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# Screening of plant and fungal metabolites in wheat, maize and animal feed using automated on-line clean-up coupled to high resolution mass spectrometry

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A wide range of plant and fungal metabolites can occur in cereals and feed but only a limited number of target compounds are sought. This screening method is using a database of over 600 metabolites to establish contamination profiles in food and feed. Extracts were injected directly into an automated turbulent flow sample clean-up system, coupled to a liquid-chromatography–high-resolution-mass-spectrometer (Orbitrap). Compound identification criteria for database searching were defined and the approach was validated by spiking plant and fungal metabolites into cereals and feed. A small survey of market samples (15) and quality control materials (9) of maize, wheat and feed was conducted using this method. Besides regulated and known secondary metabolites, fumiquinazoline F, fusarochromanone and dihydrofusarubin were identified for the first time in samples of maize and oats. This method enables clean-up of crude extracts within 18 min and screening and confirmation of a wide range of different compound classes.
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## 1. Introduction

Agricultural crops can be infected by fungi during growth, drying and subsequent storage. These fungi can lead to the production of toxic metabolites, the identity and quantity being influenced by environmental conditions. Equally plants themselves can also produce a range of naturally occurring toxins, many of which can become undesirable contaminants in the food and feed chain. An important class of fungal metabolites are mycotoxins. More than 400 mycotoxins are known already with numbers increasing with the discovery of masked mycotoxins (Berthiller et al., 2005). Mycotoxins are of worldwide concern and regulations or guidelines have been defined in around 100 countries (Van Egmond, Shothorst, & Jonker, 2007). In the European Union a defined list of some ten mycotoxins are regulated in food and feed with further recommended guideline levels defined recently for T-2 and HT-2 toxins (EC 1126/2007; EC 165/2013; EC 401/2006; EC 576/2006). Ergot alkaloids are a group of toxic metabolites produced by fungus *Claviceps purpurea* and other species in wild grasses and cereals, which are regulated based on the total weight of ergot grain but are not yet regulated on specific limits for individual toxins in food and feed. Non-regulated plant toxins such as pyrrolizidine alkaloids originating in *Boraginaceae*, *Fabaceae* and *Asteraceae* family, are of concern in food products such as e.g., herbal tea preparations, honey and pollen (Roeder, 1995).

Useful information about potential fungal contamination of food and feed can be obtained by identification of metabolic precursor compounds or degradation products (Sulyok, Berthiller, Krska, & Schuhmacher, 2006). To ensure food and feed safety, screening for a number of target plant and fungal toxins is frequently conducted. However, this can be very 'hit and miss' as the many variables controlling toxin production make prediction of what to look for very uncertain.

Due to the complexity of different chemical structures of secondary metabolites, sample preparation and detection are a challenge. An extraction solvent mixture consisting of acetonitrile/water/acetic acid (79:20:1) has been employed for plant and fungal metabolites by Sulyok et al. (2006) and has been applied for additional compounds in further methods (Abia et al., 2012; Lehner et al., 2011). Successful extraction of different types of mycotoxins has been performed, e.g., with mixtures of methanol/ethyl acetate/water (70:20:10) (Ediage, Di Mavungu, Monbaliu, Van Peteghem, & De Saeger, 2011); with acetonitrile/water mixtures (84:16) (Juan,

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Ritieni, & Manes, 2012), or a combined extraction and clean-up protocol based on QuEChERS approach by the addition of water 0.1% formic acid (FA), acetonitrile (1:1), $MgSO_4$ and NaCl (Rubert et al., 2012). Even though the trend is to simplify methods by dilution and injection of crude extracts, it is known that without sample clean-up, ion suppression or enhancement can occur and more frequent system maintenance is required (Senyuva, Gilbert, Türköz, Leeman, & Donnelly, 2012; Zachariasova et al., 2010).

An alternative approach to time-consuming and costly manual sample clean-up techniques has been described for compound classes with different chemical properties by TurboFlow™ technology. Examples of methods using turbulent flow for clean-up of different types of matrices have been described for the determination of different classes of veterinary drugs in chicken meat (Bousova, Senyuva, & Mittendorf, 2013), fluoroquinones in honey (Mottier, Hammel, Gremaud, & Guy, 2008) and *Fusarium* mycotoxins in wheat, maize and animal feed (Ates, Mittendorf, Stroka, & Senyuva, 2013).

Multi-analyte methods nowadays are based on the separation by liquid chromatography (LC), and detection either by analysis of pre-selected compounds with triple quadrupole mass spectrometer (MS/MS) or by detection in full scan mode with high resolution mass spectrometry (HRMS) systems.

Targeted analysis with LC-MS/MS operated in selected reaction monitoring (SRM) mode enables specific and sensitive detection of targeted molecules. A survey study of cereals, nuts and their products for detection of 320 fungal metabolites has been published recently. Sample extracts were diluted and injected directly into the LC-MS/MS system. In total 69 targeted compounds could be identified and confirmed using this methodology, including regulated and non-regulated toxins (Abia et al., 2012). A quantitative method has been described for the analysis of 25 mycotoxins with LC-MS/MS in cassava flour, peanut cake and maize samples, consisting of sample extraction, evaporation, reconstitution, defatting, and clean-up of the divided extract, once with glass fibre filters and once with solid-phase extraction (SPE) cartridges. This lengthy sample preparation protocol resulted in good recovery values (72–120%) and successful method validation according to predefined in-house method validation criteria (Ediage et al., 2011).

Modern HRMS instruments can be operated at resolutions up to $R = 240{,}000$ using Orbitrap technology with mass accuracy <5 ppm in full scan mode. Another advantage of HRMS is that the acquired data can even be evaluated retrospectively for additional compounds or reprocessing of raw data by another compound class database. Parallel to full scan detection, fragmentation experiments using higher energy collision dissociation (HCD) enables higher specificity by detection of fragment ions for compound confirmation where analytical standards are available. Comparable performance of HRMS to MS/MS has been demonstrated in the field of mycotoxin analysis with the additional advantage of retrospective data evaluation with HRMS (Lattanzio, Della Gatta, Godula, & Visconti, 2011). An example of using HRMS as a screening tool for the analysis of plant toxins has been described by using a QuEChERS-like extraction step and sample dilution. Accurate masses, retention times, and detection limits of 150 commercially available toxins in silage, honey, complete pig feed and food supplement have been summarised. Based on this toxin database, market samples were analysed for these target compounds. Additional compound confirmation was necessary for 34 compounds by LC-MS/MS analysis in SRM mode, due to missing fragment ion information for targeted compounds (Mol, Van Dam, Zomer, & Mulder, 2011). Another application of HRMS has demonstrated its applicability for quantitative determination. Identification and confirmation criteria using HRMS are proposed and employed in the screening of 200 fungal metabolites in parallel to the quantification of selected mycotoxins in food. Besides targeted toxins the putative identification of 13 fungal metabolites in food was reported for the first time (Lehner et al., 2011).

In the area of plant and fungal metabolites, analysis reference standards are not available most of the time, and it is therefore not possible to set up MS/MS transition parameters. HRMS is therefore the technique of choice which can overcome the problem of missing analytical standards. However it should be noted that the processing of HRMS data is very critical and identification criteria have to be defined specifically to minimise false positive and false negative results.

Determination of plant and fungal metabolites requires a generic method to overcome the challenge of missing standards and to be capable of covering a wide range of compounds with differing chemical properties. The screening method reported here, has been developed on the basis of a previously published method for the quantification of *Fusarium* mycotoxins with turbulent flow coupled to HRMS (Ates et al., 2013). The method has been transferred according to screening method requirements for compound screening using a database containing more than 600 fungal and plant metabolites. The method has been validated for representative compounds from different classes of plant and fungal metabolites, demonstrating the applicability of turbulent flow for sample clean-up.

Limitations and possibilities of this method are discussed and compound identification and confirmation criteria have been defined. Method applicability has been demonstrated by the analysis of a set of market samples of wheat, maize and animal feed.

## 2. Materials and methods

### 2.1. Reagents and solvents

Acetonitrile Optima™ grade, water Optima™ grade, methanol Optima™ grade, formic acid (FA) LC-MS grade, Pierce LTQ ESI positive ion calibration solution and Pierce LTQ ESI negative ion calibration solution, nylon filter 0.2 μm were purchased from Thermo Fisher Scientific (Langenselbold, Germany). Solvent for sample extraction was prepared by mixing 1000 mL of acetonitrile with 750 mL of water containing 0.1% FA.

### 2.2. Standards

Fungal- and plant metabolite standards of apicidin, deoxynivalenol, fumagillin, fumonisin $B_1$, fumonisin $B_2$, fusarenone X, HT-2 toxin, malformin A, sterigmatocystin, T-2 toxin, tenuazonic acid and zearalenone were purchased from Sigma–Aldrich (Taufkirchen, Germany), ergorcornine, monocrotaline and retrorsine were obtained from Römer Labs (Tulln, Austria). Stock standard solutions (100 μg/mL) were prepared individually by dissolving in methanol. Solutions were stored at −20 °C.

### 2.3. Samples

As no certified blank materials of maize, wheat and animal feed were available, a number of commercially available samples of maize, wheat and animal feed were analysed, to test if they could be used as blank material for spiking purposes. Samples were homogenised using a Waring® laboratory blender (Waring Laboratory Science, USA) and ground to a fine powder with a pestle and mortar. Survey samples of maize (two cornflakes, flour, organic flour, snack and whole maize), wheat (breakfast cereal, oat, rye and bulgur) and animal feed (mainly wheat, rye, oat, maize and grass-based) were purchased from a local market. Screening of survey samples was performed with two replicates of each sample. Quality control materials of oat flour (T-2 and HT-2 toxin,

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