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Evaluation of mycotoxins and their metabolites in human breast milk using liquid chromatography coupled to high resolution mass spectrometry

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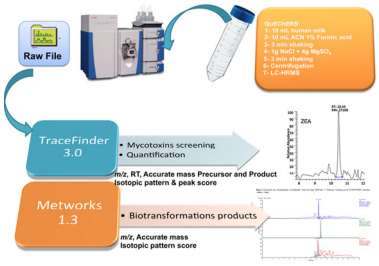
HIGHLIGHTS

- The first paper identifying and quantifying mycotoxins and metabolites in human breastmilk.
- QuEChERS–UHPLC–HRMS allowed rapid and reliable quantitative analysis.
- The exposure of mothers and infants to mycotoxins was evidenced using human milk.

GRAPHICAL ABSTRACT



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ABSTRACT

Humans can be exposed to mycotoxins through the food chain. Mycotoxins are mainly found as contaminants in food and could be subsequently excreted via biological fluids such as urine or human breast milk in native or metabolised form. Since breast milk is usually supposed as the only food for new-borns, the occurrence of mycotoxins in thirty-five human milk samples was evaluated by a newly developed method based on QuEChERS extraction and UHPLC–HRMS detection. The method described here allows the detection of target mycotoxins in order to determine the quality of this initial feeding. The method has been fully validated, with recoveries ranging from 64% to 93% and relative standard deviations (RSD, %) being lower than 20%. Using the method described, non-metabolised mycotoxins such as ZEA, NEO, NIV, ENA, ENA_1, ENB, ENB_1 and metabolites, such as ZEA metabolites, HT-2, DOM and T-2 triol were detected in human milk samples. Results obtained help to estimate the exposure of mothers and infants to mycotoxins. Moreover, to the best of our knowledge, this is the first work describing the simultaneous detection, quantification and screening of mycotoxins and their metabolites in human mature milk.

1. Introduction

Mycotoxins are substances produced by moulds that contaminate various agricultural commodities either before harvest or

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under post-harvest conditions [1]. Many attempts to study the occurrence of mycotoxins have been carried out around the world and these surveys have commonly demonstrated the co-occurrence of mycotoxins in food; in fact, results suggest that in certain situations as much as 50% of the commodities may be contaminated by mycotoxins [2]. Usually, human exposure to mycotoxins has been based on detecting and quantifying these toxins in foods, such as raw materials or cereal-based products [2,3], since the exposure occurs mostly through the intake of these food commodities.

However, relying on analytical data for determining mycotoxin exposure of human populations is difficult due to the heterogeneous distribution of mycotoxins in food commodities, the time lag between toxin intake and the development of chronic disease and the inaccuracies of dietary questionnaires for determining food intake data. Therefore, a more reliable and relevant indication of individual exposure could be provided by biomarkers measured in biological fluids. The identification of mycotoxins and their main metabolized products could therefore serve as such biomarkers and could facilitate effective exposure assessment [4–7].

Yet, in comparison to other biological fluids such as blood, plasma and urine, the database on multi-mycotoxin levels in human milk is rather small and so far practically non-existent in many countries. It is evident that breast milk is a relevant source of mycotoxins for neonates and infants since their presence in samples collected in several European countries has been documented [8–11]. During the beginning periods of human life, breast-feeding is usually major way of feeding [12–14]; therefore, the presence of mycotoxins in human milk could be a striking and serious problem.

Biomonitoring studies have also documented excretion of mycotoxins with human milk, mainly focused on aflatoxins, ochratoxin A (OTA), zearalenone (ZEA) and their metabolites [8–11]. However, these studies have not included *Fusarium* toxins and their metabolites, such as type A and B trichothecenes, enniatins or fumonisins, which have been studied in other biological fluids [4–7,15], but they have been marginalised in human milk.

Nowadays, the state of the art of analytical chemistry using high resolution mass spectrometry (HRMS) permits the use of accurate mass measurements in many fields of bioanalysis such as metabolite identification, structure elucidation, analytical toxicology, doping control and food analysis [16–18]. In the last decade, time-of-flight (TOF) and Orbitrap™ mass analysers have been mainly chosen since these techniques are becoming more affordable and they have demonstrated some advantages: identification, screening of non-target compounds and retrospective data analysis [16–18]. The applicability of Orbitrap™ mass analysers for mycotoxin analysis has been mainly demonstrated on cereals, cereal-based products, beverages and biological fluids [16–20]. Up to now, the analysis of mycotoxins and their metabolites in human mature milk has not been carried out using UHPLC–HRMS.

Biological fluids are protein-rich extracts; hence samples need more intensive clean-up prior to analysis by UHPLC–HRMS [21]. Human milk contains a mixture of essential nutrients, such as proteins, carbohydrates, fats, vitamins, and minerals. This composition could create interferences and cause signal suppression during LC–MS analysis. Interfering matrix compounds should be removed by careful sample preparation to increase the sensitivity and reproducibility of analysis. By contrast, sample preparation always means an additional working step and there is always the risk of analyte loss. A strategy for avoiding losses could be a "dilute and shoot" method, which was used for analysing 15 mycotoxins in human urine [7]. However, extraction methods that have been commonly used to human milk have been solid–liquid extraction with or without clean-up step [8–11]. These extraction methods are long and tedious, for these reasons alternatives methods, such as QuEChERS could present an alternative. Modified QuEChERS method has been applied to analyse mycotoxins in food [17] and in urine [22], providing an alternative to conventional methods.

The aim of this research was to investigate the presence of mycotoxins and their metabolites in human mature milk. For this purpose, a modified QuEChERS–UHPLC–HRMS method was optimised in order to extract simultaneously and to identify unambiguously mycotoxins and their metabolites. In this way, metabolites could be taken into account for future studies, because metabolites could be less, equal or even more active than the parent compound; in the latter case the risk of toxicity can arise. Knowledge of the chemical characteristics of all metabolites is of great importance for assessing the toxic kinetics, toxicity risks, and for developing toxicological screening procedures.

2. Experimental

2.1. Subjects and sampling

Human milk samples were collected in València (Spain) and Ethical Committee of Universitat de València approved previously this research. Thirty-five young mothers participated voluntarily in the study during one-year, from January to December 2012. Participants, after informed consent, collected human mature milk samples using an electric or manual pump and expressed milk samples into sample containers at home. Samples were then sealed, transported at 4 °C to the laboratory and immediately transferred to glass vials with Teflon-lined tops and stored at −20 °C. Samples (30–50 mL) were collected on 30th day after birth to minimize differences in milk composition. Table 1 (Supplementary data) summarizes maternal ages, weight, height, BMI, working status and residence.

All new-borns had a birth weight (BW) between the 10th and 90th percentile according to gestational age, BW ranged from 2800 to 3900 g and a normal clinical examination at sampling time-point. The sampling was based on few exclusion criteria, namely multiple pregnancies, gestational hypertension, diabetes and infections, fever, chromosomal abnormalities, metabolic diseases, diseases of the breast or central nervous system, malnutrition, maternal allergy, maternal addiction for tobacco, alcohol and abuse drugs. Newborn exclusion criteria were taken into account, such as newborns with any malformation, cardiac or haemolytic disease.

2.2. Chemicals and reagents

Standards of 3-acetyldeoxynivalenol (3-ADON), deoxynivalenol (DON), fusarenon-X (FUSX), nivalenol (NIV), HT-2 toxin, T-2 toxin, diacetoxyscirpenol (DAS), neosolaniol (NEO), aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), aflatoxin G_2 (AFG_2), aflatoxin M_1 (AFM_1), OTA, ochratoxin α (OTα), fumonisin B_1 (FB_1), fumonisin B_2 (FB_2), fumonisin B_3 (FB_3), sterigmatocystin (STER) and ZEA were supplied by Biopure (Tulln, Austria). Standard of beauvericin (BEA), α-Zearalenol (αZOL) and β-Zearalenol (βZOL) were obtained from Sigma–Aldrich (Steinheim, Germany). Enniatin A_1 (ENA_1), enniatin A (ENA), enniatin B (ENB) and enniatin B_1 (ENB_1) were purchased by Enzo Life Science (Lausen, Switzerland).

Acetonitrile and methanol, both HPLC-grade, were supplied by Merck (Darmstadt, Germany). Deionized water was prepared from a Milli-Q system (Millipore, Bedford, MA, USA). Anhydrous magnesium sulphate, sodium chloride, ammonium formate and formic acid (≥99% purity), were purchased from Sigma–Aldrich (Steinheim, Germany). Sorbent used for clean-up was octadecyl-silica (C18-E) (50 μm) bonded silica from Phenomenex (Torrance, USA).

Table 1
Mycotoxin name, elemental formula, theoretical *m/z*, retention time (RT) and pseudomolecular ion using ESI− and ESI+.

ESI negative mode					
Mycotoxin	Elemental formula	Theoretical *m/z*	RT (min)	$[M-H]^-$	$[M+HCOO]^-$
NIV	$C_{15}H_{20}O_7$	312, 1209	2.82	–	357.1191
DON	$C_{15}H_{20}O_6$	296, 1260	3.78	–	341.1242
FUS-X	$C_{17}H_{22}O_8$	354, 1315	4.83	–	399.1297
3-ADON	$C_{17}H_{22}O_7$	338, 1366	6.01	–	383.1348
ZEA	$C_{18}H_{22}O_5$	318, 1467	10.40	317.1394	–
αZOL	$C_{18}H_{24}O_5$	320, 1624	9.47	319, 1551	–
βZOL	$C_{18}H_{24}O_5$	320, 1624	9.99	319, 1551	–
ESI positive mode					
Mycotoxin	Elemental formula	Theoretical m/z	RT (min)	$[M+H]^+$	$[M+NH_4]^+$
NEO	$C_{19}H_{26}O_8$	382, 1628	5.28	–	400, 1966
DAS	$C_{19}H_{26}O_7$	366, 1779	7.90	–	384, 2017
HT-2	$C_{22}H_{32}O_8$	424, 2097	8.92	–	442, 2435
T-2	$C_{24}H_{34}O_9$	466, 2203	9.45	–	484, 2541
FB_1	$C_{34}H_{60}O_{15}N$	721.3884	9.40	722.3957	–
FB_2	$C_{34}H_{60}O_{14}N$	705.3925	10.30	706.4008	–
FB_3	$C_{34}H_{60}O_{14}N$	705.3925	9.90	706.4008	–
ENA	$C_{36}H_{63}N_3O_9$	681, 4559	11.80	–	699, 4903
ENA_1	$C_{35}H_{61}N_3O_9$	667, 4402	11,65	–	685, 4746
ENB	$C_{33}H_{57}N_3O_9$	639, 4089	11.32	–	657, 4433
ENB_1	$C_{34}H_{59}N_3O_9$	653, 4246	11.50	–	671, 4590
BEA	$C_{45}H_{57}N_3O_9$	783, 4089	11.55	–	801, 4433
AFB_1	$C_{17}H_{12}O_6$	312, 0628	8.11	313, 0707	–
AFB_2	$C_{17}H_{14}O_6$	314, 0785	7.80	315, 0863	–
AFG_1	$C_{17}H_{12}O_7$	328, 0578	7.43	329, 0656	–
AFG_2	$C_{17}H_{14}O_7$	330, 0734	7.08	331, 0812	–
AFM_1	$C_{17}H_{14}O_7$	328.0583	7.11	329.0656	–
STER	$C_{18}H_{12}O_6$	324, 0634	10.42	325, 0707	–
OTA	$C_{20}H_{18}NO_6Cl$	403, 0823	9.98	404, 0895	–
αOTA	$C_{11}H_9O_5Cl$	256.0138	7.10	257.0211	–

2.3. Extraction method

Simplified QuEChERS procedure was employed to extract mycotoxins and their metabolites from human mature milk. Homogenized and representative portions of 10 ml were deposited into a 50 mL centrifuge tube, 10 mL of 1% formic acid in ACN were added, and the mixture was vigorously shaken for 3 min. As a next step, 4 g $MgSO_4$ and 1 g of NaCl were added and the mixture was shaken for 3 min again. Once the extraction was finished, the sample was centrifuged (5 min, 10.000 rpm, 20 °C). Finally, an aliquot (1 mL) filtered through a 22 μm nylon filter before their injection into the UHPLC–HRMS.

2.4. Liquid chromatography high resolution mass spectrometry

The analytical parameters used in this study were in accordance with previous publications [16,17]. Nevertheless, in this work, two independent runs were carried out using electrospray ionization (ESI) in positive (ESI+) and negative (ESI−) modes in order to identify as high number of mycotoxins and their metabolites as possible in target and non-target analysis. Table 1 highlights ESI modes conditions and pseudomolecular ions (target mycotoxins).

An Accela UHPLC system (Thermo Scientific, San Jose, CA, USA) was used for the separation of target analytes. The chromatographic separation was performed using a Hypersil GOLD aQ™ analytical column (100 mm × 2.1 mm i.d., 1.9 μm; Thermo Fisher Scientific, San Jose, CA, USA) held at 35 °C. As mobile phase, 5 mM ammonium formate and 0.1% acid formic in water (A) and methanol (B) was used in both polarities. The gradient was as follows: start with 5% B, linear increase to 50% B in 6 min, for next 4 min another linear increase to 95% B, keep up to 15 min, switching to 5% B in 15.1 min, and column equilibration for 3 min before the next injection. The flow rate was 300 μL min^{-1}. The injection volume was 5 μL.

The operation parameters of the single-stage Orbitrap™ mass spectrometer (Exactive; Thermo Scientific, Bremen, Germany) optimised for the heated electrospray interface (HESI-II; Thermo Scientific, Bremen, Germany) were as follows: sheath gas/aux gas: 35/10 arbitrary units, capillary temperature: 300 °C, heater temperature: 300 °C, capillary voltage: +60/−50 V, and spray voltage +4/−3 kV. The system was operated in the full spectral acquisition mode in the mass range of *m/z* 100–1000 at resolving power settings of 50,000 FWHM at fixed acquisition rate of 2 spectrum s^{-1}, processed using Xcalibur 2.0, TraceFinder 3.0 and Metworks 1.3, all of them software from Thermo Scientific.

MS analyser used in this work was equipped with an HCD cell capable of generating fragmentation information in a non-selective manner. Fragmentation was achieved using collision energy of 35 eV. Full scan and all ions MS/MS spectrum acquisitions were performed in separated chromatographic runs.

The external mass axis calibration without the use of the specific lock mass was employed. For the mass accuracy calculation, mass at the apex of the chromatographic peak obtained as the extracted ion chromatogram was used.

2.5. Confirmation criteria

Confirmation criteria were based on the following items; (i) chromatographic separation: an analyte in a real sample had to elute within a ±2.5% interval of the retention time (RT) of the same analyte in a quality control sample of the same analytical batch (Table 1), (ii) mass spectrometric detection: the measured diagnostic ions with a relative intensity of >10% in the full scan mass spectrum of an analyte in a real sample had to correspond with those found in the mass spectrum of the same analyte in a quality control sample of the same analytical batch, (iii) the measured accurate mass of $[M+H]^+$, $[M+NH_4]^+$, $[M-H]^-$ or $[M+HCOOH]^-$ must fit the theoretical accurate mass with a mass tolerance set

Table 2
Validation of modified QuEChERS method: lowest Calibration Levels (LCLs), matrix effects (ME), percentage recovery (Rec., $n = 5$, %), Intra-day and Inter-day precision (% RSD) at low level (50 ng mL^{-1}) and high level (250 ng mL^{-1}).

Mycotoxin	LCL (ng mL^{-1})	ME[a]	Rec.	Intra-day[b]		Inter-day[c]	
				Low level	High level	Low level	High level
NIV	50	71	64	16	14	19	17
DON	5	71	71	11	10	12	12
3-ADON	10	80	83	12	10	14	16
FUSX	50	78	80	18	19	18	17
NEO	2	99	86	7	8	13	12
DAS	1.5	104	87	9	9	8	10
T-2	2.5	95	92	7	8	14	9
HT-2	10	96	91	9	10	11	12
ZEA	1.5	88	84	8	10	14	12
αZOL	1.5	90	88	8	9	13	10
βZOL	1.5	93	91	10	9	12	10
FB_1	10	109	89	14	9	17	12
FB_2	10	109	84	12	10	17	13
FB_3	10	104	83	15	9	18	11
ENA	1	79	82	8	10	9	10
ENA_1	1	89	80	7	7	10	8
ENB	1	90	80	8	11	10	10
ENB_1	1	82	79	10	9	17	19
BEA	1.5	70	73	11	14	16	20
AFB_1	1.25	73	75	7	9	12	10
AFB_2	1.25	69	74	8	9	12	11
AFG_1	1.25	70	71	12	10	14	13
AFG_2	1.25	77	72	13	9	15	11
AFM_1	1.25	73	69	12	13	17	17
STER	2	81	87	5	7	9	8
OTA	5	110	93	11	6	15	9
OTα	5	109	91	9	10	12	12

[a] ME%: (slope matrix matched calibration/slope standard in solvent) × 100.
[b] Number of replicates: 10.
[c] Different days: 5.

at ±5 ppm (Table 1), (iv) isotopic pattern: the relative intensities between $^{13}C/^{12}C$ isotope, expressed as a percentage of the intensity of the most intense ion, shall correspond to those of the quality control sample for the same mycotoxin and within the tolerance established in the 657/2002/CE for the percentage obtained. (v) fragment ions obtained by HCD must be present and their relative abundances with respect to $[M+H]^+$, $[M+NH_4]^+$, $[M-H]^-$ or $[M+HCOOH]^-$ ion must coincide with those of a calibration standard within ±15%.

2.6. Validation of method

Validation of the analytical method was performed according to following directive and guide on that subject [23,24]. Following parameters were studied in order to assess the efficiency of this analytical method: specificity/selectivity, matrix effects, linearity, lowest calibration level (LCL), precision as repeatability, within-lab reproducibility and recovery.

Linearity was evaluated by preparing different calibration curves (acetonitrile and human mature milk), ranged from LCL to 100 times LCL (six-point calibration) in triplicate at six concentrations levels into the dynamic range. In parallel, matrix effects were investigated in human milk by comparing the slopes of standards in solvent with the slopes of matrix-matched standards (Table 2).

Recoveries and relative standard deviations (RSDs) of mycotoxins were measured to validate the QuEChERS–UHPLC–MS/MS method by spiking the blank samples at three different concentrations (low level: 50 ng mL^{-1}, medium level: 100 ng mL^{-1}, and high level: 250 ng mL^{-1}) and then analysing them in 5 repetitions. The precision of the method was determined by the repeatability and reproducibility studies, and expressed as the RSD (%). The intra-day precision was expressed as the standard deviation of the recovery values of the spiked samples measured during the same day. The inter-day precision was determined by analysing the spiked samples for five different days.

3. Results and discussion

3.1. Optimization of modified QuEChERS method

A QuEChERS method offers different alternatives, by re-adjusting protocol according to the matrix analysed. However, there are few common basic steps: (a) homogenization of the sample, (b) addition of water, (c) addition of solvent and salts, (d) pH adjustment and (e) clean-up. Due to the aqueous nature of the matrix (90% of water) the step b – addition of water – was skipped. Moreover, the document SANCO/12495/2011 advises the addition of water only to matrices that contain less than 40% of moisture [25]. Regarding the extraction solvent, acetonitrile (ACN) has been chosen. This solvent permitted good recoveries of target mycotoxins and the partial removal of unwanted material [21]. In parallel, protein precipitation was also carried out using ACN. Consequently, the addition of 0.1%, 0.5% and 1% (v/v) of formic acid in ACN was tested. When using 0.1% and 0.5% of formic acid, lower recoveries of fumonisins, OTA, DON, OTα, ZEA and their metabolites were obtained. However, when modified QuEChERS was applied using 1% of formic acid, the recoveries for acidic and polar mycotoxins were higher and more reproducible, the acidification could increase the migration of these compounds from the aqueous phase to the organic layer. These results are in accordance to that has been reported by Lacina et al. [26], in which the addition of formic acid and pH adjustment using QuEChERS for different matrices has been described, and the efficiency of this extraction was evaluated for pesticides and mycotoxins. The next step in this study was to evaluate the need of a clean-up step. Human milk contains a high percentage of lipid content, and lipids could be transferred into the organic extract. Therefore, C_{18} sorbent (50 mg mL^{-1}) was tested as

a clean-up step for removing co-extracted compounds. According to our observations, this step did not improve sample clean-up, and was excluded from the workflow. The final modified QuEChERS method permitted a fast, easy and cheap extraction method for mycotoxins and their metabolised compounds.

3.2. Applicability of liquid chromatography coupled to high resolution mass spectrometry

The LC–HRMS has been proven as excellent tool in the screening, quantitation and confirmation of targeted mycotoxins and their metabolites. However, there are several criteria that need to be considered: mainly (I) matrix effects observed during ESI ionization of matrix containing samples and (II) compliance with regulatory requirements.

The results obtained in the matrix effect study have been interpreted taken into account the study conducted by Ferrer et al. [27]. The matrix effect was classified into three different categories attending to the calculated values. There was no matrix effect when the ME value was between 80% and 120%. A medium matrix effect was considered when the values ranged between 40% and 80% or 120% and 150%. A percentage below 40% or above 150% was classified as a high matrix effect. Following this classification with the QuEChERS–HPLC–HRMS method developed no matrix effect was found for the 63% of the analytes. None of the compounds showed high matrix effect. A medium matrix effect was obtained for the rest of the mycotoxins. This evidence is summarized in Table 2, where calculated matrix effects are shown.

The analytical method has been proven as excellent tool in the screening, quantitation and confirmation of targeted mycotoxins. However, several limitations of the direct injection of ACN were noted; on the one hand the direct injection of ACN resulted in a slightly deficient and distorted peak shape for NIV. On the other hand, matrix effects were superior for NIV as a consequence of matrix coelution and partly peak reduction, as it can be seen in Table 2. To keep in mind these premises, a compromise between the direct injection of ACN and method validation was evaluated. At the end, 27 mycotoxins were measured using direct injection of ACN, as well as, the method performance was satisfactory, although peak shape and validation data for NIV were accepted knowing that they could have been improved.

Another critical point of the applicability of HRMS in routine analysis is the expected compliance with 2002/657/EC [23]. According to the current version of the document mentioned above, as HRMS is defined as resolving power of 10,000 at 10% valley at all masses (magnetic sector instruments considered). Furthermore, criteria have not been set for mass accuracy that is the more relevant parameter with respect to the quality of obtained data. On the other hand, when Orbitrap™ analyser was employed with the HCD collision cell, it was possible to provide required number of IPs as defined by the regulation. This was achieved by simultaneous monitoring of precursor ion and product ions generated in the collision cell.

3.3. Method validation

Table 2 summarises LCLs, matrix effects (%), recoveries (%) and RSD (%) for intra-day and inter-day test values obtained for target mycotoxins in human milk.

Acceptable regression coefficients and linearity of matrix-matched curves were observed for all mycotoxins (correlation coefficients (r^2) ≥ 0.99 in all cases). LCLs values of target mycotoxins ranged from 1 to 50 ng mL^{-1}. A major drawback in the analysis of mycotoxins in complex samples by UHPLC–HRMS, such as biological fluids, is represented by the presence of matrix effects, i.e. an unexpected suppression or enhancement of the analyte response due to co-eluting sample constituents. Generally, it is supposed that the suppression or enhancement effects originate from the endogenous compounds such as fats, proteins or carbohydrates [21]. As it can be seen in Table 2, significant signal suppression and enhancement differences were observed for the different substances. Therefore, matrix-matched standards calibration was performed to compensate matrix effects and to obtain effective quantification.

Moreover, results shown in Table 2 indicated that method performance is satisfactory, meaning that recovery values (64–93%) and precision (with all RSD values below 20%) at the three fortified concentration levels were obtained.

3.4. Application to human mature milk samples

The developed analytical method allowed reliable detection of mycotoxins and their metabolites. ZEA, NEO, NIV, ENA, ENA$_1$, ENB, ENB$_1$ were detected as target and non-metabolized mycotoxins (Table 3). On the other hand, DOM, HT-2, T-2 triol and ZEA metabolites were suspected to be present in the samples. HT-2, αZOL and βZOL were undoubtedly identified because of the availability of the analytical standard. Applying above described confirmation criteria, ZEA was found in 13 samples (37%) and concentrations ranged from 2.1 to 14.3 ng mL^{-1} (Table 3); its metabolites αZOL and βZOL were only found in sample 4. On the other hand, type A trichothecenes, such as HT-2 and NEO were also detected. HT-2 was found in 10 samples (29%) and NEO in 7 samples (20%). HT-2 showed the highest concentration level for type A trichothecenes; the calculated concentration was 62.5 ng mL^{-1}. In addition, T-2 triol was found in one sample. In parallel, NIV was only found type B trichothecene and it was identified in 3 samples (9%). However, DOM, a metabolite coming from DON, was found in 1 sample (3%). Regarding other mycotoxins, two samples of human mature milk showed a low concentration of enniatins: ENA, ENA$_1$, ENB and ENB$_1$ (6%). It should be noted that the co-occurrence of ZEA and its metabolites was not observed, nor the co-occurrence of DON and its

Table 3
Occurrence of target mycotoxins or their metabolites in mature human mature milk, expressed as ng mL^{-1}.

	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	S17	S18	S19	S20	S21
ZEA			5.5		14.0	14.3	11.5	13.2	7.8					11.7		9.2	10.1	6.6	2.1	9.1	6.6
αZOL				16.7																	
βZOL				39.8																	
HT-2		46.6	60.7								62.5	34.8	37.7			15.2	55.5	20.1		12.2	19.6
NEO	36.9	14.0	18.5	20.8												10.3	13.4	11.1			
ENA	20.1														25.2						
ENA$_1$	42.1														51.1						
ENB	99.8														110.3						
ENB$_1$	101.1														90.7						
NIV				69.7						67.1									53.1		

Blank space <LCLs: lower than LCL level.

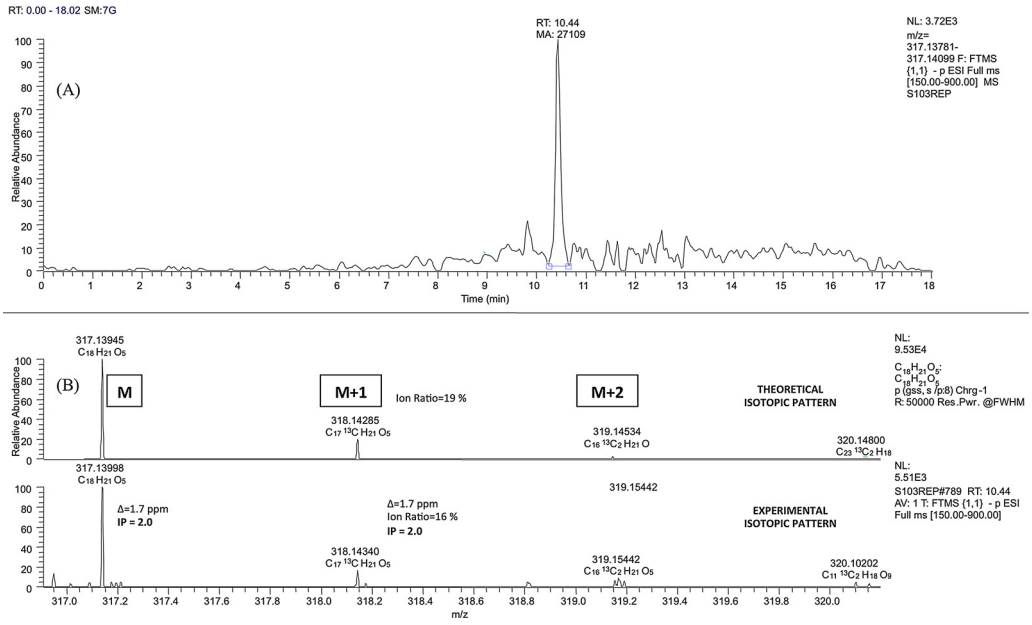

Fig. 1. ZEA confirmation. (A) HPLC-Orbitrap MS extracted ion chromatogram of ZEA resolving power 50,000 FWHM, extraction window 5 ppm. (B) Theoretical and experimental isotopic pattern of ZEA.

metabolites, DOM or phase II metabolites. This could be probably explained by their levels, which were lower than LCLs.

Since the identification and confirmation of mycotoxins and their metabolites was an important point in this research, an identity confirmation criterion was established; Fig. 1 summarizes the confirmation workflow of ZEA in human mature milk. In this example, ZEA was unambiguously confirmed using the RT, measuring precursor ion and the isotopic pattern and product ions.

Regarding routine mycotoxins analysis, the EU has established confirmatory criteria that are based on an identification points system (IPs); three points are required for confirmation of mycotoxins. In this case, for HRMS analysis 2.0 IPs are earned for each precursor ion and 2.5 IPs for each product ion [23].

In reference to a previous ZEA example the observed retention time was 10.44 min and the deviation was lower than 2.5%. The precursor ion was measured with a mass accuracy below 5 ppm. In parallel, ion ratios ^{12}C and ^{13}C were measured, obtaining 4 IPs: 2.0 IPs from ^{12}C isotope of the ion $[M-H]^-$ plus 2.0 IPs from the ^{13}C isotope of the ion $[M-H]^-$. The mass errors were calculated for ^{12}C and ^{13}C isotopes, which were 3.41 ppm and 3.60 ppm, respectively (Fig. 1B). However, 2002/657/EC does not include criteria for mass accuracy although it can be considered an additional parameter of confirmation. Therefore, based on the literature a mass accuracy <5ppm was set as a confirmatory purposes [16,17,21,23,28,29].

The next step was to study the fragmentation pathway of ZEA using all ion fragmentation MS spectrum acquisitions performed in separate chromatographic run. Peaks corresponding to the parent ion 317.1394 *m/z* $[C_{18}H_{22}O_5-H]^-$, 299.1289 *m/z* loss of water, 273.1496 *m/z* decarboxylation, and 175.0401 *m/z* were measured and fragment ions obtained and their relative abundances coincided with those of a calibration standard within ±15%, as well as this pathway was previously observed [7,20]. In this way, ZEA was unambiguously identified in human mature milk.

Subsequently, this sample was processed using MetWorks 1.3 Software (Thermo Fisher Scientific), which screens for expected biotransformations automatically. In this part, this software was applied to evaluate the presence of biotransformations of ZEA in human milk samples. ZEA samples were processed using this software and several biochemical modifications were observed. Main phase I reactions as hydroxylation and decarboxylation were identified. Fig. 2 shows extracted ions chromatograms of metabolites observed using MetWorks 1.3 Software. This figure shows that the main metabolite was produced by hydroxylation reaction, and this trend was reflected and confirmed analysing the rest of samples.

In this research ZEA and its metabolites, NEO, NIV, ENA, ENA_1, ENB and ENB_1 were detected in human milk. To our best knowledge, this is the first time that these mycotoxins are detected in this matrix. From analytical point of view, the biomonitoring programs have been commonly limited to the detection of aflatoxins and OTA up to now, since they are the most toxic mycotoxins and widely occurring mycotoxins. Secondly, QuEChERS–UHPLC–HRMS method allowed rapid and simultaneous multi-analyte determination of mycotoxins and retrospective data analysis for metabolites.

3.5. Dietary and toxicological interpretation

The distribution of xenobiotics into human milk continues to be an important issue as the community becomes more aware of the possible risks to the breast-fed infant. Transfer of xenobiotic into breast milk is most commonly quantitatively described using the milk to plasma concentration ratio [30]. In the particular case of mycotoxins, the confirmation of the presence of mycotoxins in human mature milk is in agreement with previous studies [8–10] and supports the need for monitoring mycotoxins accumulation in breast milk. Furthermore, metabolised or conjugated mycotoxins can emerge after metabolization by mammals [31]. Since many mycotoxins have lipophilic properties, and during lactation mother's fat reserves containing mycotoxins are mobilized to produce breast milk, mycotoxins may be carried into breast milk [32].

Maternal exposure to mycotoxins occurs mainly through food and drink contamination. The extent of exposure depends on several factors, such as diet composition, fungal strains occurring in the food, climatic and geographical conditions, cultivation technique and susceptibility level of host plants and crop protection. Therefore the geographical area of the study and dietary habits of mothers can influence the occurrence of mycotoxins in human milk. A recent total diet study (240 samples) was carried out in Valencia region, describing that the most frequently found mycotoxins were DON, FB_1, OTA and ZEA [33]. Analyses were performed

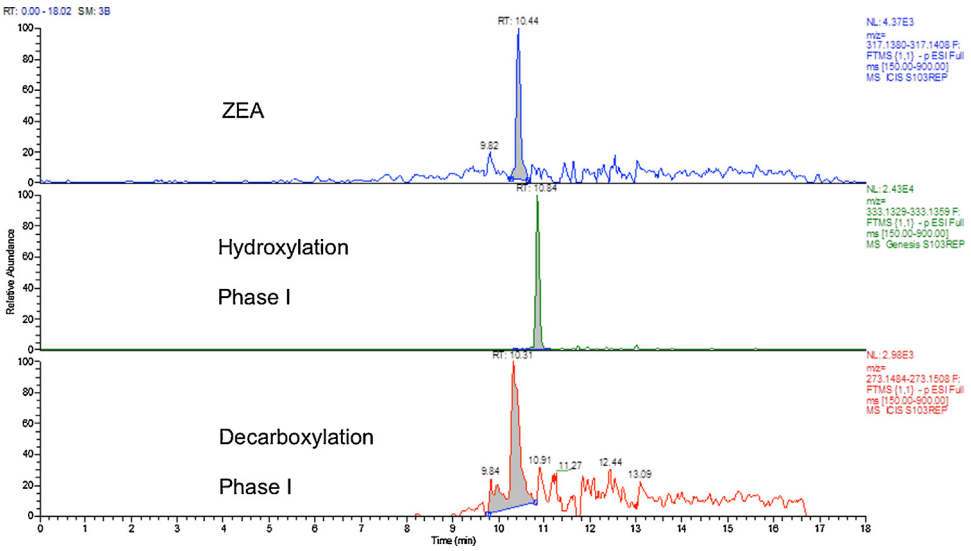

Fig. 2. Extracted ions chromatograms of metabolites observed using MetWorks 1.3 Software, resolving power 50,000 FWHM, extraction window 5 ppm.

by UHPLC coupled to a triple quadrupole mass spectrometer, and the acquisition of three SRM transitions per compound allowed positive samples to be confirmed. On the other hand, foodstuffs from the Mediterranean area were reviewed, and *Fusarium* toxins, such as fumonisins, trichothecenes a ZEA were mainly found, as well as emerging mycotoxins such as enniatins [1]. The common presence of these mycotoxins in Mediterranean foodstuffs confirms the results obtained in human milk. Moreover, the occurrence of mycotoxins and their metabolites in human milk has not been thoroughly studied.

Mycotoxin metabolism consists of the biochemical modification of compound by living organisms converting lipophilic chemical compounds into more readily excreted hydrophilic products. In general, this metabolism can be divided into two phases:

i. phase I, in which a variety of enzymes act to introduce reactive and polar groups into their substrates by transformation such as reduction, oxidation or hydrolysis.
ii. phase II or conjugation process in which modified phase I compounds are then conjugated by as glucosidation, glucuronidation or sulfatation.

In this research phase I metabolites, such as ZEA metabolites, HT-2, T-2 triol and DOM were mainly detected.

Focused on metabolism interpretation, the presence of emerging mycotoxins has been studied in pig plasma, demonstrating a big difference in oral absorption between the different enniatins. Although they have a similar chemical structure, ENB seemed to have the highest oral absorption, followed by enniatins B_1, A_1, A [15]. Furthermore, the authors concluded that elimination rate of enniatins was faster than DON. In this study, two sample presented enniatins at low concentration levels (Table 3). It appears that rapid metabolism was also carried out in humans. In fact, it has been demonstrated that the main part of absorbed *Fusarium* toxins shows a rapid elimination within 24 h after ingestion, followed by a slower excretion of small amounts [34].

T-2 was probably metabolised to T-2 triol and HT-2, and HT-2 was detected in 10 samples. These results are in agreement with previous toxicokinetic study in swine [35]. The authors described that T-2 and T-2 triol were metabolised faster than HT-2. Probably, T-2 toxin undergoes a deacetylation in C-4, producing HT-2 toxin as a phase I metabolite. However, other enzymes could react with these mycotoxins producing other metabolites. For example, it has been demonstrated in previous researches; T-2 toxin conversion to HT-2, and also T-2 metabolised to NEO as a second metabolite by carboxylesterase were studied in human blood cells [36]. Type B trichothecenes in biological fluids have been also studied in urine [4–7]. However, their presence in human milk had not been studied up to now. In this study NIV and DOM were detected. Whereas, the first one could be produced by metabolisation of FUSX [34], the second one could be produced by human microbiota [37,38].

The results presented in this paper could help to evaluate the exposure of mothers and infants to mycotoxins. Moreover, to the best of our knowledge, this is the first work describing the simultaneous detection, quantification and screening of mycotoxins in breast milk using UHPLC–HRMS approach.

4. Conclusion

The developed analytical method based on QuEChERS–UHPLC–HRMS, allowed rapid, cheap and reliable screening and quantitative analysis of mycotoxins and their metabolites in human milk. The organic extract was directly measured without clean-up step and MS detector operated in high mass accuracy and resolving power mode provided required sensitivity. Matrix-matched calibration was employed to compensate for matrix effects.

The analytical method has been applied to the analysis of 35 samples within a biomonitoring study. ZEA, NEO, NIV, ENA, ENA_1, ENB, ENB_1 were detected as non-metabolized mycotoxins while, DOM, HT-2, T-2 triol and ZEA metabolites were identified as metabolised forms.

The analytical method demonstrated to be an excellent tool for unambiguous identification of target mycotoxins and screening of non-target mycotoxins by (i) retention time, (ii) accurate mass of mycotoxin fitting the theoretical accurate mass with a tolerance set at ±5 ppm, (iii) isotopic pattern and (iiii) fragment ions obtained by HCD.

This is the first paper describing the comprehensive screening, quantification and confirmation of mycotoxins and their metabolised products in human milk samples. The presented data provide additional matter for debate about the occurrence of mycotoxins in biological fluids and their impact on human health. Even though further investigation and more broad exposure studies will be needed, the data summarized could provide a new perspective for woman dietary recommendations during pregnancy and lactation period and could support the unique role of breast feeding in infant nutrition.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aca.2013.12.001.

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