Development and validation of a methodology based on Captiva EMR-lipid clean-up and LC-MS/MS analysis for the simultaneous determination of mycotoxins in human plasma

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ABSTRACT

We report the methodology for the quantification of 19 mycotoxins in human plasma using high performance liquid chromatography-mass spectrometry (triple quadrupole). The studied mycotoxins were: deepoxy-deoxynivalenol, aflatoxins (B1, B2, G1, G2 and M1), T-2 and HT-2, ochratoxins A and B, zearalenone, sterigmatocystin, nivalenol, deoxynivalenol, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, neosolaniol, diacetoxyscirpenol and fusarenon-X. Sample deproteinization and cleanup were performed in one step using Captiva EMR-lipid (3 mL) cartridges and acetonitrile (with 1% formic acid). The extraction step was simple and fast. Validation was based on the evaluation of limits of detection (LOD) and quantification, linearity, precision, recovery, matrix effect, and stability. LOD values ranged from 0.04 ng/mL for aflatoxin

B1 to 2.7 ng/mL for HT-2, except for nivalenol, which was 9.1 ng/mL. Recovery was obtained in intermediate precision conditions and at three concentration levels. Mean values ranged from 68.8% for sterigmatocystin to 97.6% for diacetoxyscirpenol (RDS ≤ 15% for all the mycotoxins). Matrix effects (assessed at three concentration levels and in intermediate conditions) were not significant for most of the mycotoxins and were between 75.4% for sterigmatocystin and 109.3% for ochratoxin B (RDS ≤ 15% for all the mycotoxins). This methodology will be useful in human biomonitoring studies of mycotoxins for its reliability.

Keywords

Mycotoxins; Multi-detection; Human plasma; LC-MS/MS; Human biomonitoring.

Abreviations

15-ADON: 15-acetyldeoxynivalenol

3-ADON: 3-acetyldeoxynivalenol

ACN: Acetonitrile

AFB1: Aflatoxin B1

AFB2: Aflatoxin B2

AFG1: Aflatoxin G1

AFG2: Aflatoxin G2

AFM1: Aflatoxin M1

AFs: Aflatoxins

DAS: Diacetoxyscirpenol

DOM-1: Deepoxy-deoxynivalenol

DON: Deoxynivalenol

FUS-X: Fusarenon-X

HBM: Human biomonitoring

IARC: International Agency for Research on Cancer

LOD: Limit of detection

LOQ: Limit of quantification

ME: Matrix effect

NEO: Neosolaniol

NIV: Nivalenol

OTA: Ochratoxin A

OTA-d_5: Ochratoxin A-(*phenyl*-d_5)

OTB: Ochratoxin B

q: Transition of qualification

Q: Transition of quantification

RE: Relative error of the mean

RSD: Relative standard deviation

S/N: Signal-to-noise ratio

SRM: Selected reaction monitoring

STER: Sterigmatocystin

ZEA: Zearalenone

Introduction

The word *mycotoxin* is used to describe certain fungal metabolites that are toxic to humans and animals. Mycotoxins mainly enter the human body through ingestion of contaminated raw materials (cereals, fruits, etc.), their derived foods, or other foods (meat, milk, eggs, etc.) from animals fed with contaminated raw materials. Given their occurrence and toxicity, aflatoxins (AFs), ochratoxin A (OTA), fumonisins, zearalenone (ZEA), and trichothecenes are among the most important mycotoxins [1]. The International Agency for Research on Cancer (IARC) has classified aflatoxin B1 (AFB1) and naturally occurring mixtures of aflatoxins as carcinogenic to humans (Group 1) [2], and aflatoxin M1 (AFM1), OTA and sterigmatocystin (STER) as possible carcinogenic in humans (Group 2B) [3,4]. Mycotoxins are some of the most frequent causes of food safety concerns [5]. In order to protect human health, many countries have regulated maximum levels for individual mycotoxins in some raw materials and foods [6,7]. However, the most probable scenario appears to be that consumers are exposed to several mycotoxins. This has been reported by some authors and reviewed, for example, by Capriotti et al. [8].

To date, human exposure to mycotoxins has usually been studied in terms of the relationship between food contamination and consumption data, and these studies are needed to identify sources of exposure and implement guidelines that minimize human risks. However, Human Biomonitoring (HBM) measures the levels of substances in body fluids and tissues, which makes it possible to determine humans' internal exposure to chemicals, as it integrates all sources of exposure [9]. Mycotoxin HBM enriches the

chemical risk assessment in food safety and its importance will increase [10], in combination with food analysis.

Few publications currently exist in which mycotoxins are determined in human biological fluids; however, even for these mycotoxins, data on their presence in biological fluids are limited and contradictory [8]. In Europe, there is a lack of knowledge regarding the real exposure of the population to mycotoxins because it has been studied in very few human biomonitoring programs. Moreover, these programs have focused on AFs (2 programs) and DON (1 program); whereas, OTA, patulin, ZEA, fumonisin B1 and B2, T-2 toxin or HT-2 toxin have not been analyzed in any program, as reviewed in 2015 [9]. T-2 and HT-2 toxins along with nivalenol (NIV) are among the rarely studied mycotoxins in human biomonitoring [10]. However, great efforts are being carried out in order to improve this knowledge [11] and the development of adequate methodologies is needed [9]. This development is subject to certain difficulties: the wide variety of mycotoxins and their metabolites that may be present in human biological fluids and tissues, their diverse physicochemical properties and the low expected concentration levels. Metabolism of most of these mycotoxins in humans is unknown and it is necessary to define the best biomarker/s for each mycotoxin [10]. The measurement of parent compounds is commonly used as a biomarker of exposure to mycotoxins [9], although protein or DNA adducts or phase I and phase II metabolites can also be examined [10].

Also, the biological matrices used for HBM should be easily accessible without any health risk or discomfort for the sample donor. In mycotoxin studies, urine is the most frequently analyzed matrix and new methodologies for plasma and feces need to be developed and validated [10]. Presence in urine is in general a sign of recent mycotoxin intake, whereas presence in plasma is more closely linked to long-term exposure [12].

Only the development of liquid chromatography with mass detectors has given researchers a suitable tool to obtain these analyses [13]. Furthermore, the development of new modes of treatment of the sample prior to analysis is needed. They should be simple, fast and they should clean up most of the components of the matrix that may produce matrix effects. Moreover, sample treatment should not compromise adequate recovery values for all the analytes.

To date, very few methods have been developed to achieve multi-detection of mycotoxins from different mycotoxin groups in human plasma samples [12,14]. In 2015 Serrano et al. [15] developed an LC-MS/MS (QqQ) method for the determination of 4

enniatins and beauvericin in human biological fluids (plasma and urine) by extracting mycotoxins from plasma with a mixture of MeOH/H_2O (40/60 v/v) followed by a solid-phase extraction cleanup step. Although solid-phase extraction is one of the most commonly used cleanup methods, its disadvantage is that the interferents from the matrix that eluted along with the analytes of interest tend to have similar chromatographic behavior to that of the analyte and may lead to Matrix effect (ME) [16]. In 2018, Cao et al. extracted 11 mycotoxins (AFB1, AFB2, AFG1, AFG2, AFM1, STER, patulin, citrinin, fumonisins B1 and B2 and OTA) from human plasma by protein precipitation using ACN/acetic acid 1%, and mycotoxins were quantified with LC-MS/MS (QqQ). Those authors evaluated different deproteinization solvents (methanol and acetonitrile, both with and without acidification, and acid trifluoroacetic aqueous solution); the best recoveries were obtained using methanol containing acetic acid, but acidified acetonitrile was chosen because fewer matrix effects were observed when this solvent was used. Nevertheless, signal suppression/enhancement were from 69-140% [17]. Also in 2018, Slobodchikova and Vuckovic using LC-HRMS (Orbitrap) reported a multi-detection methodology for mycotoxins in human plasma [14]. Those authors studied different sample-preparation methods to counteract matrix effects and to obtain good recovery data; finally, a three-step liquid-liquid extraction procedure using ethyl acetate was selected, achieving good LODs in the determination of 17 mycotoxins in human plasma. However, the use of cleanup steps in tandem increases the risk of obtaining low recoveries and of adding interferents to the samples during the procedure [16]. In fact, the authors stated that no single method among those studied could achieve a recovery over 80% for all the mycotoxins. Moreover, the selected method gives a very low recovery for OTA, which is why this mycotoxin was excluded from method validation. In 2019, Fan et al. using LC-MS/MS (Qtrap) described a methodology able to quantify 12 mycotoxins and some of their metabolites in human plasma. Authors employed a liquid-liquid extraction procedure obtaining significant matrix effects for most of the analytes [18]. In Table 1 the published analytical methods for simultaneous mycotoxin quantification in human plasma are shown.

The aim of this paper is to present a methodology based on LC-MS/MS triple quadrupole (QqQ) analysis, which is capable of simultaneous determination of 19 mycotoxins in human plasma, as a tool for carrying-out HBM studies. The mycotoxins analyzed are 5 aflatoxins: AFB1, B2 (AFB2), G1 (AFG1), G2 (AFG2) and AFM1, 2 ochratoxins: OTA and B (OTB), T-2 and HT-2, ZEA, STER, DON and its derivatives

deepoxy-deoxynivalenol (DOM-1), 3-acetyldeoxynivalenol (3-ADON) and 15-acetyldeoxynivalenol (15-ADON); NIV, fusarenon-X (FUS-X), neosolaniol (NEO) and diacetoxyscirpenol (DAS). The sample preparation was made in one step using Agilent Captiva EMR-lipid cartridges. Validation criteria have been successfully achieved for selectivity, detection (LOD) and quantification (LOQ) limits, range, linearity, precision, accuracy, recovery, matrix effect and stability.

Experimental Section

Reagents and materials

The reagents used were: methanol (LC/MS grade) from Honeywell Riedel-de-Haën (Germany); acetonitrile (ACN) (gradient grade for LC) from Merck (Germany); formic acid (MS grade, purity > 98%) and ammonium formate (MS grade) from Fluka Sigma-Aldrich (Germany). Furthermore, deionized water (>18MΩcm resistivity) was purified in an Ultramatic Type I system from Wasserlab (Spain). Captiva EMR-lipid (3 mL) cartridges were obtained from Agilent Technologies (USA). Human plasma from three different and anonymous individuals was donated from the blood bank of the *Clínica Universidad de Navarra* (Spain) from surplus autotransfusion blood products after authorization of the Ethics Committee. EDTA was used as anticoagulant.

Mycotoxin standards

Mycotoxins (reference material, purity ≥ 98%) were obtained from Sigma-Aldrich (USA) and kept at -20°C. With the exception of OTA, which was obtained in powder form, all mycotoxins were purchased as a solution in acetonitrile. AFM1, AFG2 and AFB2: 0.5 μg/mL; AFG1 and AFB1: 2 μg/mL; ochratoxin A-(*phenyl*-d_5) (OTA-d_5) and OTB: 10 μg/mL; STER and DOM-1: 50 μg/mL; NIV, DON, 3-ADON, 15-ADON, NEO, DAS, FUS-X, ZEA and T-2 and HT-2 toxins: 100 μg/mL. OTA was prepared as a methanolic solution at 1 mg/mL and its concentration was spectrophotometrically assessed at 333 nm (UVIKON 922, Kontron Instruments SA, Madrid, Spain). These solutions were diluted in appropriate volumes of acetonitrile in order to prepare individual standard solutions.

Two mixed stock solutions in acetonitrile, containing all the mycotoxins (OTA-d_5 instead of OTA), were prepared by diluting appropriate volumes of the individual standard solutions. After preparation, an aliquot of each solution was analyzed by LC-

MS/MS and peak areas for each mycotoxin were compared in order to ensure adequate preparation. The stock solutions were stored at -20ºC after being divided into 1.5 mL vials. Prior to use, the thawed aliquot was left sitting at room temperature and in darkness for 30 minutes.

Safety precautions

Mycotoxins must be handled in solution and human exposure to dust or aerosols must be avoided. A face shield and gloves were used during the dilution of mycotoxins and extreme care was exercised when handling spiked samples. In order to prevent photodegradation of the studied mycotoxins (especially aflatoxins), samples and solutions were handled under low-light conditions.

Instrumentation and analytical conditions

The analytical method was based in those previously validated for cow milk by our group [19,20], with some modifications, especially in the gradient programs in order to shorten the chromatographic analysis time. In brief: chromatographic separation was achieved in an LC system 1200 series from Agilent Technologies (Germany). The chromatographic column was an Ascentis Express C18, 2.7 µm particle size 150 x 2.1 mm column (Supelco Analytical, USA) at 45°C. Two solutions constituted the mobile phase: solution A (5mM ammonium formate and 0.1% formic acid in water) and solution B (5mM ammonium formate and 0.1% formic acid in a 95:5 methanol-to-water ratio by volume) at a flow rate of 0.4 mL/min. Mycotoxins were separated in gradient conditions and in two different runs. In the case of mycotoxin group I (DOM-1, AFG2, AFM1, AFG1, AFB2, AFB1, OTB, ZEA, STER, OTA-d_5, T2, HT-2), the elution program was as follows: 0 min 40%B, to 72%B at 20 min, to 40%B at 20.1 min. In the case of mycotoxin group II (NIV, DON, FUS-X, NEO, 3-ADON, 15-ADON and DAS) elution program was as follows: 0 min 5% B, to 28% B at 5.0 min, to 45% B at 10.5 min, to 60% B at 11.0 min, to 90% B at 15.5 min, to 5% B at 16.0 min. In both cases, initial conditions were maintained for 10 minutes in order to re-equilibrate the column. Injection volume was 20 µL.

The detector was a 6410 Triple Quadrupole (QqQ) LC-MS/MS System from Agilent Technologies (Germany) in ESI(+) mode. Data acquisition parameters (i.e., transitions, fragmentor voltage and collision energy) for the mycotoxins were those optimized earlier by our group [19,20]. For OTA-d_5, the transition for quantification was 409.1-

239.0 and for qualification 409.1-102.1; and the other detector parameters for this compound were the same as those for OTA.

Preparation of calibration samples

Calibration samples for validating the analytical method were prepared by spiking human plasma. Adequate volumes of the mixed stock solution were poured into 15 mL polypropylene centrifuge tubes and dried in an evaporator (GeneVac, SP Scientific, England) under vacuum and at 60ºC. Then, 15 µL of ACN was added and vortexed for 5 minutes. This volume was diluted with 450 µL of human plasma and mixed using a vortex agitator for 5 minutes. Plasma samples were set aside for 10 min after being spiked and before applying the procedure for sample preparation.

Sample preparation

Human plasma (0.4 mL) was added to a Captiva EMR-lipid cartridge that contained 1.2 mL of acetonitrile (acidified with formic acid at 1%). After 5 minutes, a vacuum was applied and 0.4 mL of the effluent was put in each one of two different tubes and then evaporated until dry (60ºC). One of the tubes was reconstituted with 200 µL of 40%B-mobile phase (for analysis of mycotoxins classified as group I), while the second tube was reconstituted with 200 µL of 5%B-mobile phase (for analysis of mycotoxins classified as group II). Before chromatographic analysis, solutions were vortexed (5 min) and filtered (PVDF, 0.45 µm, Merck Millipore, Ireland).

Validation of the method

The following parameters were studied for method validation: selectivity, detection and quantification (LOQ) limits, range of concentration, linearity, precision and accuracy (within- and between-day), recovery, matrix effect, stability of the mixed stock solution at -20ºC, stability of the samples in the injector tray; and finally, stability of plasma calibration samples after three freeze-thaw cycles.

With regard to selectivity, the transition intensity ratio (q/Q in %) was calculated for each mycotoxin at three concentration levels for both standard samples (mycotoxins from the stock solution, evaporated until dry and dissolved in mobile phase) and calibration samples. The criteria for ensuring peak identity were the presence of the two transitions from the precursor ion of each one of the mycotoxins; a mean relationship (q/Q in %) with no more than a 20% difference between the two types of samples, and

comparison of the chromatographic mycotoxin retention time in both type of samples with a tolerance of ± 2.5% [21].

The concentration level in matrix-matched calibration samples with a value of signal (peak height)-to-noise (S/N) ratio of at least 3 for the qualitative transition was defined as LOD. Calculation was made using the "peak-to-peak from drift" algorithm in the Agilent MassHunter Qualitative Analysis B.06.00 software. The minimum concentration assayed in plasma with adequate precision (n=3, RSD < 20%) and accuracy (back-calculated concentration with a relative error of the mean (RE) compared to the nominal value <20%) was defined as the LOQ and included at the lowest level in the corresponding calibration curve [22].

In the determination of range and linearity of the method, eight (1, 2, 4, 6, 8, 10, 20 and 30xLOQ) calibration samples containing all the mycotoxins (OTA-d_5 instead of OTA) were prepared and analyzed on three different days. All the matrix-matched calibration curves were performed with a minimum of six points. They were accepted if the determination coefficient (R^2) was greater than 0.99, and if the back-calculated concentration for each one of the calibration samples differed (RE in %) by less than 15% from the nominal value (20% for LOQ level) [22].

Precision and accuracy of the method in within-run (or between-run) conditions were assayed by analyzing three calibration samples at LOQ, 6xLOQ and 30xLOQ for each mycotoxin calibration range, in triplicate, on one day (or on three different days). Precision was calculated as RSD in % of the concentrations obtained for each concentration level (3 or 9 for within-run and between-run, respectively). Accuracy was calculated as the RE% of back-calculated concentrations with respect to the nominal value (3 or 9 for within-run and between run, respectively). Criteria for precision and accuracy were both RSD (%) and RE (%) of less than 15% (20% for LOQ) [22].

In addition, recovery and ME were determined at three concentration levels: LOQ, 6xLOQ and 30xLOQ in the range of each mycotoxin, in triplicate and in within-run and between run conditions following the procedure indicated in Flores-Flores et al. [19,20]. For recovery, RSD should be 15% or less (20% for LOQ). On the other hand, an ME value (in %) below 80% or above 120% was considered as indicative of matrix effect: signal suppression or signal enhancement, respectively [23].

With regard to stability, calibration samples at two different levels (6 and 30xLOQ) were prepared in triplicate in order to study stability in the injector tray (4ºC). They were analyzed at 0, 24, 48, 72 hours and 1 week after being prepared. A concentration

versus-time regression analysis was performed and a slope not statistically different from 0 (p=95%) was indicative of stability. In the mixed stock solution stability at -20ºC was studied for 6 months. Stability of the mycotoxins in frozen plasma calibration samples at two concentration levels (6xLOQ and 30xLOQ) (three replicates per level in two days) and after three freeze-thaw cycles (20 h frozen at -20 ºC, 2 h thawed) was also studied. These samples were analyzed along calibration curves and stability was accepted for each mycotoxin if two-thirds of the samples presented a RE % of back-calculated concentrations with respect to the nominal less than 20% [24].

Results and Discussion

Analytical conditions

With regard to chromatographic separation, the aim of our first attempt was to determine the mycotoxins in a single analytical run. Adequate chromatographic separation and detection was obtained for all of them by applying a gradient separation starting with a 5%B in the mobile phase. However, carry-over of ochratoxin A was observed after analysis of the calibration samples. After several experiments, we concluded that OTA was retained in the injector unless 40%B was used as the initial mobile phase. In this condition, the more polar mycotoxins were not well separated and sensitivity for these mycotoxins decreased. Therefore, the mycotoxins were divided into the two groups previously mentioned. Each group was analyzed in separate runs with different gradient conditions, especially in terms of the initial composition of the mobile phase. Figures 1 and 2 show examples of the chromatograms obtained for both groups of mycotoxins in plasma samples.

During method development, it was observed that the chosen plasma for validation purposes presented levels of OTA. Therefore, OTA-d_5 was employed in order to obtain the validation parameters for OTA. It has been described that the interchange of hydrogen for deuterium can affect chromatographic behavior due to some loss of lipophilicity [25] and different retention times can cause deuterated and non-deuterated compounds to suffer from different recovery and ME [16,25]. In the presented methodology, the ratio of retention time for OTA to retention time for OTA-d_5 was 0.99 (n=10, RSD 0.07% for both compounds); therefore, similar matrix effects for both compounds has been assumed.

Sample treatment

During the development of the extraction procedure, deproteinization using ACN, acetone and methanol (acidified with 1% formic acid) were firstly assayed. Acidified ACN resulted to be the best solvent for extracting mycotoxins. Once the solvent was selected, a total of 0.4 mL of plasma was mixed with different volumes of ACN (1% formic acid). The mixture was vortexed for 2 minutes and after centrifuging (227 xg during 45 min), a fraction of the supernatant was collected and dried at 60ºC in a vacuum. In all the cases, drying process was time consuming and a residue appeared at the bottom of the tube that prevented a good redissolution in mobile phase. Hence, protein precipitation with ACN without additional cleaning was discarded. Then a cleanup step was tried using anhydrous sodium acetate. After protein precipitation, the supernatant was mixed with sodium acetate in a vortex for 2 minutes, followed by 15 minutes of centrifugation. The use of the salt achieved separation of the aqueous and organic phases from the extract and cleaned the acetonitrile phase from polar matrix components, but reduced recovery.

In recent years, the principal methodologies employed in the extraction of mycotoxins from biological matrices are liquid-liquid extraction, coupled or not with solid-phase microextraction or immunoaffinity columns [26], but new advances are commercially available. In order to improve our results, Captiva EMR-lipid cartridges were assayed. During the preliminary experiments, several volumes of acidified ACN and plasma sample were tested. In all the experiments the proportion 3:1 (ACN:plasma) was maintained. 200, 400, 500, 600 and 800 µL of plasma were added to the cartridges containing ACN (1% formic acid). Finally, a volume of 400 µL of plasma was selected because of the good recovery values obtained; the extract volume attained, adequate enough for the later analysis of both mycotoxin groups; the low evaporation time needed for the extract; and, also, in order to employ the lowest volume of plasma samples. A plasma volume of 400 µL has been considered adequate when mycotoxin biomonitoring is carried-out in humans. Afterwards, an extra ACN volume was added to the cartridge containing the precipitate, but recovery values were worse than obtained without it (from 3% for STER to 65.7% for OTB). Moreover, matrix effects were high for all mycotoxins (above 120% or under 80% as in the case of STER). For all mentioned, addition of extra volume of ACN to the cartridges was discarded. Finally, different volumes of the extract (400 and 600 µL) and for the redissolution of the residue in mobile phase (100 and 200 µL) were assayed.

The final procedure was that indicated in material and methods section, employing 400 µL of human plasma and 1.2 mL of acetonitrile with formic acid (1%). Advantages of this procedure are that precipitation of the proteins is achieved by placing acidified ACN into the cartridge and then adding plasma to it without any other preparation steps. In a short period of time (5 min) precipitation was completed and the extract was cleaned from interfering compounds when passing through the cartridge. The procedure is very easy and fast, only one step is needed in the preparation of the samples and, therefore, the possibility of error diminished. Furthermore, several samples can be prepared simultaneously using a vacuum manifold system. Additionally, very good values were obtained for recovery and matrix effect as it is explained below, whereas other authors using protein precipitation obtained high matrix effects [18] or very low recovery values with LLE, even preventing the quantification of ochratoxin A [14]. These cartridges have been used for determining mycotoxins in cheese [27] and other food matrices [28] but, to the best of our knowledge, they have not been applied in the detection of mycotoxins in human plasma.

Method validation

There is no matrix reference material available for use in the assessment of the performance of the methods for mycotoxin analysis in human plasma [10]. For this reason, spiking of human plasma samples from three individuals has been employed in order to obtain validation parameters. In our procedure, 15 µL of ACN were added to the dried residue obtained after evaporating the corresponding volume from the stock solution. This volume was then mixed with 0.450 mL of plasma. The addition of ACN was needed for improve response (higher peak areas) and diminished RSD (%). This may be because the plasma did not dissolve mycotoxin residues completely. Moreover, 15 µL represent 3% of the total plasma volume, and no disturbance of the physical characteristics of the plasma was detected.

Method validation is mandatory [29]. Regarding mycotoxins, in the European Union, Commission Regulation No 401/2006 establishes the methods of sampling and analysis for the official control of levels of mycotoxins in foodstuffs [30], but there is no equivalent for biological fluids. However, there is a Commission Decision concerning the performance of analytical methods to monitor certain substances (including mycotoxins) and residues in live animals and animal products [21]. For bioanalytical method validation there are two main guidelines, one from the FDA [22] and the other

from the European Medicines Agency [31]. In this study, the documents indicated above were consulted for validation.

Selectivity is usually evaluated by analyzing blank samples from multiple sources [22]. In our case, mycotoxins are natural contaminants and it was not possible to obtain plasma that is guaranteed to be completely free of these compounds. The FDA guideline indicates some procedures for endogenous compounds, for instance the addition of the analyte to a sample in which the initial concentration level has been evaluated, but in some cases this procedure cannot be used, e.g., in the determination of LOD or LOQ, where the concentration to add should be less than the analyte level in the "blank" sample and, moreover, it is difficult to obtain recovery and matrix effect data at low concentration levels. When mass spectrometry is used, the use of isotopically related compounds is recommended [31], but they are not available for all the mycotoxins or their metabolites; and when it is possible to buy them, their cost is high. The use of LC-MS/MS with one precursor ion and two transitions gives four points of identification, which is sufficient for confirmation [21]. In this study, the ratio of qualification peak area (q) to quantification peak area (Q) and the retention time have been recorded for each mycotoxin in calibration samples (spiked plasma) and standard samples (mycotoxins in mobile phase). The results are shown in Table 2. The relative error of the mean in % of q/Q ratios is $\leq$ 11% and the highest RE (%) value for retention times is 0.32%, both of which are lower than the values of 20% and 2.5%, respectively, established for the EU [21].

LOD values ranged from 0.04 ng/mL for aflatoxin B1 to 2.7 ng/mL for HT-2, except for nivalenol: 9.1 ng/mL. Linearity, defined as the linear relationship between analyte signals and analyte concentrations in calibration samples containing matrix components [24], has been assessed. In some of the curves, and in order to improve the adjustment, a 1/x weight factor was needed. The resulting matrix-matched calibration curves obtained for all mycotoxins met the fixed criteria: R^2 >0.99 and back-calculated concentrations presented a difference with their respective nominal values of less than 15% (<20% for LOQ). Precision and accuracy values (both within-run and between-run) are adequate and less than 15% (20% for LOQ) for all levels and mycotoxins [22]. Values obtained for one of the matrix-matched calibration curves obtained for each mycotoxin are shown in Table 3 (range, LOD and linearity parameters) and Table 4 (precision and accuracy).

Recoveries and matrix effects for all the mycotoxins have RSD (%) values less than 15% (20% for LOQ) for all concentration levels assayed in between-run conditions

(Table 5) and have, therefore, been considered satisfactory. Global recovery values (%) calculated as the mean value (n=9) ranged from 68.8% for STER to 97.6% for DAS. With regard to matrix effects, global values (n=9) ranged from 75.4 for STER to 109.3 for OTB. Matrix effect is a disadvantage of employing LC-MS/MS methods [16] and, in the case of plasma samples, the presence of phospholipids causes significant matrix effects [16,32]. Including a cleanup step that eliminates phospholipids no significant signal suppression or enhancement was observed for most of the analytes, which have a matrix effect in the range of 100±20%. Only STER has a value outside this range (75.41%). Nevertheless, matrix-matched calibration curves have been prepared for all the mycotoxins.

Finally, all the mycotoxins were stable in the mixed stock solution at -20ºC for at least 6 months and in the injector tray (after sample preparation) at 4ºC for at least 1 week. After three freeze-thaw cycles, only STER did not comply with stability requisites; however, it was stable after two freeze-thaw cycles.

Conclusions

Human biomonitoring of mycotoxins requires the development and validation of methodologies that permit multi-determination of these compounds. This will make HBM faster and more affordable. Moreover, the knowledge acquired in these HBM programs and the resulting decisions may increase safety for humans and decrease health risks. In this study, we present a validated Captiva-EMR-LC-MS/MS (QqQ) methodology that achieves the simultaneous analysis of 19 mycotoxins in human plasma. The mycotoxins studied included those of major concern for their toxicity (OTA and AFs) and also mycotoxins rarely studied in human plasma, such as trichothecenes and STER. Sample deproteinization and cleanup are performed in one step and several samples can be processed simultaneously. Although quantification of each sample is performed in two runs, the analyses of the 19 mycotoxins per sample are completed in approximately 1 hour. Good values for recoveries and matrix effects have been achieved due to the elimination of phospholipids from plasma during sample preparation and the use of matrix-matched calibration curves. LOD values ranged from 0.04 ng/mL for aflatoxin B1 to 2.7 ng/mL for HT-2, except 9.1 ng/mL for nivalenol. For all of the above, this methodology has demonstrated its reliability, being a useful tool for HBM of mycotoxins.

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Declarations of interest

The authors declare no conflict of interest.

Figure captions:

Fig. 1: Superposed typical extracted chromatograms for mycotoxins group I after extraction of a spiked plasma sample at LOQ level. A displays the quantification transition and B the qualification transition for each mycotoxin.

Fig. 2: Superposed typical extracted chromatograms for mycotoxins group II after extraction of a spiked plasma sample at LOQ level. A displays the quantification transition and B the qualification transition for each mycotoxin.

Color is not needed.

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Table 1. Analytical methods for simultaneous mycotoxin quantification in human plasma

Biomarkers	LOD (ng/mL)	Sample Preparation	Separation and Detection Technique	Ref.
AFs (B1, B2, G1, G2, M1, M2), OTA, OTα, FB1, T-2, HT-2 DON, 3-ADON, 15-ADON, DON-3-GlcA, DON-15-GlcA FUS-X, ZEA, ZAN, α-ZEL, β-ZEL, α-ZAL, β-ZAL, ZEA-14-GlcA, ZAN-14-GlcA	0.03-0.5	*LLE* 200 µL plasma + 1 mL ACN/formic acid (99/1). Centrifuge, evaporate supernatant (N_2) + 200 µL ACN/H_2O with 5 mM ammonium acetate (20:80)	*LC-MS/MS* Column:C18 (100 x 3 mm x 2.7 µm) at 40ºC Flow: 0.4 mL/min Mobile Phases: (A) MeOH; (B) H_2O/ 5mM ammonium acetate in gradient conditions Detector: ESI (±), QTrap, MRM	[18]
NIV, DON, FUS-X, 3-ADON, 15-ADON, T-2, HT-2, AFs (B1, B2, G1, G2), ZEA, α-ZEL, β-ZEL, OTA, ZAN, α-ZAL, β-ZAL	0.04-1.5	*LLE* 100 µL plasma + 150 µL Ethyl acetate (three times). Combine extracts. Centrifuge, evaporate organic phase (dryness). Reconstitute in 200 µL MeOH	*LC-HRMS* Column: C18 (50 x 2.1 mm x 2.6 µm) at 30ºC Flow: 0.3 mL/min Mobile Phases: (A) H_2O; (B) MeOH/0.1% acetic acid in gradient conditions Detector: ESI (±), Orbitrap	[14]
AFs (B1, B2, G1, G2, M1), STER, PAT, CIT, FB1, FB2, OTA	0.05-0.41	*LLE* 200 µL plasma + 50 µL β-glucuronidase (overnight 37ºC) + 1 mL ACN/acetic acid (99/1). Centrifuge, evaporate (N_2). Reconstitute in 200 µL ACN/H_2O	*LC-MS/MS* Column: C18 (100 x 2.1 mm x 2.6 µm) at 40ºC Flow: 0.2 mL/min Mobile Phases: (A) 0.2 mM acetic acid; B) ACN in gradient conditions Detector: ESI (±), QqQ, MRM	[17]
Enniatins (A, A1, B, B1) and Beauvericin	0.01-0.02	*SPE* 250 µL plasma + 25 mL MeOH/H_2O (40:60) + Carbograph clean up	*LC-MS/MS* Column: C18 (150 x 2.1 mm x 3 µm) at 30ºC Flow: 0.75-0.85 mL/min Mobile Phases: (A) H_2O; (B) MeOH both with 5 mmol/L ammonium formate and 0.1% formic acid. in gradient conditions Detector: ESI (+), QqQ, MRM	[15]

LLE: liquid-liquid extraction; ESI: Electrospray ionization; QqQ: triple quadrupole; QTrap: quadrupole- ion trap; MeOH: methanol; ACN: acetonitrile; MRM: Multiple Reaction Monitoring; OTα: Ochratoxin α; ZAN: Zearalanone; DON-3-GlcA: 3-acetyldeoxynivalenol-glucuronide; DON-15-GlcA: 15-acetyldeoxynivalenol-glucuronide; ZEL: zearalenol; ZAL: zearalanol ; PAT: Patulin; CIT: citrinin.

Table 2: Ratio qualification peak area (q)/quantification peak area (Q) and retention time (min) for each one of the mycotoxins in standard samples and calibration samples.

Mycotoxin	q/Q Standard (RSD%) n=9	q/Q Plasma (RSD %) n=9	RE (%)	RT Standard (RSD%) n=9	RT Plasma (RSD%) n=9	RE (%)
DOM-1	83.3 (5.2)	80.8 (10.0)	3.1	1.46 (0.58)	1.47 (0.70)	0.32
AFG2	74.3 (11.2)	70.2 (8.4)	5.9	2.44 (0.58)	2.44 (0.39)	0.19
AFM1	88.8 (6.7)	93.9 (14.8)	5.5	2.53 (0.53)	2.54 (0.41)	0.07
AFG1	76.2 (7.7)	76.3 (11.8)	0.2	2.98 (0.59)	2.98 (0.48)	0.09
AFB2	93.8 (5.5)	92.0 (5.3)	1.9	3.71 (0.44)	3.72 (0.48)	0.22
AFB1	61.9 (2.6)	61.3 (7.1)	1.1	4.58 (0.39)	4.59 (0.35)	0.20
HT-2	50.5 (7.5)	46.5 (16.0)	8.6	9.34 (0.19)	9.35 (0.26)	0.10
OTB	40.6 (5.2)	42.1 (8.5)	3.6	11.36 (0.27)	11.37 (0.25)	0.05
T-2	75.3 (5.2)	75.6 (14.0)	0.3	12.86 (0.19)	12.87 (0.19)	0.12
ZEA	85.4 (16.3)	84.7 (6.1)	0.8	14.51 (0.07)	14.53 (0.13)	0.15
OTA-d_5	63.5 (7.2)	65.6 (8.9)	3.3	15.53 (0.22)	15.54 (0.20)	0.09
STER	92.7 (3.7)	96.1 (12.8)	3.5	15.52 (0.09)	15.54 (0.10)	0.08
NIV	98.7 (14.0)	97.0 (16.8)	1.8	4.18 (0.21)	4.17 (0.52)	0.22
DON	89.9 (5.0)	91.1 (19.0)	1.4	5.99 (0.08)	4.98 (0.14)	0.12
FUS-X	96.7 (17.9)	97.0 (18.6)	0.3	8.02 (0.49)	8.01 (0.48)	0.06
NEO	89.5 (6.1)	86.9 (6.1)	2.9	8.68 (0.08)	8.68 (0.05)	0.04
3-ADON	72.1 (24.6)	76.0 (14.0)	5.1	9.98 (0.07)	9.98 (0.04)	0.06
15-ADON	109.1 (15.2)	97.3 (21.7)	12.1	10.11 (0.06)	10.11 (0.06)	0.06
DAS	62.9 (4.0)	63.4 (5.8)	0.8	14.07 (0.03)	14.07 (0.04)	0.01

*q: Transition of qualification. Q: Transition of quantification. RE: relative error of the mean. RT: retention time

Table 3: Linearity results for one of the matrix-matched calibration curves obtained for each mycotoxin

Mycotoxin	Range (ng/mL)	LOD (ng/mL)	LOQ (ng/mL)	Weight	R^2	Equation
DOM-1	6.0-180.0	1.35	6.0	None	0.999	y=15.73x+6.57
AFG2	0.8-24.0	0.35	0.8	1/x	0.997	y=372.45x-115.67
AFM1	0.8-24.0	0.18	0.8	1/x	0.996	y=173.50x-35.0
AFG1	0.3-9.0	0.07	0.3	1/x	0.997	y=383.88x-43.48
AFB2	0.3-9.0	0.07	0.3	1/x	0.999	y=305.03x+17.78
AFB1	0.2-6.0	0.04	0.2	1/x	0.997	y=1309.57x-123.90
HT-2	6.0-180.0	2.70	6.0	1/x	0.999	y=24.96x-57.39
OTB	1.0-30.0	0.40	1.0	1/x	0.998	y=336.57x-149.10
T-2	1.0-30.0	0.20	1.0	1/x	0.998	y=573.25x-145.93
ZEA	4.0-120.0	1.80	4.0	1/x	0.992	y=42.35x+46.90
OTA-d_5	2.0-60.0	0.40	2.0	1/x	0.996	y=93.47x-69.07
STER	1.0-30.0	0.20	1.0	1/x	0.991	y=262.55x-42.15
NIV	20.4-612.0	9.10	20.4	None	0.998	y=1.74x-13.42
DON	8.7-261.6	1.94	8.7	None	0.998	y=12.79x-22.59
FUS-X	8.7-261.6	1.95	8.7	None	0.999	y=7.01x-15.11
NEO	0.8-24.0	0.18	0.8	None	0.997	y=92.99x-26.45
3-ADON	1.8-52.5	0.70	1.8	None	0.995	y=39.57x-21.81
15-ADON	2.7-81.6	1.20	2.7	None	0.996	y=37.16x-51.05
DAS	0.7-21.0	0.15	0.7	None	0.998	y=119.37x-16.10

LOD: limit of detection. LOQ: limit of quantification

Table 4: Precision and accuracy

Mycotoxin	Precision (%RSD)						Accuracy (%RE)					
	Within-run (n=3)			Between-run (n=9)			Within-run (n=3)			Between-run (n=9)		
	LOQ*	6xLOQ	30xLOQ	LOQ	6xLOQ	30xLOQ	LOQ	6xLOQ	30xLOQ	LOQ	6xLOQ	30xLOQ
DOM-1	2.0	4.6	11.8	5.0	7.4	6.3	6.9	3.5	6.5	8.8	2.2	5.0
AFG2	6.5	5.3	2.6	7.9	9.8	8.0	1.2	5.9	6.1	7.4	5.0	1.8
AFM1	5.2	4.2	3.1	8.4	7.0	5.1	3.3	6.1	4.7	6.7	2.4	3.7
AFG1	3.5	7.7	4.0	8.2	9.4	7.3	3.3	0.7	7.9	8.9	5.0	6.9
AFB2	6.4	5.1	1.9	5.2	6.0	8.5	8.9	3.9	8.1	11.5	2.2	4.0
AFB1	5.6	5.1	3.1	6.2	7.6	8.6	10.0	1.7	8.8	14.4	4.6	7.8
HT-2	10.8	4.5	0.8	7.0	12.0	6.9	11.8	5.9	0.9	14.9	3.3	5.9
OTB	3.3	3.3	1.1	4.5	10.0	4.1	4.0	5.8	4.4	7.2	4.5	5.8
T-2	9.7	4.8	5.9	12.1	11.5	13.1	5.0	4.6	9.4	0.4	6.4	4.0
ZEA	6.3	4.7	4.7	11.6	7.0	8.1	1.3	1.2	9.3	6.3	6.2	7.8
OTA-d_5	5.4	6.7	1.8	12.2	11.5	3.9	10.7	3.9	1.5	5.6	6.7	1.8
STER	7.1	7.6	3.9	8.2	14.2	13.2	0	5.1	10.4	0.4	1.0	0.3
NIV	12.6	8.6	4.1	14.3	9.8	13.1	4.3	3.5	11.8	0.6	7.7	3.6
DON	8.0	3.0	2.2	10.4	5.1	14.3	11.9	3.0	10.3	9.7	1.1	0.8
FUS-X	11.0	5.2	9.1	11.7	7.4	13.2	8.3	5.5	11.0	10.2	3.7	5.9
NEO	9.8	2.6	2.5	15.8	5.8	10.1	11.3	0.1	7.9	4.2	5.1	2.8
3-ADON	2.9	1.2	1.4	4.7	7.7	9.0	10.2	5.3	3.1	7.9	3.0	3.1
15-ADON	3.8	7.0	2.6	9.4	6.2	6.7	2.4	0.6	3.1	0.1	5.7	3.2
DAS	6.2	3.5	3.7	6.6	12.9	7.9	3.8	4.4	3.5	4.1	4.4	2.9

*LOQ: limit of quantification. RSD: relative standard deviation. RE: relative error of the mean.

Table 5: Matrix effect and recovery data

Mycotoxin	Matrix effect (%RSD)				Recovery (%RSD)			
	Between-run (n=3)			Global (n=9)	Between-run (n=3)			Global (n=9)
	LOQ*	6xLOQ	30xLOQ		LOQ	6xLOQ	30xLOQ	
DOM-1	84.2 (6.7)	86.3 (12.0)	79.9 (7.6)	83.5 (8.7)	87.7 (18.8)	87.1 (5.1)	94.5 (11.8)	89.8 (12.0)
AFG2	103.7 (8.4)	103.2 (8.0)	97.3 (1.5)	101.4 (6.7)	92.6 (7.2)	87.2 (2.5)	90.5 (7.3)	90.1 (5.9)
AFM1	98.6 (7.9)	102.4 (6.9)	95.2 (2.9)	98.7 (6.3)	87.9 (4.1)	86.0 (1.0)	90.8 (8.6)	88.2 (5.4)
AFG1	93.1 (6.0)	94.4 (13.1)	86.7 (1.7)	91.4 (8.4)	77.0 (4.3)	81.8 (2.7)	89.4 (7.6)	82.7 (8.1)
AFB2	105.9 (7.8)	107.9 (8.5)	99.3 (0.9)	104.4 (7.0)	85.4 (11.4)	83.3 (5.6)	86.2 (6.0)	85.0 (7.2)
AFB1	88.9 (9.7)	96.5 (10.1)	88.1 (4.5)	91.2 (8.7)	80.1 (7.7)	80.9 (3.9)	86.2 (6.6)	82.4 (6.5)
HT-2	104.7 (11.2)	107.3 (8.4)	99.0 (3.7)	103.6 (8.2)	88.7 (10.8)	89.4 (3.3)	91.3 (3.7)	89.8 (6.0)
OTB	114.0 (8.4)	113.2 (9.3)	100.6 (5.4)	109.3 (9.2)	80.2 (10.0)	82.4 (5.2)	86.9 (6.5)	83.2 (7.4)
T-2	109.6 (12.4)	109.9 (8.2)	98.0 (3.2)	105.8 (9.6)	96.9 (14.0)	85 9 (2.7)	90.8 (6.7)	91.2 (9.8)
ZEA	80.7 (6.6)	90.4 (7.2)	92.5 (0.2)	87.9 (7.9)	88.2 (18.5	76.5 (10.0)	80.2 (6.2)	81.6 (13.1)
OTA-d_5	109.9 (6.8)	107.4 (10.4)	95.6 (6.9)	104.3 (9.6)	71.1 (5.9)	78.2 (8.4)	84.1 (7.4)	77.8 (9.7)
STER	78.3 (14.4)	73.6 (10.8)	74.3 (4.3)	75.4 (9.9)	68.9 (11.1)	68.2 (11.9)	69.4 (7.9)	68.8 (9.0)
NIV	88.2 (10.8)	97.8 (2.9)	85.6 (4.7)	90.5 (8.5)	86.2 (12.7)	76.1 (6.3)	82.56 (2.1)	81.6 (9.2)
DON	91.0 (16.4)	94.3 (4.2)	85.4 (3.6)	90.2 (9.7)	92.2 (4.0)	83.9 (1.5)	93.0 (5.0)	89.7 (6.0)
FUS-X	90.4 (6.3)	93.7 (2.1)	89.7 (5.6)	91.3 (4.8)	86.1 (12.2)	91.9 (1.8)	96.3 (0.6)	91.4 (7.6)
NEO	88.5 (12.5)	100.0 (7.3)	89.9 (5.4)	92.8 (9.6)	90.7 (11.4)	94.1 (2.9)	95.8 (2.8)	93.5(6.4)
3-ADON	92.2 (17.9)	98.8 (14.0)	89.0 (8.5)	93.4 (13.1)	103.1 (5.9)	87.6 (6.6)	94.5 (5.0)	95.1 (8.7)
15-ADON	96.6 (17.7)	100.1 (5.5)	90.8 (5.4)	95.8 (10.6)	90.3 (11.0)	93.4 (4.7)	94.2 (4.3)	92.6 (6.6)
DAS	98.0 (9.1)	103.1 (3.4)	95.4 (5.6)	98.9 (6.5)	106.8 (10.4)	91.3 (4.0)	94.8 (4.1)	97.6 (9.6)

*LOQ: limit of quantification. RSD: relative standard deviation