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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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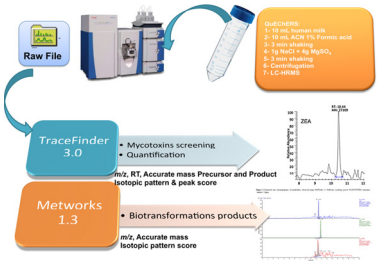
HIGHLIGHTS

- 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).