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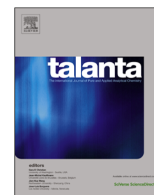

A rugged high-throughput analytical approach for the determination and quantification of multiple mycotoxins in complex feed matrices

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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.

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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 $-20\,^{\circ}$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 $\mu g\,mL^{-1}$) 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

naturally contaminated real-life samples characterized in Table S1 were used. Mycotoxins present in these materials are incorporated deeper and stronger in the matrix when compared with mycotoxins spiked on the sample surface, hence better simulation of extractability of naturally occurring mycotoxins is allowed. Dry samples were homogenized by the laboratory blender before processing. Samples with high moisture content (silages) were dried in the laboratory oven (12 h, 50 °C) before the analyses.

2.4. Development of sample preparation method

Within the extraction method development, three sample preparation procedures were tested. The model sample of maize silage was enabled for this purpose. *Method* (*i*): The QuEChERS procedure based on published method [20] consisted of extraction of 2 g sample with 10 mL of water containing 0.1% of formic acid and 10 mL of MeCN. Suspension was shaken for 30 min. After that, 1 g of NaCl and 4 g of $MgSO_4$ were added, shaken again and centrifuged. MeCN from the upper layer was analyzed. *Method* (*ii*): 2 g of sample were extracted with 10 mL of MeCN:water (50:50, v/v) mixture for 30 min. After centrifugation (5 min, 10,000 RPM), extract was analyzed. *Method* (*iii*): 2 g of sample were extracted with 10 mL of pure MeCN for 30 min. After centrifugation (5 min, 10,000 RPM), extract was analyzed. Fig. S1 illustrates the intensity of signal for particular extraction solvent mixtures used under U-HPLC–HRMS conditions (*2.7 U-HPLC–HRMS analysis*).

For matrix effects (matrix induced signal suppression/enhancement, SSE) establishment, matrix-matched standards and solvent standards (in MeCN and MeCN:water, 50:50, v/v) were prepared at 100 μg L^{-1}. The matrix effects were calculated as the matrix-matched standard to solvent standard percentage ratio. For recoveries assessment, both spikes (at 450 μg kg^{-1} prepared in three repetitions), and real-life naturally contaminated silage samples characterized in Table S1 were used.

2.5. QuEChERS method optimization

As a base, the acidic QuEChERS method published by Zachariasova et al. and Lacina et al. was taken, i.e. sample extracted with water containing formic acid and MeCN, after that, NaCl and $MgSO_4$ addition, sample centrifugation, phase separation, MeCN d-SPE purification and analysis [18,20]. Nevertheless, with regard to pH sensitive silages and other difficult feed matrices, following sub-steps were adjusted in order to get procedure rugged enough for proper co-isolation of 56 various mycotoxins. (i) pH of extraction mixture; it was modified so that would be possible to properly isolate acidic analytes from the basic matrix (basic hot-spots in silages are caused by presence of microscopic fungi); five different concentrations of formic acid in water (0.1, 0.5, 1, 2 and 5%) were examined to be sure that pH of final silage extract is as low as possible. (ii) extraction cycle duration; low-moisture samples were soaked by water containing 2% of formic acid for 1 and 30 min before MeCN addition; after addition of MeCN, the total extraction time was optimized, samples were extracted by shaking for 3, 10, 30, and 60 min. (iii) dispersive solid phase clean-up; potential of three sorbents with various sorption affinities (alumina, Envi-Carb™, and C_{18} silica) was assessed; to 2 mL of mycotoxins in solvent standard (MeCN:water, 95/5, v/v) at 50 ng mL^{-1}, 0.1 g of appropriate adsorbent and 0.3 g of $MgSO_4$ were added, and undesirable sorption of target analytes was evaluated.

2.6. Optimized QuEChERS-based procedure

Into a 50-mL polypropylene (PP) centrifugation tube were weighed 2 g of fine homogenized dry feed matrix followed by the addition of 10 mL of 2% aqueous formic acid solution. The tube was closed and the matrix was allowed to soak for 30 min. MeCN (10 mL) was then added into the tube containing the soaked matrix and shaken by laboratory shaker additional 30 min (240 RPM). The phase partition was induced by addition of 4 g of $MgSO_4$ and 1 g NaCl. The tube was immediately shaken for 30seconds to prevent coagulation of $MgSO_4$ and centrifuged (Hettich; Tuttlingen, Germany) for 5 min (10,000 RPM). For the sample clean-up, 2 mL of MeCN extract were placed into the 15-mL PP tube containing 0.1 g of C_{18} silica sorbent and 0.3 g of $MgSO_4$, mixed and centrifuged (10,000 RPM) for 1 min. The purified extract was transferred into a PP vial (Sun Sri, Rockwood, TN, USA) for the LC–MS analysis.

2.7. U-HPLC–HRMS analysis

The non-target matrix extracts profiling was realized by U-HPLC–HRMS analyses by collecting of the full spectral information according to Zachariasova et al. [21]. Chromatographic separation was performed by using the Accela 1250 U-HPLC system (Thermo Fisher Scientific, San Jose, CA, USA). The Acquity UPLC® HSS T3 analytical column (100 mm × 2.1 mm, 1.8 μm; Waters, Milford, MA, USA) held at 40 °C was used for the separation of sample components. As the mobile phases, 5 mM ammonium formate in water (A) and MeOH (B) were used. 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 100% B, keep up to 15 min, switching to 5% B in 15.1 min, and column equilibration for 5 min before the next injection. Injection volume was 5 μL, the flow rate was 0.3 mL min^{-1}. In the case of extended chromatographic runs used for the demonstration of dSPE clean-up effect, 100% of B was kept up to 50 min, then switch to 5% of B in 50.1 min was realized, and column was equilibrated for 5 min before the next injection (Fig. S2).

The orbitrap mass spectrometer (Exactive™; Thermo Fisher Scientific, Bremen, Germany) worked with atmospheric pressure chemical ionization interface (APCI; Thermo Fisher Scientific, Bremen, Germany), with the following parameters settings: sheath/aux gas: 55/10 arbitrary units, capillary temperature: 250 °C, vaporihyphen-qj;zer temperature: 320 °C, capillary voltage: +60/−50 V, discharge current: 5 μA. The system was operated in the full spectral acquisition mode in the mass range of m/z 200–2000 with resolving power set to 50,000 FWHM and acquisition rate of 2 spectra s^{-1}.

2.8. U-HPLC–MS/MS analysis

Analyses of target mycotoxins were realized by the U-HPLC–MS/MS method. Chromatographic separations were performed according to study by Lacina et al. [18]. The Acquity UPLC® system (Waters; Milford, MA, USA) was equipped with Acquity UPLC® HSS T3 column (100 × 2.1 mm, 1.8 μm, Waters; Milford, MA, USA) maintained at 40 °C, and a 10-μL sample loop. The mobile phases were different for ESI(+) and ESI(−) analysis. The 5 mM ammonium formate and 0.2% (v/v) formic acid in both Milli-Q water and MeOH was used in ESI(+). In ESI (−), 5 mM ammonium acetate in Milli-Q water and pure MeOH were used. The gradient was slightly different in both polarities: In the positive mode, the starting mobile phase composition was 10% (v/v) of organic phase (B) with flow 0.35 mL min^{-1} followed by a linear change to 50% (B) in 1 min. A slower linear gradient from 50% (B) to 100% (B) in 10 min followed, simultaneously with flow rate change from 0.35 to 0.55 mL min^{-1}. The increased flow rate compensated observed peak broadening caused by a slowdown of the gradient. The column was washed for 2 min (flow 0.7 mL min^{-1}) with 100% organic solvent and reconditioned for 2 min in the starting composition of 10% (B) (0.35 mL min^{-1}). In the negative mode, the starting mobile phase composition was 10% of (B) at the flow rate 0.35 mL min^{-1} followed by a linear change to 50% of (B) in 1 min. A linear gradient to 100% of (B) with linear increase of the flow rate to 0.5 mL min^{-1} in 6.5 min

was followed by a column washing with 100% of (B) for 2 min at 0.7 mL min^{-1}. The column was reconditioned for 2 min in the starting composition of mobile phases. A sample volume of 3 μL with the partial loop injection mode was used. The temperature in autosampler was maintained at 10 °C.

Acquity UPLC® System was coupled to QTRAP® 5500 tandem mass spectrometer (AB SCIEX; Toronto, ON, Canada), equipped with an electrospray (ESI) ion source operated in both positive and negative mode. The ESI(+) ion source parameters were as follows: needle voltage: 4500 V; curtain gas: 35 psi; nebulizer (Gas 1) and Turbo gas (Gas 2): 55 psi; turbo gas temperature: 500 °C. In the ESI (–) were needle voltage: –4500 kV; curtain gas: 35 psi; nebulizer (Gas 1) and Turbo gas (Gas 2): 55 psi; turbo gas temperature: 450 °C. Declustering potential (DP), collision (CE) and collision cell exit potential (CXP) were optimized during infusion of mixture of analytes (10–100 ng mL^{-1}) employing an automatic function of Analyst software 1.5. The MRM transitions of mycotoxins and all analyte dependent parameters are summarized in Table S2 of the supplementary materials.

2.9. Determination of matrix effects

To characterize matrix effects of the optimized QuEChERS-based method within a wide range of feed matrices, and suggest the way of their compensation, extensive validation experiments were realized. Matrix-matched standards were prepared at three different levels of 800, 450 and 100 μg kg^{-1} (160, 90, and 20 μg L^{-1}) by addition of standard solution into the sample extract for each of the 12 matrices tested; additionally to feeding cereals as wheat, barley, and oat, also the complex compound feeds for birds and calves, extracted soya and rape seeds oilcake, fermented silage feeds, malt sprouts, and wheat and maize-based dried distillers′ grains with solubles (DDGS) were included. The absolute matrix effects expressing the matrix induced signal suppression/enhancement (SSE_{abs}%) were defined as percentage ratios of matrix-matched calibration slope to solvent calibration slope (summarized in Table S3 of the supplementary materials). The relative matrix effects (SSE_{rel}) were defined as percentage ratios of calibration slope of correlated matrix to the calibration slope of reference matrix. Subsequently, all of the matrices investigated were chosen as reference, and their suitability to be used for matrix effects compensation was assessed (see Table S4 and Fig. S3).

2.10. Method validation

The method performance parameters (recoveries, repeatabilities, and limits of quantification) were determined for wheat, complex compound feed for calves, and maize silage. Wheat was chosen as a representative of common and frequently occurring feed. Complex compound feed for calves and maize silage were selected because their complexity. The analytes were quantified by an external matrix-matched calibration standards at levels 1; 2; 5; 10; 50; 100; 200 μg L^{-1} (corresponding to 5; 10; 25; 50; 250; 500; 1000 μg kg^{-1}). The repeatability of the method expressed as a relative standard deviation was assessed on matrices spiked at 250 μg kg^{-1} in seven repetitions. Limits of quantification (LOQs) were estimated as the lowest matrix-matched calibration standards which provided signal-to-noise ratio (S/N) higher than 10 at both of the quantifier and qualifier ion transitions.

3. Results and discussion

3.1. Sample preparation method development and optimization

Accurate determination of trace analytes such as mycotoxins in such ´difficult´ matrices as silages, other fermented feeds, and complex compound feeds is not an easy task. The main reason is a high amount of extractable matrix, including not only components of original plants, represented by various pigments (e.g. chlorophylls and carotenoids), waxes etc., but also products of fermentation process. Thus, fermented animal feed often contain high amounts of oligo- and monosaccharaides originated from polysaccharides, peptides, and amino acids released from proteins, various organic acids, and other products originated by enzyme catalyzed reactions. As regards complex compound feeds, various fats or free fatty acids are often added into feed formulations in order to improve their nutritional value. Considering a large diversity of possible animal feed compositions, thus the need to implement a broad scope procedure, several extraction approaches were tested in the very first phase of our study. The aim of study was to define the best extraction alternative that can enable a reliable isolation of target mycotoxins, and at the same time, also discriminate matrix co-extracts to the maximum possible extent. Additionally to the QuEChERS approach successfully employed in our earlier study concerned with analysis of *Fusarium* toxins in cereals [20], two other extraction procedures were examined; extraction with MeCN/water (contrary to QuEChERS, no partition involved), and extraction with pure MeCN. A model maize silage sample, as a representative of 'difficult' complex matrices, was used for this purpose. As illustrated in Fig. S1 depicting the overall matrix compounds contribution acquired under the full-spectral orbitrap HRMS detection, the lowest amount of total co-extracts was obtained for MeCN extraction, obviously due to a limited extraction of most polar matrix components by medium polar solvent. As regards the aqueous-MeCN extract, considerably higher amount of potentially interfering compounds was observed there. As also shown in this Figure, enabling the QuEChERS-based procedure resulted in the significant reduction of the polar matrix components remaining in water after the phase partition step (eluting in a front part of chromatogram in the reversed phase chromatography).

For all of these extraction approaches, also the SSE values obtained by target U-HPLC–MS/MS analysis were evaluated and summarized in Table 1. Contrary to the MeCN/water or QuEChERS-based extraction, the SSEs determined in the pure MeCN extract were really very low, being in a narrow range from 91% to 116% (100% = no matrix effect). From this point of view, extraction with the pure MeCN would be the best choice. However, when assessing extraction efficiency (recoveries) of target mycotoxins from the real dried silage sample, they were shown to be insufficient by using of this method. As indicated in Table 1, a maximum of 1%–10% of DON, enniatin B, and fumonisins were extracted by pure MeCN, contrary to almost 100% recoveries obtained by analyses of spikes. The importance of water addition to wet the sample, enabling releasing the analytes from the bounds to the matrix was clearly documented. This observation was independently confirmed by several other authors [18,22]. Regarding the SSE values for the QuEChERS and MeCN/water extract, the higher suppression was determined for the MeCN/water. With regard to the above mentioned facts, the QuEChERS procedure was confirmed as the best sample preparation method and was considered for further experiments.

Taking into account the very different composition of examined matrices and the broad range of mycotoxins, the extraction parameters used in the QuEChERS method were optimized in order to improve the method ruggedness. Firstly, pH value of the extraction solvent, which is crucial for the effective extraction of acidic analytes (e.g. fumonisins, MPA, PEN, CPA, etc.) was optimized. The maintaining of low pH is important especially in the case of silages, where spots contaminated fungi have typically basic pH, thus acidic analytes may occur in ionized, worse extractable form. Since the composition of silage (and fungi occurrence as well) across

Table 1
Recoveries and matrix suppression/enhancement values obtained for various extract within the sample preparation method development (analyzed by U-HPLC–MS/MS).

	Matrix supression/enhancement range[a]			Recovery range based on spikes[b]			Recovery range based on real-life silage[c]		
	QuEChERS extraction	MeCN/water extraction	MeCN extraction	QuEChERS extraction	MeCN/water extraction	MeCN extraction	QuEChERS extraction	MeCN/water extraction	MeCN extraction
Type A trichothecenes	35–240	12–189	95–99	66–103	67–99	98	*Not present*	*Not present*	*Not present*
Type B trichothecenes	103–140	66–112	98–106	82–99	78–103	99	81	83	7
Zearalenones	66–78	17–47	91–96	94–106	93–98	102	*Not present*	*Not present*	*Not present*
Fumonisins	146–289	112–295	92–101	71–93	82–95	97	88	84	1
Enniatins	80–111	43–97	98–112	72–103	79–99	104	89	83	9
Aflatoxins	45–76	22–85	95–116	95–109	96–106	98	*Not present*	*Not present*	*Not present*
Alternaria toxins	16–89	3–59	93–110	74–108	75–98	101	*Not present*	*Not present*	*Not present*
Ergot alkaloids	67–186	47–94	96–113	74–110	72–108	99	*Not present*	*Not present*	*Not present*
Penicillium mycotoxins	12–184	14–137	97–101	82–112	83–99	97	*Not present*	*Not present*	*Not present*

[a] Matrix suppression/enhancement is defined as the matrix-matched standard to solvent standard ratio; 100%=no matrix effect.
[b] Recovery based on spikes is defined as the spike to matrix-matched standard ratio.
[c] Recovery based on real-life silage (characterized in Table 1) is defined as the measured value to assigned value ratio.

the silage pit is rather uneven, five naturally contaminated 'model' silage samples collected at different positions of the pit were used for extraction process ruggedness assessment; various additions of formic acid to extraction mixture were performed. The variability of pH values of silage extracts, thus diversity of silage samples in different sampling position, is clearly documented in Table 2 (compare mainly data for low concentrations of formic acid in extraction mixture). Two visually moldy samples from a silage wall where fungal infection can more easily spread showed higher pH than those three sampled inside the silage pit, approximately two meters behind the silage wall. To achieve sufficiently stable low pH value of extract, the concentration of formic acid had to be at least 2%. The importance of pH tuning is illustrated in Table 3. The determined concentrations of fumonisins correlated with the concentration of formic acid in extraction mixture. Hence differences in fumonisins concentrations between 2% and 5% formic acid were not significant, 2% was used in the routine method.

Further parameters subjected to optimization in order to increase the recoveries of naturally occurring mycotoxins and simultaneously to keep the sample preparation as simple as possible, two other parameters were assessed. These two parameters were the time of low-moisture matrix soaking prior to adding organic solvent for improving extractability of analytes, and the overall time of

Table 2
Optimization of extraction mixture composition; demonstration on various model real-life samples.

	pH value of extract obtained by the acetonitrile/acidified water (1:1, v/v) mixture				
	0.1% HCOOH in water	**0.5% HCOOH in water**	**1% HCOOH in water**	**2% HCOOH in water**	**5% HCOOH in water**
silage 1[a]	5.57	4.53	3.91	3.89	3.81
silage 2[a]	5.83	5.10	4.59	4.23	4.03
silage 3[b]	4.71	4.28	3.72	3.66	3.61
silage 4[b]	4.36	3.89	3.64	3.42	3.01
silage 5[b]	4.99	4.66	4.11	3.96	3.88

[a] Silages 1 and 2 were sampled from the silage wall.
[b] Silages 3, 4, and 5 were sampled approx. two meters behind the silage wall.

Table 3
Comparison of concentrations of fumonisins quantified in variously extracted model silage samples; each sample was extracted in three replicates with RSD less than 6%.

	FB1* [μg kg^{-1}]	**FB2* [μg kg^{-1}]**	**FB3* [μg kg^{-1}]**
Model silage 1			
0.1% HCOOH in water	3579	501	311
0.5% HCOOH in water	3957	509	312
1% HCOOH in water	3911	514	339
2% HCOOH in water	3876	523	344
5% HCOOH in water	4012	520	349
Model silage 2			
0.1% HCOOH in water	769	116	69.1
0.5% HCOOH in water	853	123	76.9
1% HCOOH in water	853	118	64.8
2% HCOOH in water	981	119	69.9
5% HCOOH in water	1031	132	79.7
Model silage 3			
0.1% HCOOH in water	2334	340	249
0.5% HCOOH in water	2873	378	258
1% HCOOH in water	2787	365	260
2% HCOOH in water	3175	363	265
5% HCOOH in water	3206	365	263

*Calculation was based on comparison with matrix matched standard prepared from blank silage by the same extraction, as was the sample.

extraction. All these experiments were done on certified reference materials, previous proficiency test (FAPAS) samples, and naturally infected internal reference materials, because as mentioned above, using of spikes cannot to simulate the behavior of mycotoxins in real samples. The results shown in Fig. 1 clearly document the increase of analytes recoveries with the length of the soaking period, as well as with the total extraction time. Since 30 min of water soaking and 30 min of MeCN extraction provided recoveries of all of the analytes close to 100% (no improvement was observed with increased extraction time), we used these settings in the final method.

The last optimized parameter was the dispersive SPE clean-up. The suitability of various sorbents such as alumina, Envi-Carb™ and C_{18} silica were tested. Primary secondary amine (PSA), the common sorbent, in original QuEChERS method, was not considered due to known sorption of acidic compounds. Experiments based on addition of the above sorbents into MeCN/water (95:5, v/v) solution of mycotoxins, simulating the QuEChERS extract composition, showed that neither the alumina nor the charcoal was suitable for purification due to extensive sorption of target analytes. Alumina adsorbed 90%–100% of trichothecenes, zearalenones, *Alternaria* toxins, OTA, and carboxyl-containing mycotoxins, sorption of PAT and ergot alkaloids was slightly lower (50%–60%), and the only mycotoxins with no affinity to alumina were enniatins and aflatoxins with sorption of 0% and 10%, respectively. Under similar conditions, Envi-Carb™ sorbent removed between 80%–100% of OTA, zearalenones, *Alternaria* toxins, enniatins, aflatoxins, ergot alkaloids, and carboxyl-containing mycotoxins; only PAT and trichothecenes showed lower affinity (up to 20%) to Envi-Carb™. The only sorbent with insignificant effect on the recoveries of targeted mycotoxins was C_{18} silica. This sorbent influenced only enniatins (loss of 30%–40%) and ergot alkaloids (loss of 10%) due to their lower polarity; nevertheless, in overall, this was still promising result for a further work.

Subsequently, the potential of C_{18} silica to reduce matrix induced ion suppression/enhancement for multiple mycotoxins targeted in 12 various complex matrices was studied. Generally, a large extent of ionization enhancement was observed. As recently reported by Malachova et al., this phenomenon is not unusual, its occurrence depends both on the extraction method, and the LC–MS conditions [23]. The matrix induced ionization enhancement observed in our study in d-SPE C_{18} purified extracts was approx. 10%–20% higher when compared to crude, unpurified extract. Moreover, regardless the higher responses of analytes after using of C_{18} silica, thus increasing their detectability, we would recommend to employ this relatively simple and fast clean-up procedure, with respect to analytical column and the whole LC–MS system life-time. As nicely illustrated in Fig. 2 showing the overall matrix contribution measured by extended chromatographic separation and high resolution full-spectral mass spectrometric detection for wheat DDGS and silage, C_{18} d-SPE clean-up effectively eliminate the non-polar di- and triglycerides eluting in 100% MeOH between 13 and 30 min (particular di- and triglycerides were identified by the accurate mass measurement and elemental composition calculation). Under the conditions of commonly used chromatographic run in which rinsing with MeOH ends usually in 11–15 min, these co-extracts are not flushed properly and steadily accumulate on the column shortening its service life significantly. Comparison of total ion chromatograms of C_{18} d-SPE cleaned and non-cleaned extracts for all of the matrices investigated is depicted in Fig. S2 of the supplementary materials.

3.2. Matrix effects assessment

As already outlined above, the matrix induced ionization enhancement occurred for most of the analytes. The absolute matrix effects ranged between 27% and 264% for type B trichothecenes, 100%–197% for type A trichothecenes, 76%–178% for zearalenones, 150%–334% for fumonisins, 55%–144% for enniatins and BEA, 61%–319% for ergot alkaloids, 6%–551% for *Alternaria* toxins, 62%–144% for aflatoxins, 115%–136% for OTA, 63%–162% for PAT, and 5%–468% for other *Penicillium* mycotoxins (Table S3). However, considering the fact that matrix-matched standards are commonly used for matrix effects compensation, the relative matrix effects (SSE_{rel}) illustrating the interactions between the matrices mutually, are much more important. As far as SSE_{rel} are acceptably low, the use of matrix-matched calibration for quantitation of samples of different matrix is not crucial for achieving the accurate results. In our study, the values of SSE_{rel} normalized to each particular reference matrix were assessed for all of the analytes/matrices combinations, and are summarized in Table S4 (see Supplementary materials). For assessment of acceptability of results obtained, criterion of acceptance of $SSE_{rel} \pm 25\%$ was used. In other words, when the SSE_{rel} value was in

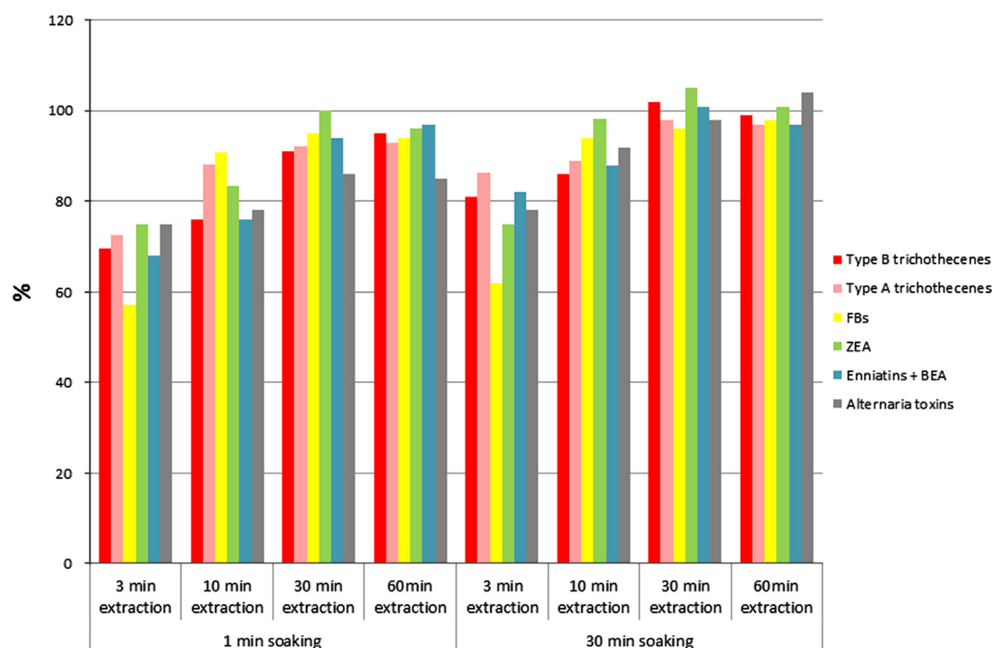

Fig. 1. Influence of the soaking period and the extraction time on recoveries of mycotoxins from certified/internal reference materials and the passed FAPAS samples.

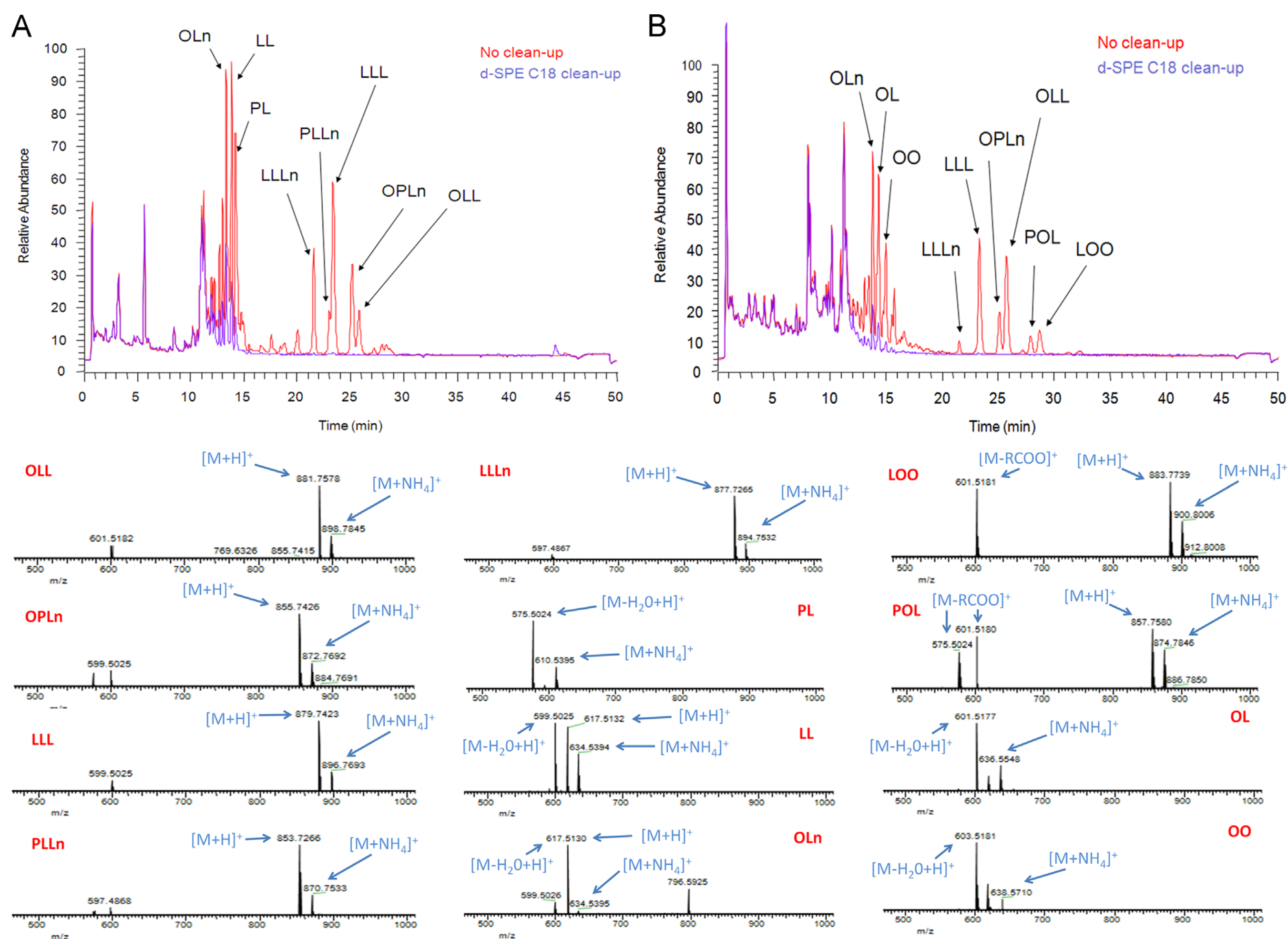

Fig. 2. Total ion current (TIC) U-HPLC–HRMS chromatograms of d-SPE C_{18} cleaned and crude extracts together with mass spectra of identified di- and triacyl glycerols; (A) – wheat DDGS, (B) – silage. O – oleic acid, $C_{18}H_{34}O_2$; L – linoleic acid, $C_{18}H_{32}O_2$; Ln – linolenic acid, $C_{18}H_{30}O_2$; P – palmitic acid, $C_{16}H_{32}O_2$.

the range 75 and 125%, the quantification bias due to calibration on not identical matrix, was considered to be still tolerable. According to our opinion, mainly the agricultural supervisory institutes and authorities, who routinely control mycotoxins in a wide range of matrices, can appreciate this approach for at least screening purposes. The outcomes resulting from this consideration are illustrated in Figs. 3 and S3. Fig. 3 shows wheat, the most common cereal matrix, as the reference one. Extended information is presented also in Fig. S3 of the supplementary materials, where the mutual influence of all of the other matrices is depicted. When taking wheat as a reference (Fig. 3), the $SSEs_{rel}$ for barley lied in the satisfactory range for 95% of analytes. Concerning oat, tolerable matrix effects were obtained for 73% analytes. Even in the case of complex compound feeds for pigs, complex compound feeds for birds, malt sprouts, wheat DDGS, and extracted soya oilcake, 89, 73, 84, 75, 80% of analytes could be quantified on wheat matrix matched standards with satisfactory quantification bias. As regards other matrices, as extracted rape oilcake, maize DDGS, complex compound feed for calves or maize silage, only 64, 61, 59, and 41% of analytes could be reasonably quantified on wheat standards with the tolerable quantification bias. The reason is probably the significantly different chemical composition of these processed and fermented matrices (high content of proteins, peptides, and other components being added into complex feedings for young animals in order to improve the nutritional value, chlorophylls, carotenoids, and fermentation products abundantly present in silages and DDGS). For these difficult feed matrices, the best option is using of the very similar animal feed for matrix effect compensation. For example, for complex compound feeds for calves, quite good reference matrices could be oat or silage, with 70 and 66% analytes lying in acceptable SSE_{rel} range (see Fig. S3). For maize DDGS, the best reference matrix would be the wheat DDGS, with 79% of analytes laying in the tolerable scope, but also extracted rape oilcake, and malt sprouts would be the good alternative. The most different matrix from the others was silage. When taking any other matrix investigated as a reference one, a number of analytes lying in the tolerable scope of matrix effects hardly exceeded 60%. From the tested matrices, the most suitable for matrix effects compensation could be only complex compound feed for calves and maize-based DDGS. But the best option for matrix effects correction would be definitely the maize silage itself.

3.3. Method validation

The thorough method validation was performed for three selected model matrices significantly differing in their chemical nature. Besides of wheat as a relatively simple matrix, also the complex compound feeds for calves, and pH sensitive silage as the more complicated ones were chosen for further validation. As shown in Table 4, recoveries of analytes calculated by using their own matrix matched calibration standards ranged between 62 and 115% for wheat, between 58% and 112% for complex feeding mixture for calves, and between 56% and 120% for silage. The lowered recoveries were obtained mainly for DON-3-Glc because of its higher polarity and thereby insufficient transfer into the

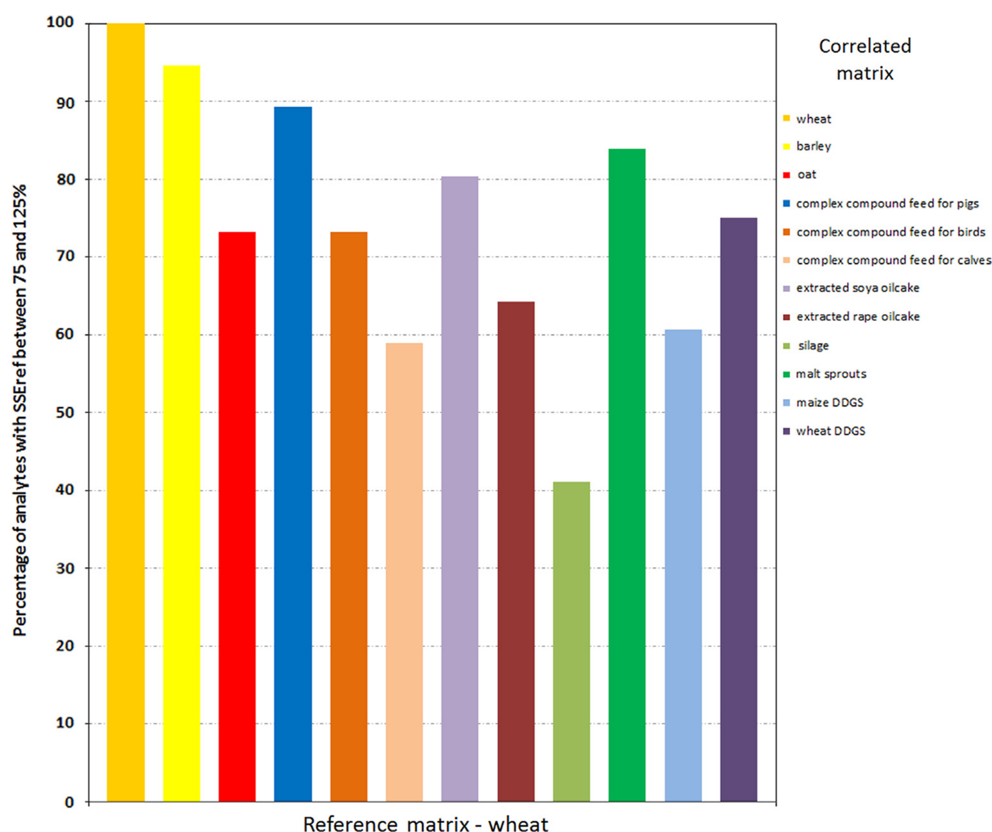

Fig. 3. Illustration of "matrix induced signal suppression/enhancement" in various matrices (reference matrix – wheat); number of SSE_{rel} occurring in the range 75%–125%. SSE_{rel} defined as percentage ratios of calibration slope of correlated matrix to the calibration slope of reference matrix. Optimized QuEChERS-based extraction/purification and U-HPLC–MS/MS determinative step were enabled.

MeCN layer when performing the QuEChERS method. Also recoveries of enniatins lied between 60% and 73%, because of their partial adsorption on the silica C_{18} d-SPE sorbent.

In Table 4, newly calculated recovery values obtained by correction of the original values for their particular SSE_{rel}, when wheat matrix was considered as the reference one, are presented. This Table provides a clear practical demonstration of the between-matrices interactions in practice. As a guide for data interpretation, SANCO document describing the requirements for method validation and establishing the acceptable recoveries for analytes in the range of 70%–120% was used [24]. In spite of the fact that this document is primarily intended for pesticides residue analysis, its principles are generally applicable for all of the food/feed contaminants. As clearly shown in Table 4, wheat as a reference matrix is not suitable for quantification of aflatoxins, Alternaria toxins (mainly ATE showed a huge bias, its recoveries based on wheat calibration were in order of thousands %), CIT, CPA, type B trichothecenes, ergot alkaloids, PAX, PEN-A, ROQ-C, STER, zearalenones, and VER (in the case of wheat-based correction of VER recovery from maize silage, the bias was also very high, even exceeding 5000%). For accurate quantification of these mycotoxins, preparation of matrix matched calibration derived from the same type of matrix, or the standard addition method, would be the best option.

Regarding the repeatability of the measurements (RSD from seven repeated analyses of spikes), it lied below 6% for approximately 70% analytes. For some mycotoxins with LOQ close to the spiking level ($250\ \mu g\ kg^{-1}$), RSDs were fairly higher, but not exceeding 25%. The lowest LOQ values were obtained for wheat; most of these LOQ values were in order of tenths and units of $\mu g\ kg^{-1}$, with exception of GLIO and VER. Concerning the complex compound feeds and silage, the LOQs were mostly slightly higher. Linearity range of analytes was dependent on intensity of responses of respective mycotoxin. The higher was the analyte response, the narrower was the linearity range. Only 37% of analytes included in the method showed linear calibration trend up to $1000\ \mu g\ L^{-1}$ (corresponds to $5000\ \mu g\ kg^{-1}$). For most of mycotoxins, incurvation of calibration curve occurred for the highest calibration points because of detector saturation. 73% of analytes showed the linearity up to $500\ \mu g\ L^{-1}$ ($2500\ \mu g\ kg^{-1}$), 100% of analytes were linear in the range of LOQ to $250\ \mu g\ L^{-1}$ ($1250\ \mu g\ kg^{-1}$).

4. Conclusions

During the presented study, the QuEChERS-based isolation approach was optimized and validated for analysis of 56 mycotoxins in 'difficult' animal feed matrices. The critical steps which might influence the performance of extraction process or U-HPLC–MS/MS separation/detection system are summarized below:

- To optimize extraction efficiency, analysis of naturally contaminated samples (i.e. samples with incurred mycotoxins) is needed.
- Soaking of low-moisture matrix with 2% (v/v) aqueous formic acid together with prolonged extraction time are essential for obtaining high recoveries in analysis of naturally contaminated matrices.
- The acidification of extraction solvent is a key condition for obtaining high recoveries of fumonisins from pH-sensitive silage samples.
- The dispersive SPE using C_{18} sorbent does not significantly influence matrix effects (suppression/reduction of target mycotoxins response), but the main benefit is the reduction of highly abundant non-polar matrix co-extracts, mainly di- and triacylglycerols, thus prolonging of the analytical column life-time.

Table 4
Performance characteristics of the optimized QuEChERS-based U-HPLC–MS/MS multi-mycotoxin method for wheat, complex compound feed for calves, and maize silage. Recovery values of the last two matrices were recalculated according to SSE_{rel}, where the wheat matrix was considered as the reference one.

Mycotoxin	Wheat		Complex compound feed for calves					Maize silage			
	recovery [%][a]	RSD [%][a]	LOQ [μg kg^{-1}][a]	recovery [%][b]	recovery [%][a]	RSD [%][b]	LOQ [μg kg^{-1}][b]	recovery [%][c]	recovery [%][a]	RSD [%][c]	LOQ [μg kg^{-1}][c]
15-ADON	92	5.9	20.0	79	85	7.5	50.0	91	347	9.1	100.0
3-ADON	109	5.7	10.0	102	103	8.2	25.0	103	106	8.0	50.0
A-clavine	94	4.6	0.5	89	78	4.5	2.5	90	129	3.8	2.5
AFB1	109	3.0	0.5	104	145	4.1	1.0	110	184	5.6	1.0
AFB2	99	5.9	1.0	91	112	7.8	1.0	95	131	9.9	5.0
AFG1	107	4.0	1.0	98	103	5.4	1.0	101	130	4.6	2.5
AFG2	106	2.6	1.0	100	113	3.5	1.0	104	143	3.0	2.5
ATE	79	2.4	1.0	81	1492	2.6	2.5	80	3025	3.6	10.0
AOH	76	3.7	1.0	84	81	1.8	1.0	109	122	2.0	5.0
AME	74	1.5	0.5	88	139	1.9	0.5	110	194	4.9	2.5
BEA	100	2.7	0.5	90	112	2.4	0.5	78	77	4.5	0.5
CIT	76	4.4	50.0	79	152	13.4	250.0	85	223	9.2	500.0
CPA	98	7.5	5.0	95	182	7.8	10.0	93	136	10.1	50.0
DON	95	1.8	50.0	90	120	5.3	100.0	85	59	6.7	100.0
DON-3-Glc	65	4.9	50.0	58	47	8.7	100.0	56	38	5.6	50.0
DAS	85	3.2	5.0	81	81	2.6	5.0	89	99	6.6	2.5
Enn-A	64	2.0	0.5	72	125	4.2	1.0	71	75	7.3	2.5
Enn-A1	68	2.8	0.5	69	117	5.4	1.0	64	70	4.9	2.5
Enn-B	62	5.7	0.5	60	73	6.3	1.0	69	58	4.7	2.5
Enn-B1	63	6.4	0.5	72	114	5.9	1.0	73	67	8.5	2.5
E-cornine	90	4.9	2.5	91	108	5.2	2.5	78	123	4.6	1.0
E-corninine	89	3.2	5.0	86	105	3.6	5.0	91	120	4.8	5.0
E-cristine	91	6.1	2.5	87	122	5.4	5.0	82	274	6.4	5.0
E-cristinine	86	3.2	10.0	89	125	3.9	10.0	84	133	4.6	10.0
E-cryptine	89	6.2	2.5	91	92	5.4	5.0	74	103	4.9	5.0
E-cryptinine	92	7.0	5.0	88	89	7.6	10.0	79	110	5.1	10.0
E-metrine	90	4.6	5.0	92	95	6.2	10.0	81	204	6.1	20.0
E-sine	89	6.3	2.5	90	81	7.4	5.0	90	167	7.3	5.0
E-sinine	78	3.0	5.0	95	85	8.4	5.0	88	164	4.7	10.0
E-tamine	93	7.6	0.5	90	137	10.2	2.5	89	166	5.9	2.5
E-taminine	85	8.1	2.5	79	121	4.2	5.0	91	170	4.8	5.0
FUS-X	115	6.7	20.0	93	87	3.9	50.0	100	94	8.6	200.0
FB1	71	4.8	10.0	76	73	5.8	20.0	76	60	6.7	20.0
FB2	93	9.7	5.0	82	83	6.4	10.0	80	72	4.7	5.0
FB3	95	11.3	10.0	92	86	8.8	20.0	80	66	3.2	20.0
GLIO	90	17.9	200.0	92	100	6.4	250.0	85	84	9.8	500.0
HT2	97	5.0	10.0	90	69	7.0	25.0	90	72	8.7	25.0
MEL	92	4.1	2.5	81	111	4.8	5.0	79	96	5.6	5.0
MPA	106	6.2	50.0	96	96	11.9	100.0	88	93	12.4	200.0
NEO	95	4.4	5.0	89	90	4.8	20.0	92	106	6.1	20.0
NIV	94	11.1	50.0	78	74	9.9	100.0	84	67	8.5	250.0
OTA	98	3.0	1.0	84	85	1.4	2.0	91	89	2.7	2.0
PAT	82	11.2	50.0	74	57	5.6	50.0	76	73	7.1	50.0
PAX	91	5.2	2.5	84	211	4.1	10.0	85	214	4.6	10.0
PEN	98	9.3	50.0	95	100	8.9	50.0	93	698	5.3	50.0
PEN-A	118	4.1	10.0	112	128	3.9	10.0	120	138	2.1	10.0
ROQ-C	111	4.7	5.0	94	135	5.8	5.0	86	140	3.9	5.0
STACH	90	4.6	10.0	85	117	3.1	10.0	80	110	7.7	20.0
STE	106	1.6	0.5	99	120	3.0	0.5	101	162	1.1	0.5
T2	88	2.4	2.5	90	96	4.0	5.0	99	107	5.6	2.5
TEN	95	3.2	2.5	90	62	5.7	2.5	88	62	4.2	5.0
VER	113	6.5	200.0	102	412	14.4	200.0	112	5356	16.4	200.0
Verruc	80	4.9	10.0	78	105	6.9	10.0	77	91	5.2	10.0
ZEA	92	3.2	0.5	94	159	3.1	0.5	109	214	2.5	1.0
α-ZOL	91	3.8	2.5	86	132	2.5	1.0	107	218	2.1	2.5
β-ZOL	90	2.8	2.5	88	146	4.1	2.5	106	227	2.0	2.5

[a] Calculated by using the wheat-based matrix matched calibration.
[b] Calculated by using the complex compound feed for calves-based matrix matched calibration.
[c] Calculated by using the maize silage-based matrix matched calibration.
Recoveries of analytes exceeding the range 70-120% are marked in yellow; significantly outlying recoveries outside the range 30-160% are marked in red.

- For high-throughput screening of multiple mycotoxins in a large set of various feed samples, the matrix effects can be compensated by using suitable model matrix/matrices for preparation of calibration standards. Wheat was proved to be the good reference matrix for majority of feed samples investigated, with exception of some fermented feed samples or the complex feeding mixtures (especially for particular analyte/matrix combinations). When aiming to quantify the problematic analyte as accurate as possible, preparing of matrix matched calibration derived from the same type of matrix, or the standard addition method, would be the best choice.

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Appendix A. Supplementary materials

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.12.064.

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