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A rugged high-throughput analytical approach for the determination and quantification of multiple mycotoxins in complex feed matrices

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ABSTRACT

We have developed and optimized high throughput method for reliable detection and quantification of 56 *Fusarium*, *Alternaria*, *Penicillium*, *Aspergillus* and *Claviceps* mycotoxins in a wide range of animal feed samples represented by cereals, complex compound feeds, extracted oilcakes, fermented silages, malt sprouts or dried distillers' grains with solubles (DDGS). From three tested extraction approaches (acetonitrile, acetonitrile/water, and QuEChERS), the QuEChERS-based method (Quick, Easy, Cheap, Effective, Rugged and Safe) was selected as the best in terms of analytes recoveries and low matrix effects. For separation and detection of target mycotoxins, method based on ultra-high performance liquid chromatography coupled with sensitive tandem mass spectrometry (U-HPLC–MS/MS) was employed. With regards to a high complexity of most of investigated feed samples, optimization of extraction/purification process was needed in the first phase to keep the method as rugged as possible. A special attention was paid to the pH of extraction solvents, especially with regard to the pH-sensitive silages. Additionally, purification of the acetonitrile extract by dispersive solid phase clean-up was assessed. Significant elimination of lipidic compounds was observed when using C_{18} silica sorbent. Matrix co-extracts were characterized by ultra-high performance liquid chromatography coupled with ultra-high resolution mass spectrometry (U-HPLC–HRMS). Large variability of matrix effects depending on the nature of examined feed was demonstrated in depth on a broad set of samples. Simple and unbiased strategies for their compensation were suggested.

1. Introduction

Nowadays, about 300–400 mycotoxins with different toxicity and economic impact have been identified in cereals and other agricultural commodities. They are the secondary metabolites produced mainly by microscopic filamentous fungi species of *Fusarium*, *Aspergillus*, *Penicillium* and *Claviceps* genus [1–4]. Concentrations of only selected mycotoxins have been regulated yet in animal feed by legislation – Commission Decision 2002/32/EC set up maximum levels for aflatoxin B1 [5], and limits for ochratoxin A, deoxynivalenol, zearalenone, fumonisin B1 and fumonisin B2 are recommended by Commission Recommendation 2006/576/EC [6]. Nevertheless, the spectrum of mycotoxins that can possibly contaminate animal feed is rather broader. Since 2008, the European Food Safety Authority (EFSA) have launched three calls for data on mycotoxins occurrence in food and feed to enable drafting of the scientific opinion on mycotoxins with respect to the food and feed safety. Type B trichothecenes (deoxynivalenol including its derivates and nivalenol), type A trichothecenes (HT-2 and T-2 toxin, and diacetoxyscirpenol), fumonisins, enniatins, beauvericin, alternaria toxins, ergot alkaloids, patulin, citrinin, sterigmatocystin, moniliformin and phomopsins have been included in the list of priority candidates for toxicological risk assessment.

For the reliable detection and quantification of these toxins in complex and difficult feed matrices, well-performed analytical methods are needed. Several studies concerned with analysis of multiple mycotoxins in feeds have been published [7–12]. However, the overall knowledge is fairly less extensive in comparison with advanced information platform on strategies applicable for control of cereals. Less effort paid to the implementation of a comprehensive analytical strategy for analysis of complex feeds was mainly due to a complexity of these matrices. Mainly fermented feeds are difficult to analyze. The other reason is also a limited transfer of most mycotoxins into edible parts of farm animals, thus low direct human health risk (aflatoxin M1 in milk and dairy products is the exception). On the other hand, adverse effects of mycotoxins on performance of farm animals resulting in economic losses, is another issue of concern. The true is that in routine practice, most of control laboratories exploit bioassays represented by ELISA (enzyme linked immunosorbent assay) for

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mycotoxins determination, however, by this approach, only several regulated mycotoxins are targeted. Currently, practically the only technique of choice for the selective and sensitive detection and quantification of multiple mycotoxins in animal feed is represented by (ultra-)high performance liquid chromatography coupled with tandem mass spectrometry, (U-)HPLC–MS/MS. The main challenge in the HPLC–MS/MS method development is the optimization of sample preparation procedure. Extraction method should allow isolation of a wide range of analytes from very different matrices with acceptable recoveries, repeatabilities, and limits of quantification (LOQs). The majority of existing mycotoxin methods is based on the acetonitrile/water extraction and many of them employ also crude extract purification. The clean-up step usually comprises solid phase extraction (SPE) with cartridges (MycoSep, C_{18}), simple liquid–liquid partition with hexane enabling defatting of extract, or much more specific immunoaffinity clean-up columns [7–9,13]. Nevertheless, beside the required reduction of matrix interferences, the purification always limits the range of analytes and prolongs analysis thus reduces sample throughput. To improve method accuracy, some of the recent methods developed for analysis of mycotoxins in animal feed used isotopically labeled internal standard surrogates [10,11]. However, their use for compensation of losses during the sample preparation, as well as matrix effects, is limited by the availability at the market, and by their cost. Worth to notice that obtaining of accurate results by this approach is only possible when for each target analyte, respective labeled analog is used. In this context, studies employing one or few internal standards for quantification of the whole set of chemically different analytes are rather controversial.

In recent years, QuEChERS method comprising extraction by acetonitrile:water mixture followed by salting-out the analytes into the acetonitrile phase to discriminate polar matrix co-extracts has become the widely used sample preparation approach. Although the QuEChERS method was originally developed and modified for analysis of pesticides in fruit and vegetables [14], it has been also successfully applied for analysis of mycotoxins by several authors [15–18]. Mol et al. were the first authors who employed the QuEChERS extraction for a simultaneous analysis of mycotoxins, pesticides and veterinary drugs in several difficult matrices, authors used sodium acetate buffer for analytes isolation, according to earlier study of Lehotay et al. [19]. Nevertheless, due to the low recoveries of fumonisins encountered, they rejected this approach in favor of the "dilute-and-shoot" approach [15]. The acetate-buffered QuEChERS was thoroughly tested also in other study dealing with analysis of 27 mycotoxins in silage, but again, recoveries of fumonisins were the same low [16]. The cause of this problem was probably the choice for experimental set-up. Omitting the use of NaCl within the method lead to the reduction of the efficiency of phase partition. Moreover, using of acetate-buffered extraction solvent showing rather high pH value ($\sim$5) was not able to extract these problematic analytes properly and repeatedly. This phenomenon was clearly documented by Lacina et al., who clearly illustrated this not only on fumonisins, but also on several acidic pesticides [18]. The suitable QuEChERS-based method showing good performance characteristics for fumonisins and other *Fusarium* mycotoxins was published by Zachariasova et al., where authors enabled the acidification of the extraction mixture with formic acid [20].

The aim of currently presented study was to critically assess the suitability of the QuEChERS method for the analysis of 56 mycotoxins produced by *Fusarium*, *Alternaria*, *Penicillium*, *Aspergillus*, and *Claviceps* fungi in a broad range of 12 'difficult' feeding matrices (feeding cereals, complex compound feeds, extracted oilcakes, fermented silages, malt sprouts or dried distillers' grains with solubles (DDGS)). To our knowledge, this is the first paper demonstrating the method optimization on real, naturally contaminated samples, which refers to the real situation much better than using of spikes. Following analytical steps were assessed: (i) composition of extraction mixture, (ii) duration of extraction process, (iii) the effect of dispersive solid phase clean-up of a crude QuEChERS extract, and (iv) strategies for matrix effects compensation. For the separation, detection, and quantitation of target mycotoxins, U-HPLC–MS/MS method was developed. For characterization of elution profiles and a nature of matrix co-extracts, ultra-high performance liquid chromatography coupled with high resolution (HR) orbitrap mass spectrometry (U-HPLC–HRMS) was applied.

2. Materials and methods

2.1. Reagents and materials

Anhydrous magnesium sulfate ($MgSO_4$), formic acid (98%), acetic acid ($\geq$99.7%), ammonium acetate (LC–MS grade), alumina (Al_2O_3), activated charcoal (p.a.), and HPLC grade acetonitrile (MeCN) were obtained from Sigma-Aldrich (Prague, Czech Republic). Methanol (MeOH) was obtained from Merck (Darmstadt, Germany). Sodium chloride (NaCl) was from Penta (Prague, Czech Republic) and Bondesil C_{18} sorbent (40 μm) for dispersive solid-phase extraction clean-up was obtained from Agilent Technologies (Santa Clara, CA, USA). Deionized water (18 MΩ) was produced by a Milli-Q system (Millipore; Bedford, MA, USA).

2.2. Analytical standards

Altogether, 56 analytical standards of mycotoxins and mycotoxin metabolites were used for experiments: *Fusarium* toxins: nivalenol (NIV), deoxynivalenol (DON), deoxynivalenol-3-glucoside (DON-3-Glc), fusarenon X (FUS-X), neosolaniol (NEO), 3- and 15-acetyldeoxynivalenol (3-ADON, 15-ADON), diacetoxyscirpenol (DAS), HT-2 and T-2 toxins (HT2, T2), verrucarol (VER), fumonisins B1, B2 and B3 (FB1, FB2, FB3), zearalenone (ZEA), α- and β-zearalenol (α-ZOL, β-ZOL), enniatins A, A1, B and B1 (Enn-A, Enn-A1, Enn-B, Enn-B1), beauvericin (BEA); 17 *Aspergillus* and *Penicillium* toxins: aflatoxins B1, B2, G1 and G2 (AFB1, AFB2, AFG1, AFG2), ochratoxin A (OTA), citrinin (CIT), cyclopiazonic acid (CPA), sterigmatocystin (STE), patulin (PAT), gliotoxin (GLIO), meleagrin (MEL), mycophenolic acid (MPA), paxilline (PAX), penicillic acid (PEN), penitrem A (PEN-A), roquefortine C (ROQ-C), verruculogen (Verruc); 12 ergot alkaloids produced by *Claviceps*: agroclavine (A-clavine), ergosine (E-sine), ergosinine (E-sinine), ergocornine (E-cornine), ergocorninine (E-corninine), ergocryptine (E-cryptine), ergocryptinine (E-cryptinine), ergocristine (E-cristine), ergocristinine (E-cristinine), ergotamine (E-tamine), ergotaminine (E-taminine), ergometrine (E-metrine) and 1 *Stachybotrys* toxin: stachybotrylactam (STACH), were obtained from Biopure (Tulln, Austria); standards of 4 *Alternaria* mycotoxins: alternariol (AOH), alternariol-monomethylether (AME), tentoxin (TEN) and altenuene (ATE) were obtained from Sigma-Aldrich (Taufkirchen, Germany). The declared purity of all standards was in the range of 96.0%–98.9%. All standards were stored in amber vials at −20 °C and brought to ambient temperature before use. Dried down standards of ergot alkaloids were stored in a mixture of MeCN:water:acetic acid (79:20:1, v/v/v), the other standards were in MeCN. For the purpose of spiking experiments, four working standards solutions were prepared, and further, a composite working standard solution (1000 μg mL^{-1}) was freshly prepared by transfer of calculated amount of each standard into an amber volumetric flask.

2.3. Samples

For realization of experiments referring to the development and optimization of the sample preparation method, certified reference materials, internal reference materials or non-spiked