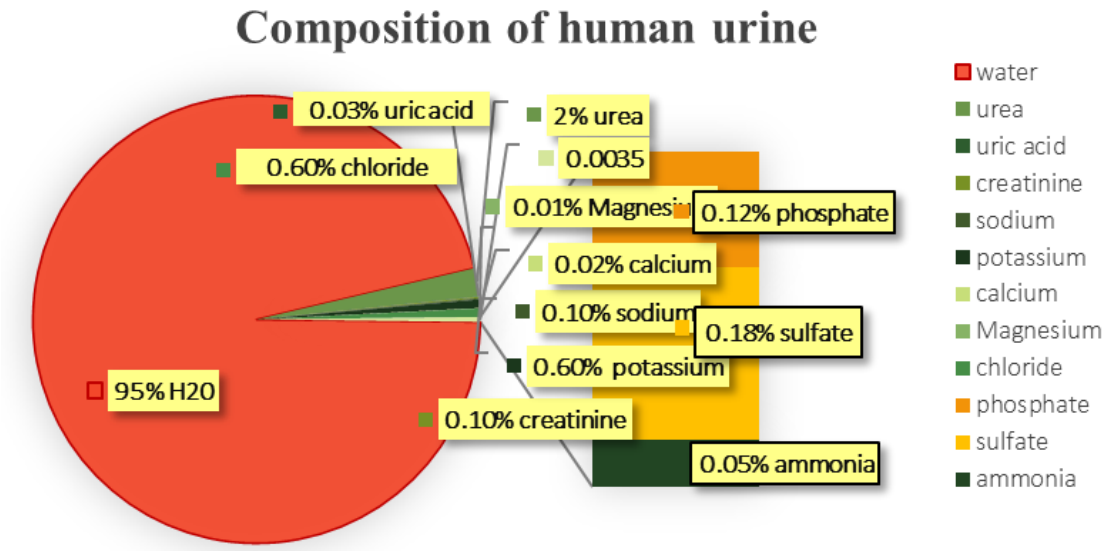


Figure 1.2. Urine composition (volume%)

1.5. Existing methods for the detection of mycotoxins in urine

To quantitate trace levels of mycotoxins, the method developed should be sensitive enough to detect the lowest levels in urine samples. Some methods, such as enzyme-linked immunosorbent assay (ELISA) or immunoaffinity columns (IAC) with HPLC-FD (High-performance liquid chromatography-fluorometric detector), can provide good detection with good LOQs (limits of quantification), but they are not applicable to analyze multiple mycotoxins. Furthermore, there is the possibility to get a false positive or negative result with ELISA.[65]

Table 1.3 summarizes the key methods used to date for the detection of multiple mycotoxins in urine biomonitoring. Most of these approaches rely on LC-MS with reversed-phase chromatography as the separation method of choice, and triple quadrupole (QqQ) or ion trap (QTRAP) as the detection method, as shown in Table 1.3. In LC-MS, multiple mycotoxins can be studied at the same time, and no derivatization is required. Selected reaction monitoring is most frequently used since it provides better LOQs. Although there is great interest in HRMS, Pallares *et al.* reported the LOQ of OTA using LC-QTOF to be about 10 ng/mL which shows the method is not sensitive enough to measure the expected levels below 1 ng/mL in real samples. [66] Pallares *et al.* tested three sample preparation techniques QuEChERS, Strata C18 SPE (solid-

phase extraction) and DLLME (dispersive liquid-liquid microextraction).[66] The low recovery of 7% and 30% was detected for OTA using SPE and DLLME, respectively. Thus, the validation performed with QuEChERS which provided recovery of 55-76% . [66]

Table 1.3 also shows that most of these methods used "dilute-and-shoot", immunoaffinity column (IAC) or QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) as the sample preparation method. Dilute-and-shoot is a fast and easy sample preparation, however the LOQs for most of analytes are not good enough as this method does not provide any sample clean-up. Hence, using other sample clean-up methods is essential to improve the LOQs. For this project LOQs of less than 0.01 ng/mL are needed for detection of mycotoxins of interest. Liu *et al.* reported good LOQs for OTA, OTα, ENNs, AOH, AME and BEA using HLB SPE as a sample preparation. However, significant ionization suppression was detected for OTA (49.0%,) FB1 (77.3%), FB2 (69.3%), OTα (12.2%) and AOH (16.4%). As a result, the reliability of LOQs for OTA, OTα and AOH is poor since such high suppression, may completely suppress the peaks of interest. High ionization suppression without the use of labelled internal standard for each analyte can also cause poor accuracy during quantitative analysis across different urine samples. According to Sarkanj *et al.,* PRIME HLB SPE has better extraction efficiency and provides better clean-up compared to Oasis HLB SPE.[67] The reported LOQs using PRIME HLB SPE for AOH, OTA, CIT and FB1 were 0.03, 0.001, 0.01 and 0.01 ng/mL, respectively. Ediage *et al.* found unacceptable limits of detection (LODs) of more than 10 ng/mL using both the dilute-and-shoot method and the dilute-evaporate-and-shoot method for almost all the mycotoxins.[68] As a result, the authors suggested two alternative sample clean-up methods to further remove urine interferences: 1) liquid–liquid extraction (LLE) with ethyl acetate/FA (99/1, v/v) followed by SAX-SPE (strong anion exchange solid-phase extraction) clean-up; 2) using HLB SPE (hydrophilic-lipophilic solid-phase extraction). The analysis of the eluate from HLB SPE showed severe ionization suppression for all the compounds evaluated, whereas SAX SPE exhibited 3- to 9-fold lower LOD compared to HLB SPE as it provided better clean-up.[68]

Escrivá *et al.* did a comparative extraction study between SALLE (salting-out liquid-liquid extraction), miniQuEChERS and DLLME (dispersive liquid-liquid microextraction).[69] Between the three sample preparation method, DLLME provided better validation results. The LOQs for ENNB and ENNB1 were about 0.1 ng/mL, and for OTA, ENNA, ENNA1 and BEA 4, 0.5, 0.3

and 1 ng/mL, respectively. Significant ion enhancemnt (116-144%) was foundfor ENNA, ENNA1, ENNB1, BEA and OTA using SALLE. In miniQuEChERS, ionization suppression was found for BEA (60.8%) and ENNB (71.2%). DLLME also showed ionization suppression for ENNA1 (72%), ENNB1 (79.2%) and BEA (70.6%).[69]Silva *et al.* showed LOQ of 10 ng/mL for both FB1 and FB2 using IAC sample clean-up. This LOQ is not enough to determine the traces of FBs in urine.[70]

In another sample preparation method proposed by Fan *et al.,* the urine sample was diluted with ACN (acetonitrile) and after evaporation, it was reconstituted in H_2O/ACN containing 5 mmol/L ammonium acetate (20/80, v/v). This method showed significant ionization suppression for OTA (58%).[71] Significant ion enhancement of about 196-335% was detected for FBs with dilute-and-shoot method by Huybrechts *et al.*.[72] Gerding *et al.* reported good LOQs for ENNB (0.0125 ng/mL), FB1 and FB2 (0.2 ng/mL), OTA (0.25 ng/mL) and OTα (0.1 ng/mL) using 10x dilution of urine samples with H_2O/ACN/FA (94/5/1 v/v/v).[73] Furthermore, ion enhancement was observed for ENNB.[73] Similarly, Warth *et al*. used an aqueous solvent (ACN/H_2O:10/90, v/v) in a dilute-and-shoot method. The LOQs for FB1 and FB2 were reported to be 2 ng/mL which is quite high.[74] In the last two studies, highly aqueous solvent was used for diluting urine samples. However, ENNs show low solubility using 40% MeOH according to Slobodchikova *et al*.[75] Therefore, LOQ levels might not be accurate and the traces of ENNs can be lost due to non-specific adsorption or poor solubility. Viegas *et al.* used high organic solvent H_2O/ACN/FA (5/95/0.1, v/v/v) for diluting urine samples.[76] The LOQs for OTA, ENNB and CIT found out to be 0.036 and 0.02 and 2 ng/mL, respectively. For detecting the trace levels of mycotoxins in urine, LOQs above 1 ng/mL are quite high.[76]

Altogether, none of these methods could provide good LOQs for the panel of 12 mycotoxins. Consequently, developing a sensitive and reliable method for assessing the lowest concentration of these mycotoxins is crucial.

Table 1.3. Summary of LC-MS methods for detection of mycotoxins in urine samples

Year	Author	Ref.	Sample preparation	LOQ (ng/ml)		LC column	Detector
2006	Pena *et al.*	77	OchraTest™ IAC	OTA = 0.02		C_{18}-Nucleosil 100 KS (250 mm × 4.6 mm i.d., 5 µm)	HPLC-FD
2010	Silva *et al.*	70	IAC	FB1 = 10 FB2 = 10		Luna C_{18} (150 mm × 4.6 mm i.d., 5 µm)	QqQ
2010	Ahn *et al.*	78	IAC	FB1 = 0.022 FB2 = 0.010	OTA = 0.004 OTα = 0.135 (β-glucuronides LLE)	Hypersil GOLD ODS (150 mm × 4.6 mm i.d., 5 µm)	QTRAP
2011	Rubert *et al.*	79	IAC MYCO 6in1™	OTA = 1.5 FB1 = 15 FB2 = 15		Gemini C_{18} (150 mm × 2.0 mm i.d., 3 µm)	QTRAP
2011	Desalegn *et al.*	80	IAC	OTs = 0.005 FBs = 0.035		-	ELISA kits
2012	Klapec *et al.*	81	LLE ($CHCl_3$/ISP) before and after enzymatic treatment	Before enzymatic treatment OTα = 0.02 OTA = 0.16 Before enzymatic treatment OTα = 0.02 OTA = 1.18		Luna C_{18} (250 mm × 3 mm i.d., 5 µm)	HPLC-FD
2012	Ediage *et al.*	68	LLE (ethyl acetate/FA (99/1, v/v))+ SAX-SPE clean-up	OTα = 0.06 OTA = 0.06	CIT = 5.76 FB1 = 0.1	ZORBAX SB-C_{18} (100 mm × 2.1 mm i.d., 3.5 µm)	QqQ (Positive mode)

Year	Author	Ref.	Sample preparation	LOQ (ng/ml)		LC column	Detector
2013	Abia *et al.*	82	Dilute-and-shoot (ACN/H_2O:10/90, v/v)	FB1 = 1.7 FB2 = 1.7 OTA = 0.17		Atlantis® T3 (150 mm × 3 mm i.d., 5 µm)	QTRAP
2014	Solfrizzo *et al.*	83	IAC MYCO 6in1™ + Oasis HLB SPE	OTA = 0.006 FB1 = 0.010		Acquity UPLC BEH column (150 mm × 2.1 mm i.d., 1.7 µm)	QqQ
2015	Huybrechts *et al.*	72	Direct method IAC clean-up for OTA, CIT	OTα = 0.2 CIT = 0.003 OTA = 0.003	FB1 = 0.2 FB2 = 0.2	UPLC HSS T3 (100 mm × 2.1 mm i.d., 1.8 µm)	QqQ
2014	Gerding *et al.*	73	Dilute-and-shoot H_2O/ACN/FA (94/5/1, v/v/v)	ENNB = 0.0125 OTα = 0.1 OTA = 0.25	FB1 = 0.125 FB2 = 0.5	NUCLEODUR C_{18} pyramid (150 mm × 2.0 mm i.d., 3 µm)	QTRAP
2014	Warth *et al.*	74	Dilute-and-shoot (ACN/H_2O:10/90, v/v)	FB1 = 2 FB2 = 2 OTA = 0.15		Atlantis® T3 (150 mm × 3 mm i.d., 3 µm)	QTRAP
2015	Gerding *et al.*	73	Dilute-and-shoot H_2O/ACN/FA (94/5/1, v/v/v)	FB1 = 0.1 ENNB = 0.005	OTα = 1 OTA = 0.075	NUCLEODUR C_{18} pyramid (150 mm × 2.0 mm i.d., 3 µm)	QTRAP
2017	Escrivá *et al.*	69	DLLME (ethyl acetate)	OTA = 4 ENNB = 0.1 ENNB1 = 0.1	BEA = 1 ENNA = 0.5 ENNA1 = 0.3	Gemini C_{18} column (150 mm × 2 mm i.d., 3 µm)	QTRAP
2019	Viegas *et al.*	76	Dilute-and-shoot H_2O/ACN/FA (5/95/0.1, v/v/v)	ENNB = 0.02 CIT = 2 OTA = 0.036		C_{18} Pyramid (100 mm × 2 mm i.d., 3 µm)	QTRAP
2018	Sarkanj *et al.*	67	Oasis HLB PRIME SPE	OTA = 0.001 FB1 = 0.01 AOH = 0.03 CIT = 0.01		HSS T3 column (100 mm × 2.1 mm i.d., 1.8 µm)	QTRAP

Year	Author	Ref.	Sample preparation	LOQ (ng/ml)		LC column	Detector
2019	Fan *et al.*	71	Protein precipitation (ACN)	OTA = 0.1 OTα = 0.2 FB1 = 0.5		UPLC Agilent Poroshell 120 EC-C_{18} (3.0 mm × 100 mm, 2.7 μm)	QTRAP
2019	Franco *et al.*	84	IAC MYCO 6in1™ + Oasis HLB SPE	OTA = 0.017 FB1 = 0.007 FB2 = 0.013		BEH C_{18} (50 mm × 2.1 mm i.d., 1.7 μm)	QqQ
2020	Qiao *et al.*	85	LLE (ethyl acetate)	AOH = 0.06 AME = 0.001		BEH C_{18} (50 mm × 2.1 mm i.d., 1.7 μm)	QqQ
2020	Silva *et al.*	86	IAC (OchraTest™)	OTA = 0.019		C_{18} Nucleosil (250 mm × 4.6 mm i.d., 5 μm)	FD
2020	Ouhibi *et al.*	87	QuEChERS	CIT = 0.2		Acquity UPLC® HSS T3) (2.1 × 100 mm, 1.8 μm)	QqQ
2020	Arroyo *et al.*	88	DMSPE (Fe_3O_4@MWCNT)	ENNA = 0.04 ENNA1 = 0.1 ENNB = 0.04	ENNB1 = 0.04 BEA = 0.04	ZORBAX RRHD Eclipse Plus C_{18} (1.8 μm, 2.1 × 100 mm)	QTOF
2022	Pallarés *et al.*	66	QuEChERS	OTA = 10		Gemini® NX-C18 (3 μm, 150 mm × 2 mm i.d,)	QTOF
2020	Liu *et al.*	89	Oasis HLB SPE	ENNB = 0.0002 ENNB1 = 0.0002 ENNA = 0.0008 ENNA1 = 0.0005 BEA = 0.0006 AOH = 0.04	OTA = 0.02 FB1 = 0.05 FB2 = 0.05 FB3 = 0.03 OTα = 0.05 AME = 0.003	CORTECS UPLC C_{18} (100 mm × 2.1 mm i.d., 1.6 μm)	QqQ

1.6. Results of biomonitoring studies for mycotoxins in urine

Knowledge of the levels of mycotoxins detected in different countries can give us an insight onto human exposure to mycotoxins from different sources. Mycotoxin levels can be also correlated to the consumption of certain foods. Examining the literature, the desirable LOQ for biomonitoring of selected mycotoxins as in urine should be as low as 0.01 ng/mL to detect the lowest level in urine sample.

A summary of the occurrence of mycotoxins and concentrations in urine from studies in different countries is presented in Table 1.4. The table provides details of the number of samples, types of mycotoxins and their detected concentrations, as well as mycotoxin concentration corrected by urine creatinine. It is worth mentioning few studies reported the mycotoxin concentrations normalized to the creatinine levels (Table 1.4).

Mycotoxins can be excreted in free or conjugated form. By doing enzymatic treatment, phase II conjugation can be removed.[89] In studies where urine mycotoxin levels were reported with and without enzymatic treatment, higher levels were generally found when enzymatic treatment was employed indicating the presence of mycotoxin metabolites. Among the mycotoxins detected in urine samples from different countries, OTA is one of the most frequently detected. Liu *et al.* detected OTA, OTα, AOH, AME and ENNB after enzymatic treatment at mean levels of 0.140, 2.38, 7.68, 0.167 and 0.0014 ng/mL respectively, in urine samples from Beijing.[89] Normalization was not performed for determination of these mycotoxins. Of the Beijing samples, about 40% were contaminated with OTA, 83% with OTα, 33% with AME and 40% with ENNB.[89] Coronel *et al*. found the maximum levels for OTA and OTα at 0.562 ng/mL and 2.895 ng/mL respectively, in urine samples that were enzymatically treated.[90] In a recent study done in Beijing, out of 2212 urine samples collected, 11.1% of them were contaminated with AOH at concentration ranging from <0.06-32 ng/mL, and 96% with AME ranging from <0.001-2.02 ng/mL.[85, 91] The maximum urine levels of OTα and CIT found in Belgium were 15 ng/mL and 6.8 ng/mL, respectively.[68] The highest level of CIT was found among samples from Nigerian volunteer donors at a concentration of 241.46 ng/mL. A recent study showed that 40% of CIT is excreted in human urine, and that is probably one of the reasons why detectable levels have been found in many countries.[35] The 85% of urine samples collected from Nigerian volunteers were positive to FB1, and the maximum level reported was 14.88 ng/mL.[67] Gerding *et al.* detected

ENNB in Haiti, Germany and Bangladesh urine with mean concentration of 0.036, 0.012 and 0.019 ng/mL, respectively.[92] Additionally, OTA was detected at mean levels of 0.109, 0.04, 0.203 ng/mL in Haiti, Germany and Bangladesh, respectively.

To date, ENNs other than ENNB were not found in urine samples with the current biomonitoring methods. Additionally, no Canadian urine biomonitoring data exists for any of 12 mycotoxins of interest. To aid high-throughput biomonitoring studies, additional method development is needed to ensure that the trace levels (<1 ng/mL) of these mycotoxins can be detected and quantitated by achieving a good LOQ.

Table 1.4. Summary of mycotoxin occurrence and concentration in urine in different countries

Ref.	Year	Authors	Number of samples tested	Number of positive samples	Mycotoxin concentration levels corrected with creatinine (ng/mg)	Mycotoxin concentration levels (ng/ml)
68	2012	Ediage *et al.*	40 Belgium	9	OTA = 0.04-0.3 OTα = 2.5-6 CIT = 4.5	OTA = ND-0.6 (3 samples) OTα = ND-15 (3 samples) CIT = ND (1 sample 6.8)
69	2017	Escrivá *et al.*	10 (5 female, 5 male) Valencia (Spain)	4 positives for ENNB 4 detectable but not quantifiable for ENNB1 2 non-detectable	-	ENNB = 0.1-0.54 ENNB1 0.1-0.34 (2 samples)
71	2019	Fan *et al.*	260 (149 male, 111 female) Nanjing (China)	144	OTA = 0.235-0.712 FB1 = 0.236-1.66	OTA = 0.153-0.557 Mean= 0.502 FB1 = 0.230-1.33 Mean= 0.871
84	2019	Franco *et al.*	162 Brazil	OTA = 44 FB1 =37 FB2 = 0	OTA = 10 -11.71 Median = 20 FB1 = 0.01-0.29 Median = 0.04	OTA = 0.01-0.027 FB1 = 0.01-0.27 FB2 = 0.01-0.27
93	2014	Ezekiel *et al.*	120 Northern Nigeria	OTA = 34 FB1 = 16 FB2 = 2	-	OTA = 0.6 (Max) Mean= 0.2 FB1 = 12.8 (Max) Mean= 4.6 FB2 = 1.0 (Max) Mean = 1.0
72	2015	Huybrechts *et al.*	32 Belgium	OTA = 69% CIT = 59%	-	OTA = 0.003-0.033 Mean = 0.0095 CIT = 0.002-0.117 Mean = 0.026

Ref.	Year	Authors	Number of samples tested	Number of positive samples	Mycotoxin concentration levels corrected with creatinine (ng/mg)	Mycotoxin concentration levels (ng/ml)
92	2015	Gerding *et al.*	Haiti (142) Germany (50) Bangladesh (95)	Haiti (OTA = 47, FB1 = 4, ENNB = 4) Germany (OTA = 15, ENNB = 7) Bangladesh (OTA = 72, ENNB = 2)	Haiti (OTA = 0.091, FB1 = 0.29, ENNB = 0.029) Germany (OTA = 0.030, ENNB = 0.017) Bangladesh (OTA = 0.207, ENNB = 0.015)	Mean (range) Haiti (OTA = 0.109 (<LOQ-0.225), FB1 = 0.440 (0.23-0.70), ENNB = 0.036 (0.021-0.065)) Germany (OTA = 0.040 (<LOQ-0.082, ENNB = 0.012 (0.010-0.014)) Bangladesh (OTA = 0.203 (<LOQ-2.010), ENNB = 0.019 (0.0119-0.0187))
67	2018	Sarkanj *et al.*	120 Nigeria	OTA = 94% FB1 = 85% CIT = 79% AOH = 8%	-	OTA = 0.003-0.31 Mean = 0.05 FB1 =0.08-14.88 Mean = 1.09 CIT = 0.015-241.46 Mean = 5.96 AOH =0.03-0.2
73	2014	Gerding *et al.*	101 (44 males, 57 females) Germany	ENNB = 20	-	ENNB < LOQ
86	2020	Silva *et al.*	85 children (41 boys, 44 girls) Portugal	OTA=79	Mean = 0.02941 Max = 0.11445	Mean = 0.020 Max = 0.052

Ref.	Year	Authors	Number of samples tested	Number of positive samples	Mycotoxin concentration levels corrected with creatinine (ng/mg)	Mycotoxin concentration levels (ng/ml)
76	2019	Viages *et al.*	25 workers 19 controls Portugal	**Workers** >LOQ CIT = 1 OTA = 1 **Controls** LOD-LOQ ENNB = 2, CIT = 1, OTA = 19		
				Workers >LOQ CIT = 1 **Controls** LOD-LOQ ENNB = 2, CIT = 10, OTA =13		
87	2020	Ouhibi *et al.*	50 from colorectal cancer patients Tunisia	CIT = 76%	Mean = 0.95 Max = 2.94	Mean = 0.45 Max = 0.96
77	2006	Pena *et al.*	60 Coimbra Portugal	OTA = 42 (70%)	-	0.021-0.105
79	2011	Rubert *et al.*	27 Valencia Spain	OTA = 11.1%	-	<LOQ
82	2013	Abia *et al.*	145 (HIV positive) Cameron	OTA = 25 FB1 = 5 FB2 = 1	OTA = 0.07 (<LOQ-1.3) FB1 = 0.33 (<LOQ-9.54) FB2 <LOQ	OTA = 0.08 (<LOQ-1.87) FB1 = 0.63 (<LOQ-14.8) FB2 <LOQ
			30 (HIV sero-negative) Cameron	OTA = 3 FB1 = 1	OTA = 0.04 (0.025–0.34) FB1 <LOQ	OTA = 0.06 (<LOQ-0.83) FB1 <LOQ
74	2014	Warth *et al.*	60 Bangkok Thailand	OTA = 1	OTA <LOQ	OTA <LOQ

Ref.	Year	Authors	Number of samples tested	Number of positive samples	Mycotoxin concentration levels corrected with creatinine (ng/mg)	Mycotoxin concentration levels (ng/ml)
94	2011	Solfrizzo *et al.*	10 Italy	Without enzymatic treatment: OTA = 5 With enzymatic treatment: OTA = 9	-	Without enzymatic treatment: OTA = 0.028 With enzymatic treatment: OTA = 0.049
78	2010	Ahn *et al.*	20 Korea	OTA = 12 FB1 = ND FB2 = ND	-	OTA = (0.013-0.093) 0.031 FB1 = ND FB2 = ND
83	2014	Solfrizzo *et al.*	52 (Southern Italy)	OTA = 52 (100%) FB1 = 29 (56 %)	-	OTA: Mean = 0.144, Median = 0.061, Max = 2.129 FB1: Mean = 0.055, Median = 0.029, Max = 0.352
89	2020	Liu *et al.*	60 Beijing (26 males, 34 females)	OTA = 24 (40%) OTα = 20 (33.3%) FB1 = 2 (3.3%) FB3 = 1 (1.7%) AOH = 17 (28.3%) AME = 50 (83.3%) ENNB = 24 (40%)		**Enzyme treatment** Mean: OTA = 0.02 <LOD~0.14 OTα = 0.22 <LOD~2.38 FB1, FB3 = 0.01 <LOD~0.07, 0.01 AOH = 0.34 <LOD~7.68 AME = 0.059 <LOD~0.167 ENNB = 0.0002 <LOD~0.0014

1.7. LC-MS methods for biomonitoring of mycotoxins in urine

1.7.1. Liquid chromatography

In LC, the type of stationary phase, mobile phase and additives, gradient, and flowrate should be optimized to achieve good separation.[95] In reversed-phase liquid chromatography, analytes can be separated from each other by interacting with non-polar stationary phase and the more polar mobile phase. The compounds elute in order of polar to more non-polar. The elution of compounds depends on their logP which shows their polarity. Also, to separate two analytes their retention factors should be different, otherwise, they co-elute.

Reversed phase LC is the most prevalent separation type used in different studies for separating mycotoxins in human urine. Table 1.2 summarise the chemical properties of each mycotoxin by including their log P and pKa values. The mycotoxins of interest ranged from mid-polar (CIT, OTα, FB1, and FB2) to non-polar (OTA, ENNs and BEA). C_{18} chromatography provides good separation of mid-polar to non-polar mycotoxins of interest. Table 1.5 briefly summarizes column type and mobile phases used for the separation of multi-mycotoxins in urine samples. Water was mostly used as mobile phase A whereas acetonitrile, methanol or a mix of both was used as mobile phase B.[67-69, 71-74, 76, 78, 83-84, 89, 92-94]. In conclusion, RP LC on C_{18} provides a suitable stationary phase to separate all these mycotoxins from each other based on their polarities and polarity differences.

Table 1.5. Summary of LC methods for separation of common mycotoxins in urine samples

Year	Chromatographic column	Solvent A	Solvent B	Reference
2014	Atlantis® T3 (150 mm ×3 mm i.d., 3 µm)	Water + 0.1% AA	ACN+ 0.1% AA	74
2011	Gemini C18 (150 mm × 2 mm i.d., 5 µm)	Water + 0.5% AA	MeOH + 0.5% AA	94
2010	Hypersil GOLD ODS (150 mm × 4.6 mm i.d., 5 µm)	Water + 0.2% FA	ACN+ 0.2% FA	78
2014	Acquity UPLC BEH (150 mm × 2.1 mm i.d., 1.7 µm)	Water + 0.5% AA	MeOH + 0.5% AA	83
2019	BEH C18 (50 mm × 2.1 mm i.d., 1.7 µm)	Water + 0.1% FA	ACN+ 0.1% FA	84
2012	ZORBAX SB-C18 (100 mm × 2.1 mm i.d., 3.5 µm)	Water + 0.3% FA	MeOH/water (94.7/5)+ 0.3% FA	68
2017	Gemini C18 (150 mm × 2 mm i.d., 3 µm)	Water+ 1% FA+ 5 mM ammonium formate	MeOH+ 1% FA+ 5 mM ammonium formate	69
2019	UPLC Agilent Poroshell 120 EC-C_{18} (3.0 mm × 100 mm, 2.7 µm)	Water + 0.1% FA	MeOH + 0.1% FA	71
2014	Atlantis® T3 (150 mm × 3.0 mm i.d., 3 µm	Water + 0.1% AA	ACN+ 0.1% AA	93
2014	NUCLEODUR C18 pyramid (150 mm × 2.0 mm i.d., 3 µm)	Water + 0.1% FA	ACN+ 0.1% FA	73
2018	HSS T3 (100 mm × 2.1 mm i.d., 1.8 µm)	Water + 0.1% AA	ACN+ 0.1% AA	67
2019	C18 Pyramid (100 mm × 2 mm i.d., 3 µm)	Water + 0.1% FA	ACN+ 0.1% FA	76
2020	CORTECS C18 (100 mm × 2.1 mm i.d., 1.6 µm)	Water + 0.2% FA	MeOH/ACN (50/50, v/v)	89

1.7.2. Mass spectrometry

1.7.2.1. Electrospray ionization

Electrospray ionization (ESI) is a soft ionization method and is applicable to liquid-phase samples. The concept of electrospray was first proposed by Malcolm Dole in 1968;[96] however, the advancement of ESI-MS and its suitability for biomolecule analysis was demonstrated by John Fenn.[97-98]

In the ESI source, a fine mist of charged droplets is produced by applying a high electric field under atmospheric pressure. As shown in Figure 1.3, an electric potential of about 2 to 5 kV is applied between the tip of the steel capillary tube and counter-electrode. The produced charged

ions are in atmospheric pressure then transferred inside a glass or steel capillary that is in high vacuum region.

Charge accumulation occurs on the surface of the liquid at the end of ESI capillary. If the voltage is high enough, it can create a Taylor cone producing droplets depending on the surface tension of the liquid (Figure 1.3, part B).[99-101] An electrochemical reaction takes place at the probe tip of the capillary. Oxidation occurs in positive mode, whereas reduction happens in negative mode.[67, 69-70] The solvent in the droplets starts to evaporate and leads to the shrinkage of drops. As the droplets' solvent evaporates, the charge density on the surface of the droplet increases until it reaches the Rayleigh limit.[101] Ions within charged droplet exceeding the Rayleigh limit can be repelled from each other due to Coulombic repulsion. Therefore, break down of droplets will typically occur before this limit.[101-103] The Equation 1.1 shows how Rayleigh limit can be calculated.

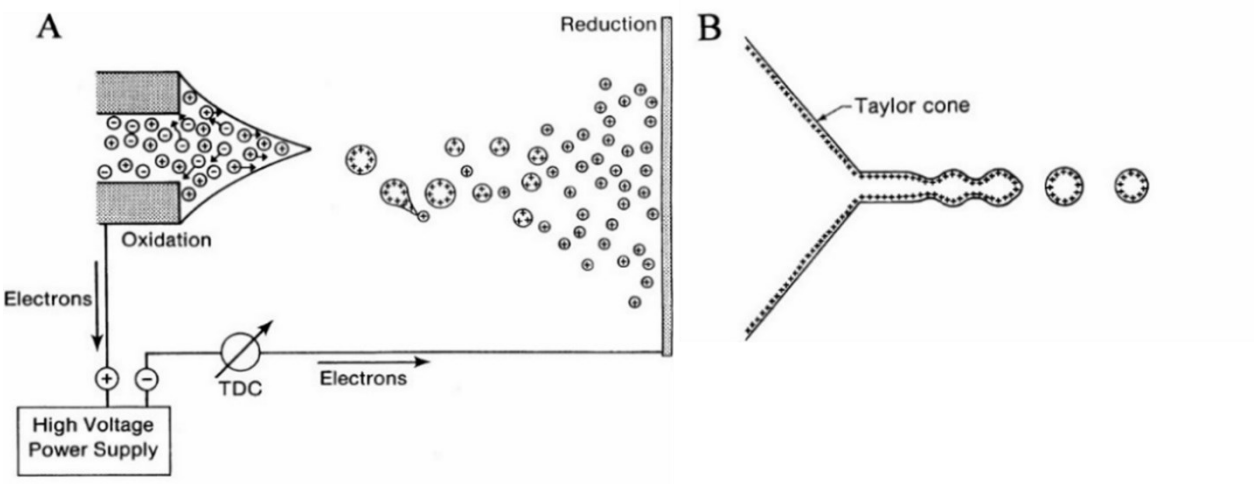


Figure 1.3 Illustration of electrochemical reaction in A, and creation of Taylor cone in ESI in B. Figure 1.3 A. reprinted with permission from Kebarle, Paul, and Liang Tang *et al.* Analytical chemistry 65.22 (1993):972A-986A. Copyright 1993 American Chemical Society. Figure 1.3 B. reprinted with permission from Kebarle *et al.* Journal of mass spectrometry 35.7 (2000): 804-817. Copyright 2000 John Wiley and Sons.[99]

Equation 1.1 Rayleigh limit equation, ε_0 shows permittivity of the environment, γ shows surface tension, and D shows the diameter of a supposed spherical droplet.

$$q^2 = 8\pi^2\varepsilon_0\gamma D^3$$

Historically two mechanisms were suggested for the production of charged ions in the gas phase from charged droplets. First one is a charge-residue model (CRM) which was mentioned by Dole *et al.*,[96] and the second one is the ion-desorption model (IDM) suggested by Iribarne and Thomson *et al.*[104] In the CRM model the solvent in charged droplets keeps evaporating and after multiple droplet fissions, the drop size becomes so small that it contains a single ion.[105] Konermann *et al*. proposed two new mechanisms, ion evaporation model (IEM) and chain ejection model (CEM), for producing gas-phase ions, whereby IEM is applicable to low molecular weight compounds, CRM for large globular species and CEM for disordered polymers.[106] IEM is applicable for mycotoxin ionization, and in this model ionized analyte is ejected from droplets to reduce charge repulsion. Ions that are commonly produced in the ESI ion source can be $[M+H]^+$, $[M+Na]^+$ and $[M+NH_4]^+$ in ESI (+), $[M-H]^-$ and $[M+acetate]^-$ in ESI (-) mode. All 12 mycotoxins of interest contain at least one site where protonation or deprotonation can take place, thus making them amenable to ESI. So far, all LC-MS/MS analyses for determining the mycotoxins of interest in urine were performed with ESI as the ion source.

One of the major drawbacks of ESI LC-MS is the occurrence of matrix effects (ME). Presence of matrix effects can affect analytical performance, and it often occurs by changing the ionization efficiency of the analytes. For example, interfering compounds in the matrix can compete with the analyte to reach the surface of the droplet, thus preventing the analyte ionization in the ESI source which leads to ionization suppression. In contrast, some compounds can increase ionization efficiency of the analyte leading to ionization enhancement. The matrix effects should be controlled otherwise, it will affect the accuracy of quantitation.[107] The acceptable maximum range for matrix effects is defined as 80% - 120%, according to FDA guidelines.[25] Matrix effects less than 80% are considered as ionization suppression, while ion enhancement is observed when matrix effects are higher than 120%. The matrix effects can be studied as relative or absolute. Absolute matrix effect is defined as the difference of analyte signal response in matrix versus neat standard prepared at the same concentration. A quantitative study of the extent of matrix

effects across different lots of the same type of matrix is defined as a relative matrix effect. To measure relative matrix effect, multiple lots of matrix must be tested.[107]

As discussed in Section 1.5, some mycotoxins suffered from significant ionization suppression in different LC-MS methods developed to date. To compensate for the matrix effects, isotopically labelled internal standards is preferred. These standards co-elute with analyte of interest and undergo similar ionization effects as the analytes depending on the composition of the matrix. However, in mycotoxin analysis, isotopically labelled internal standards are not commercially available for all mycotoxins of interest, and some of the available standards are prohibitively expensive. This drastically increases cost of analysis per sample and cost of biomonitoring studies. One way to address this issue is to develop methods with improved sample clean-up to reduce matrix effects. Thus, few ISTD can be used for entire panel of mycotoxins. To quantitate mycotoxins in urine samples, matrix effects should be minimized.

1.7.2.2. Quadrupole-time-of-flight high resolution mass spectrometer

The mass analyzer is the heart of a mass spectrometer instrument. After the ionization of analytes in the ion source, ions are separated inside the analyzer according to their mass to charge ratios (m/z). The mass analyzer which will be used for this study is a quadrupole-time of flight (QTOF) mass spectrometer. QTOF can increase the sensitivity compared to single TOF by using quadrupole to focus the ions of interest into TOF. Ions in the TOF are accelerated by applying high potential energy and separated according to the time it takes to travel inside a long tube and strike the detector. Equation 1.2 shows the kinetic energy (E) and velocity of ions (v), respectively.

Equation 1.2 Kinetic energy of ion

$$E = \frac{1}{2}mv^2$$

m in Equation 1.2 is the mass of the ion and *v* is the velocity. In Equation 1.3 *d* and *t* show the distance and time, respectively.

Equation 1.3 Velocity of ion

$$v = {}^{d}/_{t}$$

By combining these two equations Equation 1.4 is attained.

Equation 1.4 Mass of ion

$$m = \left({}^{2E}/_{d^2}\right) t^2 \rightarrow m = At^2$$

Equation 1.4 shows optimal conditions, all ions have the same E and the distance that each ion takes to reach the detector is constant, then the terms in the parentheses can be treated as a constant *A*. However, the time that the voltage is applied on the ion pulser and the time it takes for signals to be digitized is dependent on the instrument. These delays alter the apparent travel time and are referred to as t_0. Therefore, a routine mass calibration is required so that parameters such as *A* and t_0 are calculated using reference masses of known compounds that are stable, t_m is the time that each known mass (m) reaches the detector (Equation 1.5).[101, 103]

Equation 1.5 Calibration equation in TOF

$$m = A\,(t_m - t_0)^2$$

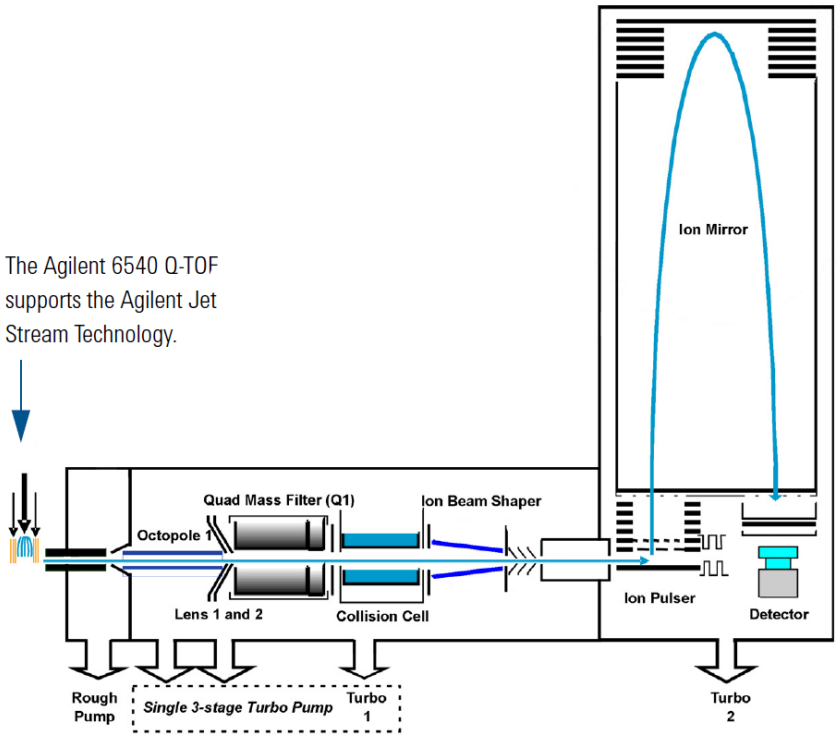


Figure 1.4 Schematic of quadrupole-time-of-flight mass spectrometer. The figure was printed from Agilent website.[108]

The ability of a mass spectrometer to discriminate between ions of differing mass-to-charge ratios is referred to as resolution and can be calculated from the apparent full width half maximum (FWHM) m/z of the peak.[103] The resolution of early TOF analyzer was about 500 FWHM. The reason for such a low resolution is that the ions, with same m/z ratios, had an initial spatial and energy spread. To minimize these effects, delayed pulsed extraction was introduced. Delayed pulsed extraction can correct for dispersion of energy in ions with the same mass by applying a high voltage after certain delay to the ions which have the same m/z ratio but initially lower kinetic energy.[109] Thus, these ions can stay less in this delay and after receiving the higher potential energy they can join the ones with same m/z ratio but with higher kinetic energy. This increases the resolution of modern QTOF to more than 40,000 FWHM. However, this method can complicate mass calibration which requires optimization and calibration within limited mass ranges. The next approach to enhance the resolution was implementation of the reflectron which

acts as an ion mirror deflecting back the ions toward the detector. Ions with same m/z with higher kinetic energy travel deeper inside the reflectron than the ions with the same m/z ratios but lower kinetic energy.[110] This method compensates for the dispersion of kinetic energy, thus resulting in higher resolution but at the cost of sensitivity. Coupling a continuous ionization system such as ESI to a pulsed analyzer such as TOF was possible by invention of orthogonal acceleration developed by O'Halloran *et al.*[111] Coupling quadrupole to TOF resulted in one of the most successful hybrid systems.[101, 103] In MS mode, the two quadrupoles shown in Figure 1.4 act as RF only, focusing a limited mass range and guiding it towards TOF analyzer. Ion optics focus the ion beam coming from the ESI into the first grid. Then, a high voltage is applied to this packet of ions to push them towards the second grid. Meanwhile, the region between ESI and the first grid refills with another packet of ions. Finally, ions in the second grid receive high potential V_{tof} and accelerate in the field-free region of tube.[101, 103]

Table 1.3 summarizes the different LC-MS methods for detection of mycotoxins used to date. As explained in Section 1.5, the most prevalent detection method for biomonitoring mycotoxins is QqQ or QTRAP both of which are low resolution mass spectrometers. These instruments can provide good sensitivity and selectivity to quantitate mycotoxins in urine because only selected ions enter the analyzer, thus the noise is decreased or removed. The main limitation of these selected reaction monitoring methods is that they are targeted, and only a priori selected analytes are measured. In contrast to low resolution MS, high resolution MS can be used for targeted and non-targeted studies. It can also be used for structural elucidation of unknown compounds and retrospective studies by taking the full scan of the entire mass range. This allows future re-examination of data for new mycotoxins that are discovered and simultaneous direct measurement of parent mycotoxin and metabolites. The selectivity in HRMS is achieved by extracting a narrow mass window around m/z of analyte of interest. However, the sensitivity of HRMS is typically lower than for SRM as increasing the intensities of ions of interest also generally increases the level of noise, ions In the HRMS (QTOF) typical resolution is more than 40,000 FWHM, while in QqQ it is about 1500 FWHM.[101]

To sum up, both high- and low-resolution MS have their own advantages and disadvantages, but both can be used to biomonitor the mycotoxins in urine samples.

1.8. Solid-phase extraction

As mentioned in Section 1.4, urine contains different salts and minerals.[58] The salts and polar compounds in urine can affect early eluting mycotoxins. Therefore, a proper sample preparation is required. Solid-phase extraction (SPE) is an extraction method whereby solid-phase sorbents such as C18 (reversed-phase), HLB (hydrophilic-lipophilic balanced sorbent), ion-exchange cartridges such as MAX (mixed-mode strong anion exchange) and MCX (mixed-mode strong cation exchange) interacts with analyte of interests, while interferences are washed away. This can clean-up samples, and it can also provide a good enrichment if the eluate from SPE evaporated and redissolved in smaller volume. A schematic of the four different steps (conditioning, loading, washing and elution) of SPE is shown in Figure 1.5.

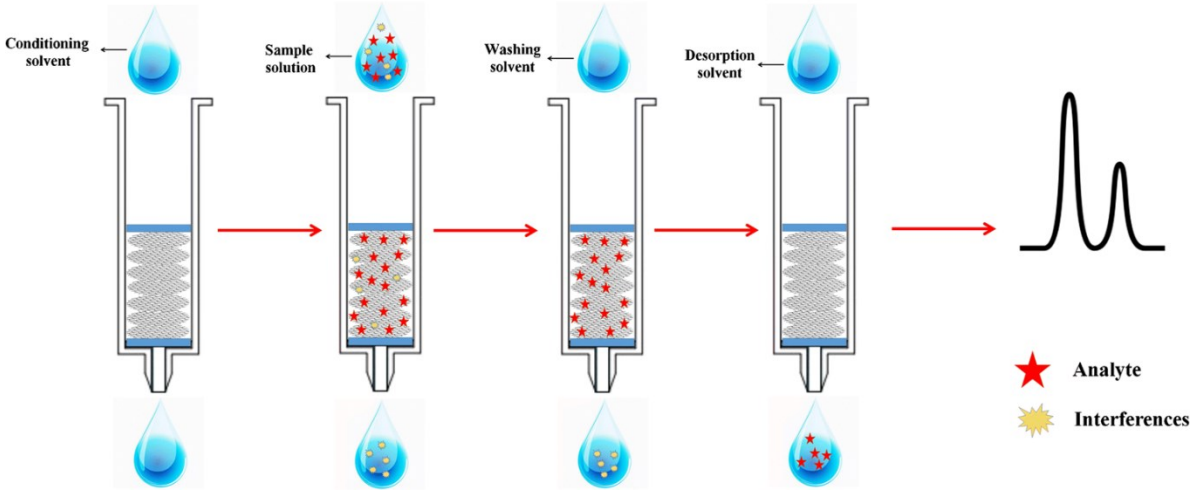


Figure 1.5 Schematic of solid-phase extraction procedure. Adopted and modified with permission from Amiri, Amirhassan, *et al.* Microchimica Acta 187.4 (2020): 1-8. Copyright 2020 Springer Nature.[112]

In the conditioning step, different solvents such as water or methanol can be used. In this step, any impurities or residues that might have remained on the cartridge during manufacture processing will be removed, and by wetting the sorbent it will facilitate analyte interactions. Optimization of the next three steps has paramount importance in giving the best clean-up, while ensuring good analyte recoveries. The mycotoxin targets shown in Table 1.2 have different polarities (logP values) and pKa. Thus, the choice of a proper SPE sorbent to cover the range of their physicochemical properties is essential. According to the types of the SPE, the pH and the

volume of the loaded samples in the loading step should be optimized. The next step, washing, is one of the main steps to clean/remove interferences coming from the matrix. The volume and composition of washing solvent should be optimized to clean only interferences and not elute analytes during this step. In the final elution step, the volume and the choice of the solvent for elution of all the mycotoxins have a great impact on their recovery. After this step, samples can be injected directly, or they can be evaporated to dryness, then reconstituted in a solvent suitable for the analysis. The addition of evaporation/reconstitution step serves to change injection solvent composition when needed and/or introduce additional enrichment factor.

1.9. Objectives

Fungal growth in an environment that is prone to global warming is inevitable. Thus, the production of fungal secondary metabolites increases steadily, with increasing the effect of climate change. The current regulations in food products can prevent human exposure to high levels of these mycotoxins.[8] However, the long-term exposure to trace levels of these mycotoxins should be monitored. Mycotoxin studies in food products are not sufficient to estimate human exposure as the diet varies according to individual preferences. In addition to food consumption, mycotoxin exposure can also take place in the work environment or in humid housing conditions. Thus, human biomonitoring is necessary to estimate the trace levels of the mycotoxins in different regions and identify sub-populations that might be at risk unacceptable levels of exposure.[8, 113] Urine samples are favored over other biological samples because urine collection is easier and constitutes a less invasive approach. Furthermore, urine can be used to monitor mycotoxins that have short half-lives in plasma. However, the urine concentration is prone to daily variation and thus the excretion of mycotoxins in the urine will also vary.[101]

The main objective of this research is to develop a sensitive and accurate liquid chromatography high-resolution mass spectrometry (LC-HRMS) method for the measurement of 12 mycotoxins in urine. The mycotoxins evaluated include those recently detected in Canadian wheat (ENNA, ENNA1, ENNB, ENNB1, BEA, CIT) and grain derived foods (AOH, AME), as well as others commonly found in many food products (FB1, FB2, OTA and its metabolite OTα).

A time-segmented polarity switching LC-HRMS method had been previously developed for 10 of these mycotoxins in human plasma.[75] To make the method applicable to urine (higher polarity

and salt content than plasma), optimization is required. The best fragmentor, ionization mode, and co-elution with other mycotoxins are investigated among the optimizations. Two sample preparation methods, “dilute-and-shoot” and SPE, are implemented in urine samples to detect trace levels of the 12 mycotoxins. The “dilute-and-shoot” method, is one of the most prevalent sample preparations for urine samples but, has significant limitations with respect to the matrix effects and the detection limits. On the other hand, SPE was investigated as it gives a better clean-up and allows enrichment. The performance of the “dilute-and-shoot” and SPE methods were compared for the biomonitoring of mycotoxins in urine samples.

Preliminary experiments found that some mycotoxins were being adsorbed onto the urine collection/storage containers. One of the first objectives was to look at the effect of the solvent for the non-specific adsorption of mycotoxins. The best recovery solvent/diluent for improving mycotoxin solubility was performed by comparing a highly aqueous solvent versus a highly organic solvent in both plastic inserts and glass vials. Adsorption affects most significantly ENNs and BEA. Therefore, we further assessed how to recover ENNs and BEA from plastic containers used to collect urine before LC-MS analysis for a more accurate quantitation. Also, different dilution factors were tested in the dilute-and-shoot method for matrix effects and the LOQ levels were compared to those reported in the literature.

The SPE preparation method was also tested for improving the LOQs and providing better sample clean-up. For better retention of the analytes and removing interferences in the SPE method, optimization of different parameters was performed. The loading pH, volume and composition of the washing solvent, and the elution volume were optimized, and various sorbents were tested. Furthermore, we evaluated the impact of the creatinine levels on the matrix effects of mycotoxins as creatinine is commonly used to normalize the concentration of urine samples. The recovery and matrix effects of the mycotoxins were evaluated with 10x and 20x enrichment with the HLB SPE method.

Chapter 2. Materials and methods

2.1. Chemicals

LC-MS grade water, methanol, and acetonitrile, acetic acid (AA, Optima® LC/MS) and formic acid (FA, Optima® LC/MS) were purchased from Fisher Scientific (Ottawa, Ontario, Canada). Human urine samples were kindly donated by students at Concordia University to use as individual and pooled samples for method development. LC-MS grade ammonium hydroxide solution was purchased from Honeywell Fluka® Canada (Montreal, Quebec, Canada).

2.2. Mycotoxin standards

$OTAd_5$ (1 mg/mL in 100% MeOH), AOH (1 mg/mL in 100% DMSO), AME and $AMEd_3$ (0.5 mg/mL in 50/50 v/v MeOH/chloroform) were purchased from Toronto Research Chemicals Inc. (Toronto, ON, Canada). FB1 (1 mg/mL in 100% MeOH), FB2 (0.5 mg/mL in 100% MeOH) and BEA were purchased from Cayman Chemicals (Ann Arbor, MI, USA). FB3 (50 µg/ml in 50% acetonitrile) and OTα (10.20 µg/ml in 50% acetonitrile) were purchased from Romer Labs (Union, MO, USA). CIT, OTA, ENNA, ENNA1, ENNB, ENNB1 were purchased from Sigma-Aldrich Canada (Oakville, Ontario, Canada). Individual standard stock solutions of all the mycotoxins were prepared at 1 mg/mL in MeOH unless otherwise specified. A combined 10 µg/ml working solution of all mycotoxins except for OTα, FB1 and FB2 was prepared in methanol and stored in aliquots at −80°C. A combined 10 µg/ml internal standard solution of $OTAd_5$, FB3 and $AMEd_3$ was prepared in methanol and stored in aliquots at −80°C. $OTAd_5$, FB3 and $AMEd_3$ were added at the final concentration of 10 ng/mL (unless otherwise specified) immediately prior to LC–MS analysis during the investigation of sample preparation techniques and matrix effects in Section 2.4.2 and 2.5.1.7. For all other experiments internal standards were added to thawed urine samples before or after sample preparation as specified in each section.

2.3. Urine creatinine measurement

Creatinine elutes at 0.85 min with solvent (Appendix, Supplementary Figure 10) and its high concentration makes LC-MS not the best choice for its analysis. Thus, the first 2 minutes of LC flow was discarded.

After collection of nine different lots of urine, the creatinine level of each lot was analyzed by CLINITEK Status® plus from Siemens® (Oakville, Ontario, Canada). CLINITEK® Microalbumin Reagent Strips were dipped into urine for measuring the creatinine level in each lot. In the presence of creatinine, a redox reaction catalyzed by copper-creatinine complex develops. The oxidation of tetramethylbenzidine (TMB) is linked with the reduction of copper in the copper-creatinine complex, resulting in a colour change from orange to green/blue. The concentration of creatinine is reported as 10, 50, 100, 200 or 300 mg/dL.[114] Table 2.1 shows a summary of information in each lot of urine: For precise measurement of creatinine for future analysis Jaffe method can be used.[115]

Table 2.1. Gender, pH and creatinine level of different lots of urine used during method development.

#Lot	Gender	pH	Creatinine level
A	Female	6.74	10 mg/dL
B	Female	6.48	50 mg/dL
C	Male	7.17	50 mg/dL
D	Female	7.17	50 mg/dL
E	Male	5.76	200 mg/dL
F	Female	5.62	200 mg/dL
G	Male	5.19	300 mg/dL
H	Female	5.58	300 mg/dL
I	Female	5.39	200 mg/dL

2.4. Development and evaluation of dilute-and-shoot method

2.4.1. Comparison of mycotoxin solubility and non-specific adsorption in two solvents using dilute-and-shoot method

2.4.1.1. Comparison of two dilute-and-shoot methods

Mycotoxins at 10 ng/mL in standard and diluted urine samples were prepared either in high organic solvent (60% MeOH with 1% FA) or high aqueous solvent (5% ACN and 1% FA) and were placed in either polypropylene tubes or glass inserts as described in Section 2.4.1.2 and

2.4.1.3. The design of the experiment is shown in Figure 2.1. The long sequence with periodic re-injections of standards and spiked urine samples were performed for overall run time of ~ 42 hours (100 injections).

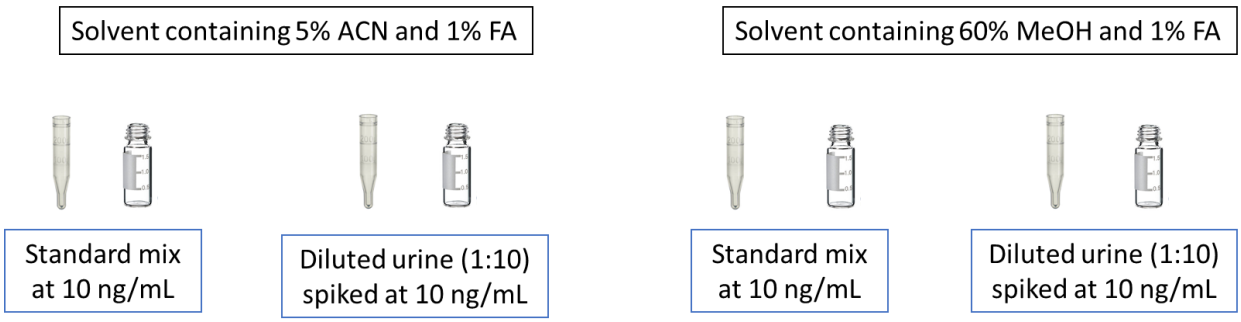


Figure 2.1 Experiment design for the comparison of dilute-and-shoot methods using two solvents and stored in glass and plastic inserts during 42 -hour LC-MS analysis.

2.4.1.2. Dilute-and-shoot method using 60% methanol (v/v) with 1% formic acid

Pooled urine sample was thawed at room temperature. 1 mL of thawed urine was centrifuged at 25830×g, (Thermo Fisher Scientific, Sorvall ST 16R centrifuge) for 20 min at 4°C. To 300 µL of the resulting supernatant, 2.7 mL of 60% MeOH containing 1% formic acid were added giving 54% MeOH (10x dilution). 1.5 mL of the diluted urine was spiked at 10 ng/mL, 200 µL aliquots were transferred to three polypropylene HPLC inserts, and the rest was transferred to a HPLC glass vial for LC-MS analysis.

2.4.1.3. Dilute-and-shoot method using 5% acetonitrile with 1% formic acid (v/v)

The same procedure as described in Section 2.4.1.2 was followed except 5% acetonitrile with 1% formic acid (v/v) was used as the dilution solvent as previously suggested by Gerding *et al.*[73]

2.4.2. Evaluation of the effect of dilution factor on matrix effects in dilute-and-shoot method

To evaluate the matrix effects, the peak area (A) of the analytes from post-extraction spike was compared to the peak area of analytes in neat solvent. To prepare post-extraction spike samples the same procedure as described in Section 2.4.1.2 was tested with different dilution factors (10x, 15x and 20x). In all cases the final composition of the extract remained at 60% MeOH and 1%

formic acid (v/v) and polypropylene plastic inserts were used for this experiment. Mycotoxin standard (10 ng/mL) in neat solvent were also analyzed, and the matrix effects were calculated according to Equation 2.1.

Furthermore, the relative matrix effect was also evaluated in six individual lots of spiked urine 4 ng/mL of mycotoxins) with different creatinine levels after 20x dilution using plastic inserts (n=1). Standards for comparison were prepared at 4 ng/mL concentration.

Equation 2.1 Calculation of ME%

$$\text{ME\%} = \boldsymbol{A}_{\boldsymbol{post\ spike}} / \boldsymbol{A}_{\boldsymbol{standard}} \times \mathbf{100\%}$$

2.4.3. Evaluation of recovery of dilute-and-shoot method in plastic and glass inserts

Recovery can be measured by comparing the area of pre-extraction spike to post-extraction spike samples. To prepare pre-extraction spike samples in the dilute-and-shoot method, urine was first spiked and then diluted 20x, whereas for post-spike samples, urine was diluted first and then spiked at the equivalent concentration. To evaluate the recovery in the dilute-and-shoot method, pooled urine sample was first centrifuged as mentioned in Section 2.4.1.2. Then, six replicates of pooled urine spiked at 200 ng/mL of mycotoxins were placed in glass or plastic inserts. Next, they were diluted 20x while keeping final organic solvent composition of 60% MeOH with 1% FA. To prepare post-extraction spike samples, the same pooled urine was diluted 20x first, and then spiked at 10 ng/mL of mycotoxins. Equation 2.2 was used to calculate recovery.

Equation 2.2 Calculation of RE%

$$\text{RE\%} = A_{\,pre\ spike} / A_{\,post\ spike} \times 100\%$$

2.4.4. Calibration curve using dilute-and-shoot method

After centrifugation of 1.0 mL pooled urine samples, a 200 µL aliquot was spiked 400 ng/mL of mycotoxins. Figure 2.2 illustrates the preparation of calibration curve in urine with 20x dilution. Serial dilution was performed by taking 50 µL of the first level added to the next level (50 µL urine with 4.4 µL of MeOH). Throughout the serial dilution MeOH composition remained constant at 8%. After that, 10 µL of each STD was diluted with 190 µL of a solvent containing

62.7% MeOH with 1.06% FA and 10.5 ng/mL mix of ISTD. The latter part was performed to make sure samples were diluted 20x and that their final composition remained constant at 60% MeOH with 1% FA. This experiment was performed using Eppendorf tubes and glass inserts. Calibration curve was prepared in the range from 20 ng/mL to 0.006 ng/mL. 1/x weighted linear regression was used to build calibration curves for all mycotoxins in solvent, and all mycotoxins in urine except ENNs and BEA that quadratic regression (1/x) was used. However, the choice of proper weighting can be confirmed later by calculating the smallest spread of weighted normalized variances.[116]

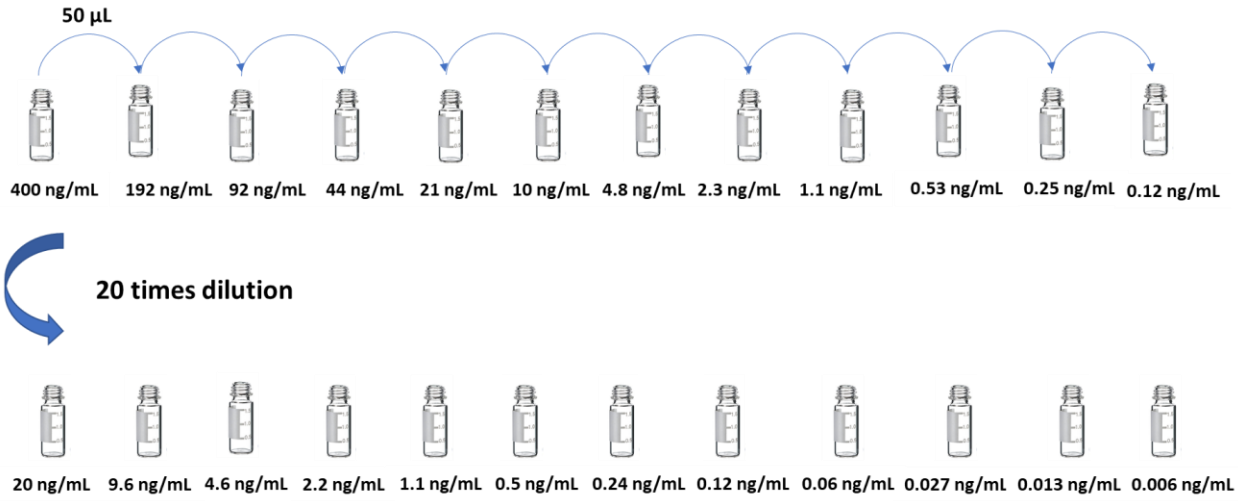


Figure 2.2 Calibration curve for dilute-and-shoot method in the range of 0.006 -20 ng/mL mycotoxins in urine. Urine was spiked at 400 ng/mL of mycotoxins and then serial dilution to 12 levels was performed as shown in top panel of figure. After that, calibration standards were diluted 20x, while keeping final composition of solvent 60% MeOH with 1% FA.

2.5. Development and evaluation of SPE method

2.5.1.1. Evaluation of the effect of urine pH on Oasis HLB SPE

Ten different lots of urine described in Section 2.3 were mixed in equal proportions to obtain pooled urine sample. The resulting pooled urine had pH of 6.0. An aliquot of this pooled urine was taken, and its pH was adjusted to 2.0 by adding 4.5% FA. Urine spiked at 10 ng/mL of mycotoxins was extracted using Oasis HLB SPE (1 cc, 30 mg, particle diameter 30 µm, Waters, Mississauga, Canada) according to the following procedure with gravity flow: i) conditioning

using 1.0 mL of MeOH and 1.0 mL of water, ii) loading of 2.0 mL spiked urine at pH 2.0 or 6.0, iii) wash with 1.0 mL of 5% MeOH, and iv) elution using 600 µL of MeOH. For each pH, four replicates were prepared. The collected eluents of two replicates of each pH were evaporated to dryness using Speedvac (Thermo, Savant SPD111V), reconstituted into 50 µL of 60% MeOH with 1% FA and transferred to HPLC plastic inserts for LC-MS analysis. The remaining two replicates of each pH were diluted to final composition of 60% MeOH with 1% FA and transferred to HPLC vials for LC-MS analysis.

2.5.1.2. Comparison of different washing solvents in SPE method

HLB SPE was conditioned and loaded as described in Section 2.5.1.1 using urine at pH 6.0 spiked with 10 ng/mL of mycotoxins. The wash volume was increased to 3.0 mL, and the following wash solvents were tested (5% MeOH, 10% MeOH with 50 mM ammonium acetate, 10% MeOH with 2% FA, 10% MeOH with 0.01% NH_4OH). Two replicates of each wash condition were prepared. In a single replicate, after washing with 3.0 mL of 10% MeOH, 1.0 mL of 20% MeOH was added. The schematic of step-by-step collection of 1 mL of 20% MeOH is shown in the Appendix, Supplementary Figure 13.Analytes were eluted using 1.5 mL MeOH. The eluent composition was adjusted to 60% MeOH with 1% FA, prior to analysis by LC-MS.

2.5.1.3. Evaluation of process efficiency, recovery, and matrix effects during method development of SPE

During initial method development experiments described in Sections 2.5.1.1 and 2.5.1.2, process efficiency of the method was tested according to Matuszewski *et al.*[107] Process efficiency (PE%) includes both recovery and matrix effects. PE% is calculated as:

Equation 2.3 Calculation of PE%

$$PE\% = \boldsymbol{A}_{\boldsymbol{pre\ spike}} / \boldsymbol{A}_{\boldsymbol{standard}} \times \mathbf{100}\%$$

To measure $A_{\ pre\ spike}$ (Area of pre-spike), urine was spiked before extraction. For the recovery test, urine was spiked with specified concentration of mycotoxins described in Section 2.5.1.4, 2.5.1.6, 2.5.1.7. Recovery (RE%) was determined according to Equation 2.2.

To evaluate the matrix effects (ME%), extracted non-spiked urine sample from SPE was spiked at 10 ng/mL of mycotoxins post-extraction and compared to the neat standard of equivalent concentration. The ME% was calculated according to Equation 2.1.

2.5.1.4. Comparison of HLB vs. C18 SPE

HLB SPE and Bond Elut C18 SPE (1 cc, 100 mg, particle diameter 40 µm, Agilent, USA) were conditioned as mentioned in Section 2.5.1.1. The 1.0 mL pooled urine (pH 6.0) spiked at 25 ng/mL of mycotoxins was loaded. For the washing step in HLB SPE, 5 and 10% MeOH, and in C18 SPE water and 2% MeOH were tested. The total volume of washing was increased to 6.0 mL. The samples were eluted with 1.5 mL MeOH. After dilution to the final composition 60% MeOH with 1% FA, the samples were transferred to HPLC vials for LC-MS analysis. The recovery and matrix effects were measured according to Section 2.5.1.3.

2.5.1.5. Comparison of MCX and MAX SPE

Oasis MCX (1 cc, 30 mg, particle diameter 30 µm, Waters) was conditioned as stated in Section 2.5.1.1. The loading, washing, and elution steps tested were the same as for HLB SPE in Section 2.5.1.4 except for the washing step where 6.0 mL of 10% MeOH was used. The eluate was diluted with water/FA to a final composition of 60% MeOH with 1%FA (v/v) and injected. The recovery and matrix effects were calculated according to the Section 2.5.1.3 at a final concentration 10 ng/mL of mycotoxins.

MAX (3 cc, 60 mg, particle diameter 30 µm, Waters) was conditioned with 3.0 mL MeOH followed by 3.0 mL water. Urine (2.0 mL, pH 6.0, spiked at 5 ng/mL mycotoxins) was loaded on the cartridge. The interferences were washed with 10.0 mL of 5% MeOH containing 50 mM ammonium acetate. Two-step elution was performed as follows: first 1.5 mL MeOH was used to elute the neutral analytes, followed by 1.5 mL MeOH containing 2% FA (v/v) to elute acidic analytes. Figure 2.3 shows the first and second eluate. 100 µL of each eluate were diluted to 60% MeOH with 1% FA and directly injected in LC-MS.

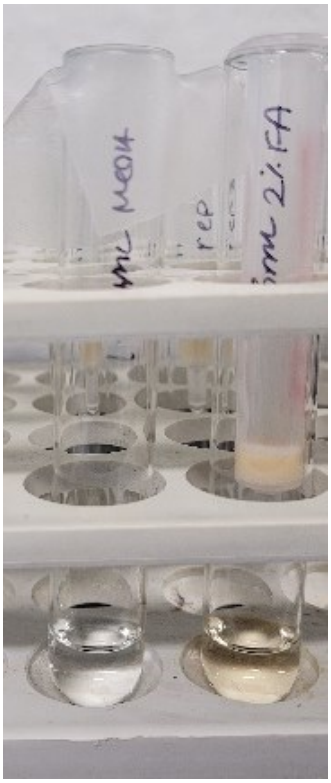


Figure 2.3 Photo of elution step during MAX SPE. The elution with MeOH (colorless) is shown on the left, elution with MeOH containing 2% FA (yellow) is shown on the right-hand side of the photo.

2.5.1.6. Optimization of evaporation-reconstitution step

Spiked pooled urine (0.5 ng/mL, 1.0 mL) was extracted using HLB SPE. The washing step was performed with 6.0 mL of 10% MeOH. After the elution step (1.5 mL, MeOH), the samples were evaporated to dryness using speedvac and reconstituted in 50 or 100 µL of 60% MeOH with 1% FA. For testing of the matrix effects, pooled urine was extracted and evaporated using the same procedure, and at the end samples were spiked at 10 ng/mL (20x urine enrichment) or 5 ng/mL (10x urine enrichment) of mycotoxins.

The above experiment was repeated in triplicate strictly using glass vials for preparation. The pooled urine was spiked at a final concentration of 0.5 ng/mL mycotoxins using glass vials for preparation. Urine (1.0 mL) was loaded twice on the HLB cartridges, and they were washed with 6.0 mL of 10% MeOH, eluted with 1.5 mL of 100% MeOH, evaporated to dryness and

reconstituted in 100 µL of 60% MeOH with 1% FA. The recovery and matrix effects were calculated according to the Section 2.5.1.3.

2.5.1.7. Modification of HLB SPE loading and evaporation step

HLB SPE cartridge was conditioned as mentioned in Section 2.5.1.1. Two replicates of spiked pooled urine samples (1.0 mL, 5 ng/mL of mycotoxins and ISTD) were loaded on the cartridge. The washing and elution step (10% and 100% MeOH) was performed as in Section 2.5.1.6. Extracted samples were transferred to Eppendorf tubes and were evaporated on speedvac for 38 minutes to reach volume less than 0.5 mL. The remaining volume in each Eppendorf tube was measured precisely and then adjusted to 600 µL by adding MeOH. Finally, they were diluted to 60% MeOH with 1% FA by adding 400 µL of water containing 2.5% FA.

To troubleshoot the loss of CIT, the same experiments were repeated with two different lots of HLB SPE, and different amount of creatinine was loaded. Figure 2.4 demonstrates the experiment design. Two replicates of pooled urine normalized to 1.2 mg (631µL, 190 mg/dL of creatinine, 5 ng/mL of mycotoxins and ISTD) were loaded on two different lot of HLB SPE. The protocol was followed a mentioned above. Also, two replicates of pooled urine at 1.9 mg (1.0 mL 190 mg/dL of creatinine, 5 ng/mL of mycotoxins and ISTD) were loaded on HLB SPE. Post-spike extraction samples were prepared by loading the non-spiked urine at an equivalent creatinine level, and after following the same HLB SPE procedure, the extracted urine samples were spiked at the correct final concentration for evaluation of recovery and matrix effects.

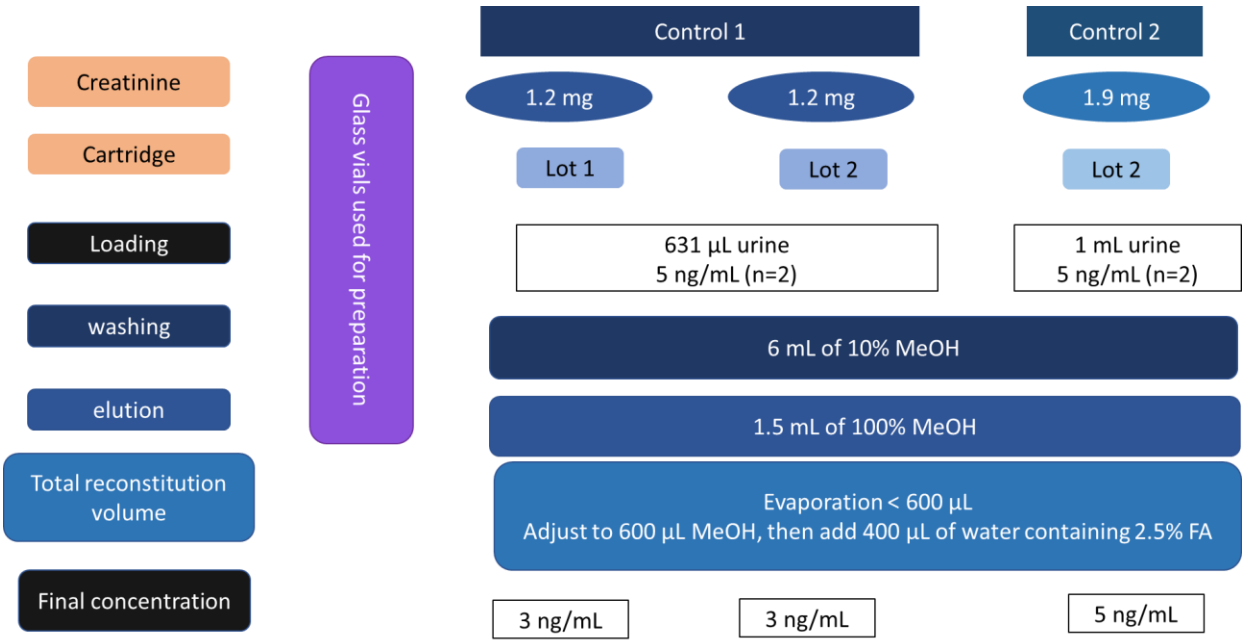


Figure 2.4 Schematic of experimental plan for testing different lots of SPE and the effect of 5% MeOH during the loading step.

2.6. Non-specific adsorption during urine collection and storage

To test non-specific adsorption during urine collection, 5 replicates of 200 µL non-spike pooled urine samples along with 5 replicates of pooled urine sample (190 mg/dL of creatinine) spiked at 5 ng/mL mycotoxins were prepared and transferred to polypropylene Eppendorf tubes. Then, samples were stored in a -80°C freezer for two weeks. After samples were thawed at room temperature, urine in both spiked and non-spiked samples was removed from the Eppendorf tube, and the Eppendorf was rinsed with 100 µL of MeOH as shown in Figure 2.5. After dilution to 60% MeOH with 1%FA, they were injected to evaluate how much mycotoxins may have adsorbed to walls during two weeks storage. Furthermore, the urine in the spiked samples was injected after five times dilution. The concentration in MeOH was calculated from calibration curve in solvent and the concentration is 10x diluted spiked urine was measured by calibration curve in urine.

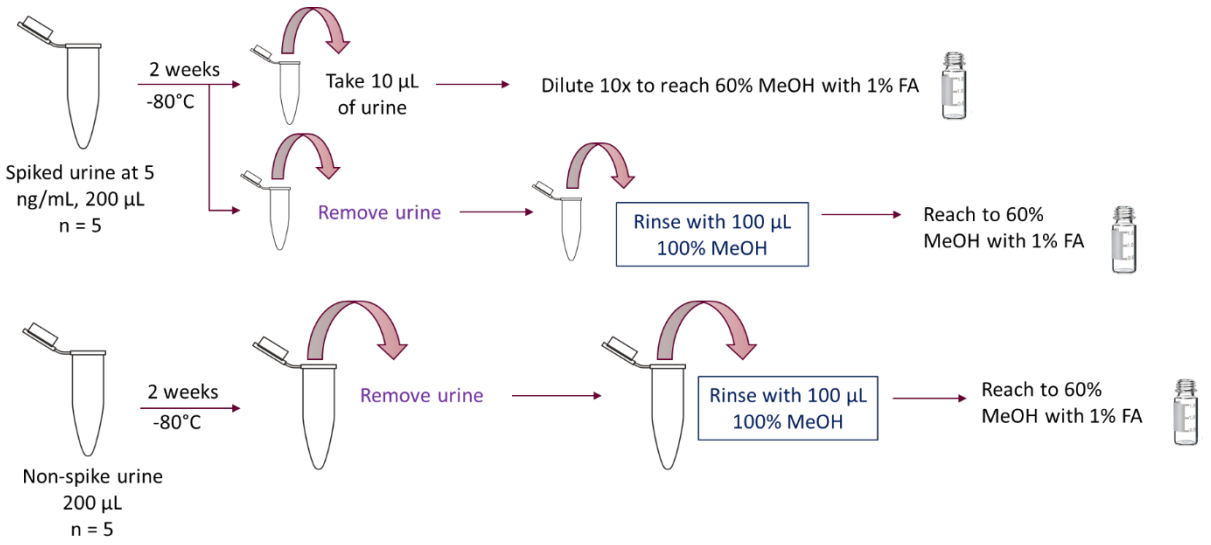


Figure 2.5 Summary of the experimental design to evaluate the mycotoxin recovery from the walls of polypropylene containers after two weeks storage.

2.6.1. Testing MeOH in the loading step in HLB SPE

To test if MeOH rinse of container and urine sample can be combined to screen for presence of mycotoxins, recovery of mycotoxins in HLB SPE was tested by adding MeOH (5% and 10%) in the loading step. Five replicates of spiked pooled urine samples (1.0 mL, 190 mg/dL, 5 ng/mL of mycotoxins and ISTD) containing 10% MeOH were loaded on the cartridge. Washing, elution and evaporation step was performed as described in Section 2.5.1.7. For post-spiked samples, five replicates of non-spiked pooled urine samples containing 10% MeOH were loaded, and the same protocol was applied. Before reaching 600 µL MeOH, they were spiked with 5 ng/mL of mycotoxins and ISTD.

Two replicates of spiked pooled urine sample normalized to 1.2 mg creatinine (631µL, 190 mg/dL, 5 ng/mL of mycotoxins and ISTD) containing 5% MeOH was loaded on HLB SPE. The washing, elution and evaporation step was done at mentioned in Section 2.5.1.7. For post-spiked samples, non-spiked urine samples were extracted by HLB SPE and before reaching to 600 µL of MeOH, were spiked at 3 ng/mL of mycotoxins and ISTD.

2.7. Final HLB SPE sample preparation

The final sample preparation protocol for 12 mycotoxins is summarized in Figure 2.6 and incorporates the rinsing step discussion in Section 3.2.2.1. All steps prior to SPE procedure should be performed using glass.

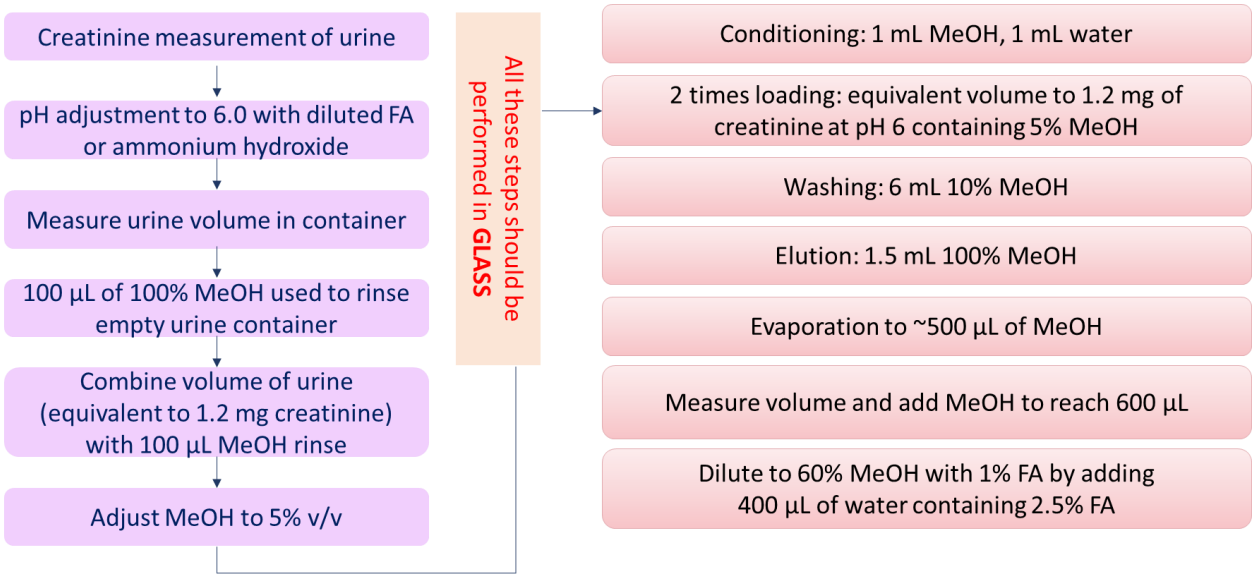


Figure 2.6 Final HLB SPE protocol for detection of 12 mycotoxins in urine sample.

2.8. LC-MS analysis

Chromatographic separation was performed using UHPLC 1290 (Agilent Technologies) with Waters CORTECS T3 Column (120 Å, 1.6 µm, 2.1 mm x 100 mm). A flow rate of 0.3 mL/min and column temperature of 40°C were used for all analyses. The final method utilized water as mobile phase A and methanol as mobile phase B, both containing 0.02% AA. The gradient specified in Table 2.2 was used to elute the analytes. For the initial 2.0 min of LC run the effluent was diverted to waste to avoid contamination of the mass spectrometer with salt. The total run time was 24.0 min. The injection volume was 10 µL.

Table 2.2. LC gradient using CORTECS T3 column:

Time	%A	%B
0.0	95	5
2.0	95	5
2.1	55	45
6.5	40	60
10.0	34	66
11.0	15	85
16.0	5	95
20.0	5	95
20.1	95	5
24.0	95	5

MS analysis was performed on Agilent 6545 QTOF with Dual Jet Stream technology ion source (AJS). MS parameters used in this study were: acquisition rate 2 spectra/s, gas temperature 195°C, drying gas 13 L/min, nebulizer 30 psi, sheath gas temperature 325°C, sheath gas flow 12 L/min, capillary voltage 3500 V and mass range 100 to 1000 m/z. The instrument was calibrated at high resolution mode (4 GHz) and mass range of 50-750 m/z. Internal mass calibration was performed using 119.03632 m/z (purine) and 980.016375 m/z (HP-0921) in negative ESI mode, and 121.050873 (purine) and 922.009798 (HP-0921) for positive ESI mode. Polarity-switching MS analysis was performed according to the following time segments in Table 2.3, which were defined according to the analytes' retention time in each mode.

Table 2.3. Time segments, ionization mode and fragmentor voltage

Time segment	ESI mode	Fragmentor (V)	Nozzle voltage (V)
0	Negative	160	2000
2	Negative	160	2000
6	Negative	200	1250
7.78	Positive	245	1250
10.15	Negative	160	2000
10.9	Positive	245	1250

Ions coming from ESI have high energy. To compensate for variation in ion energy, ion beam shaping optics and the slicer can be added to QTOF design to prevent ions with extreme velocities or spatially positioned at high or low positions from entering to slit. The position of slicer in TOF can give high sensitivity or high-resolution mode. The slit in high resolution mode is narrower, whereas in high sensitivity mode it is wider allowing more ions to enter. In this work the high-resolution mode was used. Agilent 6545 model can give a resolution up to 40,000, in scan dynamic range is about 2-15,000 transients/scan and flight tube is about 152.4 cm long.[117]

2.9. Data analysis

Data analysis was performed by using Agilent MassHunter Qualitative Analysis version 10.0 and TOF Quantitative Analysis software version10.0. The extraction window was 20 ppm, and the most intense ion for each analyte was used for quantitation as shown in Table 2.4. The retention time, molecular formula and ionization mode of each mycotoxin is also shown in Table 2.4.[75]

Table 2.4. Summary of mycotoxin retention times and m/z used for quantitation.

Mycotoxins	Molecular formula	Ion used for quantitation	Monoisotopic mass of selected ion, m/z	Retention time (min)
CIT	$C_{13}H_{14}O_5$	$[M-H]^-$	249.0763	6.88
AOH	$C_{14}H_{10}O_5$	$[M-H]^-$	257.0455	7.56
AME	$C_{15}H_{12}O_5$	$[M-H]^-$	271.0612	10.53
OTα	$C_{11}H_9ClO_5$	$[M-H]^-$	255.0060	5.42
OTA	$C_{20}H_{18}ClNO_6$	$[M+H]^+$	404.0901	9.91
FB1	$C_{34}H_{59}NO_{15}$	$[M+H]^+$	722.3963	8.04
FB2	$C_{34}H_{59}NO_{14}$	$[M+H]^+$	706.4009	11.31
ENNA	$C_{36}H_{63}N_3O_9$	$[M+Na]^+$	704.4456	14.72
ENNA1	$C_{35}H_{61}N_3O_9$	$[M+Na]^+$	690.4300	14.37
BEA	$C_{45}H_{57}N_3O_9$	$[M+Na]^+$	806.3987	13.94
ENNB	$C_{33}H_{57}N_3O_9$	$[M+Na]^+$	662.3987	13.72
ENNB1	$C_{34}H_{59}N_3O_9$	$[M+Na]^+$	676.4143	14.03

Mycotoxins (ISTD)	Molecular formula	Ion used for quantitation	Monoisotopic mass of selected ion, m/z	Retention time (min)
FB3	$C_{34}H_{59}NO_{14}$	$[M+H]^+$	706.4009	9.74
$OTAd_5$	$C_{20}H_{13}d_5ClNO_6$	$[M+H]^+$	409.1214	9.84
$AMEd_3$	$C_{15}H_9d_3O_5$	$[M-H]^-$	274.0795	10.47

Chapter 3. Results and discussion

3.1. Development of LC-HRMS method

The aim of this research was to develop an LC-HRMS method for the detection of trace levels of mycotoxins in urine. Some studies discussed in Section 1.5 used a highly aqueous solvent that could cause solubility issues for non-polar mycotoxins as ENNs and BEA or they had significant ionization suppression for analytes that make their quantitation unreliable.[73] Furthermore, no LC-HRMS method covered the entire panel of 12 mycotoxins of interest. Additionally, ENNs other than ENNB were not detected in urine samples. To address these challenges, the objective of this thesis was to develop a new method for the measurement of these analytes. To achieve sufficient sensitivity and selectivity, an effective sample preparation, LC separation and MS detection method are required to quantitate traces of mycotoxins in urine sample. The dilute-and-shoot method is one of the most prevalent sample preparations for urine samples using LC-MS.[73-74, 82] However, dilute-and-shoot do not provide sample clean-up, although dilution may reduce ionization matrix effects, it also may negatively impact LOQs achieve by this method. Thus, SPE method was tested since it can remove interferences from the sample. A LC separation and HRMS detection method was developed building upon previous LC-MS method for plasma[75] to evaluate a panel of 12 mycotoxins in urine.

3.1.1. Selection of internal standards

The primary goal of using internal standard is to increase accuracy and precision of quantitation. Implementation of isotopically labelled or structural analog internal standard (ISTD) can compensate for the variable composition of biospecimen result from matrix interferences and also correct for losses/gains that can occur during sample preparation or significant ionization suppression or enhancement. As a result, for the best quantitation performance of an LC-MS method, ISTD for each analyte should be employed. However, commercial ISTD are not available for all mycotoxins of interest. Thus, the goal of this work is to develop method with no significant matrix effects and use 2-3 ISTDs for quality control.

3.1.2. Modification of LC-MS time-segmented polarity switching method for urine biomonitoring

CIT, OTα, AOH and AME ionize better in negative mode, whereas FBs, OTA, ENNs and BEA ionize better in positive ESI mode,[75] as illustrated in Table 2.3. A time-segmented polarity switching LC-HRMS method was previously developed for 10 mycotoxins in plasma.[75] The time-segmented polarity switching permitted the analysis of all mycotoxins of interest in a single analytical run, without having to perform separate positive and negative ESI analyses of each sample extract. However, to enable the use of such a time-segmented method, adequate chromatographic resolution between the analytes which ionize better in positive versus negative ESI mode had to be achieved. The initial method was developed for plasma biomonitoring, however due to the differences in composition of urine and plasma, several modifications were made to adopt the method to urine biomonitoring. Urine contains numerous salts, minerals and polar compounds such as creatinine.[58] These components will not be retained using reversed-phase C18 chromatography. Thus, to avoid the contamination of mass spectrometer with them, the effluent from the first 2 minutes of the gradient was discarded to waste. Among the different types of C18 LC columns described in Section 1.7, the CORTECS© T3 column was chosen for this separation since it is end-capped, compatible with 100% aqueous solvent and provided the best separation of critical analytes for which ESI-polarity switching was required.[75]

Time segments and other parameters were modified according to the retention time and ionization mode of each compound. In the previous developed method, for plasma, isopropanol was used to wash away lipid the content. However, urine is not a lipid-rich matrix, thus isopropanol was removed from the mobile phase gradient. The resulting method achieved promising separation for 10 mycotoxins of interest in urine using water and methanol as the mobile phases.

By adding AOH and AME to the panel of mycotoxins, AOH and AME were found to co-elute with FB1 and FB2 in positive ESI mode (Appendix, Supplementary Figure 1). Both AOH and AME showed 10x and 6x higher intensity in negative rather than in positive ESI mode. The overlaid chromograms of AOH and AME in both ESI modes are shown in the Appendix, Supplementary Figure 2. Therefore, to achieve the best possible limits of detection, it was beneficial to separate the alternariols from the fumonisins, to enable their quantitation in negative

rather positive ESI mode. This result is in agreement with the previous study by Liu *et al.* who also showed that AOH and AME ionized better in negative ESI mode with an aqueous solvent containing 5 mmol/L ammonia acetate and 0.01% ammonium hydroxide and acetonitrile (ACN) as mobile phase A and B, respectively.[89] Thus, the gradient at the time segment where they co-elute which is between 6.50 to 10 minutes was modified. Between 6.50 to 10 minutes the elution is isocratic at 66%, thus, by changing the percentage of MeOH from 66% at 6.50 minute to 60%, FB1 and FB2 separated from AOH and AME as it is demonstrated in Figure 3.1.

In the plasma biomonitoring method, OTA eluted during time segment where negative ESI ionization was used. However, upon changing the gradient to separate the alternariols from the fumonisins, OTA now eluted during a time segment where positive ESI was used. As shown in Appendix, Supplementary Figure 11, changing from the negative to positive mode for OTA led to a 27% drop in the peak area which will not severely impact LODs and LOQs that can be achieved. Other studies such as Liu *et al.*, Gering *et al.,* Šarkanj *et al.*, Warth *et al.* detected OTA in positive mode.[67, 74, 89, 118] As a result, OTA could also be detected in positive mode. Furthermore, the change in ionization mode for OTA, also required to add an additional internal standard to evaluate the performance of analytes which were detected in negative ESI mode. Therefore, $OTAd_5$ internal standard was replaced by $AMEd_3$ to monitor compounds in negative mode. Finally, Figure 3.1 showed the separation of all 12 mycotoxins using time-segmented polarity-switching LC-HRMS method after the optimization for urine biomonitoring.

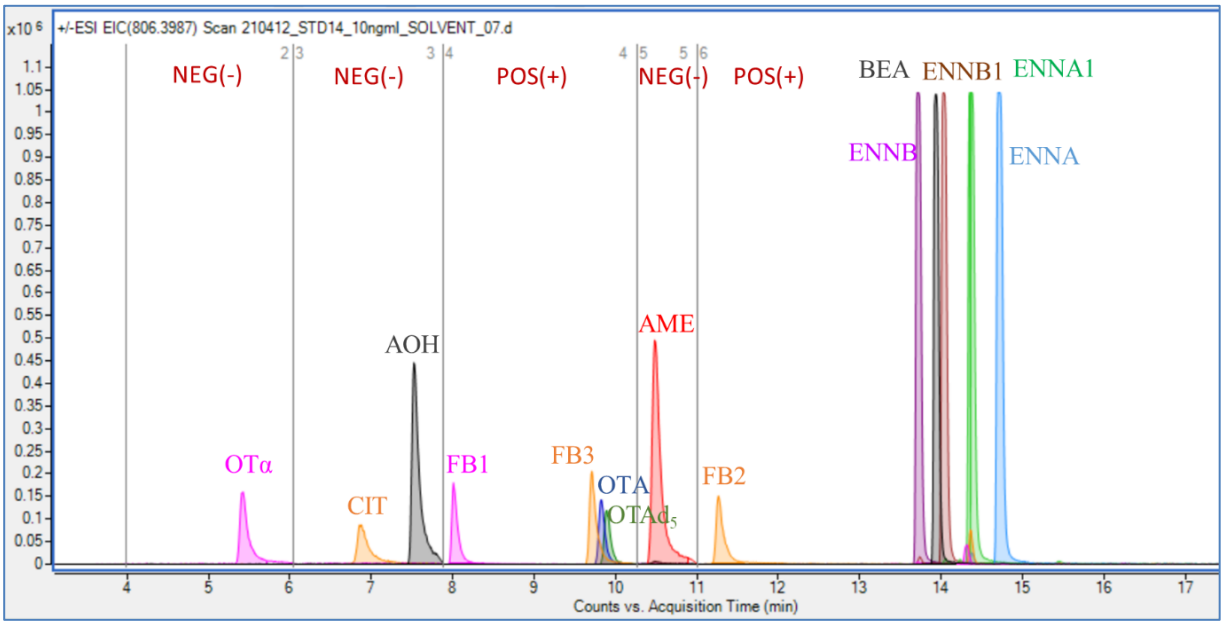


Figure 3.1 Chromatographic separation of 12 mycotoxins (10 ng/mL standard solution in 60% MeOH with 1% FA) by CORTECS T3 C_{18} column and water/methanol with 0.02% AA as the mobile phase, and the gradient described in Section 2.8.

Urine components did not interfere with the m/z of mycotoxins, as shown in Appendix, Supplementary Figure 3-Supplementary Figure 9. The extracted ion chromatogram (EIC) of mycotoxins showed that during their retention time, no interferences came from the urine.

3.1.3. Optimization of fragmentor voltage

Located between the electrospray chamber (at atmospheric pressure) and the mass spectrometer, the fragmentor voltage regulates the speed at which the ions travel through a medium-pressure (3 torr) capillary (10^{-5} torr). The required fragmentor voltage is dependent on the type of compound, thus if the voltage is increased beyond 200 V, there would be a high chance of analyte's fragmentation.[108] The best fragmentor voltages for the ten mycotoxins, excluding AOH and AME, in each mode were previously re-evaluated.[75] By adding the alternariol group to the list of target mycotoxins, the best fragmentor voltage for these compounds was evaluated. Since AOH elutes in the same time segment as CIT, the best compromise fragmentor voltage for both was chosen simultaneously. The 20 ng/mL standard mix of mycotoxins was analyzed using negative

ESI mode while optimizing the fragmentor voltage in step-wise fashion from 50 to 250 V. The best fragmentor for CIT was 200-235 V, whereas for AOH 170-200 V was optimum as shown in Figure 3.2 . Thus, 200 V was selected as final fragmentor voltage for this time segment.

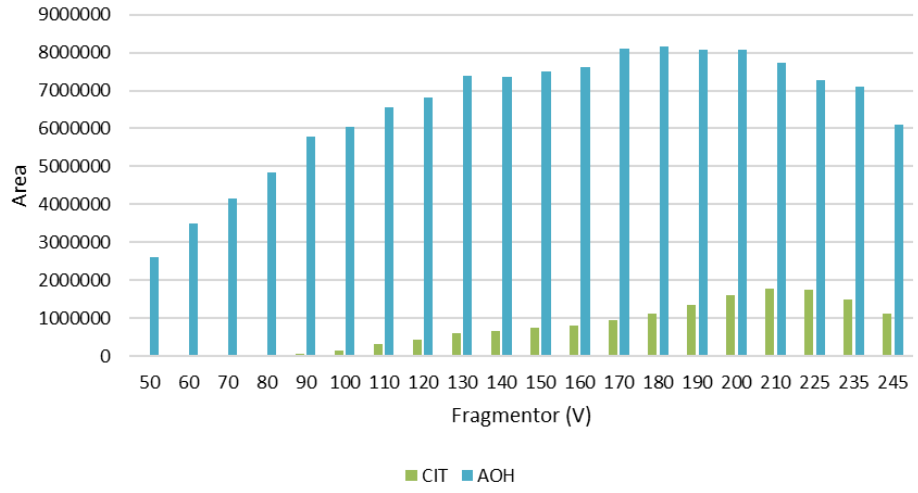


Figure 3.2 Determination of fragmentor voltage for AOH and CIT in negative ESI mode.

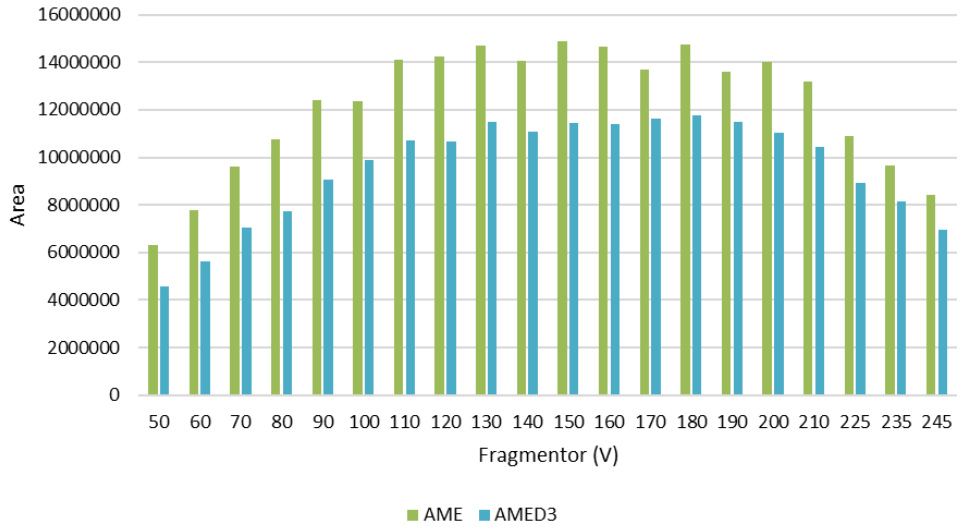


Figure 3.3 Determination of fragmentor voltage for AME and AMED$_3$ in negative ESI mode.

The results in Figure 3.3 demonstrated that the optimal fragmentor voltage for AME was at 110-200V. Thus, 160 V was selected for AME.

3.2. Development of sample preparation method for biomonitoring of 12 mycotoxins in urine

Urine is a complex mixture of salts and polar compounds as shown in Figure 1.2.[119] The 12 mycotoxins of interest for this urine biomonitoring method are diverse in terms of their structures and polarity (log P ranged from -0.67-7.27, ChemAxon). This mycotoxin panel includes acidic species such CIT and OTα, amphiphilic analytes such as FBs and OTA, basic cyclic peptide analytes ENNs and BEA and neutral analytes such as AOH and AME. Detailed information on pKa and log P is shown Table 1.2. This chemical diversity of the analytes of interest complicated the development of an appropriate sample preparation method. The dilute-and-shoot method was commonly used in different studies as mentioned earlier in Section 1.5. The SPE method could provide an additional sample clean-up and enrichment.

3.2.1. Dilute-and-shoot

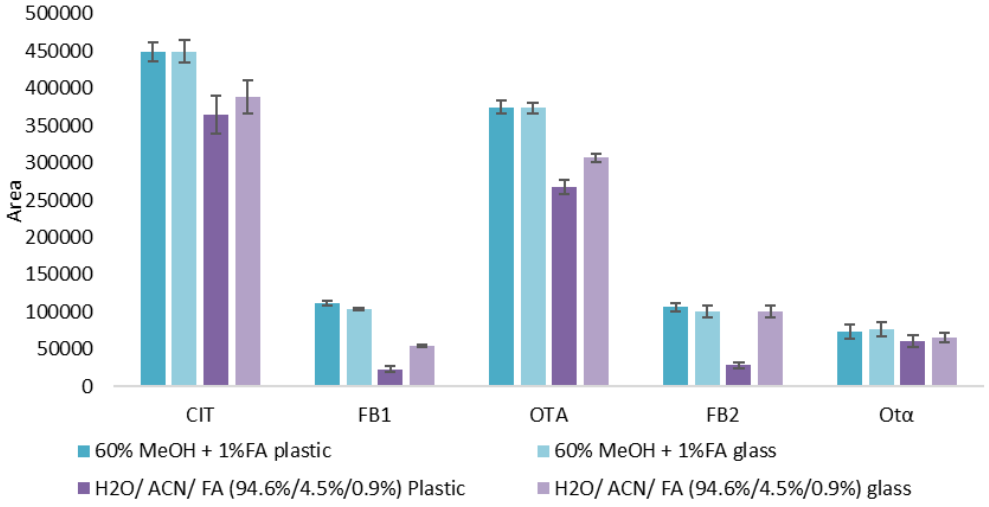
3.2.1.1. Evaluation of solubility and non-specific adsorption of mycotoxins

One of the most prevalent sample preparations used for detecting mycotoxins in urine samples is the dilute-and-shoot method.[73-74, 76, 82, 93] In the dilute-and-shoot method, three parameters should be optimized: the diluent, dilution factor, and normalization method to compensate for variations in urine composition.

Gerding *et al*. suggested a highly aqueous solvent containing 5% acetonitrile with 1% FA for diluting urine samples for a panel of mycotoxins including FBs, OTA, OTα and ENNB.[73] The method showed excellent LOQs, but our previous work indicated solubility issues for BEA and ENNs in highly aqueous solvents even those containing 40% MeOH.[75] Thus, the results from Gerding *et al.* which used even lower solvent concentrations were surprising.[73, 75] Slobodchikova *et al.* tested ENNs, BEA at 20 ng/mL with 40, 60 and 80% MeOH using plastic (polypropylene) and glass inserts. Non-specific adsorption was detected for ENNs and BEA when 40% MeOH was used. The same experiment was repeated with 60% MeOH and the results did not demonstrate adsorption losses using plastic or glass inserts.[75] Based on these previous results, our optimized solvent of 60% MeOH was compared to 5% ACN with 1% FA proposed by Gerding *et al*.[73] Thus, a detailed side-by-side comparison of two solvent systems was performed as described in Section 2.4.1. The results in Figure 3.4 show the areas of CIT, FB1, FB2, OTA

and OTα at 10 ng/mL using both solvents in glass and plastic inserts. Focusing on the results obtained in plastic inserts, mycotoxins such as CIT, OTA and OTα, showed differences of 19, 28, 17% between highly organic and high aqueous solvents. For FBs, this difference was even higher (79% for FB1 and 73% for FB2). Comparing plastic and glass inserts showed further small decreases in the mean mycotoxin areas when highly aqueous solvent was used in combination with plastic inserts. Similarly, for CIT (29%) and OTA (18%), the use of plastic inserts in combination with highly aqueous solvent for diluting urine 10-fold resulted in decrease in the mean area, indicating non-specific adsorption (Figure 3.5 (A)). This effect was not observed for OTα (3%) in diluted urine samples. The two-tailed t-test result confirmed that there is no significant difference between the intensities in diluted urines with aqueous versus organic solvent (p-value is equal to 0.432). However, the result was more pronounced for FB1 (30%) and FB2 (78%) in diluted urine (p-value < 0.0001).

A)



B)

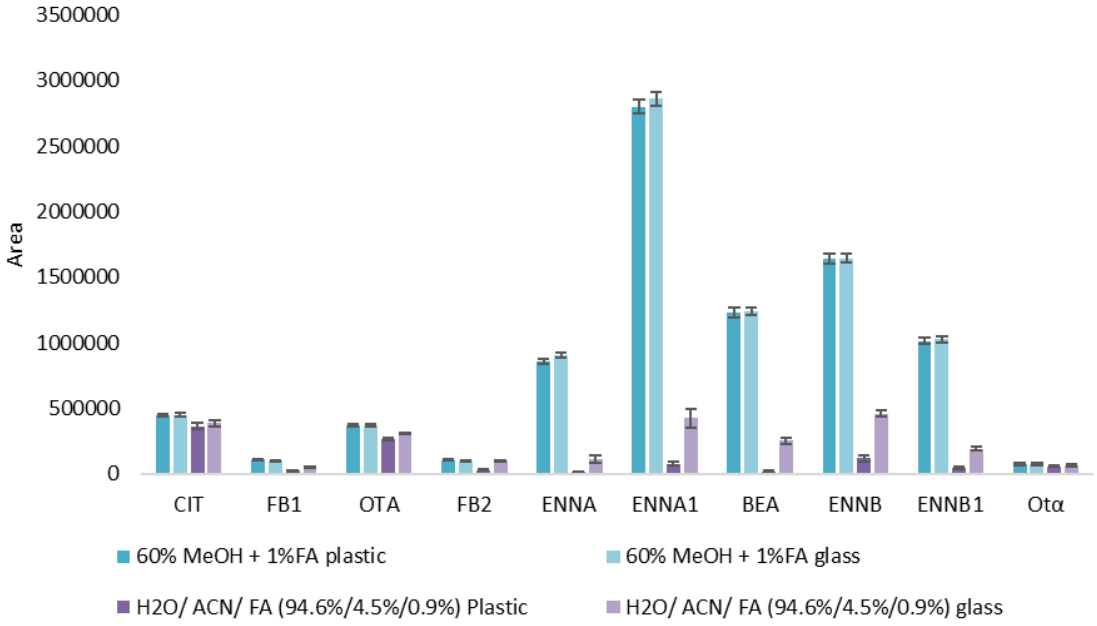
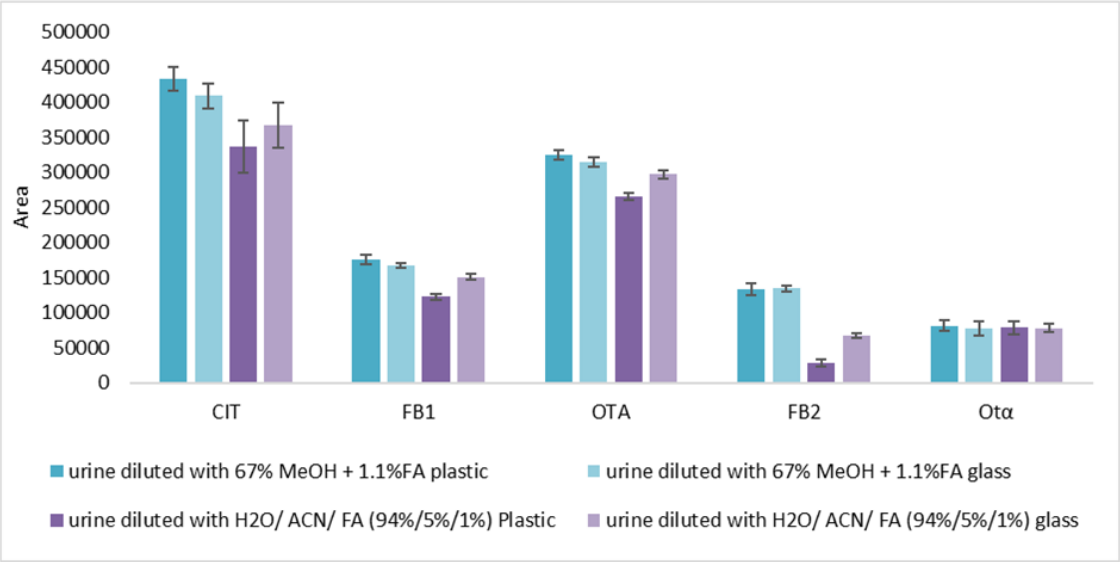


Figure 3.4. Comparison of two different solvents and container types using 10 ng/mL mycotoxin standard. Part (A) is shown on different scale, in part (B) all the data on the same scale is shown. Mean areas of 10 ng/mL mycotoxin standards (n=13) injections over 42 hours analytical sequence) prepared in either a highly organic solvent containing 60% MeOH with 1% FA or highly aqueous solvent containing 5% ACN and 0.9% FA. The standards were prepared and kept throughout the analysis in either glass or plastic inerts to check for non-specific adsorption. The error bars show standard deviations.

However, the difference between the two solvents becomes even more pronounced for ENNs and BEA as shown in Figure 3.4 (B). For ENNA1, drops of 97% and 85% in peak area were obtained when changing the solvent from highly organic to highly aqueous in plastic inserts (polypropylene) and glass vials, respectively. The same effect was observed for the rest of the ENNs and BEA. Furthermore, when urine was diluted 10-fold using the aqueous solvent, drops of 93 and 74% (plastic and glass vials, respectively) of the peak areas were seen for ENNA1 as shown in Figure 3.5 (B). This effect was also observed for all other ENNs and BEA in 10x diluted urine.

Overall, these results further confirmed our earlier study and expanded this evaluation to urine to show that ENNs and BEA are less soluble in a highly aqueous solvent, and that the highly aqueous diluent such as proposed by Gerding *et al*. should not be used for biomonitoring methods where ENNs are of interest.[73] In addition, the potential risk of non-specific adsorption to the surface of the polypropylene plastic is high, and increases in highly aqueous solvents. As a result, using highly aqueous solvent may lead to incorrect LOQ determination and poor recovery of ENNs and BEA. Based on these results, 60% methanol acidified with 1% FA was used as solvent for all working standard preparations and as injection solvent for all subsequent experiments. Using high organic solvent can cause retention time shifts or poor peak shape of early eluting mycotoxins, however, it is a compromise solvent for achieving acceptable solubility for the rest of mycotoxins.

A)



B)

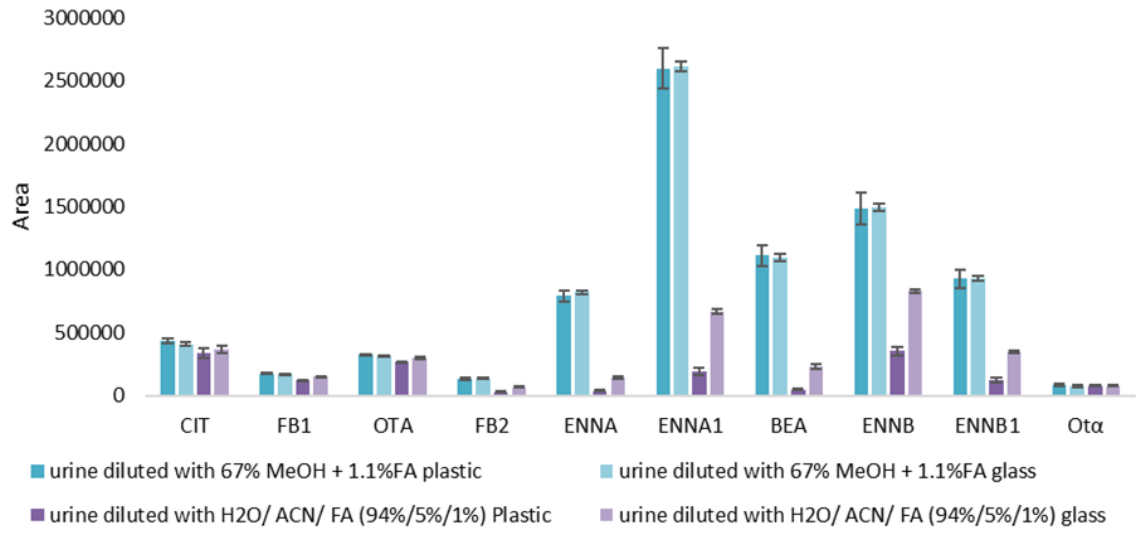


Figure 3.5. Comparison of two different solvents and container type using 10 ng/mL mycotoxins standard. Part (A) is shown on different scale, in part (B) all data on the same scale is shown. Mean areas of 10 ng/mL mycotoxin standards (n=13) injections over 42 hours analytical sequence) prepared by diluting urine 10x with high organic solvent reaching to 60% MeOH with 1% FA and high aqueous solvent reaching to 5% ACN and 0.9% FA. The diluted urine was prepared both in glass and plastic inerts to check for non-specific adsorption effect. The error bars are standard deviations.

3.2.1.2. Evaluation of matrix effects in different lots of urine

The non-analyte components of urine samples can complicate the analysis, besides the potential to contaminate the ion source with they may also create interferences for the mycotoxins of interest. Figure 3.6 shows the overlay of total-ion chromatogram (TIC) of non-spiked urine sample after 10x dilution compared to the 10 ng/mL mycotoxin mixed standard. As shown in Figure 3.6, most of the interferences coming from urine eluted between 5 to 8 minutes, so possible matrix effects are expected to be the highest for early eluting mycotoxins. Thus, a proper clean-up was required to detect the lowest level of CIT, AOH and OTα in urine samples.

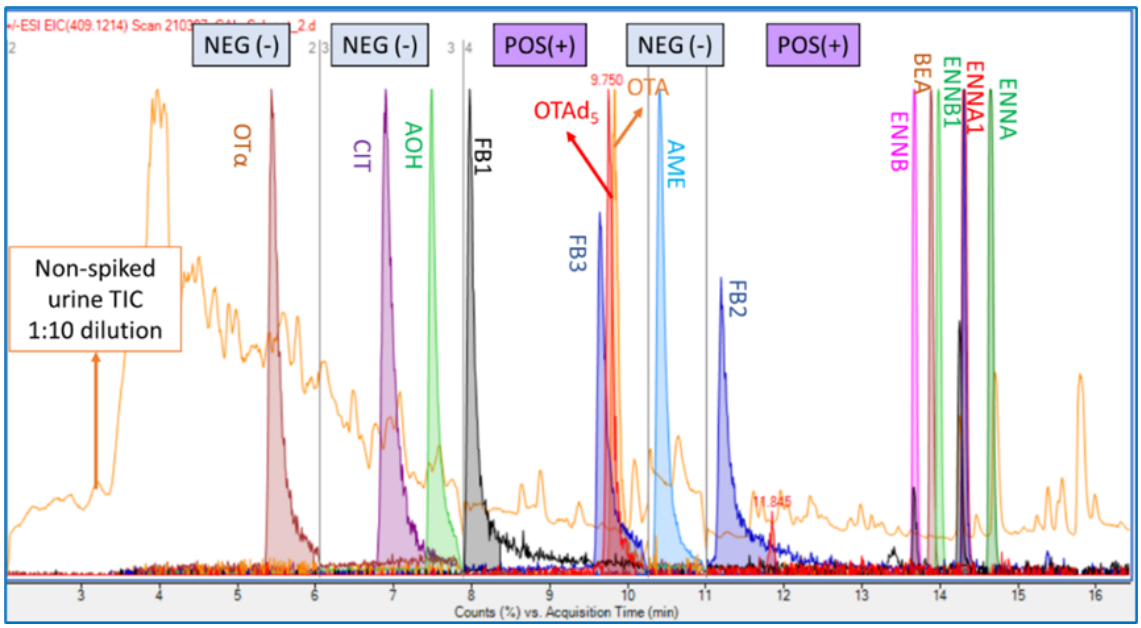


Figure 3.6. Overlay of TIC of 10x diluted non-spiked urine with EICs of 10 ng/mL mycotoxin standard in urine. 20 ppm was used as the m/z extraction window

Normal creatinine levels can vary from 39-259 mg/dL in men and 28-217 mg/dL in women depending on hydration status, diet, physical activity and so forth.[120] Figure 3.7 shows the matrix effects of analytes and ISTDs across 7 different lots of urine. Creatinine is commonly used for normalization of urine to compensate for the differences in composition. Urine samples with high creatinine levels reflect more concentrated urine and are likely to experience higher matrix effects for AOH and AME. The results demonstrated the ionization suppression of AOH (68-78%) and AME (71-78%) in all urine samples except urine B and C after 20x dilution, and the

effect appears more pronounced at higher levels of creatinine. In addition, the ion enhancement can be seen for FB1 in urine samples B, C, F and G and in FB2 for urine samples B, C and Gerding *et al.* previously detected ion enhancement for ENNB using the dilute-and-shoot method with 10x dilution with 5% ACN and 1% FA.[73] Furthermore, Silva *et al*. also reported signal enhancement for both FB1 and FB2 in maize products using UHPLC-TOF-MS.[121]

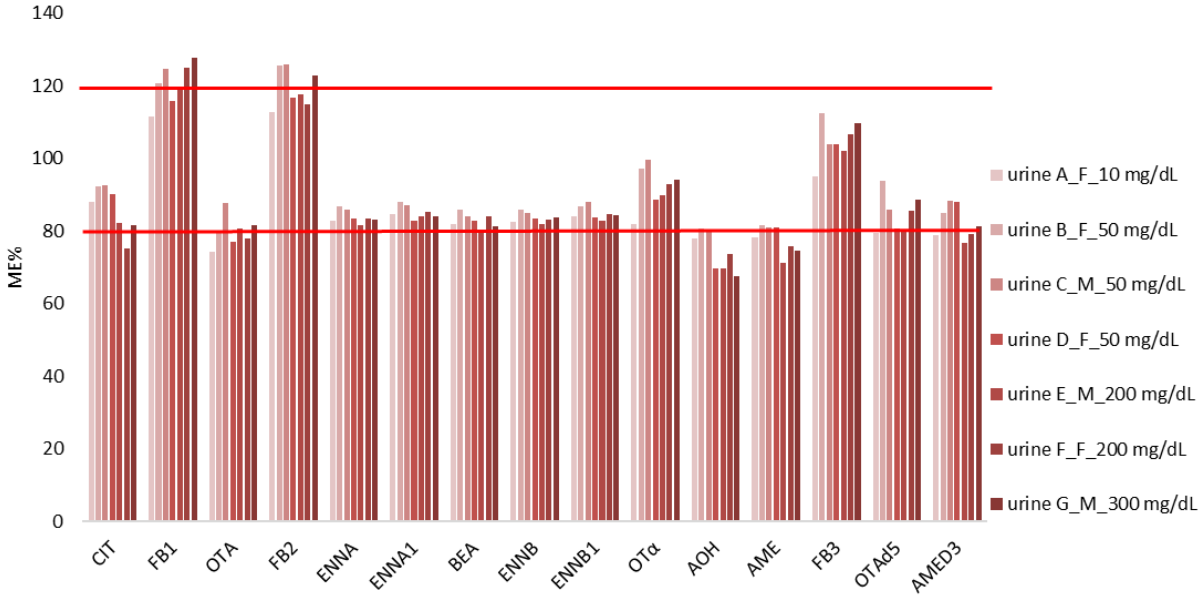


Figure 3.7. Matrix effects in urine samples (n=7 lots of urine with different creatinine levels). Urine samples diluted 20x to reach 60% MeOH with 1% FA, and spiked at 4 ng/mL of mycotoxins.

Minimizing the ME is important because it can affect sensitivity if significant ionization suppression occurs for analytes. In the most severe cases, the quantitation of certain mycotoxins may become impossible. For analytes that show consistent ionization suppression but with good peak intensity, an ISTD or a compound with similar structure and ionization properties can compensate for the differences in ionization response, otherwise it could lead to underestimation or over estimation of the analyte.[122] Thus, to investigate the matrix effects and later recovery, in both ion modes, $OTAd_5$, $AMEd_3$ and FB3 were chosen as ISTDs.

3.2.1.3. Evaluation of dilution factor

Three different dilution factors were evaluated to check which one gives the best result in terms of matrix effects in urine samples. A 1:10 dilution was the most prevalent in the reported studies. However, not all of them evaluated matrix effects.[73, 82, 118] We tested 10, 15 and 20x dilution in seven lots of urine with different creatinine levels. Dilutions exceeding 20x were not considered as they would lead to poor LOQs. The results summarised in Figure 3.8 correspond to a urine sample with a creatinine level of about 300 mg/dL. The results indicated that the matrix effects could be improved slightly for almost all the mycotoxins by increasing the dilution factor to 20-fold. However, since, this urine sample was highly concentrated significant matrix effects still persisted even at 20-fold dilution for most of the mycotoxins except FBs and OTα, suggesting a need for a better clean-up method.

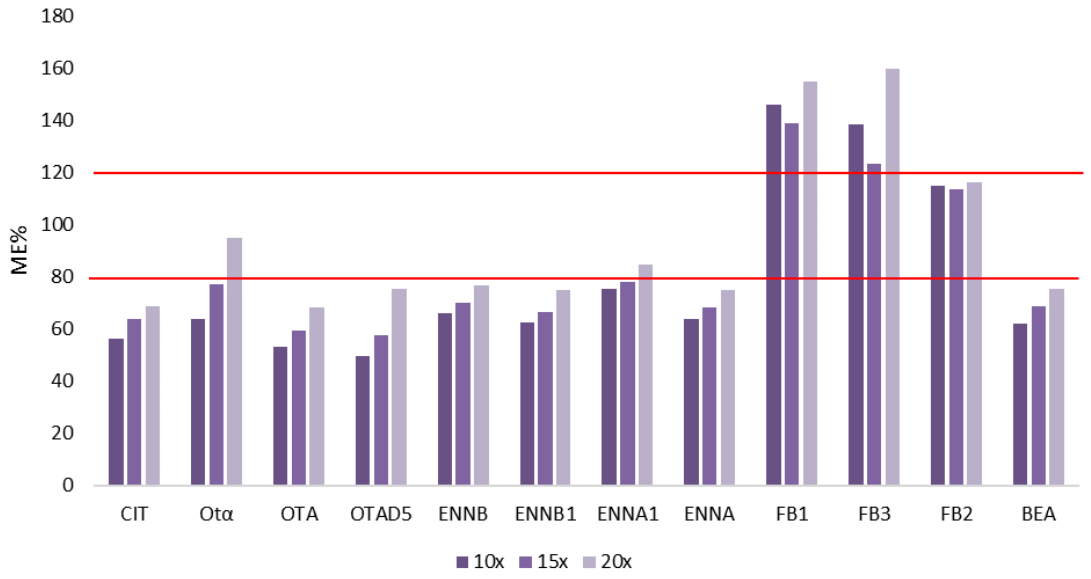


Figure 3.8. Effect of 10x, 15x and 20x dilution on matrix effects. Urine (300 mg/dL of creatinine) was spiked at 10 ng/mL of mycotoxins.

3.2.1.4. Evaluation of recovery in dilute-and-shoot method

To evaluate the recovery, spiked urine samples were prepared in either plastic or glass according to the experiment design in Figure 3.9. The amount of time that spiked urine samples remained in plastic inserts before dilution was minimized to avoid losses due to adsorption. Figure 3.10

shows the recovery of the mycotoxins evaluated in plastic inserts. Low recoveries (41-75%) were obtained for ENNs and BEA. The reason for such low recoveries could be adsorption of these compounds to the plastic walls due to their cyclic peptide structure. To investigate this possibility, the same experiment was also repeated using glass containers.

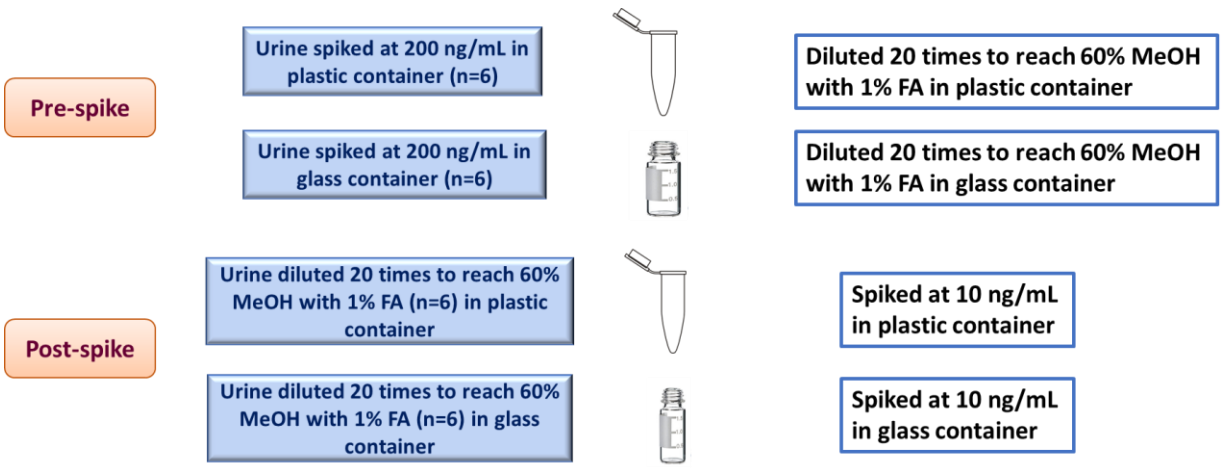


Figure 3.9 Schematic workflow of mycotoxin recovery evaluation in urine with dilute-and-shoot method using plastic (polypropylene) and glass containers. Urine diluted 20x (n=6).

The same experiment was performed using glass and showed high recoveries of ENNs and BEA (93-99%) as shown in Figure 3.11. Changing to glass inserts was more important when urine was spiked before dilution since there was a high risk of adsorption if plastic (polypropylene) was used. This is consistent with our results from Section 3.2.1.1 which also showed more pronounced losses when combination of high aqueous solvent and plastic was used. However, what was surprising in this experiment is the extent of adsorptive losses even when the time of the contact was minimized, and relatively high mycotoxin concentrations were used. In samples which were spiked after dilution to 60% MeOH with 1% FA, this effect was less pronounced. In these cases, there was enough organic solvent present to dissolve ENNs and BEA and prevent them from adsorbing to the plastic surfaces. Putting these results in the context of the entire biomonitoring method, this issue also likely occurs during collection and storage of urine samples which is typically done in sterile polypropylene cups. Therefore, this aspect needs to be addressed properly by either performing urine collection in glass containers or recovering the

adsorbed ENNs and BEA using solvent prior to further sample preparation. This issue will be further discussed in Section 3.2.2.1.

As shown in Figure 3.11, a recovery higher than 100% was detected for CIT. The apparent high recovery was not due to endogenous levels of CIT in urine because non-spiked urine was previously analyzed, and no trace of CIT was detected. Thus, this high recovery was due to the change in response from the QTOF.

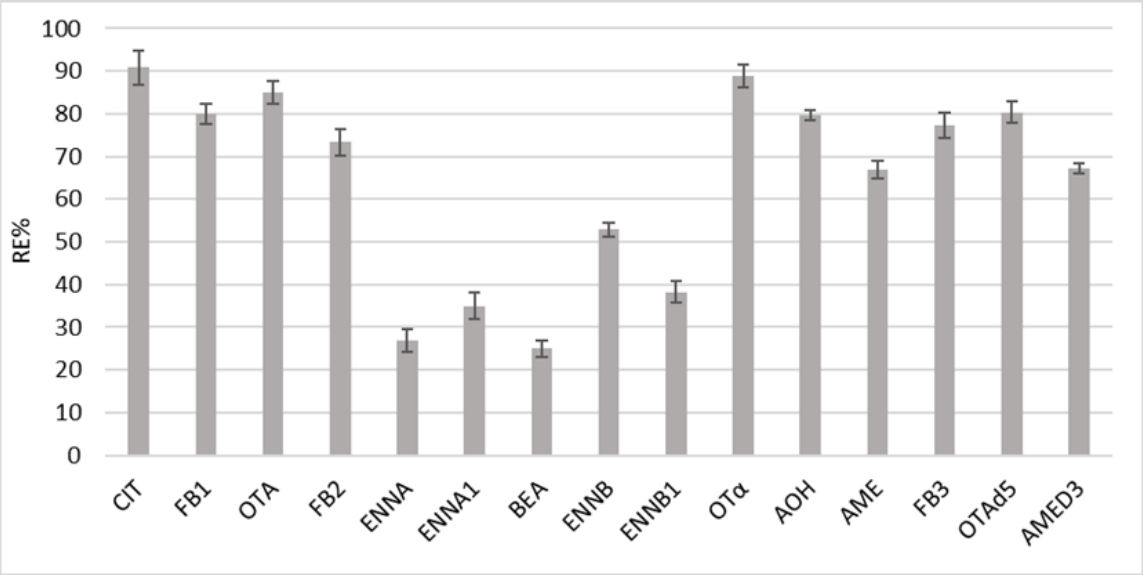


Figure 3.10 Evaluation of mycotoxin recovery in urine using 20x dilute-and-shoot method using polypropylene plastic for preparation and injection. The final concentration was 10 ng/mL of mycotoxins (n=6) . The error bars represent standard deviation

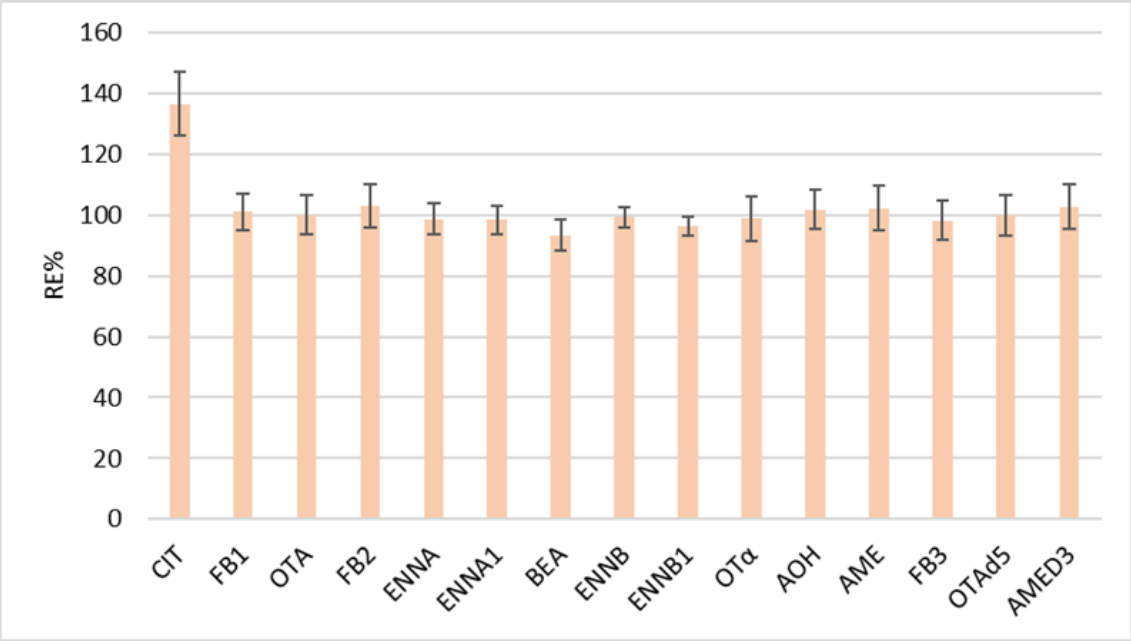


Figure 3.11 Evaluation of mycotoxin recovery in urine using 20x dilute-and-shoot method using glass inserts for preparation and injection. The final concentration was 10 ng/mL of mycotoxins (n=6). The error bars represent standard deviation

3.2.2. Determination of LOQ in dilute-and-shoot method

LOQs were evaluated for each mycotoxin in both urine and solvent. Table 3.1 demonstrated calibration curve in solvent showed a linear relationship for all the mycotoxins in the tested range of (0.0098-20 ng/mL). In 20x diluted urine this relationship was linear for all the mycotoxins except for ENNs and BEA where quadratic regression was used. The criteria for choosing the selected LOQs was S/N higher than 5 calculated by MassHunter quantitative software by taking the analyte intensity to noise intensity, and the accuracy within the range of 80-120%.

According to Table 1.4, the detected levels of mycotoxins in different countries were less than 1 ng/mL. However, urine LOQs for all the mycotoxins are above 1.2 ng/mL after 20x dilution. This shows that LOQs need to be improved to be applied in real samples, and that dilute-and-shoot method, despite its frequent use in biomonitoring, is not the best choice for this

application. In solvent, sufficient LOQs can be met for all mycotoxins except FB2 as shown in Table 3.1.

Table 3.1. LOQs obtained for dilute-and-shoot method in solvent and urine. R^2 represents coefficient of determination.

Name of compound	LOQ in solvent plastic (ng/mL)	R^2	LOQ in urine after dilution glass (ng/mL)	R^2	LOQ in urine after 20x (ng/mL) glass
CIT	**0.65**	0.997	**1.17**	0.995	**23.4**
FB1	**0.59**	0.994	**0.13**	0.995	**2.6**
OTA	**0.53**	0.998	**0.58**	0.996	**11.6**
FB2	**1.10**	0.991	**0.92**	0.992	**18.4**
ENNA	**0.04**	0.986	**0.11**	0.999	**2.2**
ENNA1	**0.02**	0.996	**0.23**	0.996	**4.6**
BEA	**0.02**	0.989	**0.21**	0.999	**4.2**
ENNB	**0.04**	0.996	**0.12**	0.999	**2.4**
ENNB1	**0.02**	0.991	**0.21**	0.999	**4.2**
OTα	**0.37**	0.993	**0.46**	0.990	**9.2**
AOH	**0.02**	0.998	**0.06**	0.997	**1.2**
AME	**0.15**	0.999	**0.11**	0.994	**2.2**

3.2.2.1. Non-specific adsorption of ENNs and BEA to polypropylene plastic tubes

Usually, urine samples are collected in a propylene container; and as previously discussed in Section 3.2.1.1, ENNs and BEA showed severe losses to the propylene plastic tubes, even when the contact time was minimized. Thus, if urine samples are collected in polypropylene containers, ENNs and BEA must be recovered prior to analysis. If this is not done, severe under-estimation of ENNs will occur and this may explain why previous biomonitoring studies did not detect ENNs and BEA.

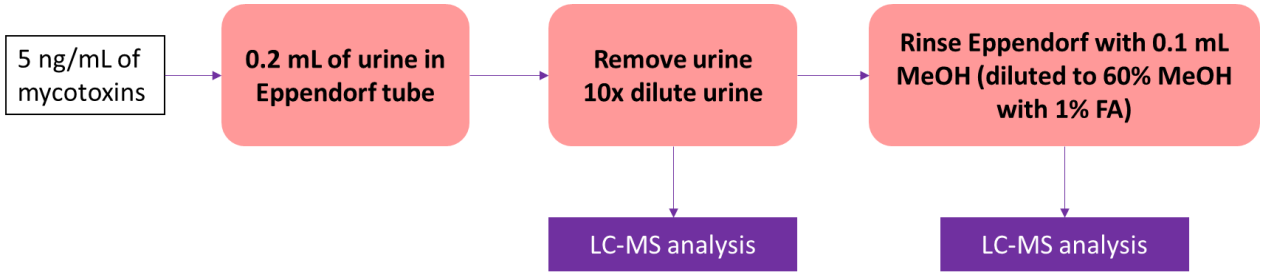


Figure 3.12 Workflow to evaluate losses of ENNs and BEA in Eppendorf tube (n=5) during two weeks storage.

To examine this issue further, recoveries were tested by storing urine samples for two weeks in the -80°C freezer to mimic typical sample collection, transportation, and storage procedure. The workflow of analysis is shown in Figure 3.12. After removing urine from the container, the Eppendorf tube was rinsed with 100 µL of MeOH as described in Section 2.6. The results in Table 3.2 show the concentration of ENNs and BEA in spiked urine that was 10-fold diluted, and in MeOH that was used for rinsing the Eppendorf tube. Among ENNs and BEA, the maximum concentration found by rinsing the Eppendorf belonged to BEA and ENNA and ENNA1 at 4.03, 3.46 and 2.68 ng/mL, respectively. This could possibly explain why they were not detected in urine samples in previous studies using different sample preparation method as discussed in Table 1.4. Figure 3.13 illustrated that 17, 30, 57, 44 and 67% of ENNB, ENNB1, ENNA, ENNA1 and BEA were recovered from the plastic wall after storage of urine samples, respectively. This result also indicated that AOH and AME were slightly susceptible to adsorption; however, the percentage of adsorption is less than 5 and 10%, respectively.

Table 3.2. Concentrations of ENNs and BEA in MeOH used for rinsing Eppendorf and in 10-fold diluted urine spiked at 5 ng/mL (n=5). After storing samples for two weeks, 10 µL of urine was analyzed after 10x dilution to reach 60% MeOH and 1%FA. After removing urine, the Eppendorf tubes rinsed with 100 µL of MeOH and then diluted to 60% MeOH With 1% FA

		ENNB	ENNB1	ENNA	ENNA1	BEA
Urine	Concentration (ng/mL)	3.45	3.04	1.97	2.52	1.78
	RE%	69%	61%	39%	50%	36%
Dilute MeOH rinse of Eppendorf	Concentration (ng/mL)	1.05	1.82	3.46	2.68	4.03
	RE%	17%	30%	57%	44%	67%

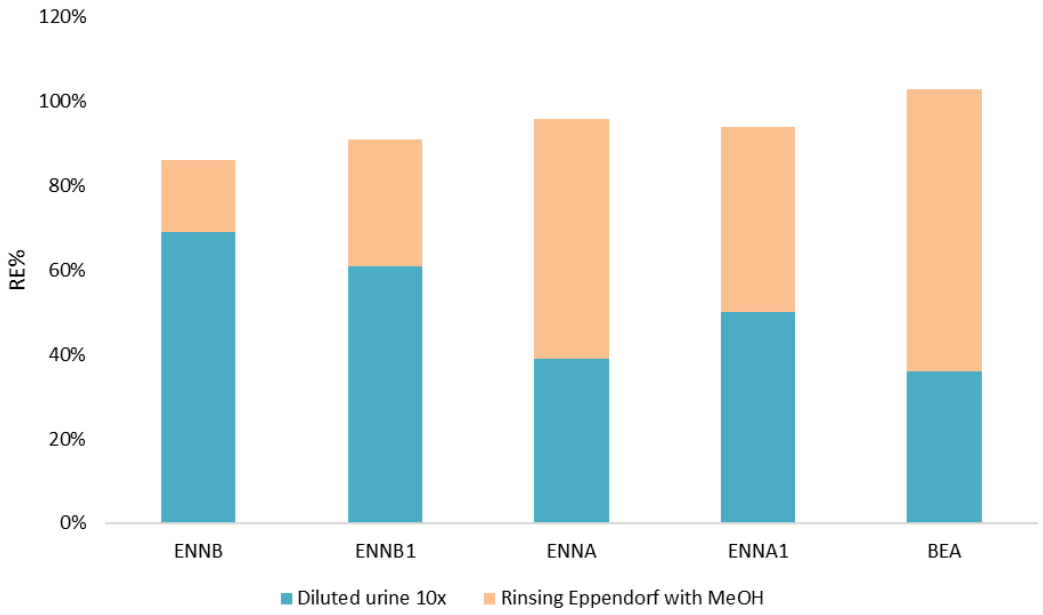


Figure 3.13 Evaluation of recovery of mycotoxins in spiked urine stored in plastic Eppendorf tube after storage for two weeks in -80°C . Recovery was measured in 10xdiluted urine and in MeOH that was used for rinsing the plastic Eppendorf tube. Pooled urine samples (200 µL) were spiked at 5 ng/mL in (n=5). After storing samples for two weeks, 10 µL of urine was analyzed after 10x dilution to reach 60% MeOH and 1%FA. After removing the urine from containers, the Eppendorf tubes were rinsed with 100 µL of MeOH and then diluted to 60% MeOH With 1% FA.

To check if one single rinse with 100 µL of MeOH was enough to completely recover ENNs and BEA, the Eppendorf tubes were rinsed for a second time with a fresh 100 µL portion of MeOH.

The concentration of ENNs and BEA of the second rinse is provided in Table 3.3. The five replicates showed different concentrations for ENNs and BEA. However, these levels were low compared to the first rinse. Therefore, either the rinsing volume should be increased, or the second-time rinsing should be applied for recovering ENNs and BEA completely.

Table 3.3. Concentration of ENNs and BEA extracted from the second-time rinsing from the Eppendorf tube (n=5). Eppendorf tubes were rinsed with second portion of 100 µL of MeOH and diluted to 60% MeOH with 1% FA before LC-MS analysis.

Second-time rising with MeOH (ng/mL)	ENNB	ENNB1	ENNA	ENNA1	BEA
Replicate 1	0.074	0.119	0.239	0.162	0.265
Replicate 2	0.024	0.049	0.113	0.070	0.125
Replicate 3	0.041	0.063	0.152	0.091	0.171
Replicate 4	0.225	0.330	0.495	0.384	0.566
Replicate 5	0.073	0.107	0.187	0.134	0.200

Consequently, to quantitate ENNs and BEA accurately, rinsing the container that was used to collect urineis essential. Alternatively, glass containers may be used during sample collection to avoid this issue.

3.2.3. Development of solid-phase extraction method for 12 mycotoxins

As demonstrated in Table 3.1, the dilute-and-shoot method could not achieve adequate LOQ levels with 20x dilution. Therefore, a strategy to both clean-up and, enrich the sample was needed. SPE method was chosen over LLE etc. Liquid-liquid extraction method would not be a good fit as several analytes such as FBs and CIT, are too polar for the efficient extraction by LLE. Among the different types of sorbents in SPE, HLB SPE was tested as it has both hydrophilic and lipophilic parts capable of retaining mycotoxins with different polarity. Furthermore, since it has a polymeric stationary phase, it has a wide range of pH tolerance of 0-14 which make it the best choice for the panel of 12 mycotoxins that their pKa are between the range of 3-18

3.2.3.1. Effect of urine pH on process efficiency (PE)

The mycotoxins of interest include acidic, neutral, and basic mycotoxins. AOH and AME have pKa ~7, ENNs ~ 18.88, and FBs and OTs ~ 3. Therefore, choosing a SPE sorbent to capable of covering these differences is of great importance. Oasis HLB SPE can retain both hydrophobic and hydrophilic mycotoxins. The extent of ionized versus neutral form of analyte depends on sample pH, for acidic, zwitterionic and basic mycotoxins.

Normal urine pH can vary from 4.5 to 7.8 and this was also observed in our set of urine samples, shown in Table 2.1.[123] This means that urine pH has to be controlled before extraction. To test the effect of pH adjustment on mycotoxin process efficiency (PE) two pHs were tested. For testing the best pH for acidic mycotoxins such as CIT and OTA with pKa around 3.0, the ideal pH should be two units below pKa, equivalent to a pH of 1.0. However, many SPE sorbents are not compatible with such low pH causing sorbent bleed that increases of MS interferences. Thus, a pH 2.0 was chosen as a compromise of the lowest pH that could be practically tested. pH 6.0 was chosen as a typical urine pH. This experiment was used to determine if pH adjustment to acidic pH may improve recovery of acidic mycotoxins.

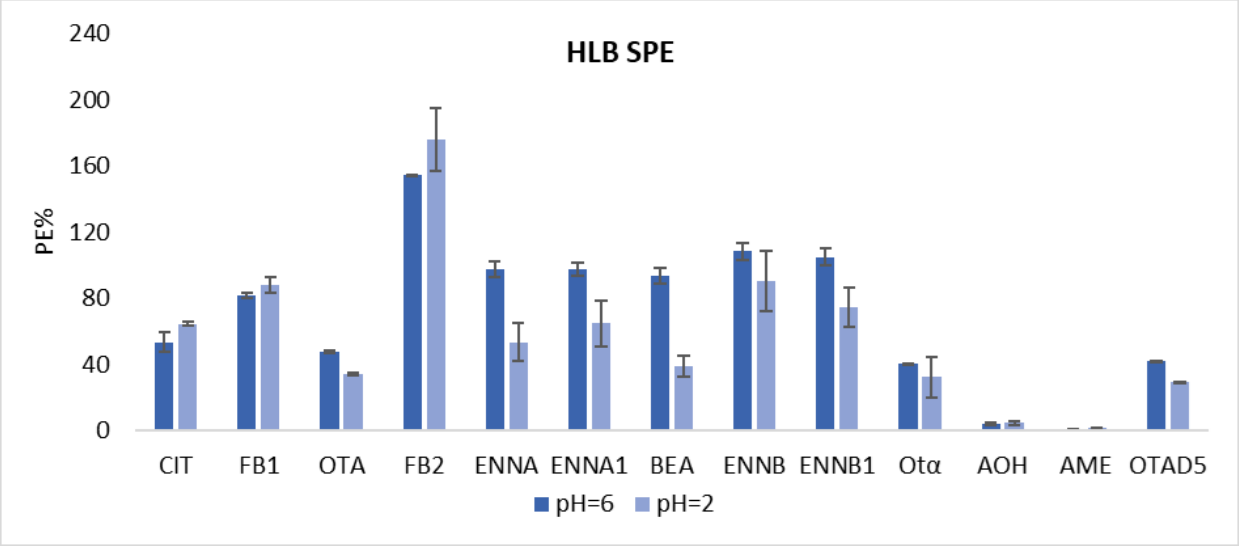


Figure 3.14 Effect of urine pH on process efficiency of mycotoxins in HLB SPE. 2 mL of urine (137 mg/dL of creatinine, adjusted to pH 2.0 or 6.0) spiked with 10 ng/mL of mycotoxins was loaded (n=2). The SPE cartridge was washed with 1 mL of 5% MeOH. Analytes were eluted with 600 µL of MeOH. Samples were diluted to 60% MeOH with 1% FA before LC-MS injection. The error bars represent standard deviation

Changing the pH from 6.0 to 2.0 decreased the process efficiency for ENNs, BEA and OTA as illustrated in Figure 3.14. However, for the rest of the mycotoxins, there were no significant differences between the two pH values. Therefore, for the rest of the optimization, pH 6.0 was used. Liu *et al.* tested pH 3.0 and 6.0, and at pH 6.0 good recoveries were achieved for FBs, ENNs, BEA, AOH, AME, OTA and OTα. However, pH 3.0 was chosen for their method due to the loss of tenuazonic acid at pH 6.0.[89]

As observed in Figure 3.14, the PE% for AOH and AME are very low at both pHs. This poor PE% could arise from either lack of recovery or matrix effects as process efficiency includes both effects. To test if recovery was the issue, 0.6 mL and 1 mL fresh portion of MeOH were used to evaluate the completeness of elution step. The results demonstrated that both AOH and AME were detected after adding either 0.6 or 1 mL of MeOH after the initial elution step, as shown in Appendix, Supplementary Figure 12. Therefore, 0.6 mL MeOH is not sufficient to elute AOH and AME and ENNs. The results in Supplementary Figure 12 demonstrate that adding either 0.6 or 1 mL of extra MeOH had a similar effect in terms of eluting AOH and AME fully. In conclusion, for subsequent experiments a total volume of 1.5 mL MeOH was used for the elution step, to ensure complete elution of all analytes.

3.2.4. Effect of washing solvent and volume on process efficiency (PE)

The next thing to check was which solvent provides the best clean-up, while maintaining good recovery of analytes. Different percentages of methanol (5%, 10% and 20%) and 10% methanol with different additives to modify pH were tested since different interferences may be removed under different pH conditions. To check for any losses of mycotoxins during the washing step, the wash solvent was collected and injected.

The results showed that using 5% and 10% MeOH as the washing solvent did not lead to any losses of mycotoxins in HLB SPE. The PE% ranged from 18 to 127%. However, when 1 mL of 20% MeOH was added after 3 mL of 5% MeOH (in total 4 mL of washing solvent), it led to a loss of 8.5% of OTα in HLB SPE (Appendix, Supplementary Figure 14). OTα is the most polar mycotoxin among the 12 mycotoxins; thus, it was the first to be affected during washing step as MeOH concentration in wash solvent was progressively escalated to increase the stringency of

the wash. Low PE% of about 7% was also detected for OTα using 2% MeOH as the washing solvent in the C18 cartridge, for similar reason.

The results presented in Figure 3.15 indicated that there were no significant differences in the PE% using different washing solvents and different pHs. However, when the washing volume was increased to 6 mL and the volume of urine loaded was decreased to 1 mL (green color), an increase in PE% of CIT, OTA, OTα, AOH and AME was observed. This result demonstrated that 3 mL for the washing step was not enough to clean-up the interferences coming from urine. Since process efficiency included both matrix effects and recovery, each one should be investigated individually to see which one led to the poor process efficiency for AOH, AME, ENNs and BEA. Based on these results, 6.0 mL wash volume and 1.0 mL urine loading were selected for all subsequent experiments.

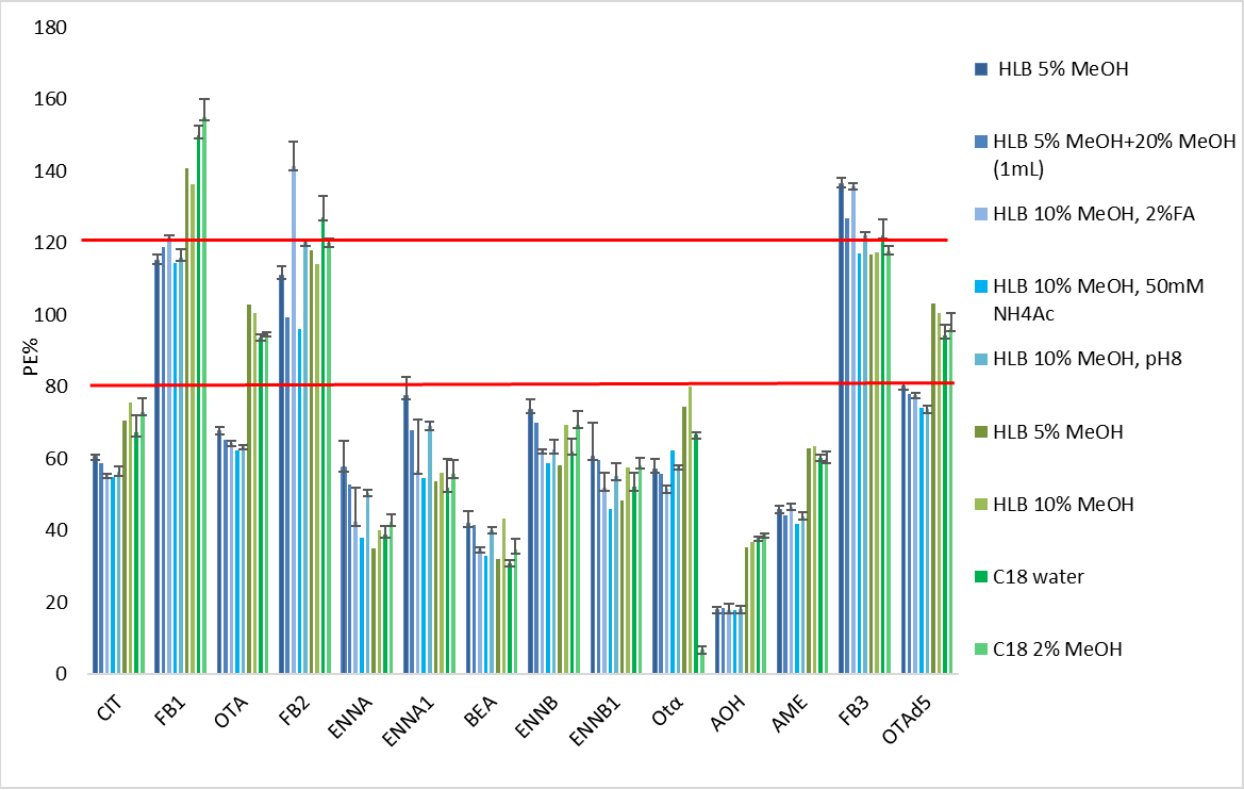


Figure 3.15. Influence of washing solvent volume and composition on mycotoxin PE% using C18 and HLB SPE. Blue color indicates loading of 2 mL spiked pooled urine (137 mg/dL of creatinine) at 10 ng/mL and use of 3 mL as the washing solvent with different pHs and percentage of MeOH (n=2). Green color represents

loading of 1 mL spiked urine at 10 ng/mL with 6 mL of washing solvent with different percentage of MeOH in HLB SPE and water and 2% MeOH in C18 SPE (n=2). The error bars represent standard deviation.

3.2.5. Comparison of HLB, C18, MCX and MAX SPE

3.2.5.1. Recovery

As mentioned in previous section, process efficiency included both recovery and matrix effects, and each should be studied individually. The recovery result is important because it shows if mycotoxins can be retained by the SPE cartridge and extracted completely without loss. Figure 3.16 demonstrates that ENN recoveries were less than 80% (33-77%) using all the sorbents. Therefore, this experiment confirms that recovery is the reason behind such poor process efficiency shown in Figure 3.15. Possible explanation for this result could be that ENNs were less soluble in urine, so had a potential risk of non-specific adsorption to the walls of propylene plastic tube before the loading step or during the SPE sample preparation.

Using 2% MeOH for washing in C18 cartridge led to the loss of OTα (the most polar analyte, see Figure 3.1, the RP LC separation on C18) during the washing step and only about 7% was recovered as is shown in Figure 3.16. Since the washing step in C18 used 6.0 mL as the total volume, eluted OTα was too diluted to be quantitated. In C18 SPE, water was the only washing solvent that could be used without resulting in loss of OTα from the sorbent during washing step. Similarly low recovery of 7% of OTA was detected by Pallarés *et al*. when Strata polymeric reversed-phase SPE was used.[66]

The low recoveries in the MCX SPE for all the mycotoxins showed that it needed more optimization in different steps. Mycotoxins were spiked in urine at pH 6.0, therefore acidic mycotoxins such as FBs, OTA, OTα and CIT were negatively charged at this pH since their pKa are 3.16, 3.17, 2.36 and 2.3, respectively. The ion exchange sites in MCX SPE are always negatively charged; thus, FBs could not be retained on the sorbent due to repulsion between carboxylate groups and anionic sites on the sorbent. In OTA, the NH group is positively charged and could interact with sulfonic part of the cartridge, and it could also be retained by the reversed-phase retention mechanism due to its LogP which was around 4.61. As shown in Figure 1.1, both CIT and OTα structures are similar, both have one carboxylic acid, and at pH 6.0 they were both negatively ionized and could not be retained on the MCX column. Hence, FBs, CIT

and OTα were detected in the washing step (Appendix, Supplementary Figure 15). One mL of extra MeOH was added after the elution step to make sure the elution volume was enough to elute all the analytes. The result in Appendix, Supplementary Figure 16 indicated that 1.5 mL was not enough to elute ENNs completely. AOH and AME were also detected in the extra mL MeOH. To elute using 1.5 mL volume, pH of elution solvent should be changed to basic pH to disrupt any ion exchange interactions. In addition, BEA was not detected in the washing or any of elution steps tested indicating very strong interaction with the sorbent.

In MAX SPE, the very strong sorbent with permanent positive charges retains acidic mycotoxins. OTA and FBs have amphoteric properties because of their carboxylic acid and amine groups. At pH 6.0, OTA (pKa 3.16) is negatively charged; therefore, it is predicted to be retained by the cartridge, and it could be recovered completely with acidic elution. FBs have pKa around 3.16, so their carboxylic acid groups were negatively charged. Moreover, they had a hydrophobic part that assisted them to interact with the reversed-phase part of the cartridge. CIT and OTα were not detected in the extra mL MeOH or the washing step. However, in this case the washing step was performed with 10 mL of 50 mM ammonium acetate which made it less possible to detect any analytes due to dilution. MeOH was used for eluting neutral and basic mycotoxins and 2% FA for acidic mycotoxins.

In conclusion, mixed-mode SPE required modifications of the loading pH, washing and elution steps and resulted in low recoveries of subset of analytes. Thus, HLB could give more promising results in terms of recovery for all the analytes of interest. However, the low recovery of ENNs and BEA requires further investigation. This will be discussed in Section 3.2.7.

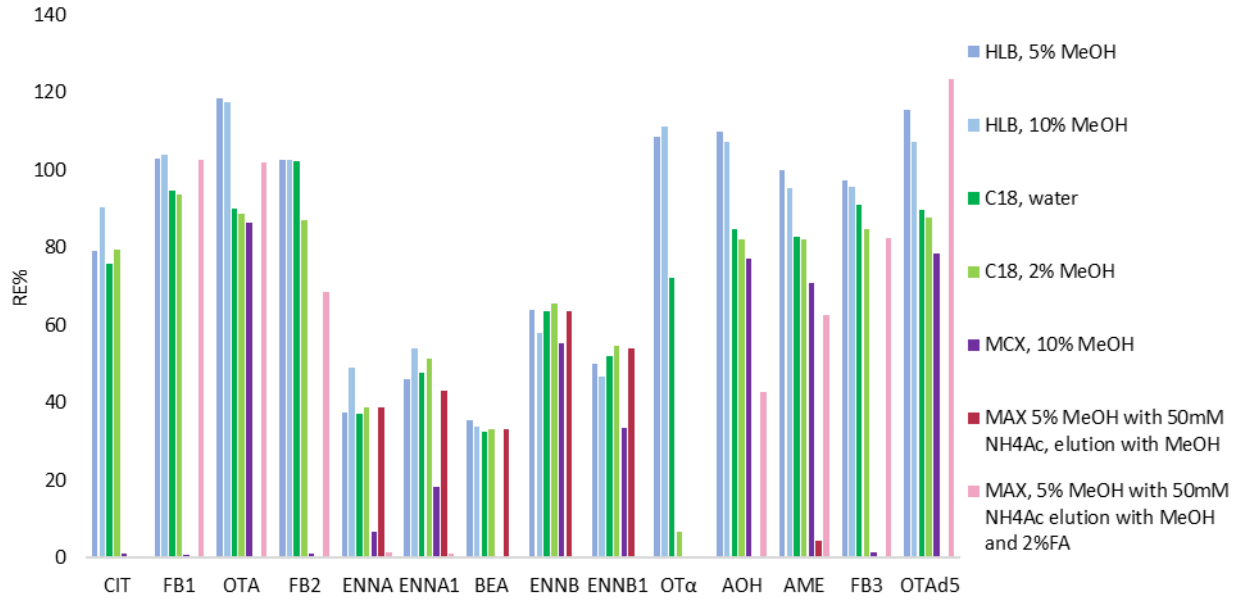


Figure 3.16 Comparison of recoveries of mycotoxins in spiked urine by HLB, C18, MCX and MAX SPE. Recovery was measured at final concentration of 10 ng/mL mycotoxins for HLB, C18 and MCX, and for MAX at 2 ng/mL. 1 mL pooled urine (137 mg/dL) at pH 6.0 was used for the loading step in HLB, C18 and MCX SPE. For the washing step, 6 mL of different solvents were tested for HLB and C18 SPE, as indicated in legend. Elution step was done with 1.5 mL of 100% MeOH. 2 mL pooled urine (137 mg/dL) at pH 6.0 was used for the loading step in MAX SPE. Washing was performed with 10 mL of 5% MeOH with 50 mM ammonium acetate. Samples were eluted with 1.5 mL of MeOH and 1.5 mL of MeOH containing 2% FA. All samples were then diluted to 60% MeOH with 1% FA before injection.

3.2.5.2. Matrix effects

As shown in Figure 3.16, recoveries of AOH and AME in HLB SPE were around (95-110%) which demonstrated that the low process efficiency that was previously mentioned in Section 3.2.4 came from the matrix effects.

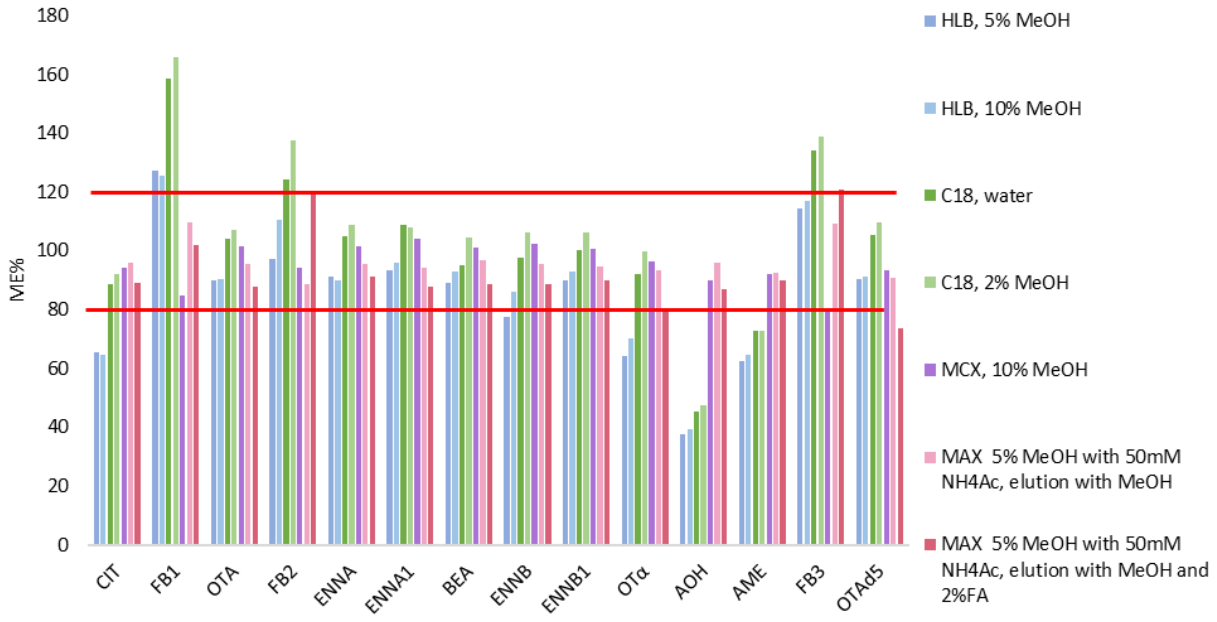


Figure 3.17 Comparison of matrix effects for HLB, C18, MCX and MAX SPE (n=1). Matrix effects was measured at final concentration of 10 ng/mL for HLB, C18 and MCX SPE, and 2 ng/mL for MAX SPE. 1 mL non-spiked pooled urine (137 mg/dL) at pH 6.0 was used for the loading step in HLB, C18 and MCX SPE. For the washing step, different solvents were tested for HLB and C18 SPE. Elution step was done with 1.5 mL of 100% MeOH. 2 mL non-spiked pooled urine (137 mg/dL) at pH 6.0 was used for the loading step in MAX SPE. Washing was performed with 10 mL of 5% MeOH with 50 mM ammonium acetate. Samples were eluted with 1.5 mL of MeOH and 1.5 mL of MeOH containing 2% FA. All samples were spiked at appropriate concentration with mycotoxins, and then diluted to 60% MeOH with 1% FA before injection.

Figure 3.17 demonstrates that in the HLB SPE there was a significant ionization suppression detected for CIT, OTα, AOH and AME using both 5 and 10% MeOH as the washing solvent. In C18 SPE, ionization suppression could be seen only for AOH. In the MCX and MAX SPE, an acceptable matrix effects were detected for all the mycotoxins. Liu *et al.* also detected a severe ionization suppression for OTα and AOH in urine samples using HLB SPE with 5-fold enrichment.[89] On the other hand, they detected matrix effects of 120% for AME.[89] Qiao *et al.* detected ionization suppression for AOH and AME in urine samples using liquid-liquid extraction. [85] Zhang *et al.* also detected a severe ionization suppression of OTα in urine samples by using protein precipitation.[124] Solfrizzo *et al.* detected significant ion enhancements for FB1

and a severe ionization suppression for OTA in urine samples using HLB SPE followed by immunoaffinity column sample preparation.[94]

To check if any interferences possibly causing ion suppression (such as plasticizer) came from the HLB SPE, 1 mL water instead of urine was loaded on HLB SPE, and after following the extraction protocol, it was spiked with mycotoxins at 10 ng/mL. The matrix effects were measured by comparing the area of extracted blank SPE spiked at 10 ng/mL of mycotoxins standard to 10 ng/mL mycotoxin standard in neat solvent. The results in Figure 3.18 showed acceptable matrix effects for all the mycotoxins in the blank HLB SPE. Thus, interferences causing ionization suppression of CIT, OTα, AOH and AME did not originate from HLB SPE, and the detected matrix effects in HLB SPE must arise from urine.

Figure 3.18 Evaluation of matrix effects arising from blank HLB SPE extraction. 1 mL water was loaded. The washing was performed with 6 mL of 10% MeOH and elution was done with 1.5 mL of 100% MeOH. Mycotoxins were spiked at 10 ng/mL. Sample was diluted to 60% MeOH with1% FA before injection.

3.2.6. Effect of creatinine on matrix effects using HLB SPE

To study the matrix effects in urine better, urine with different creatinine levels were tested similar to the relative matrix effects experiment performed with for dilute-and-shoot method and discussion in Section 2.4.2. The results in Figure 3.19 indicated that the matrix effects using different lots of pooled urine led to ionization suppression or enhancement for some of the compounds. Mycotoxins such as CIT, OTα, AOH and AME that had ionization suppression

using pooled urine with 1.37 mg did not show ionization suppression in different lots of urine with 1.17 and 1.03 mg. These results support the need to perform creatinine normalization before the loading step and LC-MS analysis. To minimize the risk of ionization suppression for CIT and OTα, 1.20 mg of creatinine should be used for the loading step.

Since AOH is susceptible to ionization suppression in pooled urine samples with different creatinine levels, $AMEd_3$ was used as an internal standard. $AMEd_3$ elutes at the same retention time as AME, and as the Figure 3.19 demonstrates at 1.17 and 1.03 mg of creatinine, the matrix effect is similar to AME as expected since isotope dilution internal standard is the gold standard method for quantitation by LC-MS. Although AOH elutes at a different retention time, its suppression follows the same trend as for AME, so $AMEd_3$ standard can be used to compensate for relative matrix effect for the structurally-related analyte as well.

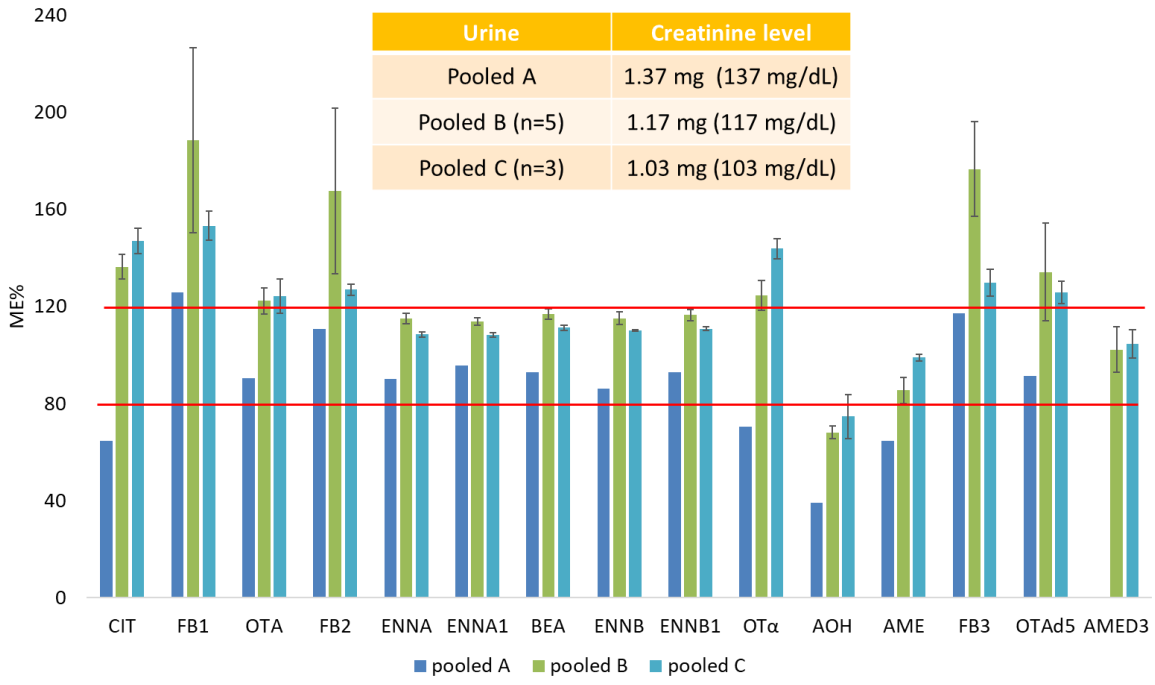


Figure 3.19 Effect of urine composition on the matrix effects in HLB SPE. The matrix effects in pooled urine A (n=1), B (n=5) and C (n=3) were measured at 10 ng/mL of mycotoxins. The washing step was performed with 6 mL of 10% MeOH and samples were eluted with 1.5 mL of 100% MeOH. Samples were diluted to 60% MeOH before injection. $AMEd_3$ was not added in pooled A urine. The error bars represent standard deviation

3.2.7. Optimization of evaporation-reconstitution step in SPE

Next, to increase enrichment and get to lower LOQs evaporation step was added to the SPE protocol. By adding evaporation step, both recovery and matrix effects were re-evaluated. Recovery should not change unless there are losses during the evaporation/ reconstitution step. In contrast, enrichment may increase matrix effects due to increase in extract concentration.

To check if evaporation of the mycotoxins could impact their recoveries, standard samples at 10 ng/mL were evaporated with and without 6 µL of glycerol. The results in Figure 3.20 showed a good recovery for all the mycotoxins with and without glycerol. High recovery (>100%) in CIT with 6 µL glycerol was due to the change in response from QTOF since in the blank solvent there were no traces of CIT found.

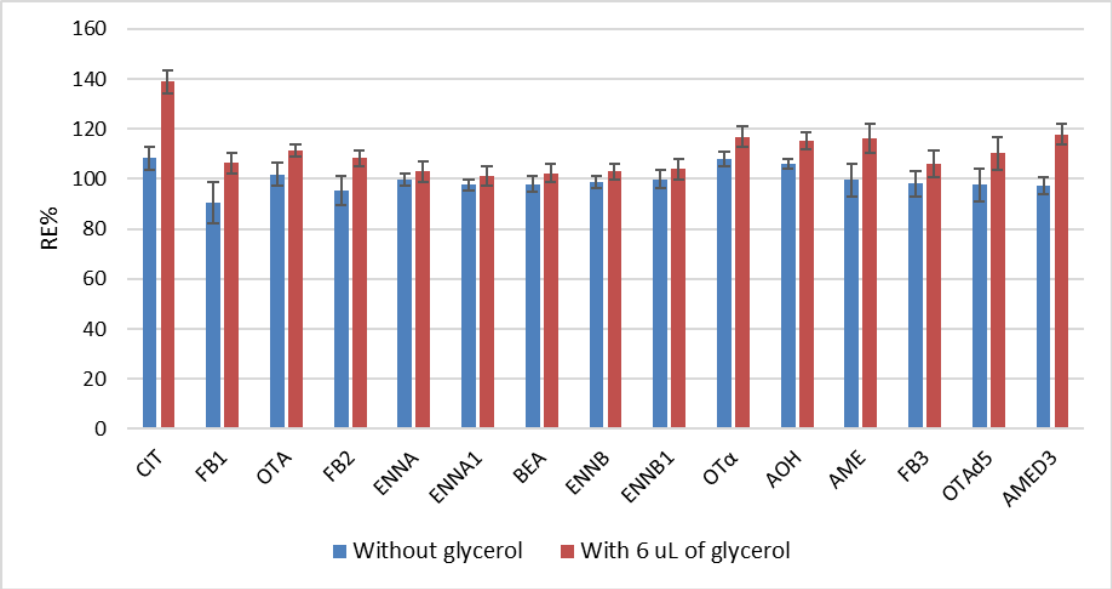


Figure 3.20 Recovery of 10 ng/mL standards evaporation with and without 6 µl glycerol (n=5). Samples were reconstituted in 400 µL of 60% MeOH with 1% FA. The error bars show standard deviation.

The reconstitution volume was one of the first parameters that was tested as described in Section 2.5.1.6. The two tested reconstitution volumes were 50 and 100 µL which gave an enrichment of 20 and 10x, respectively. The results in Table 3.4 show significant retention time shifting with 20x enrichment in urine with higher creatinine level (137 mg/dL). Some of the mycotoxins such as CIT and $OTAd_5$ were not detected with 20 and 10x enrichment. Since CIT and $OTAd_5$ eluted

close to the timing of polarity switching, it is believed this result may be due either to severe suppression which completely suppressed the signal of these analytes or significant retention time shifts which resulted in the analytes eluting during wrong ESI polarity mode. To examine the latter possibility, the same samples were run in negative mode without polarity switching. The same analytes were still not detected, or their intensities were too low to be quantitated. This result suggests severe suppression. Thus, to confirm this finding, samples with 20 and 10x enrichment were diluted 10x. The analytes were clearly observed for all the mycotoxins after 10x dilution in sample with 20 and 10x enrichment. Example of EICs of this result are shown Supplementary Figure 17. In summary, 20 and 10x enrichment in pooled urine sample with creatinine level of 137 mg/dL showed severe ionization suppression for CIT, OTα, AOH and AME. Thus, enriching the mycotoxins of interest, also resulted in similar enrichment of interferences from urine in this step.

Furthermore, the enrichment also affected the retention time of early eluting mycotoxins such as CIT and OTα. As the urine creatinine decreased to 103 mg/dL, the effect of retention time shifting improved for most of the mycotoxins, but was still observed for OTA, FB2, OTα and AME. Based on excessive retention time shifts with 20x enrichment, the rest of optimization was performed with 10x enrichment.

Table 3.4. Retention time of mycotoxins using evaporation-reconstitution step with different enrichment factor and creatinine concentrations.

Creatinine level	137 mg/dL		103 mg/dL	Standard in solvent
Dilution factor	20x/RT (min) (n=1)	10x/RT (min) (n=1)	10x/RT (min), (n=3)	
CIT	ND	ND	6.08	6.85
FB1	8.05	8.14	8.18	7.95
OTA	9.74	9.81	9.64	9.80
$OTAd_5$	ND	ND	ND	9.74
FB2	11.59	11.49	11.33	11.16
ENNA	14.72	14.73	14.64	14.65
ENNA1	14.39	14.38	14.30	14.31
BEA	13.95	13.95	13.88	13.89
ENNB	13.73	13.73	13.66	13.67
ENNB1	14.05	14.05	13.98	13.98
OTα	4.71	4.82	5.02	5.40
AOH	7.58	7.58	7.53	7.46
AME	10.58	10.56	10.39	10.32
FB3	10.18	10.00	9.84	9.61

As it was mentioned before in Section 3.2.5.1, ENNs and BEA had low recoveries in HLB SPE with direct injection, the same effect was also detected when the evaporation step was added. Therefore, to troubleshoot where exactly ENNs and BEA were lost, 5 replicates of post and pre-extraction urine samples were prepared. Pre-extraction replicates were spiked at 25 ng/mL, and their flow through were loaded two times on the same HLB SPE, to examine if too short contact time between analyte and sorbent may have caused the observed losses. In the end, these samples were diluted to 10 ng/mL without evaporation step. The flow-through after the second loading and the solvent from washing step were collected to check if anything was lost during these two steps. The results did not show any losses during the loading and washing steps. However, as shown in Figure 3.21, the recovery of ENNs increased from a range of 34-58% to 41-75%. By reloading initial sample flow-through, ENNs and BEA have more chance to interact with sorbent and be retained by it. The rest of mycotoxins have acceptable recovery (≥80%).

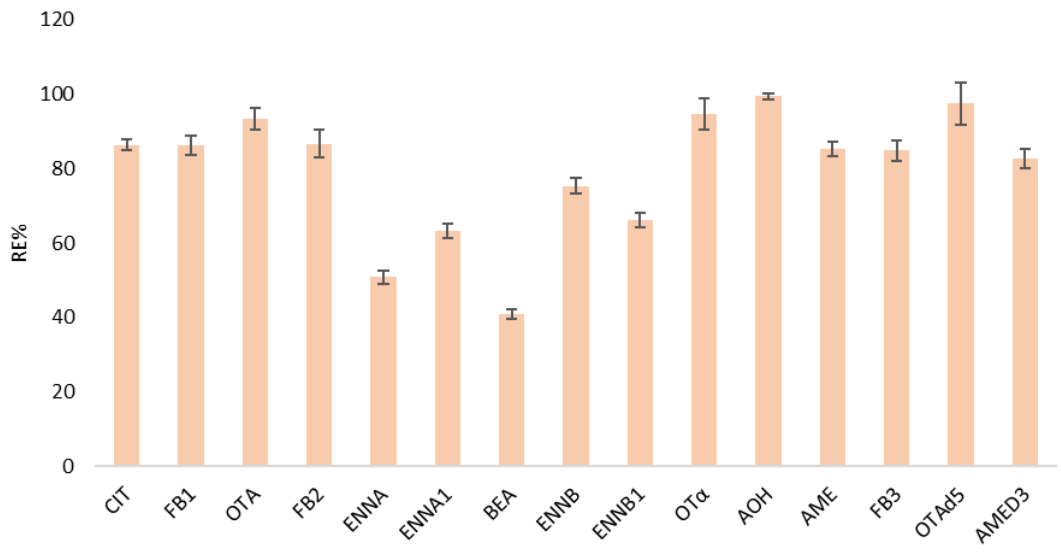


Figure 3.21 Effect of increasing contact time during sample loading on recovery of mycotoxins from spiked urine in HLB SPE (n=5). Recovery was measured at 10 ng/mL (n=5). 1 mL spiked urine (117 mg/dL) at 25 ng/mL was loaded on the cartridge. The collected flow-through was re-loaded again. The washing was performed with 6 mL of 10% MeOH, and the elution step was done with 1.5 mL of MeOH. Samples were diluted to 60% MeOH with 1% FA before injection. The error bars represent standard deviation

Another reason for the low recovery of ENNs and BEA as explained in Section 3.2.1.4 and 3.2.2.1 was the possible adsorption of these compounds to the plastic tubes. Since these samples were spiked inside the plastic tube, there was a high chance they adsorbed to the walls before loading. Therefore, to evaluate the effect of these parameters, samples were spiked in glass vials instead of polypropylene plastic tubes.

Using 10x enrichment and incorporation of two times loading, and spiking samples in glass vials recovery and matrix effects were evaluated. As shown in Figure 3.22 recoveries of ENNs and BEA increased by using glass vials and two times loading from 41-75% to 68-81%. Using glass vial minimized the chances of adsorption of these compounds during spiking. CIT was not detected this experiment. AME, $AMEd_3$ and $OTAd_5$ areas in pre-spike samples could not be quantitated due to low intensity. Similar results for ENNB (90%), ENNB1(84.1%), ENNA (71.6%), ENNA1 (77.5%), BEA (85.5%) were detected by Liu *et al.* with HLB SPE after 5x enrichment.

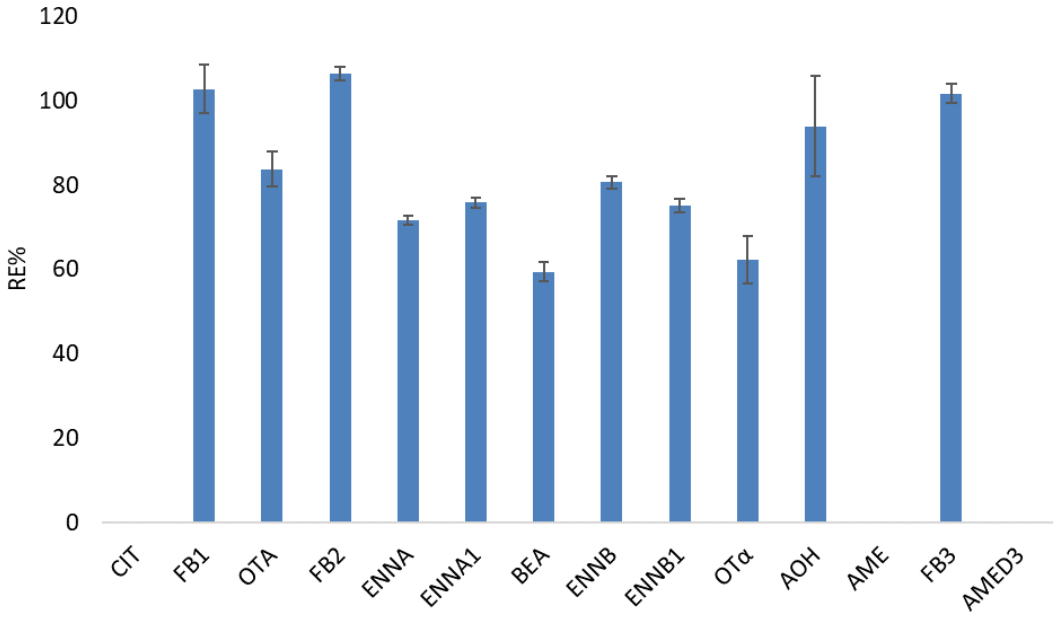


Figure 3.22 Recovery of mycotoxins from spiked urine (5 ng/mL) using HLB SPE with 10x enrichment (n=3). Spiked pooled urine sample (103 mg/dL) was loaded at 0.5 ng/mL of mycotoxins. The washing step was performed with 6 mL of 10% MeOH. Samples were eluted with 1.5 mL of MeOH, and then evaporated completely and reconstituted in solvent containing 60% MeOH with 1% FA. The error bars represent standard deviation

10x enrichment showed a huge ionization suppression for half of the mycotoxins using the HLB SPE as depicted in Figure 3.23. this explains low intensity of AME, $AMEd_3$ and $OTAd_5$ Integration of areas in $AMEd_3$ was not reproducible due to low intensity, therefore it was not included in the results. CIT was not detected in post-spike samples. Liu *et al.* also detected significant ionization suppression using HLB SPE with 5x enrichment for AOH (16.4%), OTα (12.2%), OTA (49.1%), FB1 (77.3%), FB2 (69.3%) and ion enhancement for AME (120%).[89].

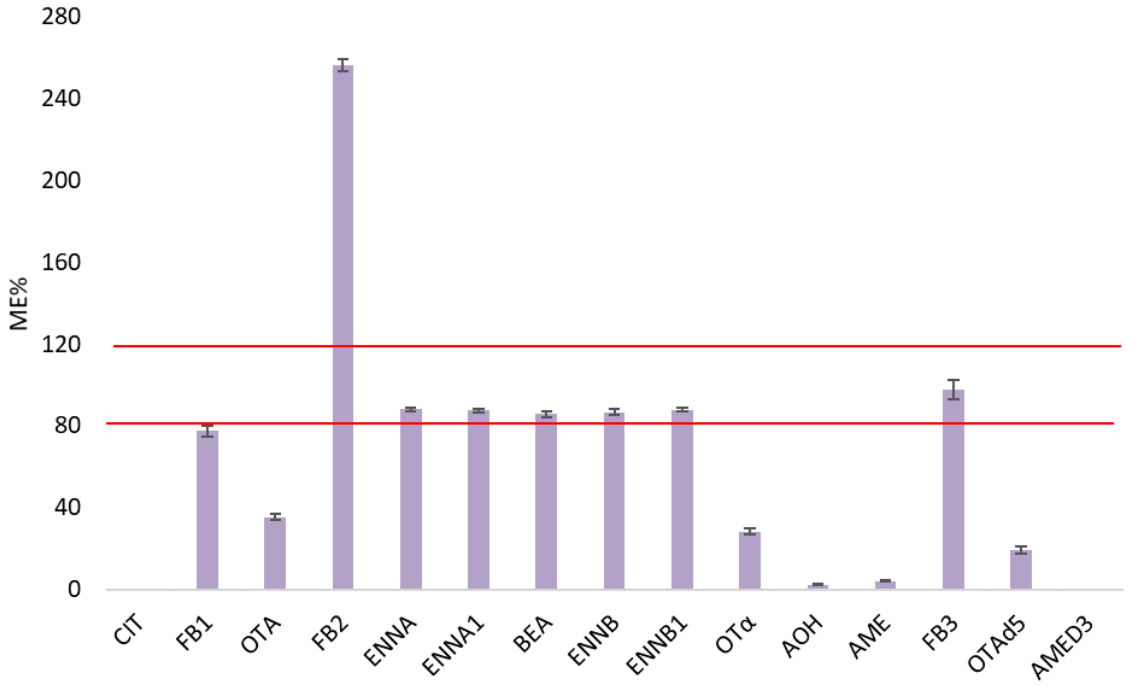


Figure 3.23 Matrix effects of mycotoxins using HLB SPE at 5 ng/mL in urine with 10x enrichment (n=3). 1 mL pooled urine sample (103 mg/dL) was loaded on HLB SPE cartridge. The washing step was performed with 6 mL of 10% MeOH. Samples were eluted with 1.5 mL of MeOH, and then evaporated completely. Samples were reconstituted in solvent containing 5 ng/mL of mycotoxins in 60% MeOH with 1% FA. The error bars represent standard deviation

Due to significant ionization suppression of CIT, OTA, OTα, AOH and AME, a modification of the procedure was required to minimize the matrix effects. As previously discussed in Section 3.2.6, urine normalization using creatinine was optimized in a way to improve the matrix effects for CIT, OTα, AOH and AME. Further optimization of washing step could not be done because by changing the strength of the washing solvent, some polar mycotoxins could be eluted. Increasing the volume of solvent in the washing step would not be effective since 6 mL of 10% MeOH is high enough to wash interferences, still ionization suppression remained. Therefore, a modification of the evaporation step was performed to minimize the matrix effects with less enrichment as described in Section 2.5.1.7. The goal of this was to allow adjustment of injection solvent strength to 60% MeOH without further dilution of samples. Instead of full evaporation, samples were evaporated to reach less than 0.5 mL.

Elimination of enrichment step improved the matrix effects for most of the mycotoxins as demonstrated in Figure 3.24. The results showed that full evaporation enriched the interferences coming from urine which led to high ionization suppression for compounds CIT, OTA, OTα, AOH and AME. Ionization suppression for AOH and AME improved tremendously compared to full evaporation from 2% to 22% for AOH and from 4% to 66% for AME. FBs show ion enhancement as discussed previously in Section 3.2.5.2. The ion enhancement was also detected for OTA and $OTAd_5$

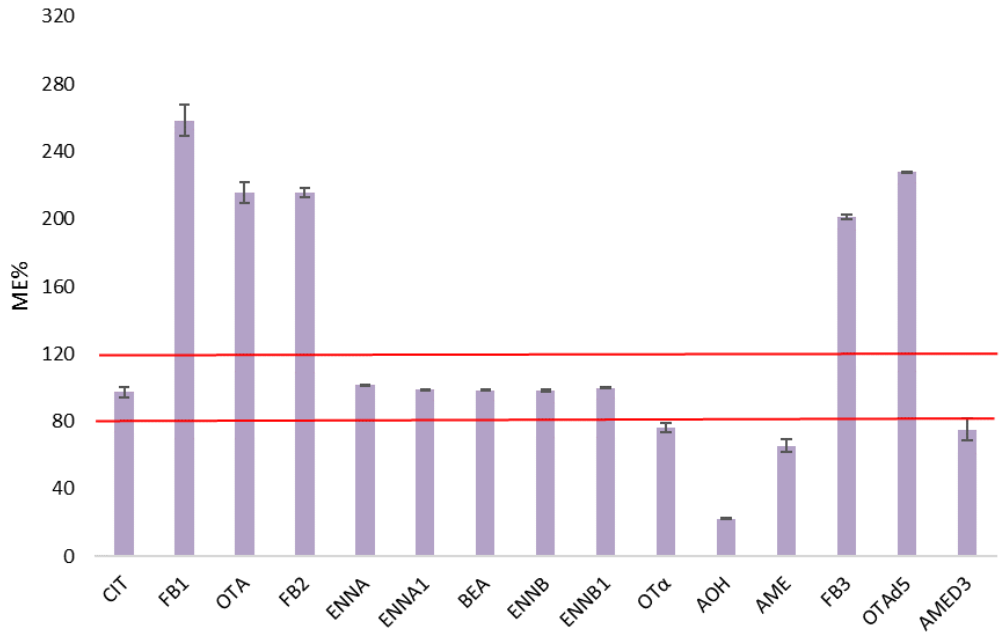


Figure 3.24 Matrix effects in urine spiked at 5 ng/mL of mycotoxins after HLB SPE with evaporation and no enrichment (n=2). 1 mL pooled urine sample (190 mg/dL) was used for the loading step. The washing was performed with 6 mL of 10% MeOH and the elution was done with 1.5 mL of MeOH. Samples were partially evaporated and were spiked at 5 ng/mL of mycotoxins. Then, samples were adjusted to final volume of 1 mL of 60% MeOH with 1% FA before injection. The error bars represent standard deviation

After modification of the evaporation step, the recovery with the same procedure was re-evaluated. Figure 3.25 showed good recovery ranging from 72 to 88% for all the mycotoxins except CIT and OTα which are the most polar mycotoxins in the panel. (see Table 1.2 and Figure 3.1 RP LC chromatogram)

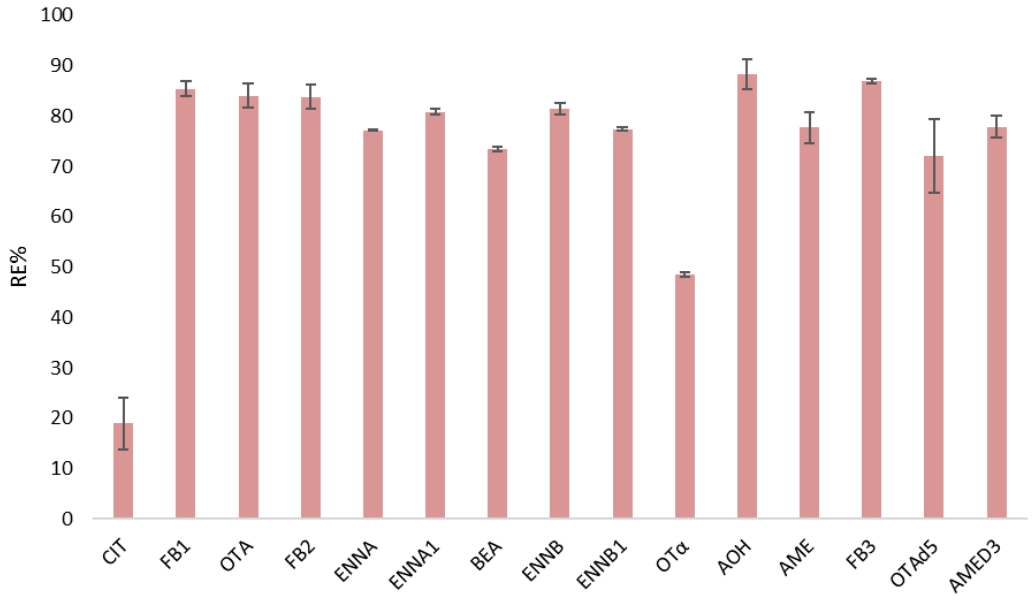


Figure 3.25 Recovery in urine spiked at 5 ng/mL of mycotoxins after HLB SPE with evaporation and no enrichment (n=2). 1 mL spiked pooled urine sample (190 mg/dL) at 5 ng/mL was used for the loading step. The washing performed with 6 mL of 10% MeOH and the elution was done with 1.5 mL of MeOH. Samples were partially evaporated and adjusted to final volume of 1 mL of 60% MeOH with 1% FA before injection The error bars represent standard deviation

A possible reason for unexpected low recoveries of CIT and OTα (in comparison to the results shown in Figure 3.16 and Figure 3.21) is the use of different lots of HLB SPE. A second possible source of low recovery was the change to a pooled urine sample to a more concentrated urine (190 mg/dL creatinine). Therefore, the effect of creatinine was also examined during the troubleshooting. To investigate the losses of CIT and OTα, the same experiment was performed with old and new SPE batches with normalizing creatinine to 1.2 mg (by loading 0.63 mL of urine with 190 mg/dL of creatinine), and another test was done with 1.9 mg (by taking 1.0 mL of urine with 190 mg/dL of creatinine) in new SPE batches for comparing the recovery and matrix effects. The results in Figure 3.26 (A) demonstrated that low recoveries detected in CIT (40%) and OTα (61%) were due to using new SPE lot. CIT was not detected in new lot of SPE with 1.2 mg creatinine. The old SPE lot showed recoveries of 71 and 88% for CIT and OTα, respectively which is consistent with the previous results.

This finalized method was applied to non-spiked urine. After the sample extraction with the procedure described in Section 2.5.1.7, ENNA and ENNA1 were detected at concentrations close to 0.01 ng/mL, as presented in Appendix, Supplementary Figure 18. This result showed that our method can be potentially applied for the detection of mycotoxins in urine.

Results in Figure 3.26 (B) show that AOH and AME were more susceptible to matrix effects when creatinine levels varied. Changing the creatinine from 1.9 mg to 1.2 mg improved in matrix effects from 21 to 40% for AOH and 57 to 65% for AME, respectively. Ionization suppression was also detected for OTα using the new lot of SPE about 70%, and for CIT (69%) using old SPE lot as shown in Figure 3.26(B).

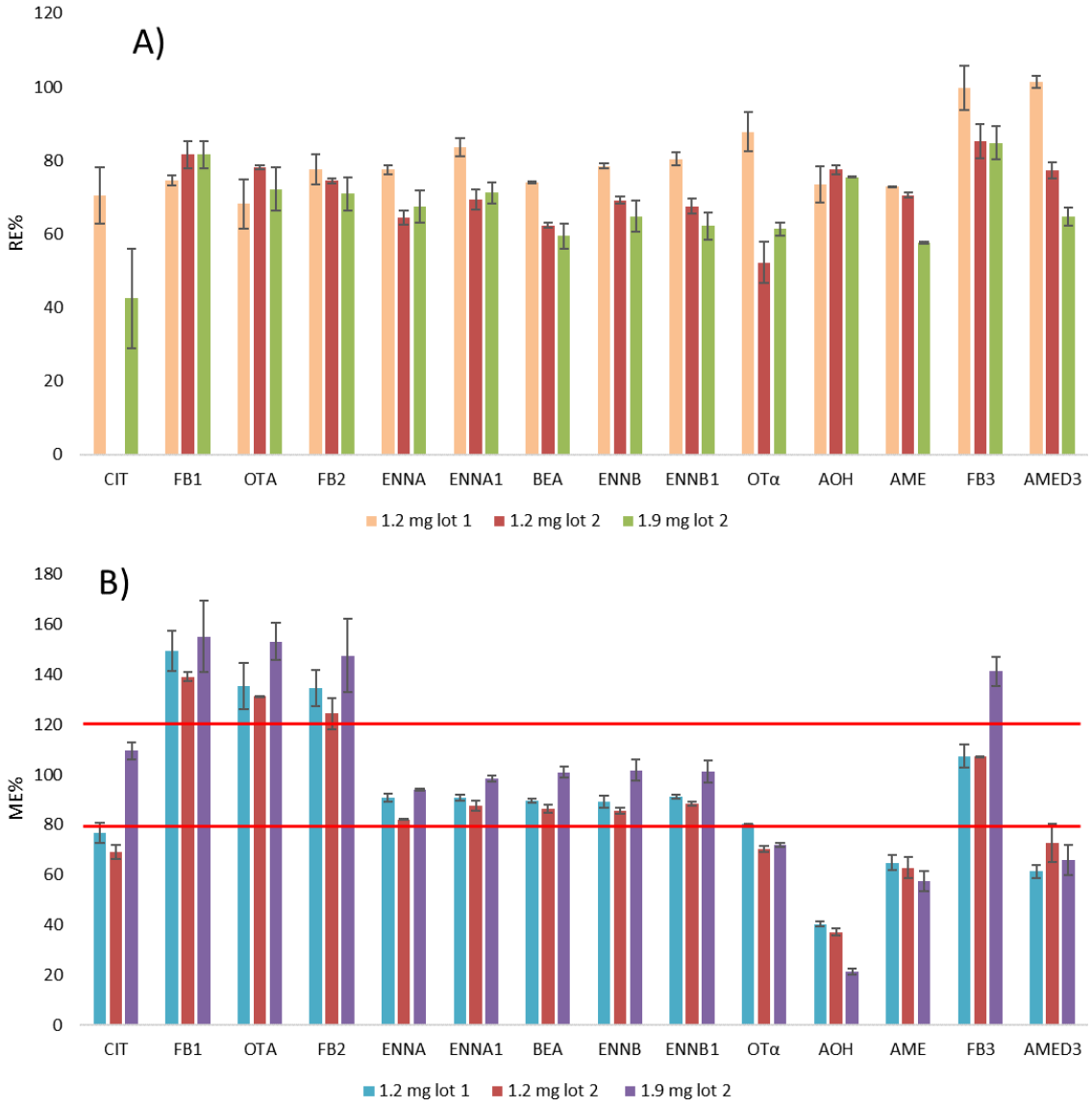


Figure 3.26 Recovery (A) and matrix effects (B) of mycotoxins in spiked urine using new and old HLB SPE lot. 0.63 mL of spiked urine sample (190 mg/dL) at 5 ng/mL of mycotoxins was loaded on two lots of HLB SPE (n=2). 1 mL spiked urine sample at 5 ng/mL (190 mg/dL) was also loaded on lot 2 of HLB SPE (n=2). The washing step was performed with 6 mL of 10% MeOH and the elution step was done with 1.5 mL of MeOH. Samples were partially evaporated and adjusted to final volume of 1 mL of 60% MeOH with 1% FA before injection. The error bars represent standard deviation

3.2.8. HLB SPE finalized method

Urine samples are usually collected in sterile polypropylene containers. As it was shown in Figure 3.13, ENNs and BEA were prone to non-specific adsorption. Therefore, to check if the step to recover ENNs and BEA from plastic containers by rinsing the urine plastic container with 100 µL of MeOH could be added to HLB SPE procedure for future studies, the effect of MeOH during loading was examined. Implementing 10% MeOH in the loading step led to a decrease in recovery of FB2, ENNA, ENNA1, BEA and $OTAd_5$ as demonstrated in Figure 3.27 (A). A two-tailed t-test was performed to determine if the differences in the recovery with and without 10% MeOH in the loading step were significant. The t-test confirmed significant differences in the recovery for FB2, ENNA, ENNA1, BEA and $OTAd_5$. The reason significant difference was detected in $OTAd_5$ not OTA is that $OTAd_5$ had less points across its peak area. The p-values for each mycotoxin are provided in the Table 3.5 below. Low recovery of CIT is due to the usage of new lot of SPE cartridges, since previously used an old lot was finished.

Table 3.5. The p-values of each mycotoxins using t-test.

Mycotoxin	P-value (two-tailed)	Statistic significantly different
FB1	0.180	No
OTA	0.273	No
FB2	0.021	Yes
ENNA	0.007	Yes
ENNA1	0.023	Yes
BEA	0.011	Yes
ENNB	0.059	No
ENNB1	0.104	No
OTα	0.625	No
AOH	0.414	No
AME	0.535	No
FB3	0.688	No
$OTAd_5$	0.036	Yes
$AMEd_3$	0.611	No

As expected, similar matrix effects were observed for the mycotoxins in presence of MeOH during the loading step. The ionization suppression in AOH (22%) and AME (66%) is due to

level of creatinine (Figure 3.27(B)). Significant ion enhancement was detected for FB1 (258%), OTA (215%), FB2 (216%), FB3 (201%) and $OTAd_5$ (228%) for both samples. FBs have consistently shown ion enhancement in urine throughout this project.

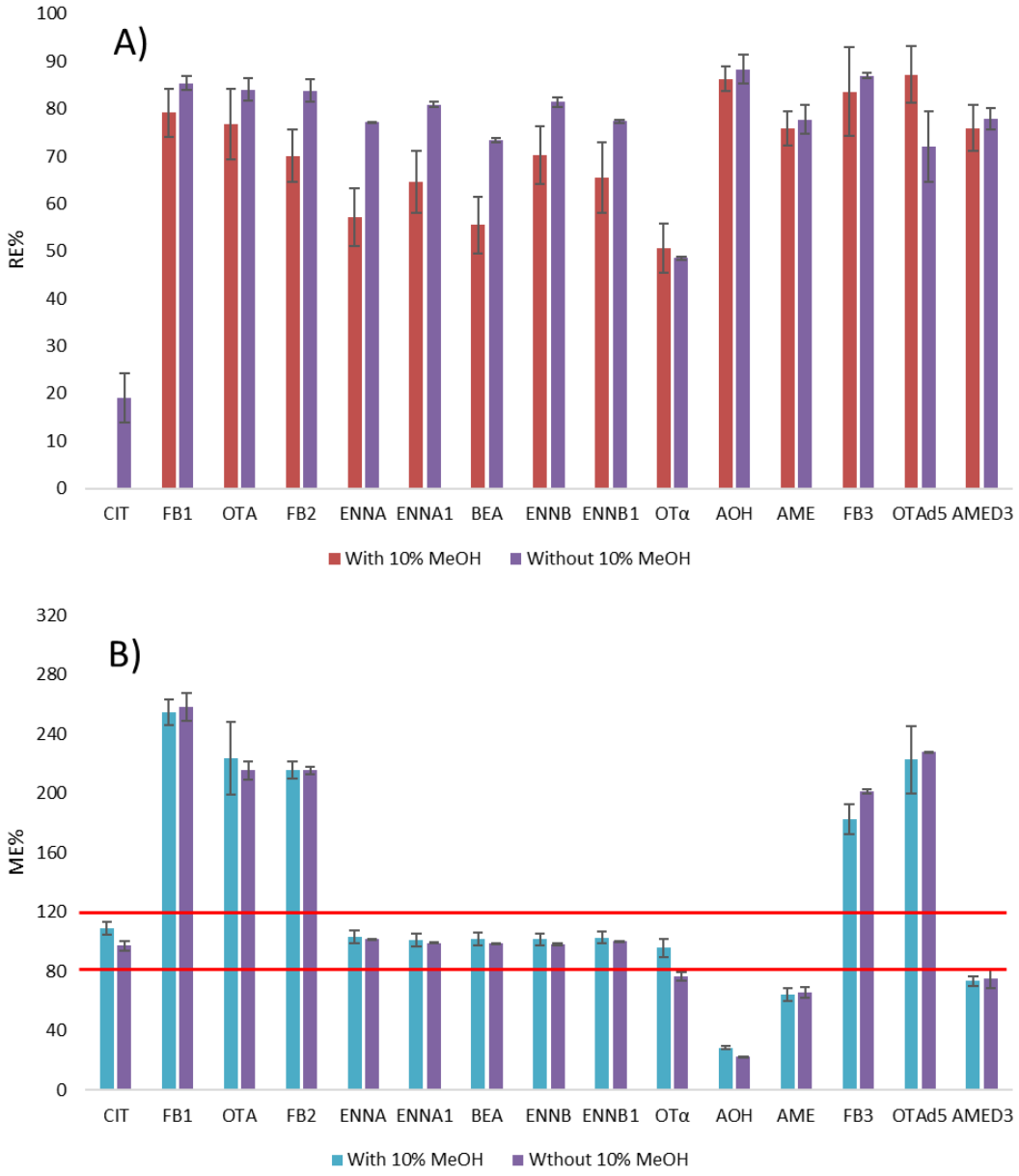


Figure 3.27 Effect of 10% MeOH during sample loading on recovery (A), and matrix effects (B) of mycotoxins using HLB SPE. 1 mL of spiked urine sample (190 mg/dL) at 5 ng/mL was used with 10% MeOH (n=5), and without 10% MeOH (n=2) in the loading step. The washing was performed with 6 mL of 10% MeOH and the elution was done with 1.5 mL of MeOH. Samples were partially evaporated and adjusted to final volume of 1 mL of 60% MeOH with 1% FA before injection. The error bars represent standard deviation

To make sure that rinsing urine containers with MeOH before extraction with HLB SPE could be incorporated in SPE procedure without affecting the recoveries and precision, 5% MeOH in the loading step was tested. The results shown in Figure 3.28 confirmed acceptable recovery with and without 5% MeOH except for AOH and AME. shows that urine containing 5% MeOH would not result in loss of mycotoxins during the HLB SPE loading step. The two-tailed t-test result in Table 3.6 showed a significant difference between with and without using 5% MeOH in the loading step for AOH and AME. The CIT was not detected using new lot of SPE. OTα low recoveries with (53%) and without 5% (52%) MeOH were due to low retention of polar compounds on new lot of HLB SPE as previously confirmed in Figure 3.27.

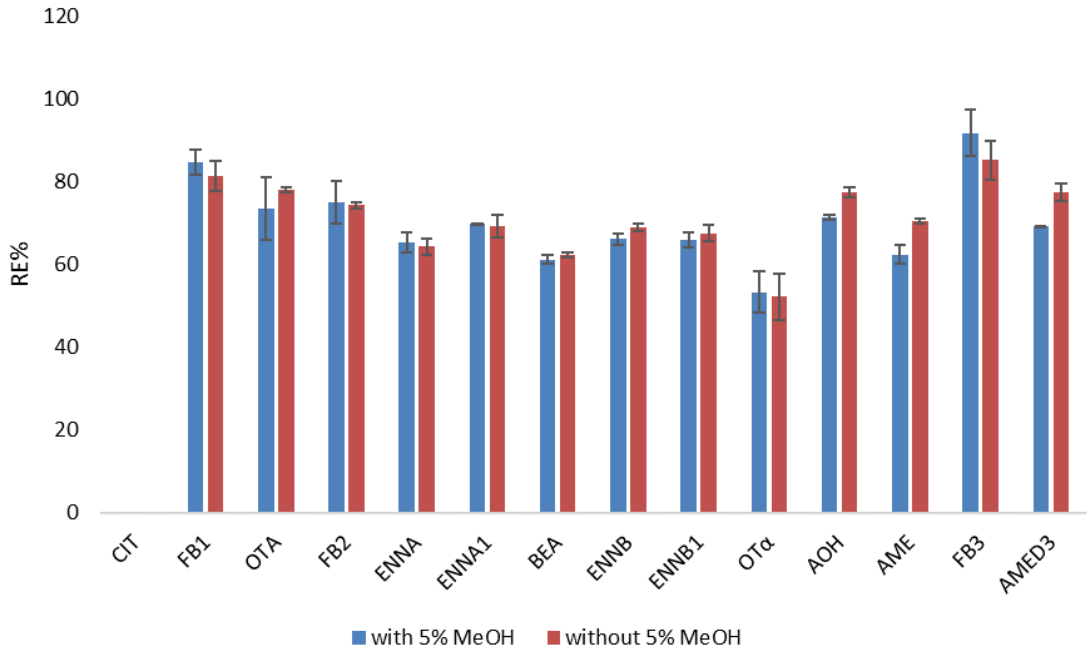


Figure 3.28 Effect of 5% MeOH during sample loading on recovery of mycotoxins using HLB SPE. 0.63 mL of spiked urine sample (190 mg/dL) at 5 ng/mL of mycotoxins was loaded on new lot of HLB SPE with and without 5% MeOH (n=2). The washing was performed with 6 mL of 10% MeOH and elution with 1.5 mL of MeOH. Samples were partially evaporated and adjusted to final volume of 1 mL of 60% MeOH with 1% FA before injection. OTAd_5 result was removed due to its low peak intensity. CIT was also not detected. The error bars represent standard deviation

Table 3.6. The p-values of each mycotoxins using 5% MeOH in the loading step using t-test.

Mycotoxin	P-value (two-tailed)	Statistic significantly different
FB1	0.461	No
OTA	0.630	No
FB2	0.813	No
ENNA	0.697	No
ENNA1	0.654	No
BEA	0.376	No
ENNB	0.123	No
ENNB1	0.394	No
OTα	0.867	No
AOH	0.020	Yes
AME	0.046	Yes
FB3	0.305	No
$AMEd_3$	0.036	Yes

Matrix effects results provided in Figure 3.29, showed acceptable matrix effects for all the mycotoxins except AOH (44%) and AME (69%) using 5% MeOH. Significant ion enhancement was detected for FB1(151%), and OTA (143%) when 5% MeOH was used during the loading step. Similar ion enhancement was detected for FB1 (139%), OTA (131%), FB2 (124%) without using 5% MeOH in the loading step. However, ionization suppression was detected for CIT (69%), OTα (70%), AOH (37%) and AME (53%) in samples without 5% MeOH in the loading step. Similar effect was detected for ISTDs (FB3, $AMEd_3$) which confirms that they can be used to compensate for matrix effects.

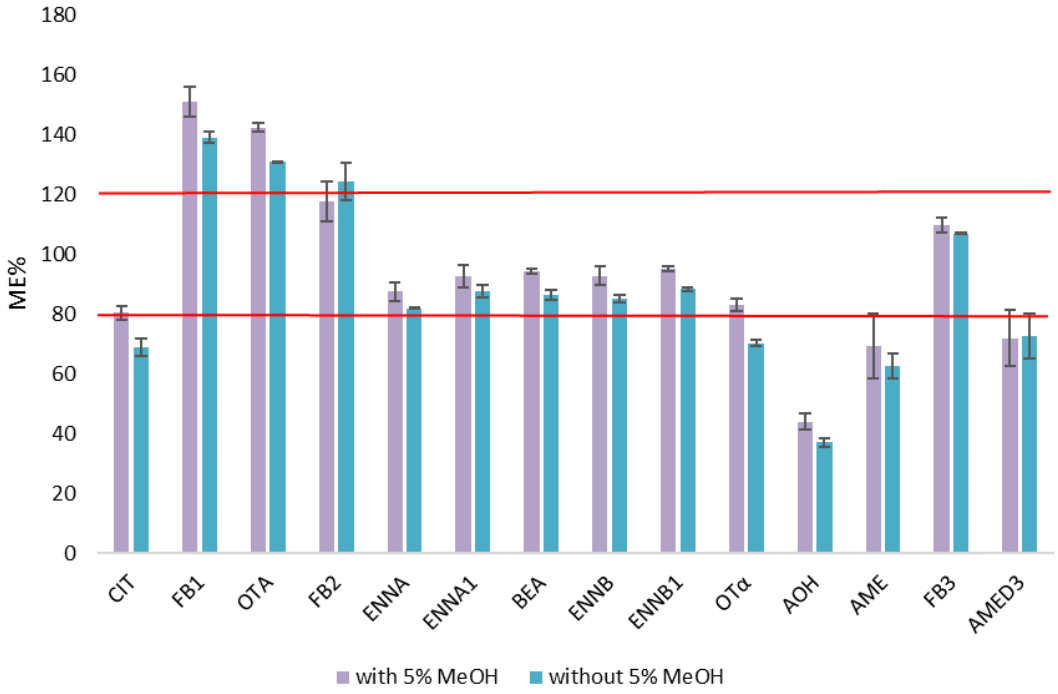


Figure 3.29 Matrix effects of mycotoxins in urine with and without using 5% MeOH during sample loading on HLB SPE. 0.63 mL of urine sample (190 mg/dL) was loaded on new lot of HLB SPE with and without 5% MeOH (n=2). The washing was performed with 6 mL of 10% MeOH and elution was done with 1.5 mL of MeOH. Samples were partially evaporated and adjusted to final volume of 1 mL of 60% MeOH with 1% FA before injection. $OTAd_5$ result was removed due to its low peak intensity. The error bars represent standard deviation

To sum up, 10x enrichment led to significant ionization suppression. Thus, enrichment was removed from the SPE protocol to evaluate the matrix effects with. AOH and AME were prone to matrix effects even with urine normalization to 1.2 mg of creatinine. Using 5% and 10% MeOH in the loading step did not affect the matrix effects, as expected.

Using 10% MeOH during the HLB SPE loading led to the decrease in recoveries of ENNA, ENNA1, BEA, FB2 and $OTAd_5$. In contrast, the use of 5% MeOH led to good recoveries for all the mycotoxins. Therefore, adding an extra rinsing step after the adjustment to 5% MeOH to the SPE HLB method (as described in Section 3.2.2.1) is feasible for future studies.

Liu *et al.* detected good LOQs in urine samples by using HLB SPE with 5x enrichment for 11 out of the 12 mycotoxins we covered. However, creatinine normalization was not performed in

their study, and they observed severe ionization suppression for OTA, FB1, FB2, AOH and OTα. For the last two (AOH and OTα) the ME was less than 16%, indicating severe ionization suppression with adverse impact on LOQ. We also observed ion suppression for AOH and AME, but the matrix effects were 40% and 65%, respectively with our method. In addition, developing HRMS method is advantageous to do retrospective studies.

Chapter 4. Conclusions and future work

The climate change around the world promotes the growth of fungi during food production and storage. Sensitive and accurate measurement methods are required to tackle this issue and to have an estimate of the level of mycotoxins in human biological samples such as urine. In this research, a polarity switching HRMS method previously developed for plasma[75] was applied for the detection of 12 mycotoxins in urine samples with some modifications.

Urine samples were assessed with the dilute-and-shoot and the SPE preparation methods for the detection of the mycotoxins. Two types of diluents, one highly aqueous and other highly organic, were initially tested with the dilute-and-shoot preparation to assess for solubility and non-specific adsorption of mycotoxins. The test showed that ENNs and BEA can be adsorbed to the plastic Eppendorf tubes due to their low solubility in an aqueous solvent. Our findings could potentially explain why most studies do not find detectable levels of ENNs and BEA. LOQ levels resulting from the dilute-and-shoot preparation method were above 1.2 ng/mL for all the mycotoxins tested, proving the method unsuitable as minimal LOQs of less than 0.5 ng/mL are needed.

Therefore, the SPE method was tested to provide clean-up and enrichment. The SPE method with a HLB sorbent showed better recoveries for all the mycotoxins tested. Good recoveries were obtained by loading at pH 6.0, using 6 mL of 10% MeOH as the washing step and elution with 1.5 mL 100% MeOH. However, the 10x enrichment step was not added to the final method since severe ionization suppressions, was detected for CIT, OTA, OTα, AOH and AME. This suggests that the interfering species are also being retained and preconcentrated along with the analytes. Noticeably, the normalization of creatinine to 1.2 mg in HLB SPE can improve the ME for CIT, OTα and OTA. Less enrichment in the HLB SPE method showed improvement in the ME for all the mycotoxins except AOH (22%) and AME (66%); in this case an internal standard specifically $AMEd_3$, can compensate for the ME. The HLB SPE method proved to be promising with our optimizations as trace levels of ENNA and ENNA1 down to ~0.01 ng/mL were detected in non-spiked urine samples (refer to Supplementary Figure 18).

A tendency to adsorption of ENNs and BEA to the plastic containers was observed after two weeks of storage in the freezer. Rinsing the container with at least 0.1 mL of MeOH was

necessary to recover them from the propylene tubes. We propose that this additional rinsing step can be incorporated to the HLB SPE procedure for future experiments with real samples. The proposed methodology for the subsequent validation and experiments with clinical samples is shown in Figure 4.1. In further detail, samples from a single extraction using the HLB SPE method can be run in two separate subsets to reduce LOQs, one analyzed by triple quadrupole (QqQ) and the other by QTOF MS (refer to Figure 4.1).

All mycotoxins can also be run by QqQ, however, using the QTOF gives the advantage of doing retrospective studies in the future. Early eluting mycotoxins and those susceptible to ionization suppression as CIT, OTA, OTα, FBs, AOH, and AME can be diluted 10 after the evaporation and reconstitution steps, and they can be run on the triple quadrupole MS.

Never before detectable levels of ENNs (except ENNB) and BEA were found in urine, probably because of we successfully overcame the adsorption to the containers during collection and sample preparation. A 20x enrichment can substantially improve the LOQs for ENNs and BEA as they do not show any suppression even after enrichment. Running these samples on QTOF allows to quantitate the levels of ENNs and BEA not previously detected in urine, plus the obtained data can also be used for future retrospective analysis when searching for similar mycotoxins. The diagram below illustrates the proposed procedure step-by-step. Additionally, other metabolites of these 12 mycotoxins could be analyzed in urine with our developed LC-MS method.

It is difficult to make a single method proper for different classes of mycotoxins, however, combining the recent developed method with Irina's method[125] can provide monitoring of (12+17=29) mycotoxins and more than 200 of their metabolites in both urine and plasma. Separate approaches for neutral, acidic, and basic mycotoxins may be considered in the future to expand the panel that may be successfully monitored.

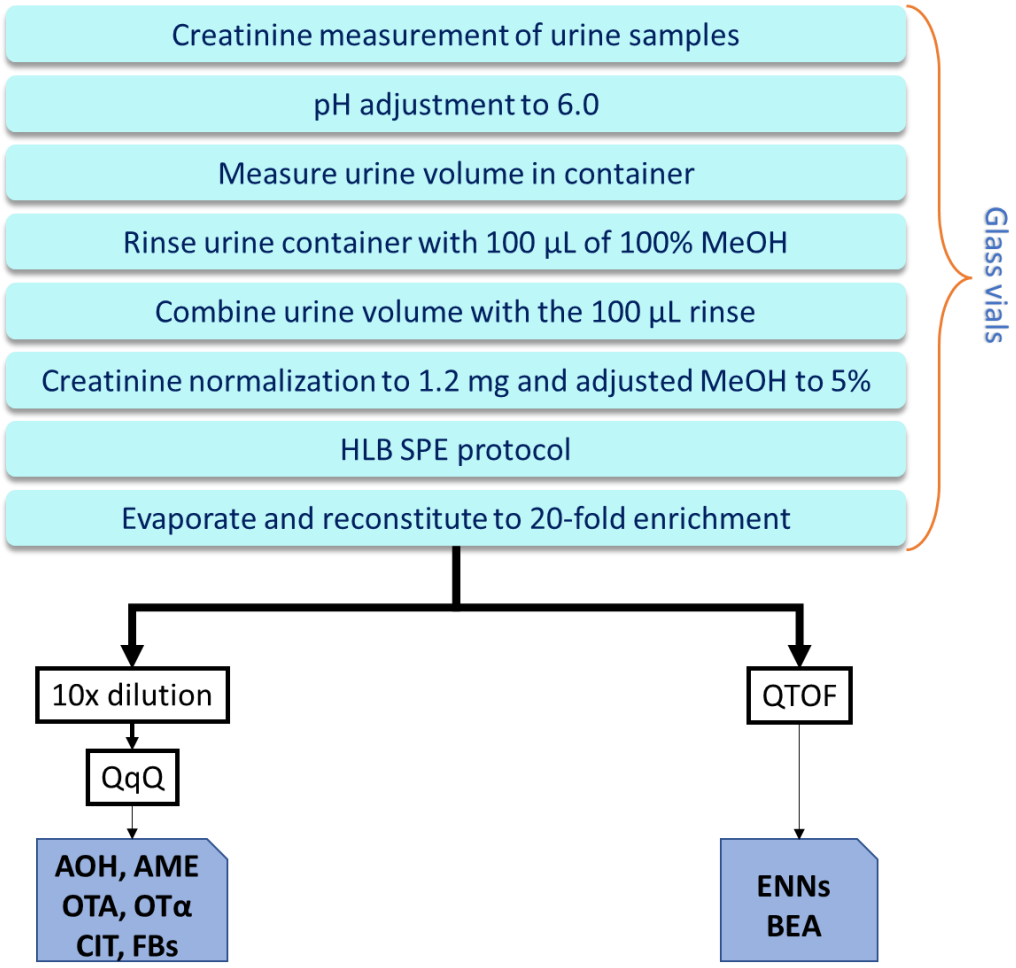


Figure 4.1 Proposed experimental approach for reducing mycotoxin LOQs

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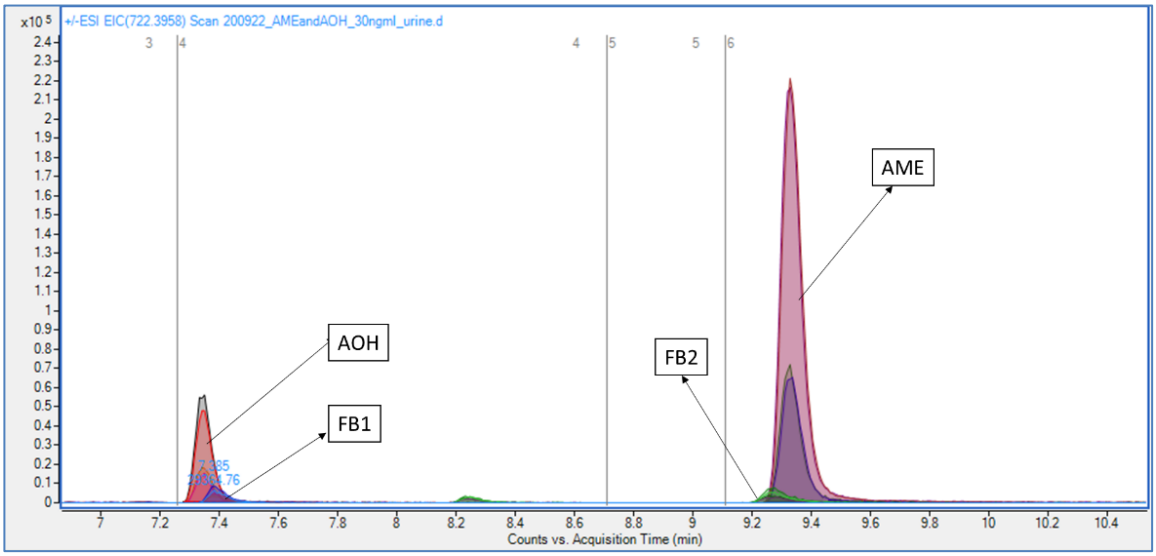
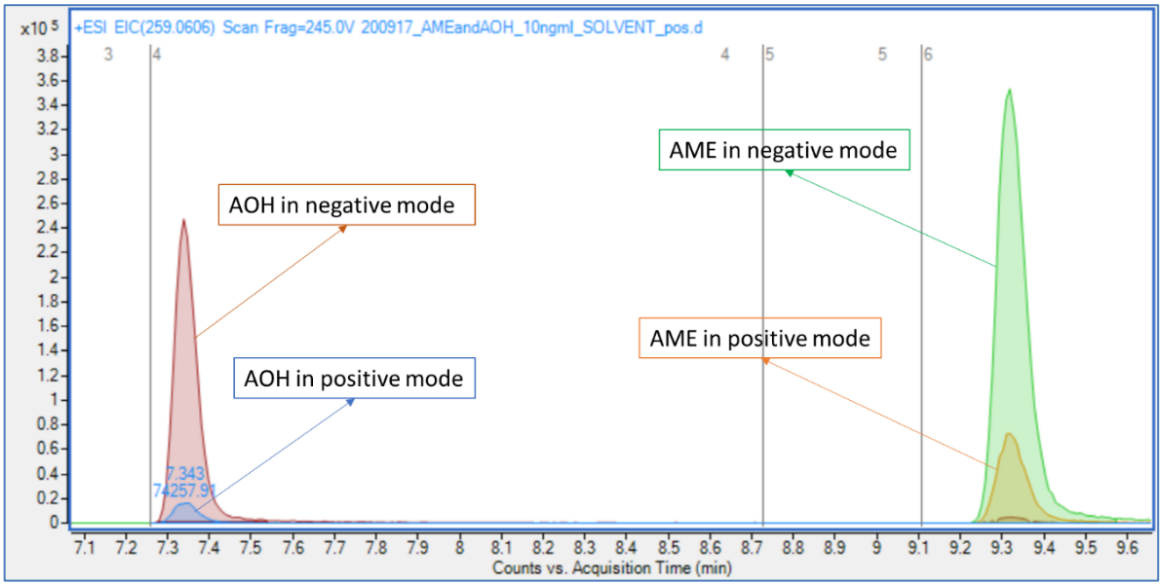
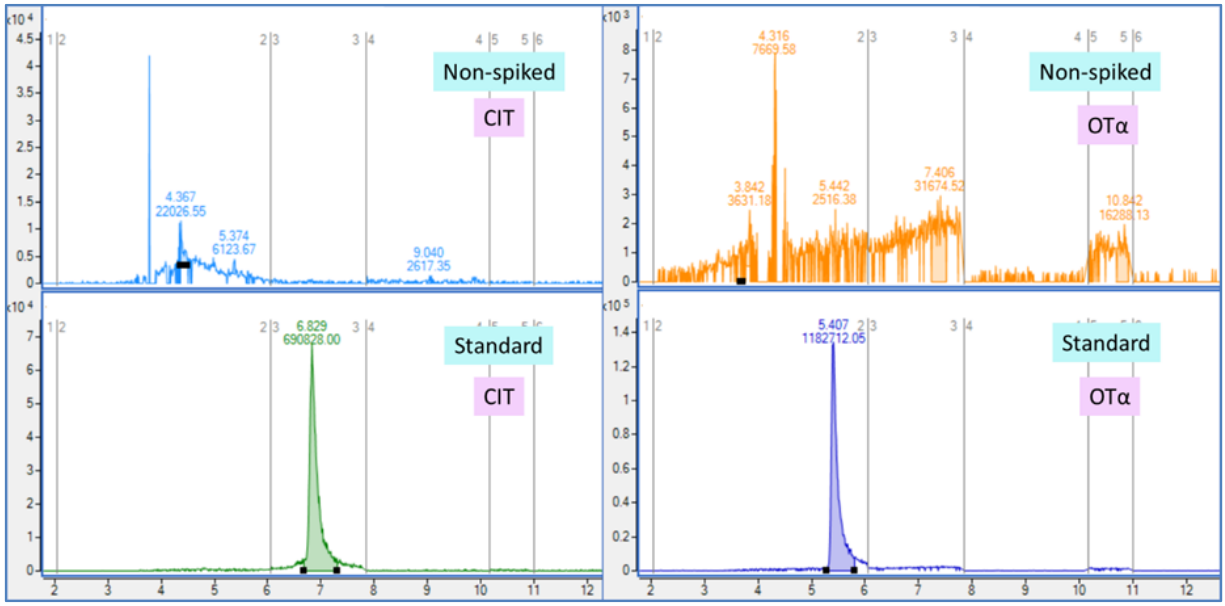
Appendix



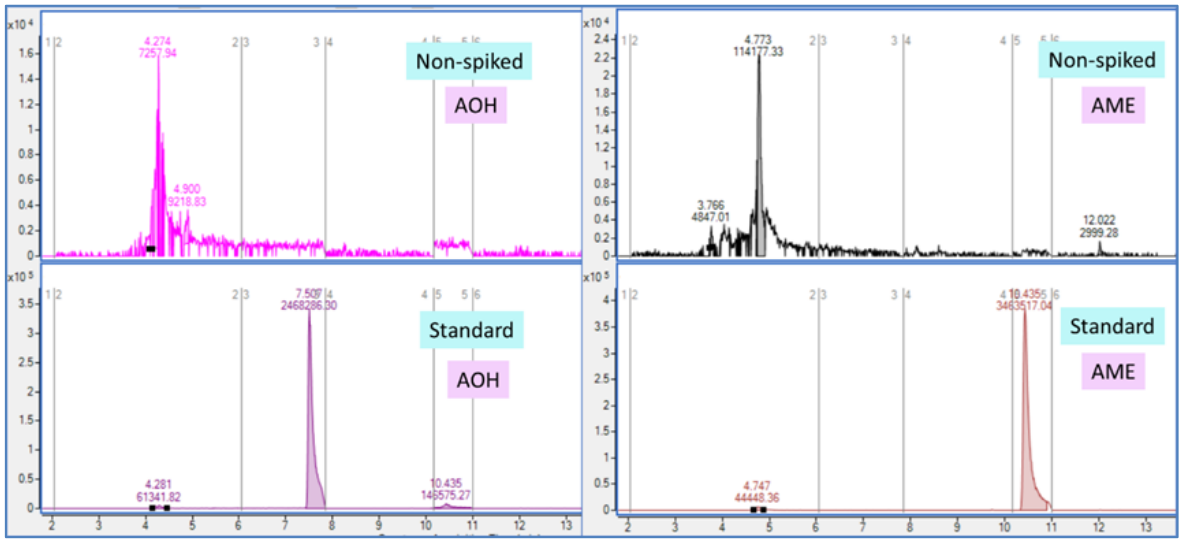
Supplementary Figure 1 Co-elution of fumonisins with alternariols in positive mode at 10 ng/mL using CORTECS T3 column with water and MeOH with 0.02% AA as the mobile phase A and B, respectively..



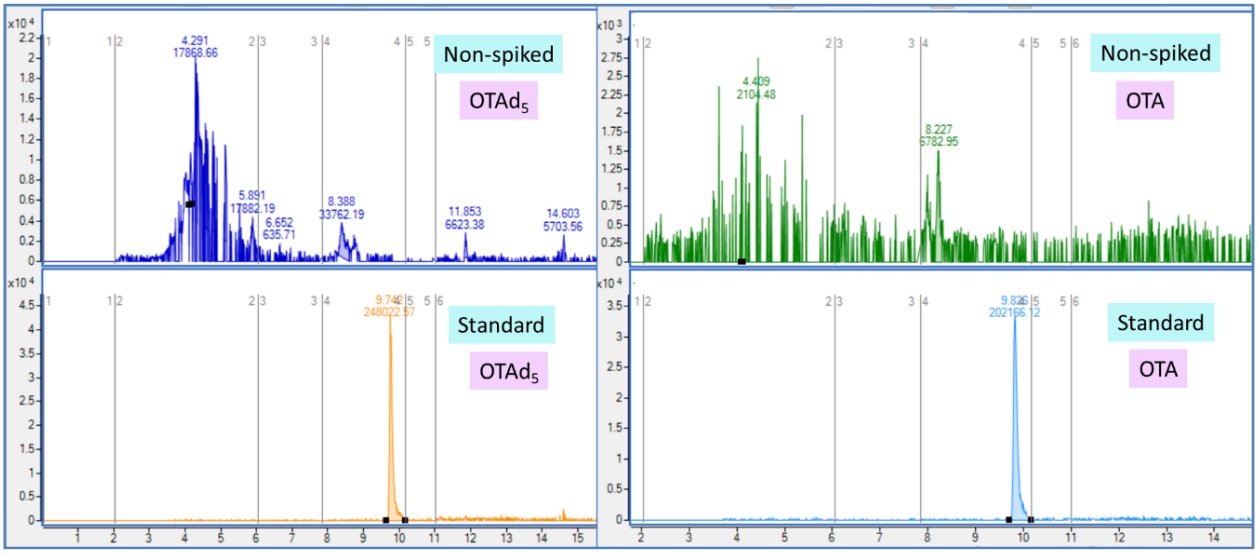
Supplementary Figure 2 Comparison of negative and positive ESI modes for 10 ng/mL standard of AOH and AME.



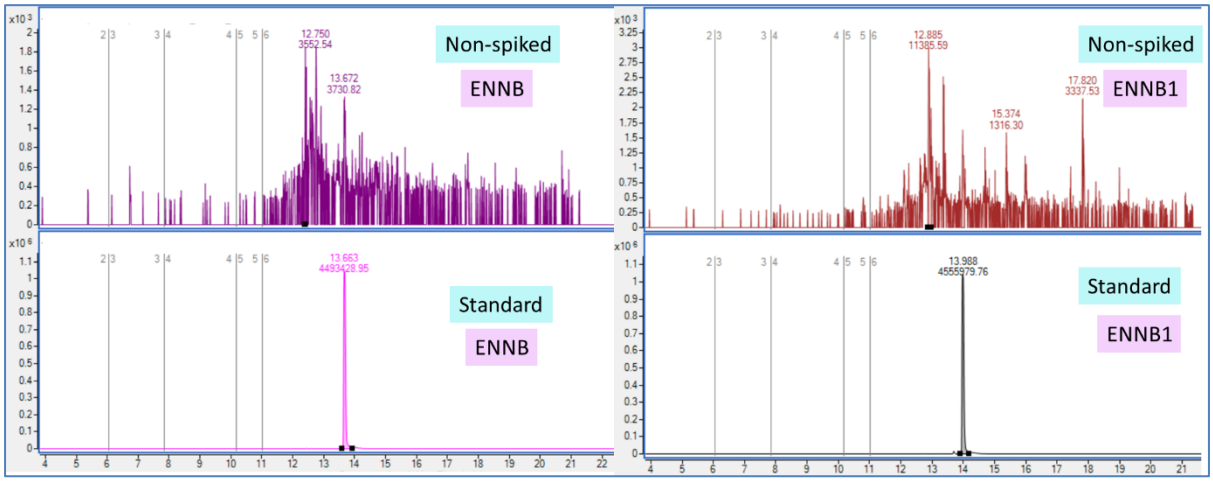
Supplementary Figure 3 EIC of CIT (left panel) and OTα (right panel) in 10x diluted non-spiked urine versus standard (10 ng/mL).



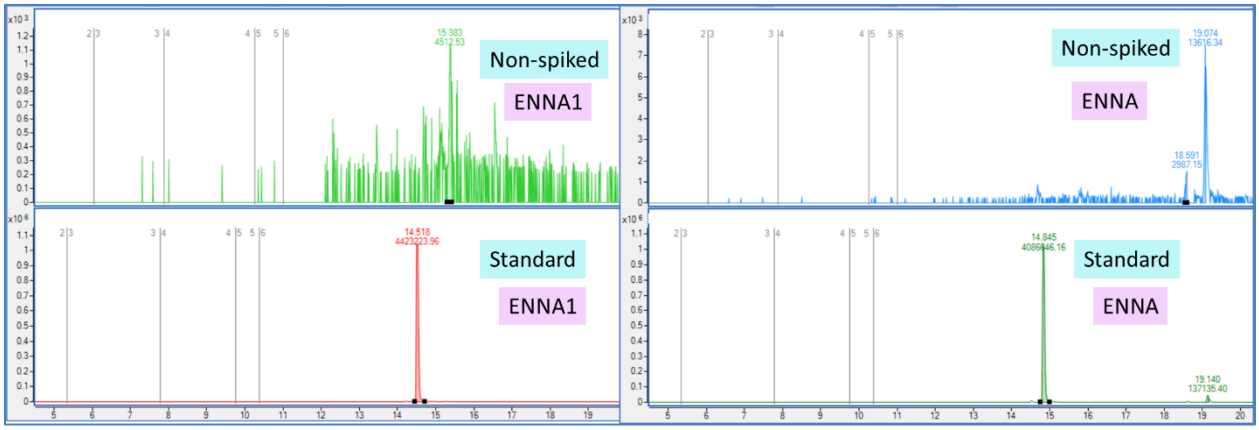
Supplementary Figure 4 EIC of AOH (left panel) and AME (right panel) in 10x diluted non-spiked urine versus standard (10 ng/mL).



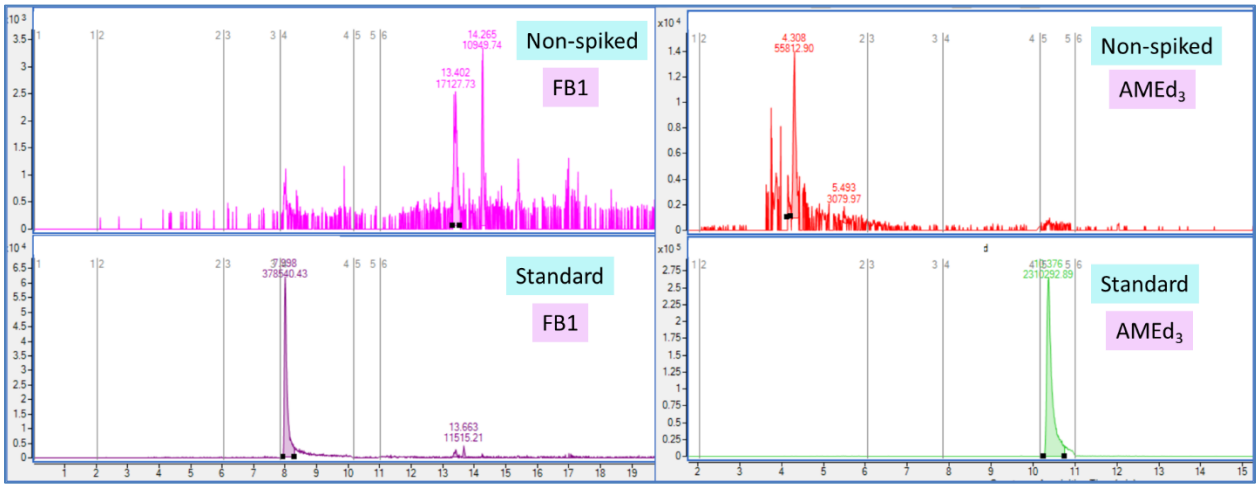
Supplementary Figure 5 EIC of $OTAd_5$ (left panel) and OTA (right panel) in 10x diluted non-spiked urine versus standard (10 ng/mL).



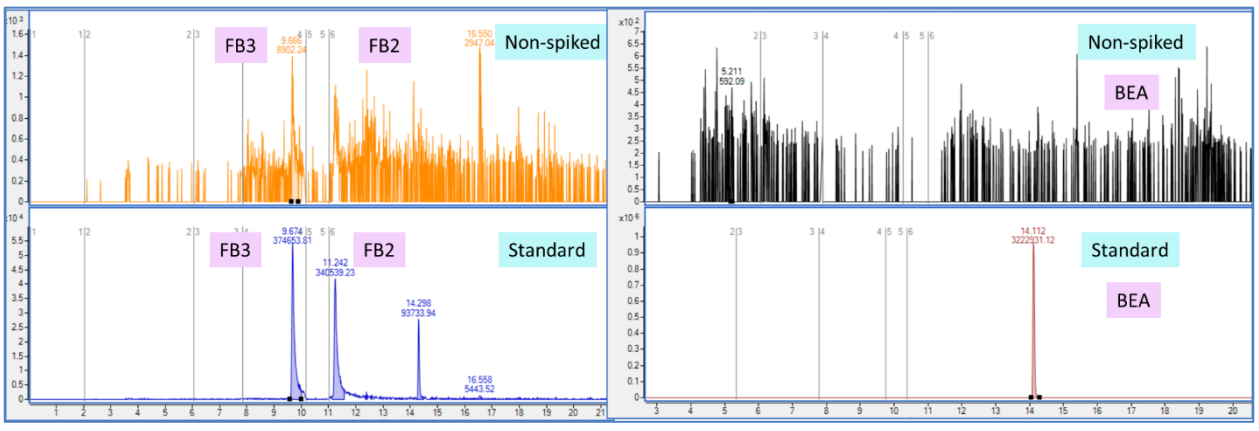
Supplementary Figure 6 EIC of ENNB (left panel) and ENNB1 (right panel) in 10x diluted non-spiked urine versus standard (10 ng/mL).



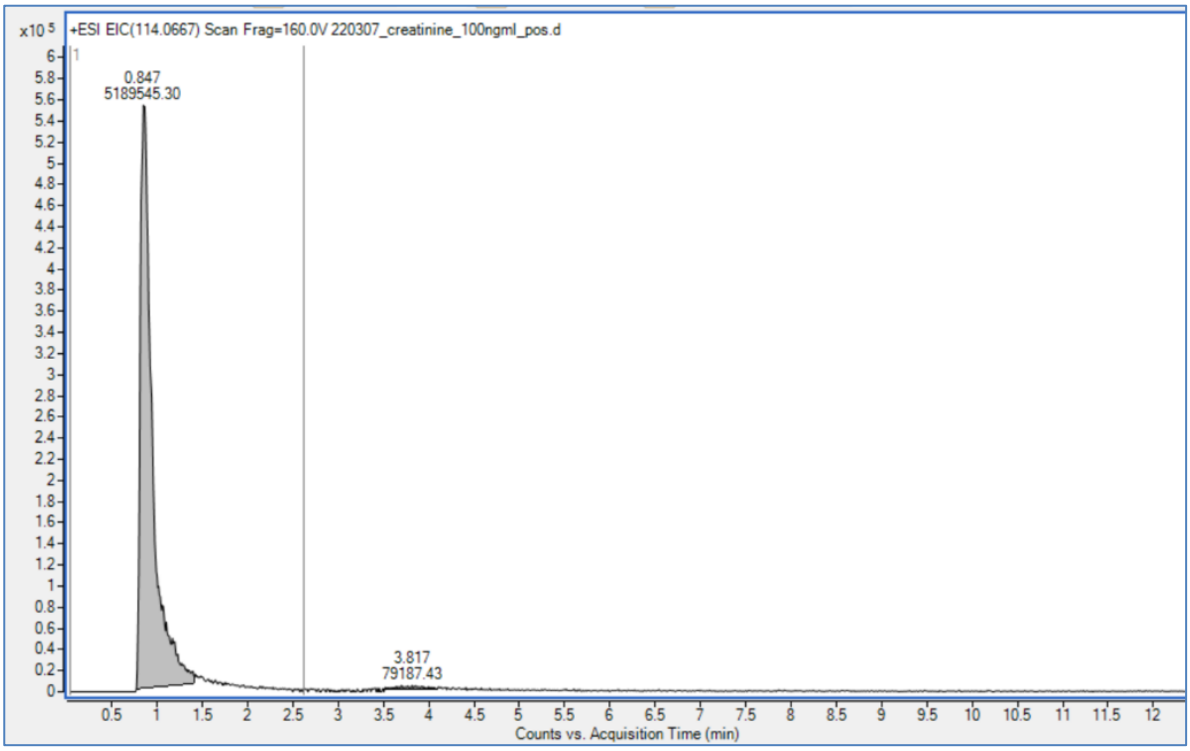
Supplementary Figure 7 EIC of ENNA1 (left panel) and ENNA (right panel) in 10x diluted non-spiked urine versus standard (10 ng/mL).



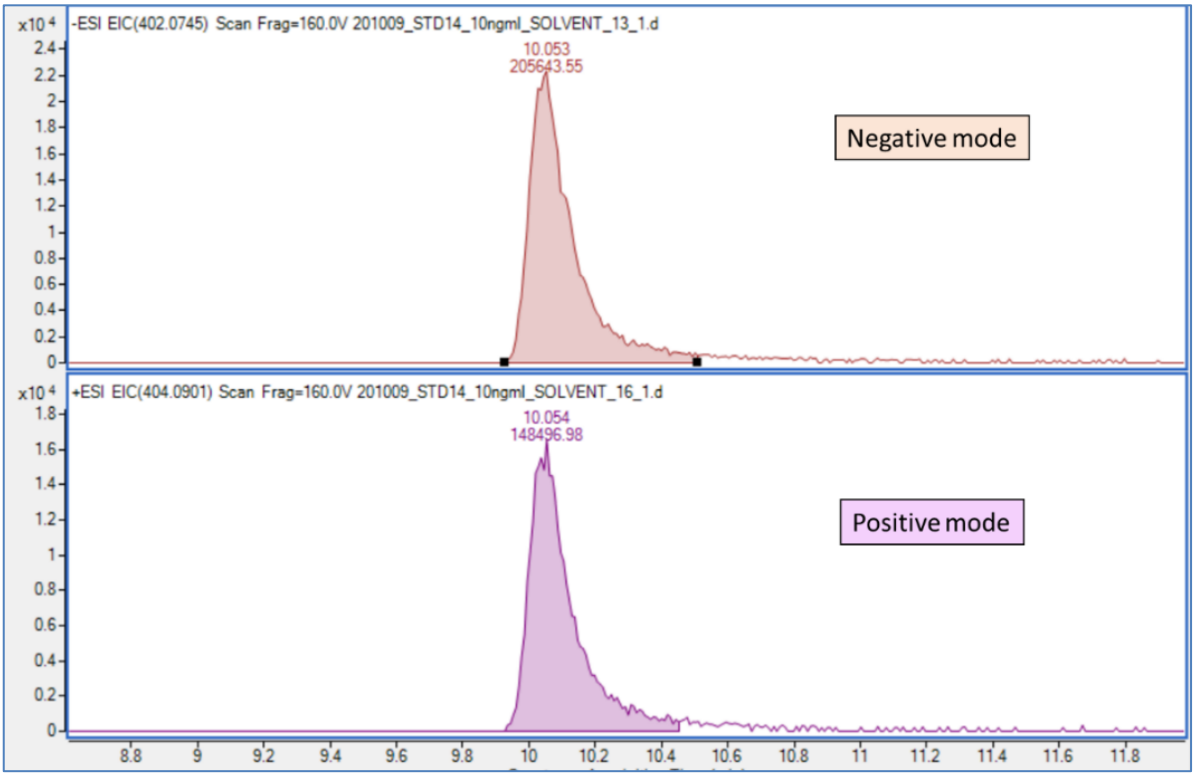
Supplementary Figure 8 EIC of FB1 (left panel) and AMEd$_3$ (right panel) in 10x diluted non-spiked urine versus standard (10 ng/mL).



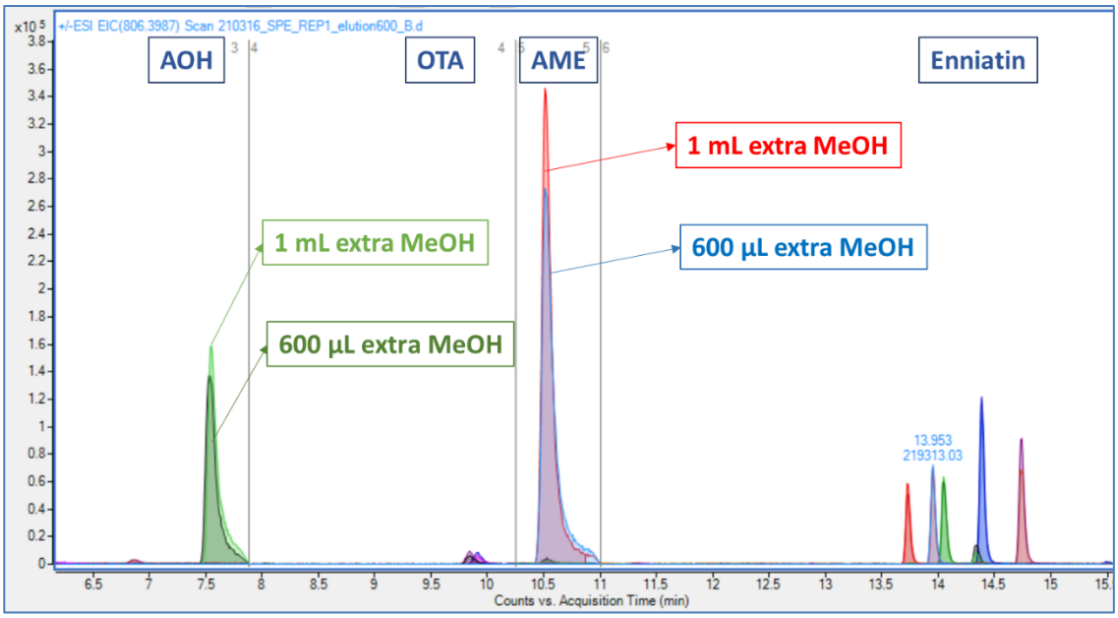
Supplementary Figure 9 EIC of FB3 and FB2 (left panel) and BEA (right panel) in 10x diluted non-spiked urine versus standard (10 ng/mL).



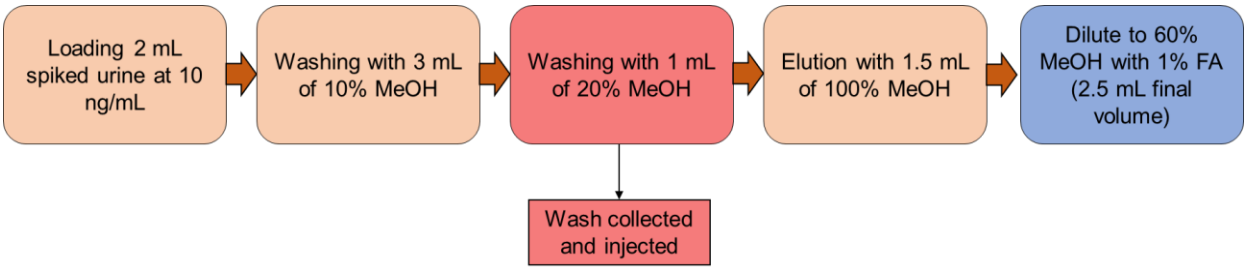
Supplementary Figure 10 Creatinine standard at 100 ng/mL in water in positive ESI mode.



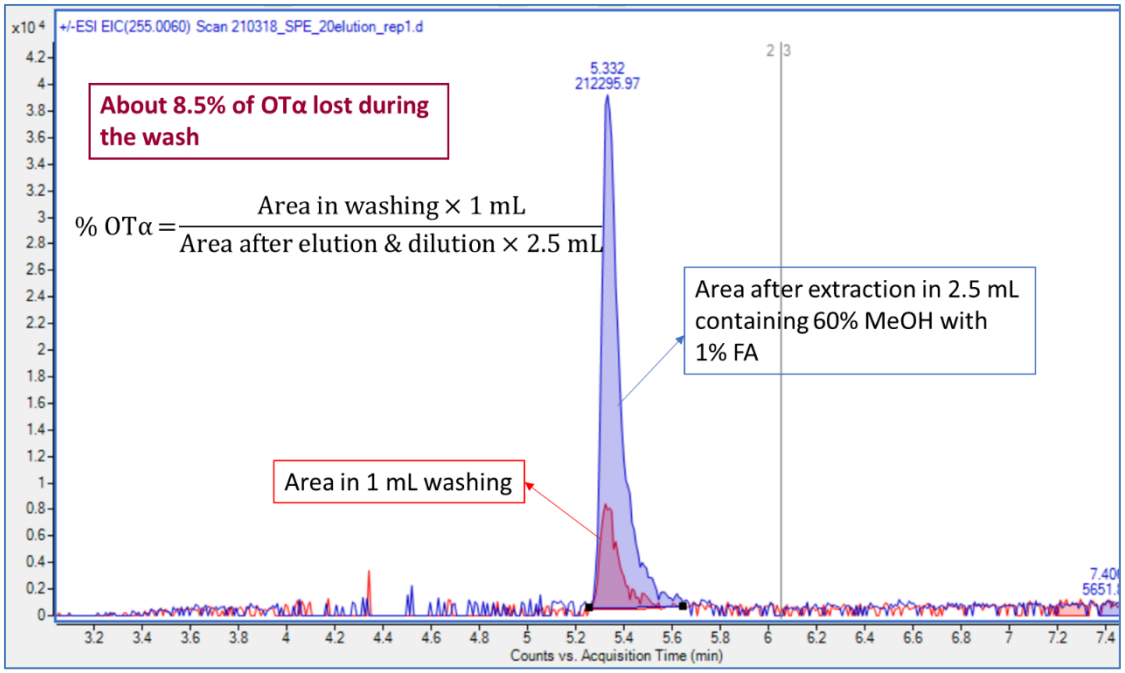
Supplementary Figure 11 Intensity of OTA in different modes. 10 ng/mL OTA standard in 60% MeOH with 1% FA in negative and positive ESI mode using CORTECS T3 column. The intensity difference between positive and negative mode is 27%.



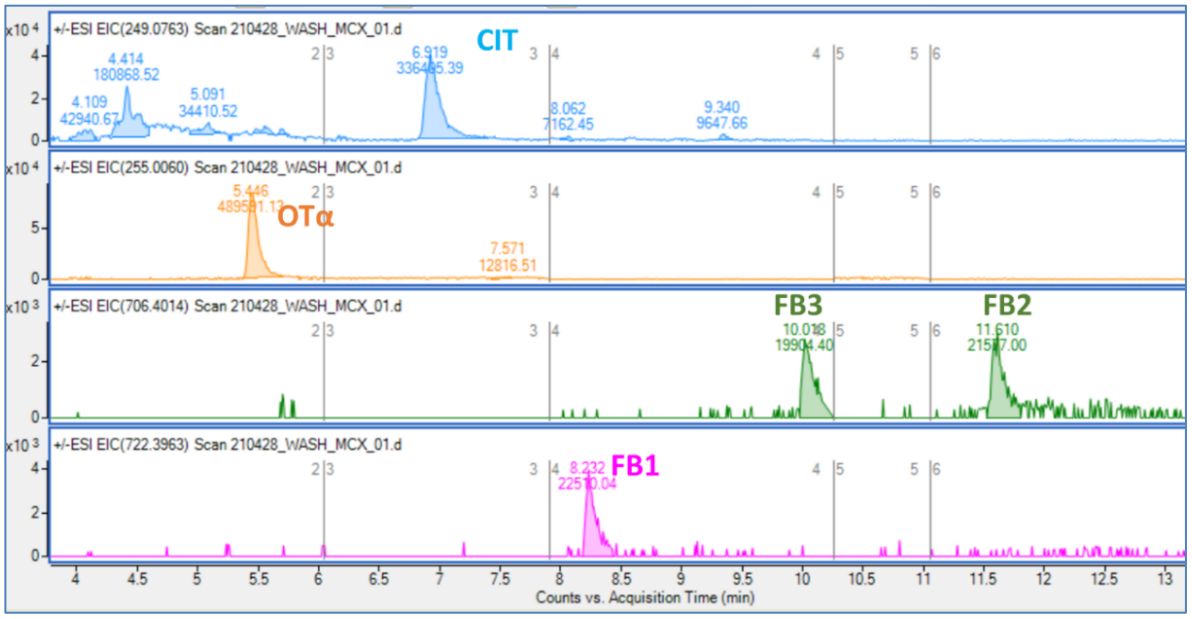
Supplementary Figure 12 Elution of AOH and AME with adding 0.6 and 1 mL of extra methanol after elution step in HLB SPE. 2 mL spiked urine at 10 ng/mL of mycotoxins (137 mg/dL of creatinine) at pH 2 and 6 was loaded (n=2). Interferences of HLB SPE was washed with 1 mL of 5% MeOH. Analytes were eluted with 600 µL of MeOH. Samples were diluted to 60% MeOH with 1% FA before LC-MS injection.



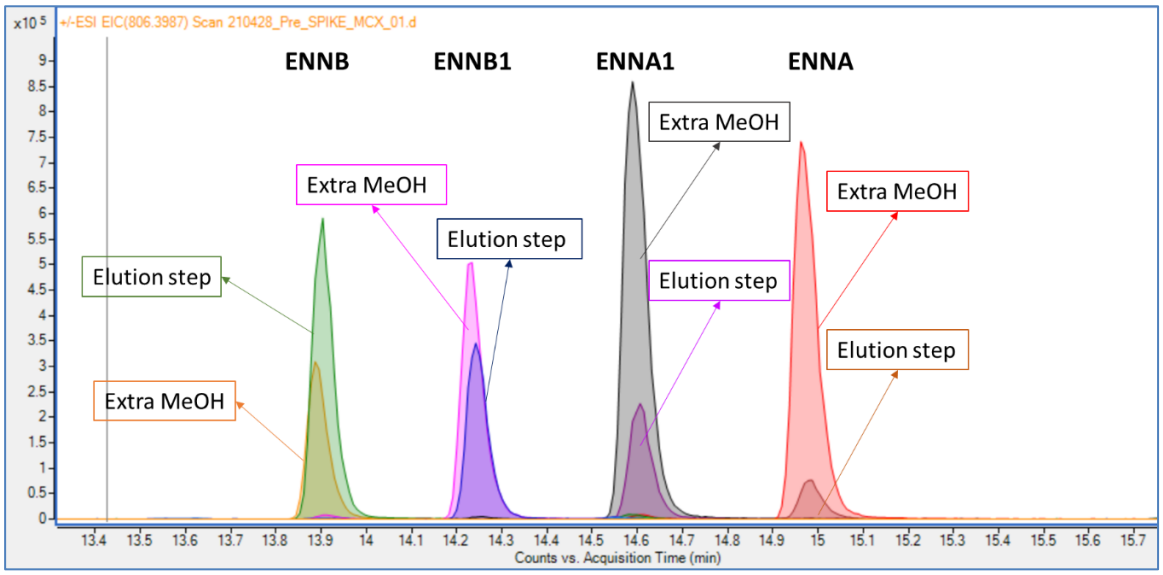
Supplementary Figure 13 Flowchart procedure for collection of wash eluate. The 20% MeOH washing solvent was collected after adding 3 mL of 10% MeOH in HLB SPE.



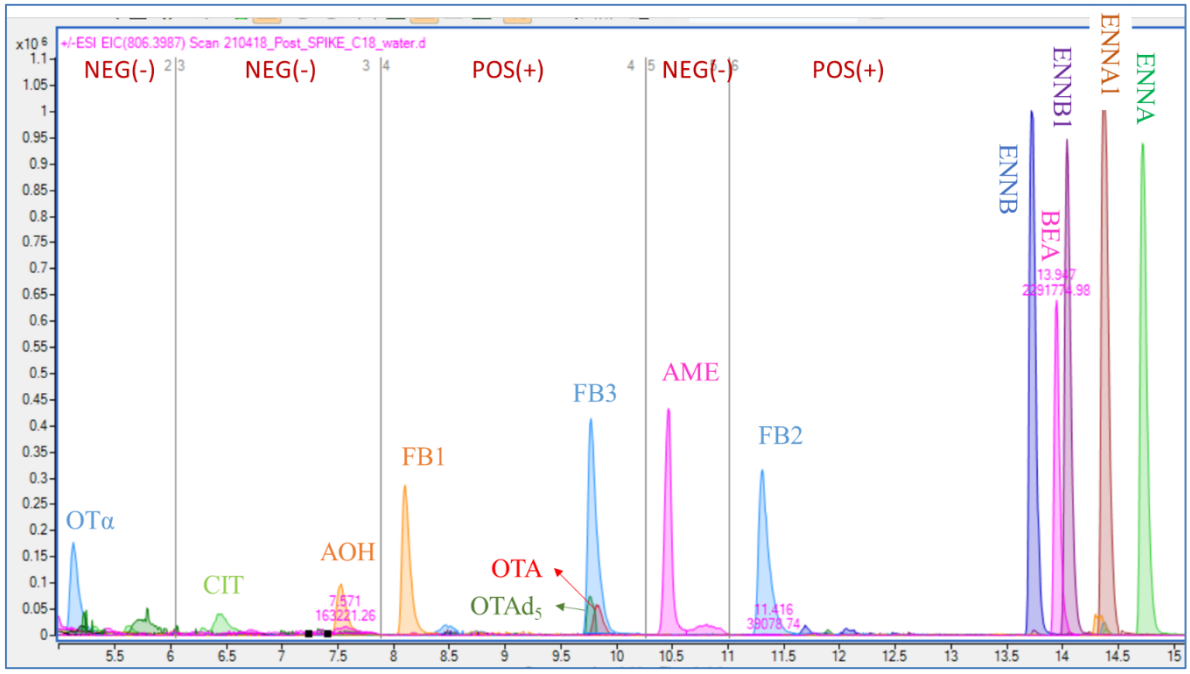
Supplementary Figure 14 The loss of OTα during washing step with 1 mL of 20% MeOH in HLB SPE. 2 mL of spiked urine at 10 ng/mL (137 mg/dL) was loaded on HLB SPE. Washing step was performed with 3 mL of 10% MeOH. Then, 1 mL of 20% MeOH was added and collected separately. Analytes were eluted with 1.5 mL of MeOH and diluted to 60% MeOH with 1% FA by adding 1 mL of water containing 2.5% FA. Blue color shows the peak in the elution step after dilution to 60% MeOH with 1%FA, and the red color shows the peak found in the remaining of 20% MeOH as the washing solvent.



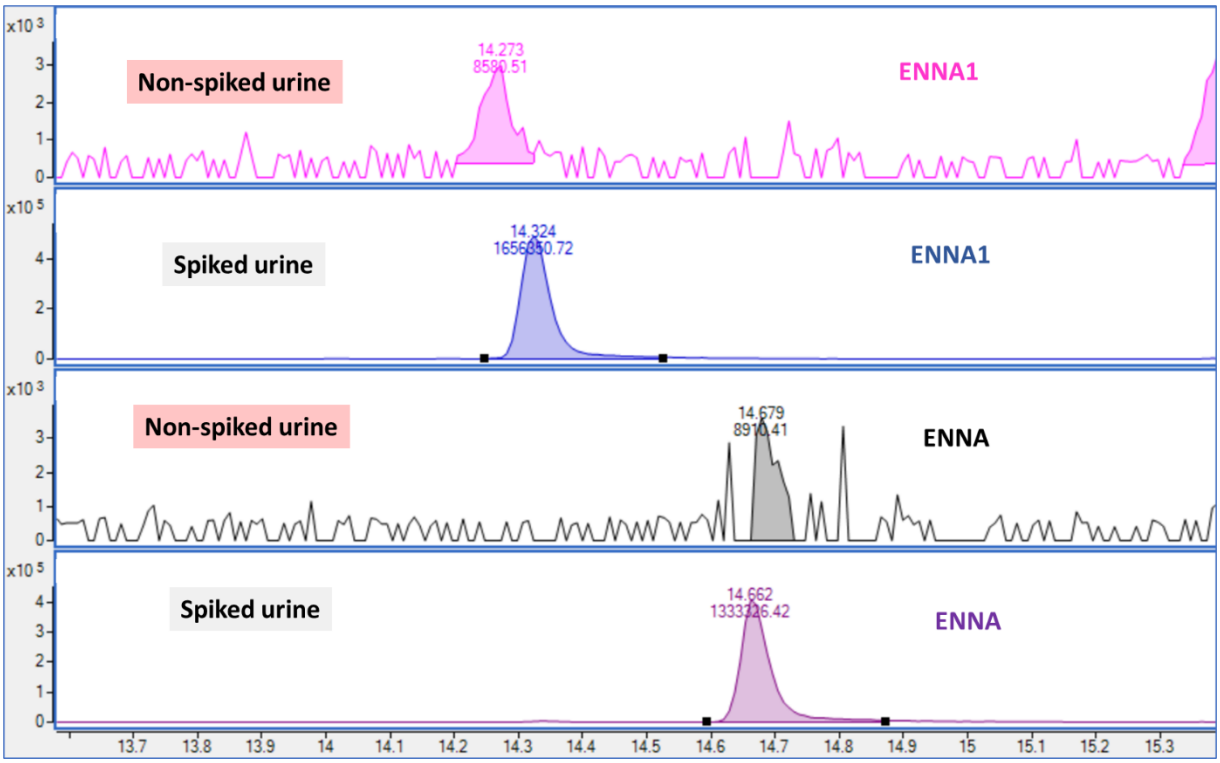
Supplementary Figure 15 Losses of polar to mid polar mycotoxins (CIT, OTα and FBs) in the washing step in MCX SPE. 1 mL of spiked urine at 25 ng/mL (137 mg/dL) was loaded on MCX SPE. Washing step was performed with 6 mL of 10% MeOH and analytes were eluted with 1.5 mL of MeOH. Samples were diluted to 60% MeOH with 1% FA before analysis.



Supplementary Figure 16 EIC of ENNs in MCX SPE after two steps of elution. Second elution was perfomed by adding extra mL of MeOH. 1 mL of spiked urine (137 mg/dL) at 25 ng/mL was loaded on MCX SPE. Washing was performed with 6 mL of 10% MeOH and the analytes were eluted with 1.5 mL of MeOH. The eluates were then diluted to 60% MeOH with 1% FA before analysis.



Supplementary Figure 17 EIC of mycotoxins in urine spiked at 10 ng/mL using HLB SPE with 20x enrichment. Urine sample spiked at 10 ng/mL (137 mg/dL) was loaded on HLB SPE. The washing step was performed with 6 mL of 10% MeOH and mycotoxins were eluted with 1.5 mL of MeOH. Samples were evaporated to dryness and reconstituted in 50 µL of 60% MeOH with 1% FA. After analysis, 10 µL of the sample was diluted 10x



Supplementary Figure 18 Detection of ENNA and ENNA1 in noon-spiked pooled urine HLB SPE. 631 µL of non-spiked pooled urine (normalized to 1.2 mg creatinine) was loaded on HLB SPE. The washing step was performed with 6 mL of 10% MeOH and mycotoxins were eluted with 1.5 mL of MeOH. Sample was partially evaporated and adjusted to final volume of 1 mL of 60% MeOH with 1% FA before injection.