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Multiclass analysis of mycotoxins in biscuits by high performance liquid chromatography–tandem mass spectrometry. Comparison of different extraction procedures

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ABSTRACT

A sensitive, simple and rapid method for the simultaneous determination of 19 mycotoxins in biscuits (a dry matrix containing cereals and egg) has been developed using high performance liquid chromatography coupled to tandem mass spectrometry with electrospray source working in both positive and negative mode.

Due to the matrix complexity and the high amount of contaminants, a solid phase extraction method using graphitized carbon black was optimized for an effective clean-up step. Accuracy was carried out in the selected matrix using blank samples spiked at three analyte concentrations. Recoveries between 63 and 107% and relative standard deviations lower than 12% were obtained. For all considered mycotoxin classes, i.e. thricotecenes A and B, zearalenone and its metabolites, fumonisins, ochratoxin A, enniatins and their structurally related beauvericin, the method was validated in terms of linearity, recovery, matrix effect, precision, limit of detection and limit of quantification. Matrix-matched calibration was used for quantification purposes, in order to compensate for matrix effect. The coefficients of determination obtained were in the range of 0.9927–1. The limits of quantification, ranging from 0.04 $\mu g\,kg^{-1}$ for enniatin B1 to 80.2 $\mu g\,kg^{-1}$ for nivalenol, were always lower than maximum permitted levels for every regulated mycotoxin by the current European legislation.

1. Introduction

Mycotoxins are a group of naturally occurring toxic compounds produced by the secondary metabolism of many filamentous fungi (mainly *Penicillium*, *Fusarium* and *Aspergillus* genera) [1,2]. Both fungal growth and mycotoxin production depend on a variety of factors. Whenever several physical, chemical, and biological conditions take place, mycotoxin contamination may occur. High temperature processes cause varying degrees of reduction of mycotoxin concentrations, but most mycotoxins are moderately stable in most food processing systems thus they can still be found even in finished products [3]. The presence of mycotoxins in food may cause mycotoxicosis, which comprises many different adverse effects, such as the induction of acutely toxic, immunosuppressive, mutagenic, teratogenic, oestrogenic and carcinogenic effects; for these reasons mycotoxin contamination of food and feed is a worldwide problem with great relevance to human and animal health [4–7].

As far as mycotoxin contamination of cereals and their products is concerned, current European Union (EU) food safety legislation regulates the content of some mycotoxins by means of the Regulation (CE) 1881/2006 [8] and its subsequent amendments (Regulation (CE) 1126/2007 and Regulation (UE) 165/2010) [9]. Ochratoxin A maximum level (ML) is set at 3 $\mu g\,kg^{-1}$, deoxynivalenol ML ranges between 500 and 750 $\mu g\,kg^{-1}$, depending on the type of cereal intended for direct human consumption (the lower level is for bread, including small bakery wares, pastries, biscuits, cereal snacks and breakfast cereals); zearalenone ML ranges between 50 and 75 $\mu g\,kg^{-1}$, depending on the type of cereal intended for direct human consumption (the lower level being as previously described for deoxynivalenol); fumonisins (FBs), the levels of which are regulated only for maize, maize-based breakfast cereals and maize-based snacks, have a ML set at 800 $\mu g\,kg^{-1}$ for the sum of $FB_1 + FB_2$. T-2 and HT-2 toxins are named, but the

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value of their limit is not indicated. All the other considered mycotoxins, such as less common trichothecenes A and B, zearalenone metabolites, enniatins and beauvericin, are not legislated yet but we decided to consider them because they can be found in maize [10] and because they can be considered as recently "emerging" mycotoxins [11,12], due to the increasing attention for their toxicity [13].

For multiclass mycotoxin analysis, in recent years there is a growing tendency to develop rapid LC-mass spectrometry (MS) methods with minimum sample treatments. Approaches commonly employ solid–liquid extraction (SLE) [14–18], solid phase extraction (SPE) [19,20], and/or liquid–liquid extraction (LLE) with the subsequent direct injection to LC-MS instrumentation and/or immunoaffinity-column (IAC) clean-up [21–28].

Nowadays, one of the main objectives in food contaminant analysis is the development of methods with minimum sample treatment. In many works the QuEChERS methodology (quick, easy, cheap, effective, rugged and safe) has also been recently employed for the determination of multiclass mycotoxin analysis in different food matrices [1,12,23,29] but its effectiveness is still under examination, with results depending on the type of matrix. The development of a multi-mycotoxin analysis method, which does not comprise any purification step for the quantification of analytes having great differences in structures and physicochemical properties, is still challenging, especially for matrices, such as cereal- and egg-derivative products, having high content of fats and other interfering substances. Therefore, despite the potentiality of LC-MS/MS techniques, in some cases a purification step cannot be omitted, in order to avoid significant matrix effects (MEs) which decrease sensitivity and could lead to quantification errors.

The aim of this study was the development of a sensitive and reliable confirmatory multiclass procedure, based on SPE clean-up followed by LC/ESI-MS/MS, for the simultaneous determination of 19 selected mycotoxins in common worldwide cookies. The effectiveness of two direct methods (i.e. SLE and QuEChERS) was initially evaluated by high performance liquid chromatography (HPLC) coupled to a triple quadrupole mass spectrometry. Then we developed a method with a sample clean-up using graphitized carbon black (GCB) which allowed the selective isolation of analytes from complex matrices due to various types of interactions (i.e. hydrophobic, electronic and ion-exchange interactions).

The developed method can be applied to the determination of all the selected mycotoxins and, as far as the legislated *Fusarium* mycotoxins (deoxynivalenol, zearalenone, T-2 toxin, HT-2 toxin and fumonisins B1 and B2) and ochrathoxin A is concerned, it allows their determination below their MLs, as regulated by EU [9,30,31].

2. Experimental

2.1. Chemical and reagents

Standards of (i) *Fusarium* toxins, major acetylated conjugates and other products of transformation, namely 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON), deoxynivalenol (DON), diacetoxyscirpenol (DAS), fusarenon-X (FUSX), HT-2 toxin (HT-2), neosolaniol (NEO), nivalenol (NIV), T-2 toxin (T-2), zearalenone (ZEA), α-zearalenol (α-ZOL), β-zearalenol (β-ZOL); (ii) ochratoxins: ochratoxin A (OTA); (iii) fumonisins: fumonisin B1 (FB1), fumonisin B2 (FB2); and (iv) enniatins and their structurally related beauvericin: beauvericin (BEA), enniatin A (ENA), enniatin A1 (ENA1), and enniatin B1 (ENB1) were purchased from Sigma–Aldrich (St. Louis, MO, USA). 3-ADON, 15-ADON, FUS-X, NEO and NIV (100 μg mL^{-1} in acetonitrile) were purchased in solution (analytical standard grade) whilst the other mycotoxin reference standards were supplied as powder (Premium Quality Level and/or assay ≥98%).

Sulfamethoxazole (SMX) was used as volumetric internal standard (IS). Full names, abbreviations, chemical formula and precursor ions of the selected compounds are reported in Table 1.

All reagents were of analytical reagent grade, solvents were LC-MS grade. Formic acid, acetonitrile, methanol, ammonium formate, dichloromethane, hydrochloric acid, magnesium sulfate ($MgSO_4$), and sodium chloride (NaCl) were obtained from Sigma–Aldrich. Ultrapure water (resistivity 18.2 MΩ cm) was obtained by an Arium water purification system (Sartorius, Florence, Italy).

2.2. Standard solutions

Individual analyte stock solutions were prepared at 100 μg mL^{-1} in acetonitrile. Fumonisin B1 and B2 were dissolved in acetonitrile/water (50:50, v/v). All of the standards were stored at −20 °C.

A composite standard working solution was prepared considering the intensity response (i.e. sensitivity in the LC-MS/MS measurement) of the target analytes and, for the legislated ones, their allowed MLs. According to this, the composite standard working solution was prepared by combining aliquots of each individual stock solution and diluting with acetonitrile to obtain the final concentration of 5 μg mL^{-1} for DON, NIV and FUSX; 2 μg mL^{-1} for 3-ADON, 15-ADON, HT-2, α-ZOL, β-ZOL, FB1, and FB2; 0.5 μg mL^{-1} for DAS, NEO, T-2, ZEA; 0.02 μg mL^{-1} for OTA, ENA, ENA1, ENB1, and BEA.

All the above solutions were stored at −20 °C in amber glass vials and kept in the dark at room temperature (20–25 °C) before use. Working standard solutions were prepared by suitable dilution of stocks with acetonitrile. These solutions were kept at 4 °C and renewed weekly.

2.3. Samples

As no blank certified reference materials are available, a number of samples of cookies (digestive type) of brands commonly found in stores and purchased randomly from Rome area retail markets, were checked to evaluate their contamination level to be used as blank material for spiking purposes (Fig. S1 in supplementary data). To ensure representative sampling, ten packages (10× 400 g) were collected and ground to a fine powder using a mortar and pestle. After that, a 500 g mycotoxin-free pooled powdered sample was randomly taken from the homogeneous fine powder and stored in the dark at 4 °C until analysis and used as blank materials for the validation study. Thereafter, sub-samples of 1 g were weighed for analysis.

All samples used for method development were kept in a dark and dry location at room temperature (20–25 °C) until handled.

2.4. Sample preparation

2.4.1. Extraction and clean-up apparatus

A model ST ultrasonic bath at a frequency of 50 ± 3 Hz from Stimin (Milan, Italy), and an ALC (Milan, Italy) multispeed refrigerated centrifuge PK131R were used.

Polypropylene tubes, polyethylene frits, and a vacuum manifold were from Supelco (Bellefonte, PA, USA); Carbograph-4 was purchased by LARA (Rome, Italy). Carbograph-4 is a GCB with a surface area of 210 m^2 g^{-1} and particle size range of 120–400 mesh, similar to Carboprep 200 (Restek, Bellefonte, PA, USA) and Envicarb X (Supelco).

Carbograph-4 cartridges were prepared by placing 500 mg of the adsorbent inside 6 mL polypropylene tubes between two