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Determination of deoxynivalenol and deoxynivalenol-3-glucoside in wheat and barley using liquid chromatography coupled to mass spectrometry: On-line clean-up *versus* conventional sample preparation techniques

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ABSTRACT

In this study, we compared the performance of conventional sample preparation techniques used in mycotoxin analyses against automated on-line sample clean-up for the determination of deoxynivalenol (DON) and its conjugated derivative, deoxynivalenol-3-β-D-glucoside (D3G), in cereal grains. Blank wheat and barley samples were spiked with DON and D3G, extracted with a mixture of acetonitrile:water (84:16, v/v) and processed by one of the following: extract and shoot, MycoSep® 227 clean-up columns, MycoSep 227 with an additional acetonitrile elution step and centrifugal filtration, followed by analysis with liquid chromatography tandem mass spectrometry. Based on method performance characteristics and poor recoveries (<30%) obtained for the polar D3G with some techniques, the extract and shoot approach was chosen for the inter-laboratory method comparison study. Thus, the same spiked samples were analysed in parallel by another laboratory with an in-house validated on-line sample clean-up method, utilising TurboFlow™ chromatography coupled to high resolution mass spectrometry. Method validation was performed by determination of specificity, linearity, recovery, intra-day precision and the limits of detection and quantification. Matrix-matched linearity ($R^2 > 0.985$) was established in the range of 100–1600 and 20–320 μg/kg for DON and D3G, respectively. Average recoveries (%RSD) were acceptable with both methods for wheat and barley, ranging between 73% and 102% (3–12%) for DON and 72% and 98% (1–10%) for D3G. The benefit of using automated sample clean-up in comparison to extract and shoot is the ability to inject directly pure extracts into the mass spectrometer, offering faster analyses and improved sensitivity with minimum system maintenance.

1. Introduction

Crops are susceptible to fungal infection that can lead to the production of mycotoxins. Deoxynivalenol (DON) is a *Fusarium* mycotoxin primarily produced by strains of the cereal pathogens *Fusarium graminearum* and *Fusarium culmorum* [1]. DON is one of the most prevalent food safety-associated mycotoxins and according to current worldwide survey data, more than 50% of food and feed samples analysed were found positive, with the highest levels observed in wheat, maize and oats [2]. This mycotoxin belongs to the structurally related sesquiterpenes, denoted as trichothecenes, containing an olefinic group, variable numbers of hydroxyl/acetoxy groups and a stable epoxide ring that is critical to their toxicity [3]. The epoxide group allows these compounds to bind to ribosomes, resulting in ribosomic stress and subsequent inhibition of protein synthesis in eukaryotes, along with other cytotoxic effects [4]. Studies have demonstrated the ability of DON to disrupt gastrointestinal processes, the immune system, the endocrine system and functions of the brain [5]. The most frequent manifestations of acute exposure to DON include emesis, anorexia, haemorrhage and circulatory shock, as well as impaired reproduction and development [6]. However, limited information is available regarding the chronic effects of continuous exposure to low concentrations of this mycotoxin and its derivatives [7].

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The toxic effects of DON are not restricted to mammals as this mycotoxin is also phytotoxic, causing a variety of symptoms in plants such as growth retardation, inhibition of germination, chlorosis and necrosis, while being associated with *Fusarium* head blight (FHB) in cereals [4]. Nevertheless, similarly to other living organisms, plants are capable of detoxifying mycotoxins into metabolites that are less toxic to them, by conjugation with polar substances such as sugars, sulphate and amino acids, the so-called 'masked mycotoxins' (reviewed by [8]). These conjugates are defined as 'biologically modified mycotoxins' in a more recent paper [9]. Deoxynivalenol-3-β-D-glucoside (D3G) is a plant phase II detoxification product of DON, formed by a major *in planta* detoxification pathway that was first isolated from *Zea mays* suspension cultures treated with DON [10]. Poppenberger et al. [11] reported a significant reduction in the ability of D3G to inhibit protein synthesis *in vitro* compared to DON. Additionally, in FHB-resistant wheat lines DON was effectively converted to D3G as a detoxification metabolite, revealing a co-relation between the D3G/DON ratio and FHB resistance [12]. The natural occurrence of D3G was first reported in contaminated wheat and maize [13]. Since then, D3G has also been detected in barley, oats and cereal-derived products [14–16].

Due to the toxic potential and high prevalence of DON, the European Commission (EC) established regulatory limits for this mycotoxin in cereal grains and cereal-based products intended for human consumption, by adopting regulation EC No 1881/2006 [17] and amending regulation EC No 1126/2007 [18]. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) after a thorough risk assessment, proposed a provisional maximum tolerable daily intake (PMTDI) for DON of 1 μg/kg b.w. per day, including its acetylated derivatives, 3-acetyl-DON (3ADON) and 15-acetyl-DON (15ADON) as additional contributing factors to dietary exposure to DON [19]. Even though D3G was not included in the PMTDI, due to the lack of toxicological data, JECFA stressed the possibility that this conjugated form may be hydrolysed back to its native form in the mammalian gastrointestinal tract during digestion. It was recently confirmed, by both *in vitro* and *in vivo* studies, that D3G can be hydrolysed by the action of intestinal microbiota and potentially exert toxic effects on local epithelial cells [20–22].

Nevertheless, D3G is not the only biologically modified form of DON, as a number of new metabolites have recently been reported, including DON-glutathione, DON-S-cysteine, DON-S-cysteinyl-glycine and several DON-oligoglucosides [23,24]. The continuous discovery of new modified forms of mycotoxins, with unknown health implications for animals and humans, may lead to re-assessment of the toxicological significance of parent mycotoxins. As a consequence, there is an increasing demand for the development of analytical methods for sensitive and reliable quantification of mycotoxins and their derivatives, thus allowing a more accurate exposure assessment to these compounds.

The majority of analytical methods for the determination of modified mycotoxins, rely on liquid chromatography (LC) often coupled to a mass spectrometer (MS). To date, several analytical methods based on either conventional sample clean-up techniques or the 'dilute/extract and shoot' approach have been tested for the simultaneous determination of DON and D3G. Vendl et al. [25] tested the application of unspecific sample preparation techniques including MycoSep® 226 and 230 clean-up columns, as well as specific approaches such as immunoaffinity columns (IACs) for analysis of DON, zearalenone (ZEN) and a number of their modified derivatives. The authors concluded that from the methods tested, none other than the extract and shoot approach was suitable to accommodate the wide range of analyte polarities. Clean-up with MycoSep 227 cartridges has also been evaluated for analysis of DON and D3G in wheat [26] and maize [27] with varying results depending on the matrix. In another study, IACs were successfully used for DON and D3G determination [28]. However, IACs are not generally appropriate for multi-mycotoxin analysis, due to the high degree of specificity of the antibodies comprising them. As a consequence, most LC–MS methods employ variations of the dilute/extract and shoot approach, regardless of its drawbacks as a sample pre-treatment technique including differences in chromatographic behaviour of analytes among various types of samples, severe matrix effects and lower sensitivities.

TurboFlow™ chromatography is an on-line sample clean-up and pre-concentration technique that enables direct injection of liquid samples or extracts that are purified on specialised TurboFlow (TLX) columns and transferred onto the analytical column, which is connected to an MS. A detailed description of the principle, along with method development processes with TurboFlow chromatography, has been presented for pesticides [29]. The applicability of TurboFlow chromatography in food analysis has already been demonstrated, for example, in the determination of *Fusarium* mycotoxins in cereals and animal feed [30], plant and fungal metabolites in cereals and animal feed [31], veterinary drug residues in chicken meat [32] or melamine determination in infant formula [33]. Its main advantages are its versatility in the analysis of different matrices, since the matrix is removed, as well as a drastic reduction in the sample preparation time required due to automation and minimal manual sample handling.

The aim of this study was to compare the performance of conventional sample preparation techniques with that of an on-line sample clean-up system for the determination of DON and its polar conjugate D3G in cereal grains. During method development, sample preparation techniques previously described for the determination of DON in cereals were tested for their applicability in D3G analysis with liquid chromatography tandem mass spectrometry (LC–MS/MS). The selection of the sample pre-treatment techniques was based on two criteria: (i) their potential applicability in multi-residue analysis and (ii) their proven efficiency for DON detection found in literature. Following evaluation of method performance characteristics obtained by each sample preparation technique, an LC–MS/MS method was developed and validated in-house. The same samples were analysed in parallel, as part of an inter-laboratory method comparison study, by an automated on-line sample preparation clean-up method utilising TurboFlow chromatography coupled to high resolution mass spectrometry (HRMS). Method validation parameters for specificity, linearity, recovery, intra-day precision, the limit of detection (LOD) and limit of quantification (LOQ) were based on European guidelines [34,35].

2. Materials and methods

2.1. Chemicals and reagents

Acetonitrile (HPLC grade) and ammonium hydroxide (25%) were purchased from J.T. Baker (Deventer, the Netherlands). Ultra-pure water was produced by a Milli-Q Plus system (Millipore, Espoo, Finland). Acetonitrile (Optima™ grade), methanol (Optima grade), water (Optima grade), formic acid (LC–MS grade) and Pierce LTQ ESI positive ion calibration solution, used in the on-line clean-up analysis, were purchased from Thermo Fisher Scientific (Langenselbold, Germany). DON and a standard solution of D3G (50 μg/mL in acetonitrile) were purchased from Sigma–Aldrich (Steinheim, Germany). A stock standard solution of DON (100 μg/mL) was prepared by dissolving the analyte in acetonitrile. All solutions were stored at −20 °C.