Title: Liquid Chromatography – High Resolution Mass Spectrometry Method for Monitoring of 17 Mycotoxins in Human Plasma for Exposure Studies

Authors: Irina Slobodchikova, Dajana Vuckovic

PII: S0021-9673(18)30323-6
DOI: https://doi.org/10.1016/j.chroma.2018.03.030
Reference: CHROMA 359269

To appear in: *Journal of Chromatography A*

Received date: 15-9-2017
Revised date: 12-3-2018
Accepted date: 14-3-2018

Please cite this article as: Irina Slobodchikova, Dajana Vuckovic, Liquid Chromatography – High Resolution Mass Spectrometry Method for Monitoring of 17 Mycotoxins in Human Plasma for Exposure Studies, Journal of Chromatography A https://doi.org/10.1016/j.chroma.2018.03.030

Liquid Chromatography – High Resolution Mass Spectrometry Method for Monitoring of 17 Mycotoxins in Human Plasma for Exposure Studies

Irina Slobodchikova[1], Dajana Vuckovic[1]

[1]Department of Chemistry and Biochemistry, Concordia University, Montréal, Québec, Canada

Corresponding author: D. Vuckovic, Department of Chemistry and Biochemistry, 7141 Sherbrooke Street West, Montréal, Québec, Canada, H4B 1R6
Email: dajana.vuckovic@concordia.ca
Telephone: 514-848-2424 ext. 3981
Fax: 514-848-2868

Highlights

- First LC-MS assay for 17 mycotoxins was developed and validated in human plasma.
- Liquid-liquid extraction minimized absolute matrix effects to improve quantitation.
- 0.02% acetic acid enhanced sensitivity of mycotoxin analysis in negative ESI.

Mycotoxins are secondary metabolites produced by filamentous fungi. Primary route of human exposure to mycotoxins is the intake of the contaminated food. Minimizing mycotoxin exposure is important for population health, as their chronic toxic effects have been associated with kidney and liver diseases, some types of cancer and immunosuppression. The objective of this work was to develop and validate a multi-class mycotoxin method suitable for exposure monitoring of mycotoxins in human plasma. A sensitive liquid chromatography – mass spectrometry method was developed for 17 mycotoxins: nivalenol (NIV), deoxynivalenol, fusarenon X, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, T-2 toxin, HT-2 toxin, aflatoxin B1, aflatoxin B2, aflatoxin G1, aflatoxin G2, zearalenone, α-zearalenol (α-ZOL), β-zearalenol, zearalanone, α-zeranoland, and β-zeranol. The method relies on three-step liquid-liquid extraction with ethyl acetate to eliminate the need for immunoaffinity extraction and minimize ionization matrix effects. Chromatographic separation of mycotoxins, including all isomers, was achieved with pentafluorophenyl column and water/methanol mobile phase. Mycotoxin detection and quantitation were performed using high-resolution mass spectrometry on LTQ Velos Orbitrap, in both positive and negative electrospray ionization (ESI(+) and (ESI(-)). The use of 0.02% acetic acid as mobile phase additive for ESI(-) resulted in significant increase in ionization efficiency ranging from 1.7 to 26 times for mycotoxins that ionize better in ESI(-). The optimized method was validated according to FDA guidance procedures. LOQs of all mycotoxins ranged from 0.1 to 0.5 ng/ml, except NIV which resulted in LOQ of 3 ng/ml because of low extraction recovery of this highly polar mycotoxin. Mean intra-day accuracy ranged from 85.8% to 116.4%, and intra-

day precision (n=6) ranged from 1.6% to 12.5% RSD for all mycotoxins except α-ZOL where mean accuracy ranged from 72.9% to 97.2%. Inter-day accuracy and precision were 85.6% to 111.5% and 2.7 to 15.6% RSD respectively, showing good analytical performance of the method for biomonitoring.

Keywords: mycotoxins, high-resolution mass spectrometry, human plasma, pentafluorophenyl**,** biomonitoring, validation

1. Introduction

Mycotoxins are fungal metabolites that can be toxic to animal and human populations. Mycotoxin exposure may contribute to a variety of adverse health effects, as specific mycotoxins may have hepatotoxic, nephrotoxic, carcinogenic, cytotoxic, immunosuppressive, inflammatory, neurological, estrogenic and/or teratogenic effects[1],[2]. Direct contact and inhalation represent minor routes of exposure, while the majority of human exposure to mycotoxins occurs through diet via intake of contaminated food. In fact, 25% of grain supply worldwide is estimated to be contaminated with mycotoxins, and the contamination of Canadian food chain is well documented[3],[4],[5],[6],[7]. Although food contamination and consumption data can be used to estimate human exposure levels, such approaches raise concerns about the accuracy of the estimated levels especially for at risk populations such as infants and children. In addition, inter-individual variability in adsorption, distribution, metabolism and excretion of mycotoxins can contribute to higher exposure of particular individuals or consumer groups. Thus, the direct monitoring of mycotoxin levels in biological fluids such as urine or blood is crucial for the most accurate estimate of human exposure to these toxins and for further refinement of diet-based models.

The majority of existing mycotoxin methods for biomonitoring studies focus on the quantitation of one or few mycotoxins of similar chemical properties, with ochratoxin A (OTA) being the most commonly studied in biological fluids due to its high toxicity, long lifetime in blood and high prevalence worldwide with 90-100% incidence in samples tested[8],[9]. The use of such single analyte or class-specific methods[10],[11] makes large scale studies of multiple mycotoxins cost prohibitive. Ideally, accurate methods for monitoring trace quantities of mycotoxins in both urine and plasma are required to obtain complementary information on both short-and medium-term exposure, depending on toxin's half-life in plasma, as well as metabolism and excretion rates of these species. Depending on the mycotoxin of interest, monitoring of either plasma or urine specimens can provide different advantages. For example, for OTA, which has plasma life of 35 days[12], monitoring of plasma is beneficial as it can provide evidence of medium-term and time-weighted average exposure. This mycotoxin is also present at higher concentrations in plasma versus urine which further supports use of this matrix[13]. For aflatoxin B1 (AFB1), analysis of plasma also reflects medium-term exposure due to AFB1 binding to plasma albumin[14]. On the other hand, monitoring of mycotoxins such as fumonisin B1 (FB1) and T-2 toxin (T-2) in plasma provides evidence of short-term exposure due to their shorter lifetimes of 18 min and 8.1 hour in rat blood, respectively[15] [16].

The importance of direct biomonitoring studies is illustrated by findings of higher prevalence than expected of some mycotoxins. For example, citrinin and its main metabolite were detected in 90% of urine samples in Belgian study[17]. Another study found that 16-69% and 1% of Belgian population may have exceeded tolerable daily intakes of deoxynivalenol (DON) and OTA, respectively[18]. Gerding *et al.* [19] also found 16% of urine samples collected in Germany exceeded tolerable daily intake of DON whereas 94% and 40% of study participants in southern Italy exceeded tolerable daily intakes for OTA and DON[20]. These studies clearly establish the need and the importance of direct biomonitoring of mycotoxins. To support multi-mycotoxin biomonitoring studies and reduce the cost of such studies, the availability of liquid chromatography - mass spectrometry (LC-MS) assays that can measure as many species as possible simultaneously and without the need for immunoaffinity enrichment is critically needed. For example, Wallin *et al*. used extensive sample preparation including enzymatic hydrolysis step, sequential immunoaffinity and reversed-phase solid phase extraction (SPE) to quantitate 5-10 mycotoxins in human urine[21]. Although the method achieved good limits of detection for human biomonitoring, the use of immunoaffinity increases the cost per sample of the assay and/or restricts the method for analytes recognized by the antibodies utilized. To avoid these limitations, several LC-MS multi-mycotoxin methods that omit immunoaffinity enrichment have been reported and validated in urine[22],[23],[24]. These simple direct injection or dilute-and-shoot methods were recently further extended to enable (semi-)-quantitation of total of 32 mycotoxins[17] or 23 mycotoxins[19], respectively. Heyndrickx *et al.* [18] combined two LC-MS methods, one direct filter-and-shoot and one with extensive sample clean-up (liquid-liquid extraction (LLE) with ethyl acetate/formic acid followed by strong ion exchange SPE) to permit monitoring of total of 33 mycotoxins, out of which 9 were detected in urine samples from children and adult Belgian population[18]. In addition, two methods combining QuEChERS (abbreviation name originates from the first letters of **Q**uick, **E**asy, **C**heap, **E**ffective, **R**ugged, and **S**afe) extraction and LC-MS for monitoring of 27 mycotoxins in human breast milk and 30 mycotoxins in animal milk were also developed[25],[26].

In contrast to urine biomatrix, suitable multi-class LC-MS methods for biomonitoring of human plasma or serum are currently not available due to high matrix complexity, physicochemical diversity of mycotoxins of interest and the need for exceptional analytical sensitivity. Thus, for the determination of multiple mycotoxins in plasma, most methods to date focused on analysis of structurally-related mycotoxins belonging to a single family, for example zearalenone and its metabolites[27] or enniatins and beauvericin[28]. To address these limitations, Devreese *et al.* [29] developed simple acetonitrile solvent precipitation method in combination with LC-MS for measurement of 13 mycotoxins in pig plasma suitable for toxicological studies, but the limits of quantitation (2-10 ng/mL)[29] do not make this method adaptable to human biomonitoring. Similarly, De Santis *et al.* [30] combined pronase treatment, acidified ethyl acetate LLE and QuEChERS with LC-MS detection for the analysis of 8 mycotoxins, but the method showed insufficient limit of quantifications (LOQs) for accurate quantitation (mean values for positive samples below LOQ) and significant susceptibility to matrix effects for several of the analytes despite extensive clean-up. Tolosa *et al.* [31] developed dispersive liquid-liquid microextraction method using ethyl acetate in combination with LC-MS/MS for

measurement of 15 mycotoxins in fish plasma. However, the method LOQs ranged from 1 to 17 ng/ml which is not sufficient for detection of mycotoxins in human plasma. Osteresch *et al.* [32] proposed a method that combines simple solvent extraction using water/acetone/acetonitrile (30:35:35, v/v/v), evaporation/reconstitution and LC-MS/MS analysis using multiple reaction monitoring mode for dried blood spots or dried serum spots. This method enables the quantitation of the largest panel of mycotoxins to date in blood (27 mycotoxins and their metabolites) with LOQs ranging from 0.005 – 5.0 ng/mL which makes it suitable for human blood biomonitoring. However, significant stability and matrix effect issues were observed. For example, matrix effects ranged from 13 to 842% and from 14 to 939% for dried blood spot matrix and dried serum spot matrix, respectively, whereas the values of 80-120% indicate the absence of significant matrix effect. In summary, none of the existing methods adequately meet the needs for human biomonitoring in terms of sensitivity, coverage, accuracy and matrix effects. Matrix effect is a complex and analyte-specific phenomenon that can be compensated by the addition of isotopically-labelled internal standards. These internal standards can compensate not only for the losses during the procedure, but also any changes in ionization due to presence of matrix interferences. However, for the mycotoxins of interest in this work there are very few commercially available isotopically-labelled standards, and using multiple ^{13}C isotopically labelled mycotoxin standards makes the method prohibitively expensive to implement for population monitoring studies. Therefore, one of the objectives of this study was to develop a sample preparation method that results in minimal absolute matrix effect while achieving high analyte recovery (> 80% was preferable) for this application.

The objective of this study was to develop and validate a sensitive multi-residue mycotoxin LC-MS method in human plasma suitable for mycotoxin exposure biomonitoring without need for immunoaffinity enrichment. The mycotoxins selected for method development are mycotoxins routinely monitored and detected in North American, and specifically Canadian food supply[33]. The effect of sample preparation (solvent precipitation, SPE and LLE) and LC separation on simultaneous analysis of all important/prevalent mycotoxin classes using a single small volume sample of human blood was investigated in detail. Special attention was also paid to minimizing matrix effects as isotopically-labelled internal standards for many of our mycotoxins of interest are currently not commercially available. The final method was validated for the quantitation of 17 mycotoxins of interest. The main novelty of this work is that the method drastically increases the number of analytes that can be measured in human plasma, while ensuring good limits of quantitation without the need for immunaoffinity enrichment and accuracy/precision of the method. In addition, the method reduces ionization matrix effects to ensure acceptable method accuracy even when isotopically labelled internal standards are not available. The availability of this optimized and rigorously validated method opens up new avenues for mycotoxin biomonitoring while minimizing cost per sample and allowing for retrospective examination of high resolution MS data for other mycotoxins that may become of particular health interest in future.

2. Materials and methods

2.1 Chemicals

LC-MS grade water, methanol and acetonitrile, and HPLC grade ethyl acetate were purchased from Fisher Scientific (Ottawa, Ontario, Canada). Acetic acid (AA, meets specifications of American Chemical Society grade, 99.7%), formic acid (FA, for mass spectrometry, 98%), and magnesium sulfate (anhydrous, ReagentPlus®, ≥99.5%) were purchased from Sigma-Aldrich Canada (Oakville, Ontario, Canada). Pooled human plasma with sodium citrate as anti-coagulant was purchased from Bioreclamation Inc. (Baltimore, MD, USA).

2.2 Mycotoxin standards

Nivalenol hydrate (NIV), DON, fusarenon X (FUS-X), 3-acetyldeoxynivalenol (3-AcDON), 15-acetyldeoxynivalenol (15-AcDON), AFB1, aflatoxin B2 (AFB2), aflatoxin G2 (AFG2), α-zearalenol (α-ZOL), β-zearalenol (β-ZOL), OTA and zearalanone (ZAN) were purchased from Sigma-Aldrich Canada. Aflatoxin G1 (AFG1), T-2, HT-2 toxin (HT-2), α-zeranol (α-ZAL), β-zeranol (β-ZAL), and ochratoxin A-d_5 ($OTAd_5$) were purchased from Toronto Research Chemicals Inc. (Toronto, ON, Canada). FB1, fumonisin B2 (FB2) and zearalenone (ZEN) were purchased from Cayman Chemicals (Ann Arbor, MI, USA). Individual standard stock solutions of all mycotoxins at 1 mg/ml concentration were prepared in 100% MeOH and kept at -80^{o}C. Fumonisin B3 (50 µg/ml in 50% acetonitrile) and ^{13}C-Zearalenone (^{13}C-ZEN, 25.5 µg/ml in acetonitrile) were purchased from Romer Labs (Union, MO, USA). 3-acetyl-d_3-deoxynivalenol solution (3-$AcDONd_3$, 100 µg/ml in acetonitrile) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). A combined 10 µg/ml working solution of all mycotoxin standards was prepared in methanol every 6 months and stored in aliquots at -80°C. Isotopically labelled standards were diluted to 10 µg/ml for $OTAd_5$ in methanol and FB3 in acetonitrile, 4 µg/ml for 3-$AcDONd_3$ and 1 µg/ml for ^{13}C-ZEN in acetonitrile. 25 ng/ml concentration of $OTAd_5$ and FB3 was added immediately prior to LC-MS analysis while investigating different types of sample preparation techniques. Internal standards, 3-$AcDONd_3$ and ^{13}C-ZEN were used during final method validation at 10 ng/ml and 3 ng/ml final concentration. During method validation, internal standards were used to monitor injection volume, signal stability and ionization matrix effects. For application of this final method to exposure studies, it is recommended to add internal standards to plasma prior to the extraction to also monitor extraction recovery in study samples as positive quality control.

2.3. Method development of sample preparation: comparison of LLE, SPE and protein precipitation methods

2.3.1 Protein precipitation. 300 µl of acetonitrile was added to 100 µl of plasma and mixed on vortex (Fisher Scientific Vortex Mixer) for 20 min. Samples were then centrifuged at 25830 x g, (Thermo Fisher Scientific, Sorvall ST 16R centrifuge) for 10 min at 4°C. The 300 µl of supernatant was aspirated into a new polypropylene extraction tube, evaporated to dryness using Speedvac (Labconco CentriVap 7812013) and reconstituted into 200 µl of 20% methanol containing $OTAd_5$ and FB3 internal standards. This solution was transferred into polypropylene HPLC inserts for analysis.

2.3.2 Three-step LLE procedure with ethyl acetate. 100 µl of plasma and 150 µl of ethyl acetate were vortexed for 20 min and centrifuged at 25830 x g, 4°C for 10 min. The organic layer (100 µl) was transferred into another centrifuge tube. Plasma residues were re-extracted two more times using fresh portions of 150 µl of ethyl acetate followed by vortexing, centrifugation and collection of the organic layers into the polypropylene collection tube. Collected organic phase (300 µl) was evaporated to dryness using Speedvac, reconstituted into 200 µl of 20% methanol containing internal standards, and transferred into polypropylene HPLC inserts for LC-MS analysis.

2.3.3 Two-step LLE procedure with ethyl acetate. The same procedure as described for three-step LLE (section 2.3.2) was used but only 2 x 150 µL portions of ethyl acetate were used.

2.3.4 One step LLE procedure. 100 µl of plasma and 300 µl of ethyl acetate were vortexed for 20 min and centrifuged at 25830 xg, 4°C for 10 min. The collected organic layer (200 µl) was transferred into polypropylene centrifuge tube, evaporated to dryness using Speedvac, reconstituted into 20% methanol containing internal standards, and transferred into polypropylene HPLC inserts for LC-MS analysis.

2.3.5 One-step LLE of acidified plasma with ethyl acetate or methyl tert-butyl ether (MTBE). 100 µl of plasma was acidified with 1% (FA) to pH 4, and then extracted using ethyl acetate or methyl tert-butyl ether (MTBE) as described in one-step LLE extraction (section 2.3.4).

2.3.6 Complex four-step LLE with ethyl acetate, and sequential salt addition and acidification. Two-step LLE was performed as described in two-step LLE (section 2.3.3), then $MgSO_4$ (0.0241 g) was added to plasma residue and extracted with fresh portion of ethyl acetate (150 µl) followed by vortexing, centrifugation and collection of the organic layer into the collection tube. After third LLE step, formic acid (50 µl) was added to plasma residue and this was then extracted using a 150 µL portion of ethyl acetate. Collected organic phase (400 µl) was evaporated to dryness using Speedvac, reconstituted into 200 µl of 20% methanol containing internal standards, and transferred into polypropylene HPLC inserts for LC-MS analysis.

2.3.7 HLB SPE procedure. Hydrophilic-lipophilic-balanced sorbent (Oasis HLB) SPE (3 cc, 60 mg, average particle diameter 29.2 µm, Waters, Massachusetts, USA) was performed as follows: (i) conditioning with 3 ml of methanol and 3 ml of H_2O, (ii) loading of 1 ml of plasma, (iii) washing of interferences with 1 ml of 5% methanol, and (iv) eluting mycotoxins with 1 ml of methanol. Collected eluents (700 µl) were then evaporated to dryness, reconstituted into 200 µl of 20% methanol with internal standards, and transferred into HPLC inserts for LC-MS analysis. HLB SPE was chosen after preliminary comparison with C18 and SAX [data not shown] because it provided the highest overall method recoveries across the analytes of interest

2.3.8 Method comparison and selection of optimum sample preparation method

Recovery experiments were performed for each sample preparation technique in order to find an appropriate method with the highest recovery across different mycotoxin classes

tested (Supplementary Figure S1). Blank plasma samples were spiked with 100 ng/ml of mycotoxins, incubated for an hour and extracted according to the procedures described in sections 2.3.1 to 2.3.7 (n=3 replicates). The amount of analyte in each sample was determined using calibration curve prepared in reconstitution solvent (20% methanol) according to the formula RE% = C_m/C_{th}*100%, where RE% is the recovery, C_m is the measured concentration in the injection solvent and C_{th} is theoretical concentration in injection solvent which includes the correction for differences in volumes transferred in specific procedures. This determination is equivalent to overall method recovery as it includes the effects of both extraction recovery and matrix effects due to ionization suppression/enhancement.

In addition, ionization matrix effect was also evaluated for best method from each sample preparation approach. Blank plasma samples were extracted according to the procedures described in sections 2.3.1, 2.3.2 and 2.3.7 (n=3), and then were spiked with 25 ng/ml (n=3) of mycotoxin mixture during reconstitution step. Matrix effect was determined according to the formula signal intensity % = $A_{plasma}/A_{std.}$*100%, where A_{plasma} is the measured peak area of a given mycotoxin in post-extracted spiked plasma, and $A_{std.}$ is the measured peak area of the same mycotoxin in standard solution prepared at the same concentration in 20% methanol. The values above 120% indicate significant ionization enhancement, while values below 80% indicate significant ionization suppression.

2.4 Final optimized three-step LLE procedure with ethyl acetate used for method validation

Final three-step LLE used for validation was modified as follows from the protocol used during method development. Collected organic volumes after each extraction step were changed to 70 µl, 100 µl and 200 µl after first, and second and third extraction steps, respectively. Reconstitution volume after evaporation was changed to 400 µl to prevent early aging of the column. Finally, the evaporation process was strictly time-controlled (320 min) in order to avoid losses during re-solubilisation and achieve consistent recovery across different days.

2.5 LC-MS analysis

2.5.1 LC-MS development

Initial method development experiments compared the performance of core-shell Kinetex C18 and PFP columns (2.6 µm, 100 Å, 50 x 2.1 mm, Phenomenex, Torrance, California, USA) in combination with various methanol and acetonitrile gradients in order to separate all mycotoxins of interest. The critical pair for this separation was found to be 3-AcDON and 15-AcDON. PFP column in combination with methanol provided good separation of all isomers, so was selected for all further experiments. Next, the effect of mobile phase additives on mycotoxin signal intensities was investigated using PFP column and methanol gradient containing different additives (2 mM ammonium acetate, 0.1% FA or 0.1% AA). The effect of these additives on ionization in ESI(+) and ESI(-) was determined by comparing signal intensities obtained for high concentration mycotoxin standard in order to find out which additive provided the highest signal intensity for given mycotoxins. Finally, for ESI (-), the concentration of the best additive (AA) was

also optimized after testing 0.1%, 0.02% and 0.006% v/v AA. The final optimized conditions for LC-MS method are given in detail in 2.5.2 below.

2.5.2 Final validated LC-MS method

Chromatographic separation was performed using HPLC 1100 (Agilent Technologies, Santa Clara, CA, USA) with Phenomenex Kinetex Pentafluorophenyl (PFP) column (2.6 µm, 100 Å, 50 x 2.1 mm, Torrance, California, USA) and guard column (security guard ultra-cartridge for 2.1 mm ID columns) of the same type. The flow rate of 0.3 ml/min and the column temperature of 30^{o}C (CERA Column Temperature controller for liquid chromatography heater 250, Cera Inc, Baldwin Park, California, USA) were used for all analyses. For positive electrospray ionization (ESI(+)), the mobile phases A and B were water and methanol containing 0.1% AA (v/v). The following step gradient was used: 5% B for the first 1.0 min, increase to 50% B from 1.0 min to 3.0 min, keep isocratic at 50% B for 7 min, from 10 to 10.1 min increase to 70% B, from 10.1 to 17.5 min keep isocratic at 70% B, from 17.5 to 17.6 min increase to 98%, from 17.6 to 26.0 min keep isocratic at 98% B, and finally re-equilibrate the column at 5% B for 7 min. For negative electrospray ionization (ESI(-)), the mobile phases A and B were water and methanol, both containing 0.02% AA (v/v). The step gradient conditions were 5% B for the first 1.0 min, increase to 50% B from 1.0 min to 3.0 min, keep isocratic conditions at 50% B for 7 min, from 10.0 to 10.1 min increase to 70% B, from 10.1 to 17.5 min keep isocratic at 70% B, from 17.5 to 17.6 min, increase to 98% B, from 17.6 up to 30.0 min keep isocratic at 98% B, and finally re-equilibrate the column at 5% B for 7 min. The washing step is longer in ESI(-) method because the reduction in the amount of acetic acid in mobile phase increases retention of many analytes by several minutes, so longer wash step ensures any lipids are completely washed away before next injection. Injection volume for all analyses was 10 µL.

High-resolution MS analysis was performed using LTQ Velos Orbitrap equipped with HESI electrospray ionization source (Thermo Scientific, San Jose, CA, USA). The following ESI(+) parameters were used: source voltage 4 kV, capillary temperature 275^{o}C, source heater temperature 300^{o}C, sheath gas flow 20, S-lens RF 62% and auxiliary gas flow 5. For ESI(-): source voltage 3.5 kV, capillary temperature 350^{o}C, source heater temperature 300^{o}C, sheath gas flow 20, S-lens RF 63% and auxiliary gas flow 10 were used. For both ESI(±), mass range from 280 m/z up to 500 m/z, automatic gain control target 1 x 10^{6} ions, and resolution of 60,000 were used.

All analytical batches included analysis of appropriate extraction and solvent blanks, plasma calibration curves at the beginning and end of the analytical batch, one solvent calibration curve and injection of quality control samples every 6-10 sample injections to ensure LC-MS stability throughout the run.

For data acquisition and processing, Xcalibur software 2.7 SP1 was used. Mycotoxins were quantitated using the most intense ions which were extracted with ±5 ppm window. Mycotoxins that ionized efficiently in ESI(+) are aflatoxins and fumonisins with abundant protonated ions $[M+H]^{+}$, HT-2 with abundant ammonium adduct $[M+NH_4]^{+}$, T-2 and 15-AcDON

with abundant sodium adduct $[M+Na]^+$. Mycotoxins that exhibit better limits of detection in ESI(-) are type B trichothecenes including NIV, DON, FUS-X, and 3-AcDON producing abundant adducts with AA, $[M+CH_3COO-H]^-$ and ZEN, ZAN, β-ZAL, β-ZOL, α-ZAL, α-ZOL demonstrating highly intense deprotonated molecular ion $[M-H]^-$. OTA is the only mycotoxin that gave similar intensities in both ionization modes with the (de)protonated molecular ion. Supplementary Table S1 summarizes the monoisotopic masses of the most intense ions and retention times of all mycotoxins.

2.6 Method validation

The final fully optimized method was validated according to the procedures described in FDA guidance for bioanalytical method validation[34]. The main parameters for validation were selectivity, linearity, absolute recovery, accuracy, precision, stability and LOQ. Matrix-matched calibration curves were prepared each day for the quantification of mycotoxins in plasma in the range from 0.039 ng/ml to 10 ng/ml, except NIV for which range was 3x higher, 0.117 ng/ml to 30 ng/ml. Blank plasma was spiked with combined mycotoxin standard to yield 10 ng/ml concentration of each mycotoxin, except NIV at 30 ng/mL. Then, two fold serial dilution with blank plasma was used to prepare eight more standard concentration levels, followed by mixing and incubation for 1 hour at 4^{o}C. The calibration curve samples were then treated with three-step LLE (section 2.4) in the same way as validation samples and analyzed using LC-MS. 1/x weighted linear regression was used for all mycotoxins to build calibration curves from lower limit of quantification (LLOQ) to upper limit of quantification (ULOQ) for each analyte. 3-AcDONd_3 was used as internal standard in ESI(+) for all mycotoxins, while 3-AcDONd_3 and ^{13}C-ZEN were used for early- and late-eluting mycotoxins respectively in ESI(-). Absolute recovery was examined using three concentration levels: 0.5, 3 and 8 ng/ml (n=3) for 16 mycotoxins, except for NIV where 9 and 24 ng/ml were used. For NIV, only two concentration levels were used because of higher LLOQ for this mycotoxin (3 ng/mL) and the expectation that NIV levels in real samples will not exceed 30 ng/mL. Therefore, two selected concentrations are sufficient to characterize method performance over narrow range of 3-30 ng/mL. For absolute recovery, blank plasma samples were spiked before extraction and analysed against standard curve prepared using post-extracted spiked plasma. LOQ, intra-day accuracy and precision were measured using validation samples (n=6 replicates per concentration level) prepared by spiking blank plasma at seven concentration levels, 0.1, 0.2, 0.3, 0.5, 1, 3 and 10 ng/ml for all mycotoxins, except NIV where 3x concentration levels were used (3 ng/ml, 9 ng/ml and 30 ng/ml), for the same reasons as described for recovery. Inter-day precision and accuracy were evaluated at the same concentration levels with one replicate per day (n=5 days) measured against fresh plasma calibration curve prepared on that day. Inter- and intra-day precision was calculated using relative standard (RSD) formula for the concentrations determined from the calibration curves. Inter- and intra-day accuracy was calculated according to formula: accuracy= C_m/C_a*100%, where C_m is measured concentration in validation sample and C_a is the actual value[34]. LOQ was defined as the lowest concentration that meets minimum signal-to-noise ratio of 5 and the requirements for precision of ≤ 20% RSD and accuracy in the range of 80 – 120% based on inter- and intra-day

runs. The selectivity of the method was investigated using human plasma samples from 10 different biological sources to ensure no interferences. The stability of plasma samples spiked at 0.5 ng/ml and 3 ng/ml for all mycotoxins except for 9 ng/mL NIV was evaluated under following conditions (n=3 replicates per condition): autosampler at 4^{o}C, freeze/thaw (3 cycles), 3 h and 6 h bench stability at room temperature.

3. Results and discussion

3.1 Development of a sensitive LC-MS method

The main objective of this study was to develop a sensitive and reliable LC-MS multi-mycotoxin assay to allow simultaneous detection and quantification of common toxicologically important mycotoxins and their metabolites frequently found in Canadian food supply and thus of possible interest for biomonitoring. In order to develop LC-MS assay of suitable sensitivity for this application, method optimization included development of LC separation (Section 3.1.1), MS optimization (Section 3.1.2.) and detailed comparison of sample preparation techniques (Section 3.2) for a total of 20 mycotoxins. The final optimized method was fully validated according to procedures described in FDA Bioanalytical Method Validation guidelines[34] for seventeen mycotoxins: AFB1, AFB2, AFG1, AFG2, ZEN, α-ZOL, β-ZOL, ZAN, α-ZAL, β-ZAL, T-2, HT-2, DON, NIV, 15-AcDON, 3-AcDON and FUS-X in human plasma. The proposed method is not suitable (i) for OTA, FB1 and FB2 due to poor extraction recovery by LLE and (ii) irreproducible retention time of FB1 and FB2 on PFP LC.

3.1.1 Development of LC separation. The LC-MS method development focused on the isomer separation and achieving low limits of detection by optimization of mobile phase additives. For LC separation of all mycotoxins and particularly isomers, different columns (C18 and PFP), different solvents (methanol and acetonitrile), mobile phase modifiers (FA and AA), and gradients were manipulated to provide suitable separation. It was found that pentafluorophenyl column and methanol mobile phases provided the best separation of all mycotoxins and isomers, especially the separation of two isomeric compounds 15-AcDON and 3-AcDON which co-eluted when using acetonitrile-based mobile phases. The chromatographic separation of all mycotoxins is shown in Supplementary Figures S2 and S3. Isomers, including α-ZAL and β-ZOL, and α-ZOL, β-ZOL and ZAN are baseline separated except 3-AcDON and 15-AcDON where resolution of 0.7 is routinely obtained. In addition, 3-AcDON and 15-AcDON also show different ionization behaviour allowing their accurate quantitation. 3-AcDON ionized better in ESI(-) whereas 15-AcDON preferentially formed sodium adduct in ESI(+), permitting accurate quantitation. Our results of better chromatographic separation of these isomers on PFP versus C18 column are similar to Breidbach [35] and Baker *et al.* [36] findings. In addition, Qi *et al*. [37] also applied PFP column for the separation of four aflatoxins and OTA.

During subsequent sample preparation method development and validation, the performance of both PFP and F5 columns was tested. The manufacturer describes the columns as having the same chemistry, but the surface coverage of 2.4 µmol/m^2 for F5 versus 3.3 µmol/m^2

for PFP column. In our experiment, the selectivity of the two columns was considerably different, and F5 showed poor isomer separation even after gradient re-optimization. In addition, F5 showed build-up of triglycerides during long analytical batches which caused significant shifts in retention time of three mycotoxins (α-ZOL, ZAN, and ZEN) and loss of chromatographic resolution between critical pair of α-ZOL and ZAN when running long analytical batches (Supplementary Figure S4). Based on these results, F5 column is not recommended for this application, and PFP columns from other manufacturers should be carefully examined for similar issues if opting for PFP columns other than the recommended Kinetex PFP. These results are in agreement with Tamura *et al.* [33] who also found differences in isomer separation of selected mycotoxins when using PFP columns from different manufacturers.

3.1.2 Effect of mobile phase additives on ionization efficiency. The effect of additives (FA and AA) on ESI ionization efficiency was evaluated. The results showed that the intensity of 13 out of 15 tested mycotoxins increased from 1.4x up to 26x (for 3-AcDON) when using AA instead of FA [Supplementary Figure S5]. No significant improvement was observed for NIV and T-2. Taking into account only mycotoxins for which ESI(-) gives better limits of detection, the average improvement obtained by using AA as additive was 4.5x (not including 3-AcDON) showing it is important to use different mobile phases for positive and negative ESI analysis for this application. For the same mycotoxins, an additional improvement of signal intensity (ranging from 33% up to 89%) was achieved by decreasing AA concentration from 0.1% to 0.02% (v/v) in ESI(-) mode [Supplementary Figure S6]. Further decrease of AA concentration (0.02% versus 0.006%, v/v) showed an additional improvement of signal intensities ranging from 38% to 112%. However, 0.006% AA resulted in poor precision for all mycotoxins that form AA adduct (NIV, DON, FUS-X, and 3-AcDON). Area RSD% of NIV, DON, FUS-X, and 3-AcDON (n=6) were 28%, 51%, 46%, and 48%, respectively for 0.006% of AA versus 9%, 4%, 5%, and 4% for 0.02% of AA. In addition, the other mycotoxins detected in ESI(-) showed the same trend of poorer but still acceptable precision (range of RSD% was from 5-12% for 0.006% of AA versus 1-4% for 0.02% AA). Therefore, it is not recommended to decrease concentration of AA in mobile phase for mycotoxin analysis below 0.02% (v/v).

The influence of mobile phase additives on mycotoxin ionization efficiency has not been investigated in detail to the best of our knowledge. Variety of mobile phase additives are used for mycotoxin analysis, including FA, AA, ammonium formate, and ammonium acetate, but often only the final choice of additives is mentioned [38],[29],[39],[40],[41]. Among these studies, Huybrechts *et al.*[21] used 0.1% AA in ESI(-), and stated that both AA and acetate buffer mobile phases gave similar S/N ratios (similar sensitivity). Devreese *et al.* [28] used 0.1% AA and 0.01% AA for their multi-mycotoxin and zearalenone class-specific methods, respectively and state that these modifiers provided the best sensitivity[27], but the extent of improvement was not reported. Osteresch *et al.* [30] proposed the use of AA gradient and showed it provided better separation and S/N ratios than FA, but different gradients were tested for the two modifiers precluding direct side-by-side comparison. However, the potential of AA to increase signal intensity in ESI(-) was previously shown for other types of compounds. For example, Wu *et al.*

[38] examined the effect of four mobile phase additives: formic, acetic, propionic, and n-butyric acids and their concentrations on the signal intensities of four androgen modulators without acidic functional groups in ESI(-). The maximum improvement of ionization efficiency (about 30%-50%) of four chosen compounds was obtained with 1 mM AA (equivalent to 0.006% v/v)[42]. Zhang *et al.* [39] examined FA, AA ammonium acetate, and ammonium fluoride and their concentrations on the ESI(-) ionization efficiency of 26 different standards and untargeted metabolomics of urine. 1 mM AA provided the highest ionization efficiency for 23 out of 26 standards and the highest metabolite coverage/intensity for untargeted metabolomics method. Although more fundamental research on this topic is needed, it is proposed that the observed good performance of AA for ESI(-) is due to a combination of factors including optimal pH of droplet environment (different than bulk pH), facilitating electrochemical reduction which in turn may improve droplet charging, high gas phase proton affinity of weak acid anions and sufficiently small molecular volume so that the additive itself does not suppress analyte ionization[42],[43].

In summary, final optimized LC-MS method uses pentafluorophenyl column and the mobile phase containing water/methanol with 0.1% AA and water/methanol with 0.02% AA for ESI(+) and ESI(-), respectively to achieve the best possible limits of detection for all mycotoxins of interest.

3.2 Development of sample preparation method for multi-mycotoxin analysis

Sample preparation is a crucial step for the development of this multi-class mycotoxin method. The mycotoxins of interest in this work are chemically diverse compounds with acidic, neutral and/or basic properties, and cover wide polarity scale (logP from -1.9 to 4.74[44],[45],[46],[47]). High recovery was important for this application since mycotoxins are expected to be present in low concentrations (~ pg/mL). In order to obtain high analyte recovery, good selectivity, and to minimize matrix effects in complex matrix such as plasma, several types of sample preparation techniques were initially investigated as summarized in Supplementary Figure 1. Among these, solvent precipitation with acetonitrile, Oasis HLB SPE, and LLE (ethyl acetate versus methyl tert-butyl ether with or without acidification and salting out) were selected for further detailed evaluation as described in section 2.3.

The simplest procedure, protein precipitation with acetonitrile similar to the method proposed by Devreese *et al.* [29] for pig plasma, resulted in low method recoveries of all aflatoxins (less than 45%). Aflatoxins do not show significant matrix effects confirming that this is due to low extraction recovery (Figure 1). Recovery below 80% was observed for NIV, 15-AcDON, α-ZOL and ZAN, but matrix effect evaluation for these mycotoxins indicates that this is due to ionization suppression rather than poor extraction recovery (Figure 1). Overall, our results for extraction recovery using protein precipitation matched well Devreese *et al.* [29], after taking into account matrix effects except for AFB1. The difference in recovery observed for this mycotoxin may be due to different anticoagulant used (heparinized plasma versus citrated plasma), different species of plasma or the differences in chromatographic separation.

SPE methods, and especially Oasis HLB SPE were successfully applied for the mycotoxin analysis in various matrices, such as aqueous environmental samples[48], food[49] and urine samples[50],[20]. The wide-spread application of Oasis HLB sorbent is explained by its capability to retain different classes of mycotoxins, covering wide range of polarity. However, this wide selectivity can also result in insufficient sample clean-up and/or requirement to extract highly hydrophobic compounds prior to SPE in order to increase the extraction efficiency of mycotoxins. For example, food supplement samples treated using combination of LLE with ethyl acetate and Oasis HLB SPE required removal of nonpolar compounds with hexane prior to SPE[49]. Solfrizzo *et al*.[46] developed multi-analyte LC-MS/MS method for 7 mycotoxins in urine, which required two sequential SPEs, multi-toxin immunoaffinity column and Oasis HLB SPE in order to get proper clean up, high recovery, and repeatability for all stated mycotoxins. Oasis HLB SPE method (Figure 1a) developed in this work did not provide high overall recovery of ZEN (21%) and its metabolites (α-ZOL (8%) and β-ZOL (45%)), ZAN (10%), NIV (57%) and 15-AcDON (55%), OTA (16%), and AFB1 (69%) [Figure 1a]. Matrix effect experiment [Figure 1b] showed significant suppression for: ZEN (57%) and α-ZOL (17%), β-ZOL (46%), ZAN (32%), NIV (54%), 15-AcDON (29%), 3-AcDON (58%), FUS-X (76%), β-ZAL (78%), α-ZAL (71%). For majority of mycotoxins ionization suppression resulted in poor overall recovery, except for AFB1 and OTA where low extraction efficiency also played a role. It should be noted that SPE method presented here incorporates an enrichment step versus what was used for LLE, which significantly contributes to the observed ionization suppression. We also evaluated overall recovery for HLB SPE after 5x-dilution (without inclusion of evaporation/reconstitution step) to match LLE, and we still observed low overall recovery for AFG2, AFG1, OTA, α-ZOL, ZAN and ZEN. Further optimization of SPE wash and elution solvents may further improve selectivity of SPE, but was not further explored in this study as LLE provided acceptable performance and lower cost per sample as discussed below.

To examine the recovery of mycotoxins using LLE, selectivity of ethyl acetate versus methyl tert-butyl ether (MTBE) was first investigated. The results obtained showed that ethyl acetate provided higher recovery for all mycotoxins of interest [Supplementary Figure S7b]. The number of extraction steps required for complete recovery was investigated next [Supplementary Figure S8]. Two-step LLE showed significant increase in recovery as expected theoretically, while third step provided noticeable gains in recovery for NIV, DON, FUS-X, 15-AcDON, HT-2 and T-2. Based on these results, three-step LLE was selected. Considering the recovery of acidic mycotoxins was very low, the effect of plasma acidification prior to LLE was also investigated. Considering pKa values of FB1 (pKa 3.16[44]), FB2 (pKa 3.16[44]) and OTA (pKa 4.2-4.4[51]), pre-spiked plasma samples were acidified with 1% of FA to pH of 4 before one-step LLE to increase extraction efficiency. As expected, this enhanced the recovery of OTA (19.9%), FB1 (4.9%) and FB2 (8.9%), but unfortunately also significantly reduced the recovery of FUS-X, AFG1, AFB1, β-ZOL, T-2, HT-2, α-ZOL, ZAN and ZEN [Supplementary Figure S7a]. Supplementary Figure S9 shows that doing acidification of plasma before or after two-step LLE results in improved extraction recovery of acidic mycotoxins (OTA, FB1 and FB2) but lowers the recovery

of all other mycotoxins so lower pH values were not further explored. Finally, it was decided not to include pH adjustment step in the final method.

Salting out effect was also investigated to improve the recovery for polar compounds, such as NIV. Addition of salts at high concentration can induce extraction of polar compounds from aqueous phase to organic one. $MgSO_4$ was selected for this purpose based on the study by Song *et al*. [39]. They investigated the influence of different salts on extraction efficiency of 12 mycotoxins using a salting-out assisted LLE that included addition of salt to urine samples before the extraction with ethyl acetate followed by addition of acetonitrile to the remaining sample. The highest recovery was achieved with $MgSO_4$ and it helped to improve recoveries of all mycotoxins, especially polar mycotoxins such as NIV, DON, and FB1 recovery of which increased from a few percent to almost 100% [39]. The use of $MgSO_4$ in our LLE method improved the recovery of the most polar mycotoxin NIV from 24% up to 34% [Supplementary Figure S9], but reduced overall recovery of all other mycotoxins. Sequential acidification followed by salt addition [Supplementary Figure S9] also did not result in acceptable recovery of both acidic and neutral/basic mycotoxins. Based on all of these results, three step LLE using ethyl acetate without pH or salt adjustment was selected as the best LLE method for this application.

Tolosa *et al.* [31] and Escrivá *et al.*[52] also showed that ethyl acetate provided better extraction recovery than chloroform for various mycotoxins in plasma and urine samples, respectively using dispersive liquid-liquid microextraction format. Qi *et al*. [37] compared the extraction efficiency of ethyl acetate and toluene for four aflatoxins and OTA from snus and smokeless tobacco products, whereas Belhassen *et al.* [53] used ethyl acetate for extraction of six zeranols from human urine samples with high recovery. Three type of sample preparation techniques, reversed phase SPE, LLE with chloroform and LLE with mixture of acidified acetonitrile and ethyl acetate, were also compared for the extraction of five aflatoxins and OTA from milk[54]. Both LLE methods resulted in better recovery than SPE, with the highest recovery provided by mixture of acidified acetonitrile and ethyl acetate [54].

Comparing the results of all sample preparation techniques tested showed that there was no single method that could provide the recovery of all mycotoxins above 80% [Figure 1a]. Figure 1 summarizes only the results of the three best methods from each technique; protein precipitation with ACN, three-step LLE with ethyl acetate, and Oasis HLB SPE. Based on the fact that three-step LLE showed no significant matrix effect for any of the mycotoxins and achieved recoveries above 80% for FUS-X, 15-AcDON, 3-AcDON, AFG2, AFG1, AB2, AFB1, α-ZOL, ZAN, and ZEN mycotoxins, it was selected as the best method for this application. The presence of plasma co-eluting compounds with mycotoxins can enhance/supress their signal intensities resulting in an inaccurate quantification, and effecting reproducibility and accuracy of the method. In addition, plasma samples without proper sample clean-up can cause early LC column aging. Therefore, the advantages of using more selective sample preparation methods such as LLE for this application are multi-fold. However, the chosen method provides unacceptably low recovery for OTA (0.6%), FB1 (0.7%), and FB2 (0.6%). Therefore, it is not recommended to monitor these

analytes using the current method and OTA, FB1 and FB2 were excluded from method validation. A separate method that can provide high recovery and reduce matrix effect has to be developed for these analytes.

3.3 Results of method validation

The primary goal of method validation is to assess the method performance for intended application. There is no specific validation guidance established for exposure monitoring studies, so to the method performance in this study was evaluated using the procedures set by FDA for the evaluation of drugs and their metabolites in biological matrices using LC-MS, but slightly wider acceptance criteria of 80-120% accuracy and ≤20% RSD was applied for this biomonitoring method due to measurement of very low concentrations of interest, close to instrumental LLOQ. The method validation was performed for 17 out of 20 mycotoxins, OTA and fumonisins were excluded based on their unacceptable recovery during method development. The main parameters evaluated during method validation included: linearity, recovery, accuracy, precision, LOQ, selectivity and stability.

Matrix-matched calibration curves were linear for all mycotoxins in the range of LOQ to 10 ng/ml and LOQ to 30 ng/ml for NIV with the average correlation coefficients in the range from 0.995 to 1.000. Mean absolute recoveries of 16 mycotoxins using the finalized method ranged from 74% to 113%, except NIV (17%) [Supplementary Figure S10]. The low absolute recovery of NIV led to higher LOQ (3 ng/ml) than for other mycotoxins and also necessitated higher spiking concentration levels for validation samples for NIV.

LOQ was determined based on the results of precision and accuracy obtained during intra- and inter-day experiments to meet the following requirements ≤ 20% RSD and the range of 80-120%, respectively (Supplementary Figure 2). Two compounds that did not meet the FDA requirements for LOQ are α-ZOL and ZEN. Their intra-day accuracy were 146% for α-ZOL with 31% RSD% and 124% for ZEN with 6.5% RSD. The results obtained are summarized in Table 1.

The mean intra-day accuracy ranged from 85.8% to 116.4%, and intra-day precision ranged from 1.6% to 12.5% RSD for all the concentration levels higher than LOQ except for α-ZOL [Supplementary Figure S11]. This meets requirements of 80-120% accuracy and %RSD ≤ 20% for 16 out of 17 mycotoxins. α-ZOL showed poorer accuracy for 1 ng/ml (72.9% with 16.8% RSD). Inter-day accuracy and precision results are shown in Figure 2 and Supplementary Table 2. Mean accuracy ranged from 85.5% to 111.5%, while precision ranged from 2.7 to 15.6% RSD. The only exception was α-ZOL at 1 ng/ml with acceptable accuracy of 93.3% but poor % RSD of 17.7%. In summary, intra- and inter-day accuracy and precision results show that this method performed well for the analysis of trace concentrations of mycotoxins, and that accuracy of 80-120% and precision of ≤20% RSD can be achieved for all mycotoxins except α-ZOL.

The selectivity and detailed matrix effect experiments show that the poorer (and variable) precision and accuracy observed for α-ZOL were primarily due to matrix effects and could not fully be compensated by ^{13}C-ZEN standard which elutes at different retention time

(Supplementary Figure S12). The selectivity experiment showed that there was a co-eluting highly intense peak at m/z 277.1447 that can interfere with α-ZOL and impact its accuracy/precision during filling of the Orbitrap. The detailed investigation of absolute matrix effects in the same 10 individual plasma samples that were spiked at low mycotoxin concentrations (close to LOQ), show the influence of matrix on signal intensity [Figure 3]. The experiment revealed that the zeranols could be prone to suppression in individual samples. All zeranols were suppressed more in female than male plasma samples, possibly due to suppression by female sex hormones which are structurally similar and expected to elute in similar retention time window. Among all zeranols, α-ZOL was the most affected by the plasma components, with signal intensity dropping up to 29% (mean for female samples). The best solution to compensate and to monitor matrix effect for zeranols, and to have accurate quantification, especially for α-ZOL is to use a labelled internal standard for this analyte. Another option, in the absence of labelled standard, is to re-analyze α-ZOL positive samples using a single-point standard addition to obtain more accurate concentrations of this mycotoxin, if required. A detailed study by Fabregat-Cabello *et al.*[55] compared different calibration approaches for mycotoxins in food and feed, and found single-point standard addition is the most efficient option of accurate quantitation when an isotopically labelled internal standard is not available[55]. Finally, the observed matrix effects in some of the individual plasma samples will slightly impact the LOD of the method in individual samples for zeranol class of mycotoxins. The highest impact will be for α-ZOL, where 2-3x higher LODs as well as LOQs may be observed for the samples with severe matrix interference and suppression, while for other zeranols the effect on LOD or LOQ will be slight to negligible. For other mycotoxin classes, Figure 3 shows that there were no significant absolute matrix effects detected across various individual samples. This confirms that the method will be able to provide highly accurate and precise results for these mycotoxins even if individual internal standards for each mycotoxin are not available.

The investigation of stability, namely, prepared extract stability on autosampler at 4^{o}C for 96 h and stability during 3 freeze/thaw cycles showed that all analytes are stable at these conditions except DON where significant increase in recovery was observed for 96 h 0.5 ng/mL sample (Supplementary Figures S13 and S14). 96 h stability shows that very long analytical batches, suitable for exposure monitoring studies, can be accommodated using current method. However, tests of 3 h and 6 h bench stability of plasma samples at room temperature revealed that two compounds out of 17, AFG2 and AFG1 were not stable at these conditions [Supplementary Figure S13]. This result is in agreement with the study by Diaz *et al.*[56]. They found that AFG2 and AFG1 stability depends on temperature and composition of the solvent[56]. Huge losses of AFG2 and AFG1 were observed when they were dissolved in organic solvent with any amount of water and kept for 24 hours at 20^{o}C. However, no significant decrease was noticed for AFG2 and AFG1 dissolved in organic solvents containing more than 20% of organic solvent and kept at 5^{o}C. Since, it is important to have 20% organic solvent evaluation of autosampler stability in this paper was done using 20% organic solvent and showed similar stability of these

compounds. For the purposes of this method, unacceptable benchtop stability of AFG1 and AFG2 in plasma means that plasma should be thawed on ice and processed immediately after thawing.

The method developed and validated in this work provides better sensitivity than the multi-class mycotoxin method proposed for pig plasma (2-10 ng/mL)[29] and analyte-specific methods proposed in the same work (0.5 -5 ng/mL). In comparison to multi-mycotoxin method for dried serum spots, our assay provides better LOQs for T-2, ZAN, ZEN and HT-2, similar LOQ for AFG2 and slightly poorer LOQs for other aflatoxins, but it should be noted that S/N of 10 criteria was used for the determination of LOQ without stringent accuracy/precision requirements applied in current study[32].

The LLOQs for aflatoxins in our work were 0.1 ng/ml for AFB1 and 0.2 ng/mL for the remaining aflatoxins. These results are better or similar to the class-specific methods used for the direct measurement of aflatoxins in plasma or serum. For instance, reported LLOQs for aflatoxins using LC-MS/MS analysis ranged from 1 ng/mL for ethyl acetate LLE [57], 0.21-0.43 ng/mL for dilute-and-shoot method by Cao *et al*. [58], and 0.13-0.42 ng/mL for HLB SPE [59]. It should be noted that all three of these studies used S/N ratio for LLOQ determination, while our study used the more stringent accuracy and precision requirements. Corcuera *et al*. obtained LLOQ of 2 ng/mL for AFB1 in combination with UHPLC with Postcolumn Fluorescence Derivatization (UHPLC-FLD) analysis using similar LLOQ criteria to our study [60]. For type A trichothecenes, HT-2 and T-2, we established LLOQs of 0.2 ng/mL. This is significantly better than reported LLOQs in literature for animal plasma matrices which ranged from 1 ng/mL for pig plasma [61], 2.5 ng/mL for chicken plasma [61] and 1-2 ng/mL for pig plasma [62]. Our results are comparable to analyte-specific method for AFB1 and T-2 where LOQ of 0.05 ng/mL was obtained in rat plasma after protein precipitation and SPE and using S/N criterion of 10 [16].Type B trichothecenes had LLOQs of 0.2 ng/mL (3AcDON and 15AcDON) and 0.3 ng/mL (DON) in our study. In contrast, Broakert *et al*. reported LLOQs for 1-2 ng/mL for chicken plasma and 0.1-1 ng/mL for pig plasma for the same three analytes when using acetonitrile protein precipitation [63], de Baere *et al*. reported 1 ng/mL for pig plasma (DON), 1.25 ng/mL for chicken plasma for DON when using a combination of protein precipitation and HLB SPE[61], and Brezina *et al*. reported 0.45 ng/mL using HLB SPE for DON [41]. For other members of this family, NIV and FUS-X we did not find any relevant literature for comparison. For zearalenone class, our LLOQs ranged from 0.1 – 0.5 ng/mL. Songsermsakul *et al*. reported 0.5-0.6 ng/mL for determination of this class in horse plasma [64], whereas Brezina *et al*. reported LLOQs between 0.08 to 2.37 ng/mL using HLB SPE in pig serum [41]. In addition, LOQs obtained for ZEN and its metabolites are better in current study than for analyte-specific method that relied on protein precipitation (0.2-5 ng/mL) in chicken and pig plasma [27].Overall, the LLOQs for our multi-mycotoxin method compare well with class-specific methods previously reported in literature and provide similar or significantly better LLOQs while expanding the number of mycotoxins that can be evaluated. In addition, for many of the mycotoxins under study, this is first method developed for their measurement in human plasma.

For the mycotoxins included in current method, there is very limited data available on their concentrations and occurrence in plasma or serum. In a study of serum from 213 children, De Santis *et al.* [29] reported range from <LOD to 27.9 ng/mL for DON (19.5% positive), ZEN <LOD to 3.9 ng/mL (5.4% positive) and <LOD to 0.73 ng/mL for AFB1 (22.9% positive). Based on these

reported concentrations, our method should be suitable for human biomonitoring studies and provides better LOQs for two out of these three mycotoxins, while providing capability to simultaneously monitor additional 14 mycotoxins.

The simultaneous analysis is critical aspect of this work. During biomonitoring studies, it is not known *a priori* which mycotoxins may be present. The availability of a method that can accurately measure large number of mycotoxins that commonly occur in the food supply reduces the cost of analysis per sample over methods that would focus only on a single mycotoxin. It allows monitoring of large number of samples (hundreds or thousands) to identify sub-populations that may exceed recommended exposure guidelines. The use of high-resolution Orbitrap mass spectrometry and the extraction/separation methods which can accommodate mycotoxins of various polarity, also permits the use of this method for screening of additional mycotoxin metabolites for later inclusion in the panel. These mycotoxin metabolites can be missed by targeted strategies relying on multiple reaction monitoring, and thus an underestimation of mycotoxin prevalence and concentrations can be reported. This has been illustrated well in recent studies on urine, where direct monitoring of urine samples for parent compounds resulted in low detection of positive study samples (0-8%) for ZEN and its metabolites, whereas inclusion of enzymatic hydrolysis and immunoaffinity cleanup step showed much higher prevalence across all studies ranging from 17-100% depending on geographic location and analyte[65]. In addition, high-resolution MS data can also be retrospectively examined for other mycotoxins that may become of particular health interest. The main disadvantages of high-resolution MS for this application are large file size, high cost of instrumentation and data analysis time required for retrospective analysis.

4. Conclusions

The goal of this study was to develop a sensitive and reliable LC-MS based multi-mycotoxin assay allowing simultaneous detection and quantification of common toxicologically important mycotoxins and their metabolites. The method was successfully developed and validated for 17 out of 20 initially stated mycotoxins, with 15 of these mycotoxins meeting accuracy and precision of 80-120% and ≤20% RSD at all concentrations tested including LLOQ. High sensitivity of the method was achieved through careful optimization of sample preparation technique, chromatographic separation and mobile phase additive selection. With newer models of Orbitrap, additional shortening of analysis time and improved limits of detection can be anticipated. The cost per sample of the method was kept low by employing LLE and minimizing absolute matrix effects which permits the use of limited number of isotopically labelled internal standards for quality control purposes. This makes the proposed method cost-effective for implementation in large-scale population monitoring efforts. The main disadvantage of our method is that additional IS for α-ZOL is highly desirable and should be incorporated in future studies whenever possible (or alternately, standard addition method should be used for α-ZOL positive samples). Due to the use of high-resolution MS, the method can also be used for screening of the presence of additional mycotoxins and their metabolites for future inclusion in

the panel and to study mycotoxin metabolism in humans in more detail. It will also allow to study temporal and inter-individual differences of mycotoxin concentrations as insufficient data exists for the mycotoxins in our panel. To the best of our knowledge, this is the first LC-MS method for highly sensitive analysis and quantification of 17 mycotoxins in human plasma samples. The availability of this method opens up new and exciting opportunities for direct exposure monitoring of these common contaminants.

ACKNOWLEDGMENTS

This work was supported by Fonds du Recherche du Quebec [FRQNT grant number 2014-NC-172924 and FRQS grant number 33273] and the Centre for Biological Applications of Mass Spectrometry at Concordia University. I.S. also gratefully acknowledges scholarship support from the Centre of Biological Applications of Mass Spectrometry at Concordia University.

REFERENCES

[1] A.L. Capriotti, G. Caruso, C. Cavaliere, P. Foglia, R. Samperi, A. Laganà, Multiclass mycotoxin analysis in food, environmental and biological matrices with chromatography/mass spectrometry, Mass Spectrom. Rev. 31 (2012) 466–503. doi: 10.1002/mas.20351. Epub 2011 Nov 7.

[2] H. Fromme, M. Gareis, W. Völkel, C. Gottschalk, Overall internal exposure to mycotoxins and their occurrence in occupational and residential settings - An overview, Int. J. Hyg. Environ. Health. 219 (2016) 143–165. doi:10.1016/j.ijheh.2015.11.004.

[3] G.A. Lombaert, P. Pellaers, V. Roscoe, M. Mankotia, R. Neil, P.M. Scott, Mycotoxins in infant cereal foods from the Canadian retail market, Food Addit.Contam. 20 (2003) 494–504. doi:10.1080/0265203031000094645.

[4] V. Roscoe, G. Lombaert, V. Huzel, G. Neumann, J. Melietio, D. Kitchen, S. Kotello, T. Krakalovich, R. Trelka, P.M. Scott, Mycotoxins in breakfast cereals from the Canadian retail market: a 3-year survey, Food Addit. Contam. Part A. Chem. Anal. Control. Expo. Risk Assess. 25 (2008) 347–355. doi:10.1080/02652030701551826.

[5] G. Lombaert, P. Pellaers, G. Neumann, D. Kitchen, V. Huzel, R. Trelka, S. Kotello, P.M. Scott, Ochratoxin A in dried vine fruits on the Canadian retail market, Food Addit. Contam. 21 (2004) 578–85. doi:10.1080/02652030410001687681.

[6] L. Ng, M. Mankotia, P. Pantazopoulos, R.J. Neil, P.M. Scott, Ochratoxin A in wine and grape juice sold in Canada, Food Addit. Contam. 21 (2004) 971–981. doi:10.1080/02652030400000653.

[7] S.T. Tran, T.K. Smith, G.N. Girgis, A survey of free and conjugated deoxynivalenol in the 2008 corn crop in Ontario, Canada, J. Sci. Food Agric. 92 (2012) 37–41. doi:10.1002/jsfa.4674. Epub 2011 Oct 14.

[8] K. Muñoz, M. Blaszkewicz, G.H. Degen, Simultaneous analysis of ochratoxin A and its major metabolite ochratoxin alpha in plasma and urine for an advanced biomonitoring of the mycotoxin, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 878 (2010) 2623–2629. doi:10.1016/j.jchromb.2009.11.044.

[9] E. Märtlbauer, E. Usleber, R. Dietrich, E. Schneider, Ochratoxin A in human blood serum - Retrospective long-term data, Mycotoxin Res. 25 (2009) 175–186. doi:10.1007/s12550-009-0025-z.

[10] N.W. Turner, S. Subrahmanyam, S.A. Piletsky, Analytical methods for determination of mycotoxins: A review, Anal. Chim. Acta. 632 (2009) 168–180. doi:https://doi.org/10.1016/j.aca.2008.11.010.

[11] N.W. Turner, H. Bramhmbhatt, M. Szabo-Vezse, A. Poma, R. Coker, S.A. Piletsky, Analytical methods for determination of mycotoxins: An update (2009-2014), Anal. Chim.

Acta. 901 (2015) 12–33. doi:10.1016/j.aca.2015.10.013.

[12] E. Heyndrickx, I. Sioen, M. Bellemans, M. De Maeyer, A. Callebaut, S. De Henauw, S. De Saeger, Assessment of mycotoxin exposure in the Belgian population using biomarkers: Aim, design and methods of the BIOMYCO study, Food Addit. Contam. - Part A. 31 (2014) 924–931. doi:https://doi.org/10.1080/19440049.2014.900192.

[13] N. Ali, K. Muñoz, G.H. Degen, Ochratoxin A and its metabolites in urines of German adults—An assessment of variables in biomarker analysis, Toxicol. Lett. 275 (2017) 19–26. doi:https://doi.org/10.1016/j.toxlet.2017.04.013.

[14] P.F. Scroll, J.D. Groopman, Long-term stability of human aflatoxin B1 albumin adducts assessed by isotope dilution mass spectrometry and high-performance liquid chromatography-fluorescence, Cancer Epidemiol Biomarkers Prev. 17 (2008) 1436–1439. doi:10.1158/1055-9965.EPI-07-2926.Long.

[15] G. Shephard, P. Thiel, E. Sydenham, Initial studies on the toxicokinetics of fumonisin B1 in rats, Food Chem. Toxicol. 30 (1992) 277–279. doi:https://doi.org/10.1016/0278-6915(92)90004-5.

[16] Z. Han, Z. Zhao, S. Song, G. Liu, J. Shi, J. Zhang, Y. Liao, D. Zhang, Y. Wu, S. De Saeger, A. Wu, Establishment of an isotope dilution LC-MS/MS method revealing kinetics and distribution of co-occurring mycotoxins in rats, Anal. Methods. 4 (2012) 3708. doi:10.1039/c2ay25891a.

[17] B. Huybrechts, J.C. Martins, P. Debongnie, S. Uhlig, A. Callebaut, Fast and sensitive LC–MS/MS method measuring human mycotoxin exposure using biomarkers in urine, Arch. Toxicol. 89 (2015) 1993–2005. doi:10.1007/s00204-014-1358-8.

[18] E. Heyndrickx, I. Sioen, B. Huybrechts, A. Callebaut, S. De Henauw, S. De Saeger, Human biomonitoring of multiple mycotoxins in the Belgian population: Results of the BIOMYCO study, Environ. Int. 84 (2015) 82–89. doi:10.1016/j.envint.2015.06.011.

[19] J. Gerding, N. Ali, J. Schwartzbord, B. Cramer, D.L. Brown, G.H. Degen, H.-U. Humpf, A comparative study of the human urinary mycotoxin excretion patterns in Bangladesh, Germany, and Haiti using a rapid and sensitive LC-MS/MS approach, Mycotoxin Res. (2015) 127–136. doi:10.1007/s12550-015-0223-9.

[20] M. Solfrizzo, L. Gambacorta, A. Visconti, Assessment of multi-mycotoxin exposure in southern Italy by urinary multi-biomarker determination, Toxins (Basel). 6 (2014) 523–538. doi:10.3390/toxins6020523.

[21] S. Wallin, L. Gambacorta, N. Kotova, E. Warensjö Lemming, C. Nälsén, M. Solfrizzo, M. Olsen, Biomonitoring of concurrent mycotoxin exposure among adults in Sweden through urinary multi-biomarker analysis, Food Chem. Toxicol. 83 (2015) 133–139. doi:10.1016/j.fct.2015.05.023.

[22] E. Njumbe Ediage, J. Diana Di Mavungu, S. Song, A. Wu, C. Van Peteghem, S. De Saeger, A direct assessment of mycotoxin biomarkers in human urine samples by liquid

chromatography tandem mass spectrometry, Anal. Chim. Acta. 741 (2012) 58–69. doi:10.1016/j.aca.2012.06.038.

[23] B. Warth, M. Sulyok, P. Fruhmann, H. Mikula, F. Berthiller, R. Schuhmacher, C. Hametner, W.A. Abia, G. Adam, J. Fröhlich, R. Krska, Development and validation of a rapid multi-biomarker liquid chromatography/tandem mass spectrometry method to assess human exposure to mycotoxins, Rapid Commun. Mass Spectrom. 26 (2012) 1533–1540. doi:10.1002/rcm.6255.

[24] B. Warth, M. Sulyok, R. Krska, LC-MS/MS-based multibiomarker approaches for the assessment of human exposure to mycotoxins, Anal. Bioanal. Chem. 405 (2013) 5687–5695. doi:10.1007/s00216-013-7011-1.

[25] J. Rubert, N. León, C. Sáez, C.P.B. Martins, M. Godula, V. Yusà, J. Mañes, J.M. Soriano, C. Soler, Evaluation of mycotoxins and their metabolites in human breast milk using liquid chromatography coupled to high resolution mass spectrometry, Anal. Chim. Acta. 820 (2014) 39–46. doi: 10.1016/j.aca.2014.02.009.

[26] Z. Zhao, N. Liu, L. Yang, Y. Deng, J. Wang, S. Song, S. Lin, A. Wu, Z. Zhou, J. Hou, Multi-mycotoxin analysis of animal feed and animal-derived food using LC-MS/MS system with timed and highly selective reaction monitoring, Anal. Bioanal. Chem. 407 (2015) 7359–7368. doi:10.1007/s00216-015-8898-5.

[27] S. De Baere, A. Osselaere, M. Devreese, L. Vanhaecke, P. De Backer, S. Croubels, Development of a liquid-chromatography tandem mass spectrometry chromatography high-resolution mass spectrometry method for the quantitative determination of zearalenone and its major metabolites in chicken and pig plasma, Anal. Chim. Acta. 756 (2012) 37–48. doi:10.1016/j.aca.2012.10.027.

[28] A.B. Serrano, A.L. Capriotti, C. Cavaliere, S. Piovesana, R. Samperi, S. Ventura, A. Laganà, Development of a rapid LC-MS/MS method for the determination of emerging fusarium mycotoxins enniatins and beauvericin in human biological fluids, Toxins (Basel). 7 (2015) 3554–3571. doi:10.3390/toxins7093554.

[29] M. Devreese, S. De Baere, P. De Backer, S. Croubels, Quantitative determination of several toxicological important mycotoxins in pig plasma using multi-mycotoxin and analyte-specific high performance liquid chromatography-tandem mass spectrometric methods, J. Chromatogr. A. 1257 (2012) 74–80. doi:10.1016/j.chroma.2012.08.008.

[30] B. De Santis, M.E. Raggi, G. Moretti, F. Facchiano, A. Mezzelani, L. Villa, A. Bonfanti, A. Campioni, S. Rossi, S. Camposeo, S. Soricelli, G. Moracci, F. Debegnach, E. Gregori, F. Ciceri, L. Milanesi, A. Marabotti, C. Brera, Study on the association among mycotoxins and other variables in children with autism, Toxins (Basel). 9 (2017) 1–20. doi:10.3390/toxins9070203.

[31] J. Tolosa, G. Font, J. Mañes, E. Ferrer, Multimycotoxin analysis in water and fish plasma by liquid chromatography-tandem mass spectrometry, Chemosphere. 145 (2016) 402–408. doi:10.1016/j.chemosphere.2015.11.085.

[32] B. Osteresch, S. Viegas, B. Cramer, H.U. Humpf, Multi-mycotoxin analysis using dried blood spots and dried serum spots, Anal. Bioanal. Chem. 409 (2017) 3369–3382. doi:10.1007/s00216-017-0279-9.

[33] G. Schatzmayr, E. Streit, Global occurrence of mycotoxins in the food and feed chain: facts and figures, World Mycotoxin J. 6 (2013) 213–222. doi:10.3920/WMJ2013.1572.

[34] Food and Drug Administration, Draft Guidance for Industry Bioanalytical Method Validation, 2013. doi:http://www.labcompliance.de/documents/FDA/FDA-Others/Laboratory/f-507-bioanalytical-4252fnl.pdf.

[35] A. Breidbach, The Impact of Superficially Porous Particles and New Stationary-Phase Chemistries on the LC-MS Determination of Mycotoxins in Food and Feed, LC GC. 34 (2016) 10–14.

[36] D. Baker, C. Titman, N. Loftus, J. Horner, Multi-residue analysis of 18 regulated mycotoxins by LC-MS / MS, ASMS 2017 TP-185, 2017: pp. 1–8.

[37] D. Qi, T. Fei, H. Liu, H. Yao, D. Wu, B. Liu, Development of Multiple Heart-Cutting Two-Dimensional Liquid Chromatography Coupled to Quadrupole-Orbitrap High Resolution Mass Spectrometry for Simultaneous Determination of Aflatoxin B1, B2, G1, G2, and Ochratoxin A in Snus, a Smokeless Tobacco Product, J. Agric. Food Chem. 65 (2017) 9923–9929. doi:10.1021/acs.jafc.7b04329.

[38] B. De Santis, F. Debegnach, E. Gregori, S. Russo, F. Marchegiani, G. Moracci, C. Brera, Development of a LC-MS/MS method for the multi-mycotoxin determination in composite cereal-based samples, Toxins (Basel). 9 (2017) 169. doi:10.3390/toxins9050169.

[39] S. Song, E.N. Ediage, A. Wu, S. De Saeger, Development and application of salting-out assisted liquid/liquid extraction for multi-mycotoxin biomarkers analysis in pig urine with high performance liquid chromatography/tandem mass spectrometry, J. Chromatogr. A. 1292 (2013) 111–120. doi:10.1016/j.chroma.2012.10.071.

[40] F. Soleimany, S. Jinap, F. Abas, Determination of mycotoxins in cereals by liquid chromatography tandem mass spectrometry, Food Chem. 130 (2012) 1055–1060. doi:10.1016/j.foodchem.2011.07.131.

[41] U. Brezina, H. Valenta, I. Rempe, S. Kersten, H.U. Humpf, S. Dänicke, Development of a liquid chromatography tandem mass spectrometry method for the simultaneous determination of zearalenone, deoxynivalenol and their metabolites in pig serum, Mycotoxin Res. 30 (2014) 171–186. doi:10.1007/s12550-014-0200-8.

[42] Z. Wu, W. Gao, M.A. Phelps, D. Wu, D.D. Miller, J.T. Dalton, Favorable Effects of Weak Acids on Negative-Ion Electrospray Ionization Mass Spectrometry, Changes. 76 (2004) 839–847. doi:10.1016/j.biotechadv.2011.08.021.Secreted.

[43] X. Zhang, M.R. Clausen, X. Zhao, H. Zheng, H.C. Bertram, Enhancing the power of liquid chromatography-mass spectrometry-based urine metabolomics in negative ion mode by

optimization of the additive, Anal. Chem. 84 (2012) 7785–7792. doi:10.1021/ac3013835.

[44] D.S. Wishart, D. Tzur, C. Knox, R. Eisner, A.C. Guo, N. Young, D. Cheng, K. Jewell, D. Arndt, S. Sawhney, C. Fung, L. Nikolai, M. Lewis, M.A. Coutouly, I. Forsythe, P. Tang, S. Shrivastava, K. Jeroncic, P. Stothard, G. Amegbey, D. Block, D.D. Hau, J. Wagner, J. Miniaci, M. Clements, M. Gebremedhin, N. Guo, Y. Zhang, G.E. Duggan, G.D. MacInnis, A.M. Weljie, R. Dowlatabadi, F. Bamforth, D. Clive, R. Greiner, L. Li, T. Marrie, B.D. Sykes, H.J. Vogel, L. Querengesser, HMDB: The human metabolome database, Nucleic Acids Res. 35 (2007) 521–526. doi:10.1093/nar/gkl923.

[45] Http://www.hmdb.ca/, "HMDB: The Human Metabolome Database", http://www.hmdb.ca/ date accessed 2017/08/02, Http://www.hmdb.ca

[46] E. Lim, A. Pon, Y. Djoumbou, C. Knox, S. Shrivastava, A.C. Guo, V. Neveu, D.S. Wishart, T3DB: A comprehensively annotated database of common toxins and their targets, Nucleic Acids Res. 38 (2009) 781–786. doi:10.1093/nar/gkp934.

[47] "The Toxin and Toxin Target Database (T3DB)", http://www.t3db.ca/, date accessed 2017/08/02

[48] J. Schenzel, R.P. Schwarzenbach, T.D. Bucheli, Multi-residue screening method to quantify mycotoxins in aqueous environmental samples, J. Agric. Food Chem. 58 (2010) 11207–11217. doi:10.1021/jf102737q.

[49] J. Diana Di Mavungu, S. Monbaliu, M.-L. Scippo, G. Maghuin-Rogister, Y.-J. Schneider, Y. Larondelle, A. Callebaut, J. Robbens, C. Van Peteghem, S. De Saeger, LC-MS/MS multi-analyte method for mycotoxin determination in food supplements, Food Addit. Contam. Part A. Chem. Anal. Control. Expo. Risk Assess. 26 (2009) 885–895. doi:10.1080/02652030902774649.

[50] M. Solfrizzo, L. Gambacorta, V.M.T. Lattanzio, S. Powers, A. Visconti, Simultaneous LC-MS/MS determination of aflatoxin M 1, ochratoxin A, deoxynivalenol, de-epoxydeoxynivalenol, α and β-zearalenols and fumonisin B 1 in urine as a multi-biomarker method to assess exposure to mycotoxins, Anal. Bioanal. Chem. 401 (2011) 2831–2841. doi:10.1007/s00216-011-5354-z.

[51] E. Anil, I.M. Alkis, Ochratoxin A and Brewing Technology: A Review, J. Inst. Brew. 116 (2010) 23–32. doi:10.1002/j.2050-0416.2010.tb00394.x.

[52] L. Escrivá, L. Manyes, G. Font, H. Berrada, Mycotoxin analysis of human urine by LC-MS/MS: A comparative extraction study, Toxins (Basel). 9 (2017). doi:10.3390/toxins9100330.

[53] H. Belhassen, I. Jiménez-Díaz, R. Ghali, H. Ghorbel, J.M. Molina-Molina, N. Olea, A. Hedili, Validation of a UHPLC-MS/MS method for quantification of zearalenone, α-zearalenol, β-zearalenol, α-zearalanol, β-zearalanol and zearalanone in human urine, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 962 (2014) 68–74. doi:10.1016/j.jchromb.2014.05.019.

[54] P.D. Andrade, J.L.G. da Silva, E.D. Caldas, Simultaneous analysis of aflatoxins B1, B2, G1,

G2, M1 and ochratoxin A in breast milk by high-performance liquid chromatography/fluorescence after liquid-liquid extraction with low temperature purification (LLE-LTP), J. Chromatogr. A. 1304 (2013) 61–68. doi:10.1016/j.chroma.2013.06.049.

[55] N. Fabregat-Cabello, P. Zomer, J.V. Sancho, A.F. Roig-Navarro, H.G.. Mol, Comparison of approaches to deal with matrix effects in LC-MS/MS based determinations of mycotoxins in food and feed, World Mycotoxin J. 9 (2016) 149–161. doi:10.3920/WMJ2014.1872.

[56] P.A.M. Gonzalo J.Diaz, Sandra M. Cepeda, Stability of aflatoxins in solution, J. AOAC Int. 95 (2012) 1084–8. doi: https://doi.org/10.5740/jaoacint.11-017

[57] A. Santini, R. Ferracane, G. Meca, A. Ritieni, Comparison and improvement of the existing methods for the determination of aflatoxins in human serum by LC-MS/MS, Anal. Methods. 2 (2010) 884. doi:10.1039/b9ay00316a.

[58] X. Cao, X. Li, J. Li, Y. Niu, L. Shi, Z. Fang, T. Zhang, H. Ding, Quantitative determination of carcinogenic mycotoxins in human and animal biological matrices and animal-derived foods using multi-mycotoxin and analyte-specific high performance liquid chromatography-tandem mass spectrometric methods, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 1073 (2018) 191–200. doi:10.1016/j.jchromb.2017.10.006.

[59] Z.-Y. Chen, S. Ying, J.-H. Liu, P.-P. Zhan, Y.-G. Zhao, PRiME pass-through cleanup for the fast determination of aflatoxins in human serum by using LC-MS/MS, Anal. Methods. 8 (2016) 1457–1462. doi:10.1039/C5AY03099D.

[60] L.A. Corcuera, M. Ibáñez-Vea, A. Vettorazzi, E. González-Peñas, A.L. de Cerain, Validation of a UHPLC-FLD analytical method for the simultaneous quantification of aflatoxin B1 and ochratoxin a in rat plasma, liver and kidney, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 879 (2011) 2733–2740. doi:10.1016/j.jchromb.2011.07.039.

[61] S. De Baere, J. Goossens, A. Osselaere, M. Devreese, V. Vandenbroucke, P. De Backer, S. Croubels, Quantitative determination of T-2 toxin, HT-2 toxin, deoxynivalenol and deepoxy-deoxynivalenol in animal body fluids using LC-MS/MS detection, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 879 (2011) 2403–2415. doi:10.1016/j.jchromb.2011.06.036.

[62] Y. Sun, G. Zhang, H. Zhao, J. Zheng, F. Hu, B. Fang, Liquid chromatography-tandem mass spectrometry method for toxicokinetics, tissue distribution, and excretion studies of T-2 toxin and its major metabolites in pigs, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 958 (2014) 75–82. doi:10.1016/j.jchromb.2014.03.010.

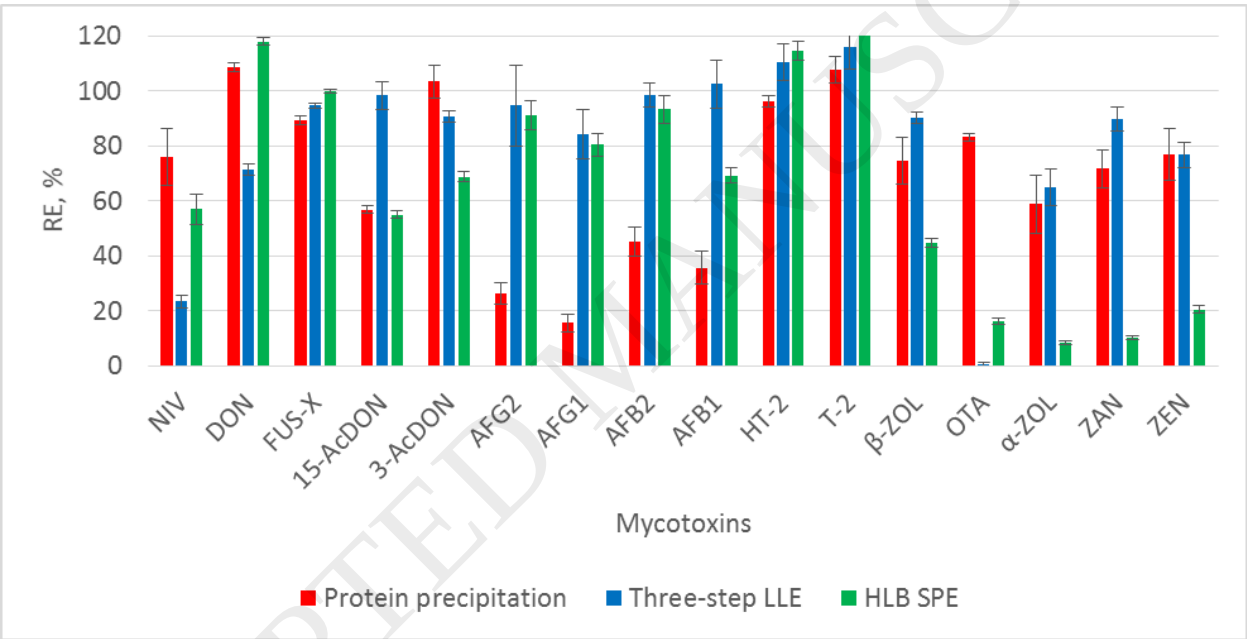
[63] N. Broekaert, M. Devreese, T. De Mil, S. Fraeyman, S. De Baere, S. De Saeger, P. De Backer, S. Croubels, Development and validation of an LC-MS/MS method for the toxicokinetic study of deoxynivalenol and its acetylated derivatives in chicken and pig plasma, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 971 (2014) 43–51. doi:10.1016/j.jchromb.2014.09.012.

[64] P. Songsermsakul, G. Sontag, M. Cichna-Markl, J. Zentek, E. Razzazi-Fazeli, Determination of zearalenone and its metabolites in urine, plasma and faeces of horses by HPLC-APCI-MS, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 843 (2006) 252–261. doi:10.1016/j.jchromb.2006.06.012.

[65] A. Mally, M. Solfrizzo, G.H. Degen, Biomonitoring of the mycotoxin Zearalenone: current state-of-the art and application to human exposure assessment, Arch. Toxicol. 90 (2016) 1281–1292. doi:10.1007/s00204-016-1704-0.

List of Figure Captions

*Figure 1. Comparison of (a) overall method recoveries (RE% = C_m/C_{th}*100%, where RE% is the recovery, C_m is the measured concentration in the injection solvent and C_{th} is theoretical concentration in injection solvent) and (b) absolute matrix effects observed for mycotoxins in human plasma using protein precipitation with acetonitrile, 3-step LLE with ethyl acetate, and Oasis HLB SPE. For (a) plasma (n=3) was spiked pre-extraction with 20 ng/mL of mycotoxins for LLE and SPE and 100 ng/mL for protein precipitation and analyzed against standard curve prepared in reconstitution solvent (20% methanol). β-ZAL and α-ZAL standards were not available at the time experiment (a) was performed. For (b) extracted plasma (n=3) was spiked post-extraction with 25 ng/mL of mycotoxins; peak areas of mycotoxins in plasma were compared to the peak areas in solvent (20% MeOH) to estimate matrix effect. The results show mean values while error bars show standard deviation of three replicate determinations.*



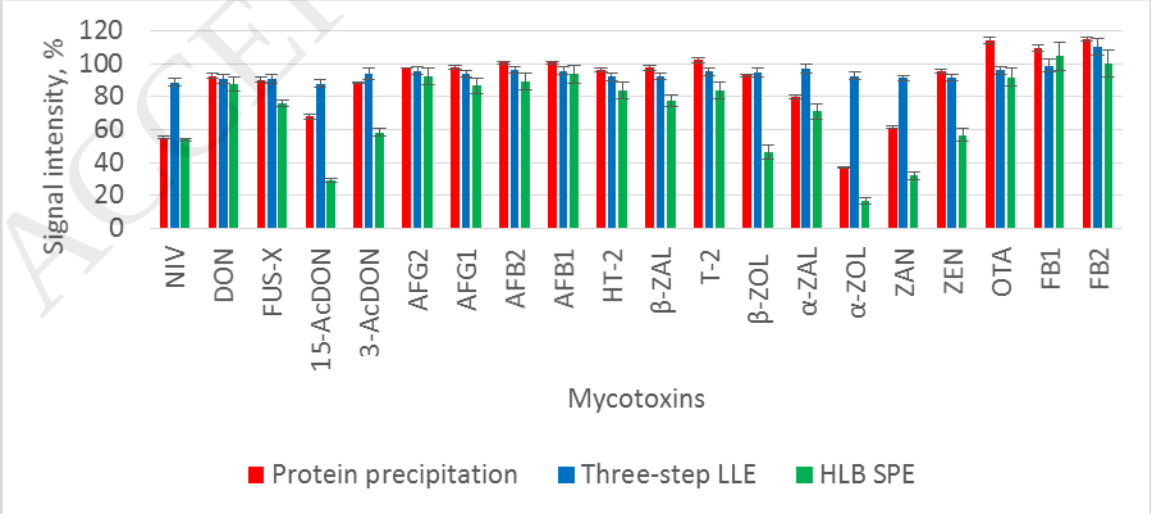
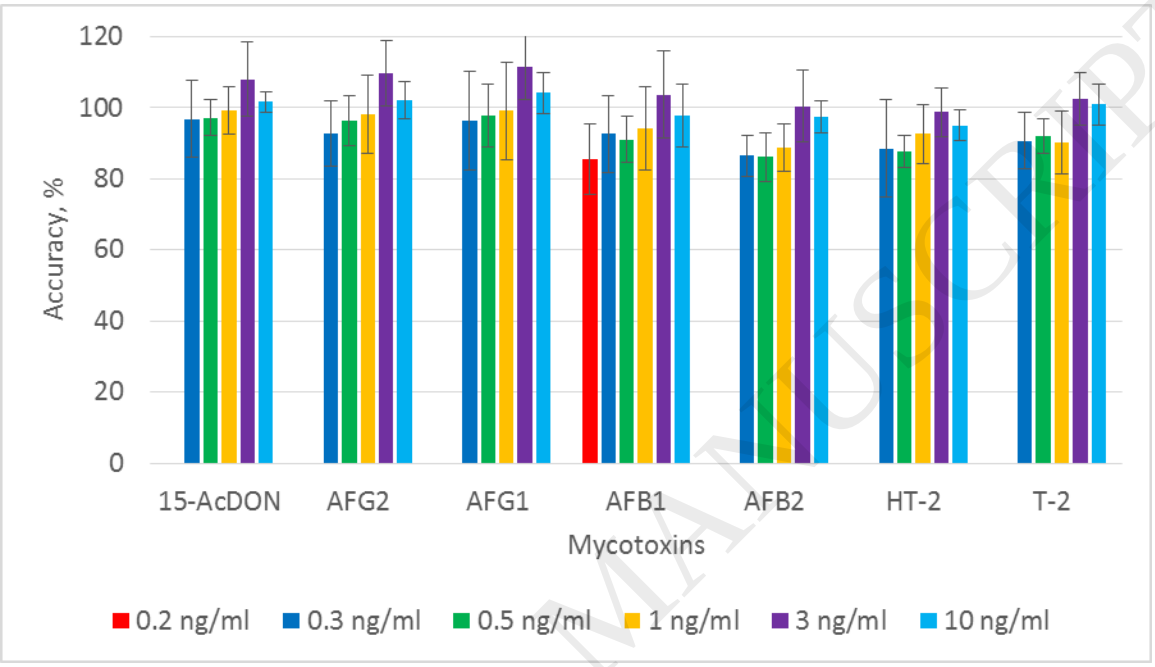


Figure 2. Inter-day accuracy and precision for mycotoxins detected in ESI(+) (a) and ESI(-) (b). y-axis shows mean accuracy, and standard deviation (n=5) is shown as an error bar. Inter-day precision and accuracy determination was performed using 0.2, 0.3, 0.5, 1, 3, and 10 ng/ml validation plasma samples (n=5 days), except for NIV where 3x the stated concentrations were used (denoted with). Standard curve in plasma was prepared on each day to analyze validation samples. For each mycotoxin, all concentrations above its LOQ are shown.*



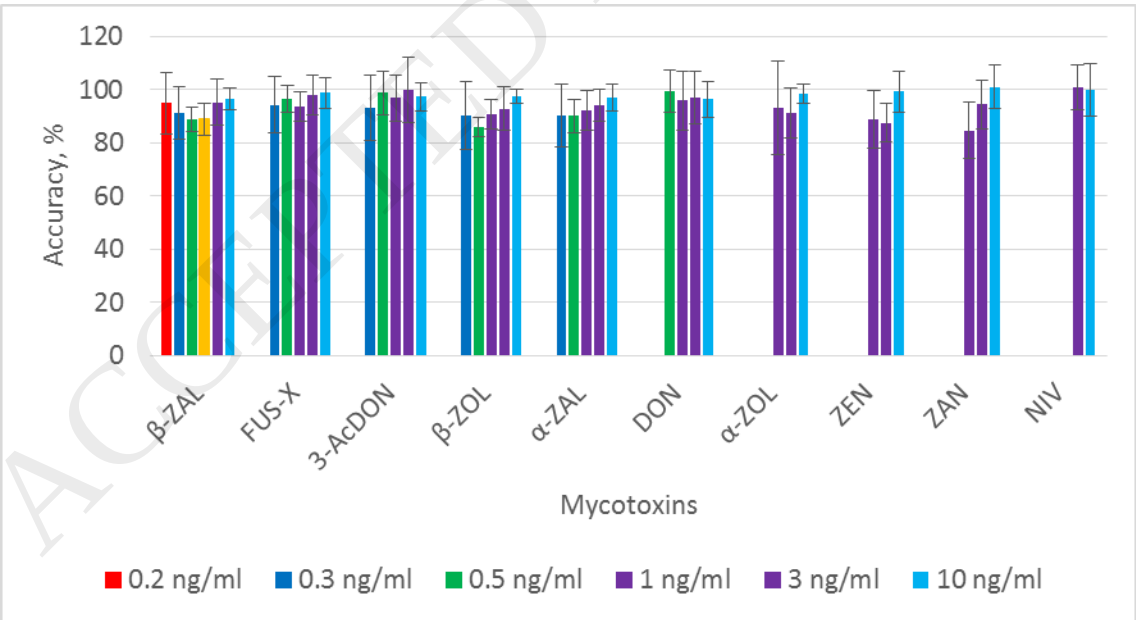
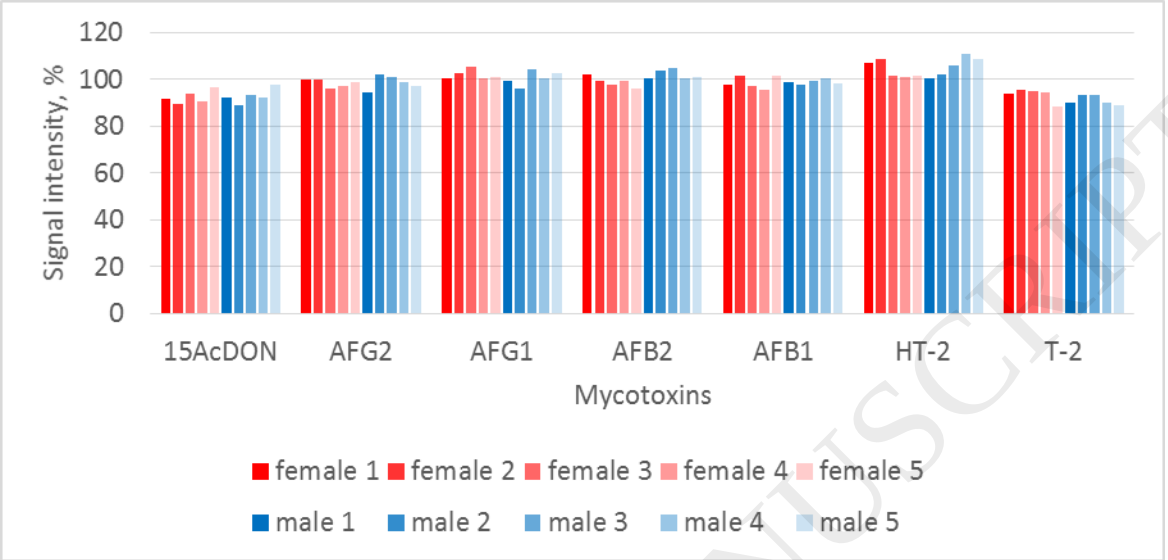


Figure 3. Investigation of absolute ionization matrix effect in 10 individual plasma samples using ESI(+) (a) and ESI(-) (b). Plasma samples were spiked with mycotoxin mixture to have concentration 0.3 ng/ml for 15-AcDON, AFB1, AFB2, AFG1, AFG2, HT-2, T-2, 3-ACDON, FUS-X, β-

ZAN, β-ZOL, α-ZAN, 1.5 ng/ml for DON, α-ZOL, ZEN, ZAN and 9 ng/ml for NIV. The area of post-extraction spiked individual plasma was compared to the area of the standard solution prepared in 20 % MeOH in order to determine absolute matrix effect.



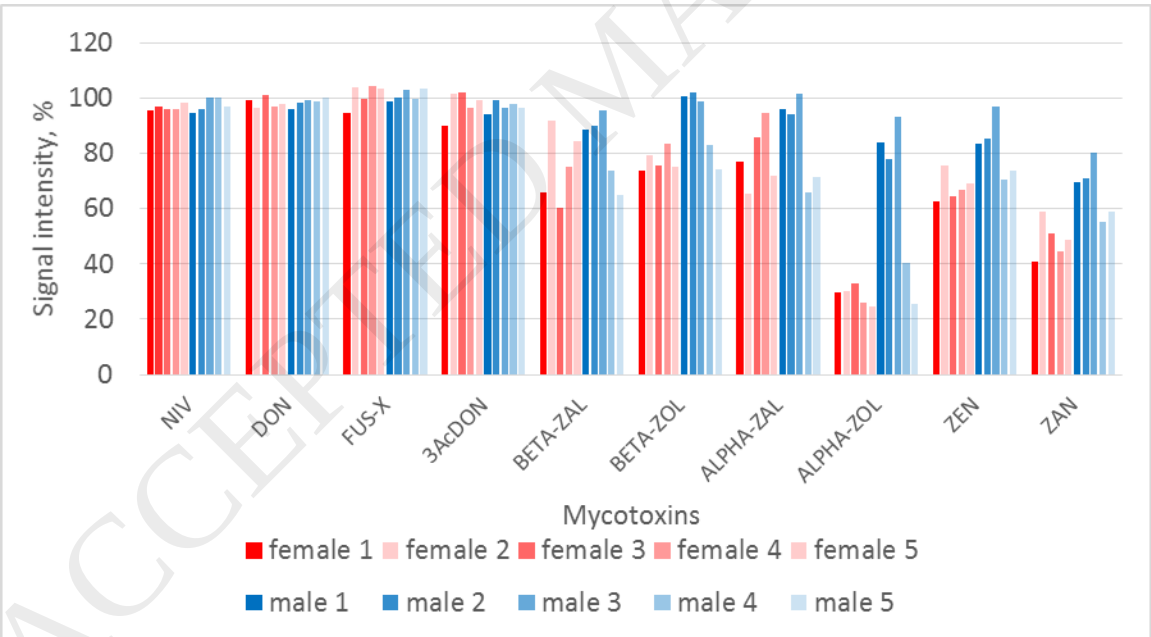


*Table 1. LOD and LOQs of all mycotoxins and inter- and intra-day accuracy and precision obtained at LOQ level. *Analyte does not meet FDA requirements.*

Mycotoxin	LOD (ng/ml)	LOQ (ng/mL)	LOQ (ng on-column)	ESI(-), intra-day		ESI(-), inter-day	
				Accuracy (%)	RSD%	Accuracy (%)	RSD%
β-ZAL	0.04	0.1	0.001	105.6	7.4	105.4	10.4
FUS-X	0.08	0.2	0.001	99.4	5.2	97.3	15.7
3-AcDON	0.07	0.2	0.001	88.4	8.3	97.1	16.7
β-ZOL	0.08	0.2	0.002	104.1	7.6	88.3	16.9
α-ZAL	0.08	0.2	0.002	100.7	6.7	84.2	11.7
DON	0.08	0.3	0.003	109.0	5.6	87.9	20.6
α-ZOL	0.3	0.5	0.005	146.0*	31.0*	91.4	15.4
ZEN	0.2	0.5	0.005	124.0*	6.5	86.5	11.8
ZAN	0.2	0.5	0.005	117.3	5.7	86.4	16.9
NIV	1.5	3.0	0.03	98.9	16.5	99.6	9.8
Mycotoxin	**LOD (ng/ml)**	**LOQ (ng/ml)**	**LOQ (ng on-column)**	**ESI(+), intra-day**		**ESI(+), inter-day**	
				Accuracy (%)	**RSD%**	**Accuracy (%)**	**RSD%**
15-AcDON	0.07	0.2	0.001	100.3	6.4	100.5	17.3
AFG2	0.07	0.2	0.002	102.9	6.1	89.6	11.4
AFG1	0.07	0.2	0.002	100.8	10.5	87.1	19.7
AB1	0.04	0.1	0.001	87.2	13.2	84.1	13.1
AFB2	0.08	0.2	0.001	96.2	9.1	86.0	14.7
HT-2	0.08	0.2	0.002	93.6	8.9	84.8	12.6
T-2	0.08	0.2	0.002	105.2	4.0	91.7	11.2