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The use of immunoaffinity columns connected in tandem for selective and cost-effective mycotoxin clean-up prior to multi-mycotoxin liquid chromatographic–tandem mass spectrometric analysis in food matrices

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ABSTRACT

This paper describes the use of two immunoaffinity columns (IACs) coupled in tandem, providing selective clean-up, based on targeted mycotoxins known to co-occur in specific matrices. An IAC for aflatoxins + ochratoxin A + fumonisins (AOF) was combined with an IAC for deoxynivalenol + zearalenone + T-2/HT-2 toxins (DZT); an IAC for ochratoxin A (O) was combined with a DZT column; and an aflatoxin + ochratoxin (AO) column was combined with a DZT column. By combining pairs of columns it was demonstrated that specific clean-up can be achieved as required for different matrices. Samples of rye flour, maize, breakfast cereal and wholemeal bread were analysed for mycotoxins regulated in the EU, by spiking at levels close to EU limits for adult and infant foods. After IAC clean-up extracts were analysed by LC–MS/MS with quantification using multiple reaction monitoring. Recoveries were found to be in range from 60 to 108%, RSDs below 10% depending on the matrix and mycotoxin combination and LOQs ranged from 0.1 ng/g for aflatoxin B_1 to 13.0 ng/g for deoxynivalenol. Surplus cereal proficiency test materials (FAPAS®) were also analysed with found levels of mycotoxins falling within the satisfactory range of concentrations (Z score $\leq \pm 2$), demonstrating the accuracy of the proposed multi-mycotoxin IAC methods.

1. Introduction

The introduction of commercial immunoaffinity columns (IACs) in the mid-1980s led to a step-change in the analysis of mycotoxins in food and feed. Initially IACs were only for clean-up of aflatoxin B_1, B_2, G_1 and G_2 (aflatoxins), but over the past 20 years the range of mycotoxin IACs has increased to include aflatoxin M_1, ochratoxin A (OTA), deoxynivalenol (DON), T-2 and HT-2 toxins, fumonisins ($B_1 + B_2$) and zearalenone (ZON) [1,2]. Methods for regulated mycotoxins have been adopted as Official methods by AOAC International and CEN and these are predominantly based on IAC clean-up, prior to LC with fluorescence detection [3–8]. In recent years there has been a general recognition, that in some instances, where regulations are enforced for more than one mycotoxin in the same commodity, it makes sense to have IACs containing multiple antibodies. Initially columns were introduced for simultaneous clean-up of aflatoxins and OTA (AflaOchra), which is of particular relevance for the analysis of dried fruit, spices and cocoa, where co-occurrence is common [9]. Columns for aflatoxins and OTA were employed for the analysis of ginseng and ginger [10], with the method being subsequently validated by an inter-laboratory study [11] and accepted as an AOAC First Action Method [12]. Subsequently IACs became available containing combinations of three antibodies such as aflatoxins, OTA and ZON (AOZ) [11]; DON, ZON and T-2/HT-2 toxins (DZT) [14] and aflatoxins, OTA, and fumonisins (AOF). One company has developed columns containing six antibodies (AOFDZT) and these columns have been used for the analysis of wheat and maize, corn flakes and maize snacks [15] and breakfast cereals [16]. Notable with the use of this column containing six antibodies was the need to employ matrix-matched calibration in LC–MS/MS, as column eluant was found to exert an influence over calibration through ion suppression or enhancement [16].

Where cereals, particularly maize needs to be analysed for a range of *Fusarium* toxins, HPLC analysis with fluorescence detection is no longer viable, as some target mycotoxins such as DON and fumonisins lack fluorescence. It is therefore generally accepted that LC–MS/MS is a more suitable approach. There are arguments that MS/MS offers such high specificity, that no clean-up is required at

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all, and background interference can be compensated for, if matrix matching is used to correct for ion suppression or enhancement [17,18]. However, Romagnoli et al., [14] and others have shown that there are advantages in combining IACs and LC–MS/MS in achieving efficient removal of matrix interferences, simple chromatographic outline, high selectivity, low detection limits and separation of a wide range of mycotoxins with different physico-chemical properties in a single run. It has been clearly shown that without IAC clean-up, there can be shoulders and interference peaks close to the target mycotoxins, which adversely impact on the measurement of ion ratios for confirmation of identification [19]. Even with alternative clean-up techniques (such as QuEChERS) prior to LC–MS/MS, for unequivocal measurement and to achieve the necessary sensitivity the use of IAC clean-up is still recommended [20].

It is clear that there are benefits in employing multi-mycotoxin IACs for LC–MS/MS and ideally a diverse range of commercial columns containing different combinations of anything between two and six antibodies would meet these needs. In reality only a limited range of commercial IACs are available, and it is unrealistic to expect the large number of potential combinations of mycotoxins to be marketed commercially in a diversity of columns. It is worth noting that employing an AOFDZT column is only relevant for maize-based products [15,16], and when other cereals are analysed with these columns the fumonisin antibody on the column is redundant.

To achieve a range of target combinations of clean-up for mycotoxins, we have investigated whether it might be possible to use existing IACs in tandem. Use of columns in tandem has the potential to offer a range of desired combinations of mycotoxins in accordance with European regulations, so that analytical clean-up can focus on target mycotoxins known to co-occur in specific matrices. In this paper we have therefore demonstrated the feasibility of using OTA and DZT columns in tandem (ODZT) for the analysis of wholemeal bread, AOF and DZT columns in tandem for the analysis of a range of maize and maize products including infant foods, and AflaOchra and DZT columns (AODZT) for the analysis of oat-based muesli containing dried fruit and nuts. The recoveries and precision were determined in a variety of spiked matrices at different levels. The accuracy of the methods were demonstrated by analysis of surplus proficiency test materials (FAPAS®) comparing found values with assigned values and the range of levels deemed as satisfactory.

2. Materials and method

2.1. Chemicals and reagents

Immunoaffinity columns OCHRAPREP®, DZT MS-PREP®, AOF MS-PREP® and AFLAOCHRA PREP® were obtained from R-Biopharm Rhone Ltd (Glasgow, UK). Columns were connected in tandem using Supelco SPE Tube Adaptors, 57020-U, (Sigma–Aldrich Ltd., Gillingham, UK). The first column was connected below the glass barrel, with the second column connected below the first column. The way in which the columns are connected is shown in the Supplementary information.

Methanol (HPLC grade) was from Fisher Chemical (Loughborough, UK), ammonium formate was Fluka ≥99.0% (Sigma–Aldrich Ltd., Gillingham, UK); formic acid was Optima LC/MS grade (Fisher Scientific, Loughborough, UK). Deionised water was prepared using Millipore Elix followed by Milli-Q Academic to produce 18.2 MΩ water.

Phosphate buffered saline (PBS) tablets were obtained from R-Biopharm Rhone Ltd (Glasgow, UK), one tablet was dissolved in 100 mL water to give sodium chloride 8.0 g/L, potassium chloride 0.2 g/L, Di-sodium hydrogen phosphate 1.15 g/L, potassium dihydrogen phosphate 0.2 g/L, pH 7.3 ± 0.2 at 25 °C.

Mycotoxin standards of DON (1 mg), ZON (10 mg), T-2 toxin (5 mg), HT-2 toxin (5 mg), fumonisin B_1 (1 mg), fumonisin B_2 (1 mg) were supplied as dry films for reconstitution and obtained from Sigma–Aldrich Ltd. (Gillingham, UK). The solids were initially dissolved in 1 mL of acetonitrile and then serial dilutions were carried out to prepare 50 μg/mL DON, 5 μg/mL ZON, and combined T-2 and HT-2 toxin solution (40 μg/mL total) and 160 μg/mL combined fumonisins total. OTA standard solution at a concentration of 1000 ng/mL was OCHRASTANDARD from R-Biopharm Rhone Ltd (Glasgow, UK). Aflatoxin solution containing 250 ng/mL of each of aflatoxins B_1, B_2, G_1 and G_2 was AFLASTANDARD from R-Biopharm Rhone Ltd (Glasgow, UK).

2.2. Mixed working solution

Mixed AOFDZT working solution was prepared by removing 1.25 mL from 10 mL of water:methanol (50:50 v/v), and then adding:-

- 100 μL of 160 μg/mL fumonisin solution to give 1600 ng/mL.
- 50 μL aflatoxins mixture containing 0.25 μg/mL each to give 1.25 ng/mL each.
- 50 μL of 1 μg/mL OTA solution to give 5 ng/mL.
- 500 μL of 50 μg/mL DON solution to give 2500 ng/mL.
- 500 μL of 5 μg/mL ZON solution to give 250 ng/mL.
- 50 μL of T-2 and HT-2 toxin solution (40 μg/mL total) to give 200 ng/mL total.

2.3. Mixed calibration solution

Mixed AOFDZT calibration solutions were prepared from AOFDZT working solution (1 mL) by sequential dilution with (1 mL) water:methanol (50:50 v/v) to give calibration points in the ranges of concentrations for DON of 2.4–1250 ng/mL; each aflatoxin of 0.01–0.625 ng/mL; fumonisin B1 of 1.25–625 ng/mL; fumonisin B2 of 0.35–175 ng/mL; T-2/HT-2 toxins each of 0.1–50 ng/mL; OTA of 0.08–2.5 ng/mL; ZON of 0.5–125 ng/mL.

2.4. Samples

Muesli, (800 g) containing toasted malted wheat flakes (38%), rolled oats (36%), raisins (14%), sultanas (4.5%), whey powder and nibbed hazelnuts (3.0%) containing a background level of 2.0 ng/g of T-2 toxin, and wholemeal bread containing background levels of DON of 16 ng/g and HT-2 toxin of 2.0 ng/g were purchased from a local supermarket. Wholemeal rye flour (1 kg) containing none of the mycotoxins detectable above the LODs and white maize meal (5 kg) containing background levels of FB1 and FB2 of 38 and 9 ng/g respectively were obtained directly from Specialist Foods (London, UK). FAPAS® surplus proficiency test materials contaminated with mycotoxins: T04246 maize containing aflatoxin B_1, OTA, DON, ZON, fumonisins and T-2/HT-2 toxins; and T04244 cereal-based infant food containing aflatoxin B_1, OTA, DON, ZON and T-2/HT-2 toxins were obtained from the Food & Environment Research Agency (York, UK).

For spiking the homogenised sample (25 g) was weighed into a flask and an appropriate amount of aflatoxin standard was pipetted onto the sample, allowing solvent evaporation and permeation of toxins into the matrix. Spiking of rye flour, maize flour and muesli was at 2 ng/g for each individual aflatoxin (8 ng/g total aflatoxins) and at 0.1 ng/g for each individual aflatoxin in maize and infant foods. Spiking with DON was at 1000 ng/g for rye and maize flour, 500 ng/g for bread and muesli and 200 ng/g for maize flour and infant food. Spiking with ZON was at 100 ng/g for rye and maize