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Application of single immunoaffinity clean-up for simultaneous determination of regulated mycotoxins in cereals and nuts

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ABSTRACT

A rapid and sensitive analytical strategy for the simultaneous determination of twelve mycotoxins (aflatoxins, fumonisins, zearalenon, deoxynivalenol, ochratoxin A, T-2 and HT-2 toxins) using ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC–MS/MS) was developed and validated. The method was validated for peanuts, barley and maize-breakfast cereals; selected as they represent the matrices most often contaminated by mycotoxins. The method is designed for fast and reliable analyses of mycotoxins in regulatory, industrial and private laboratories. Multi-target immunoaffinity columns containing antibodies for all mycotoxins studied herein were used for sample clean-up. Method optimization was predominantly focused on the simplification of extraction and clean-up procedure recommended by column producers. This newly developed and simplified procedure decreased both the sample preparation time and the solvent volumes used for their processing. The analysis of all regulated mycotoxins was conducted by a newly developed UHPLC–MS/MS method with a sample run time of only ten minutes. The method trueness was tested with analytical spikes and certified reference materials, with recoveries ranging from 71% to 112% for all of the examined mycotoxins.

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1. Introduction

Mycotoxins are widespread natural chemical contaminants which can significantly influence the hygienic–toxicological quality of various agricultural commodities. Their presence in foods can cause adverse health effects to consumers, humans and/or animals [1]. In many countries, regulatory limits have already been established for mycotoxins (the overview of world legislation limits is available and summarized on web page www.mycotoxins.org), and a number of other countries are setting or improving their actual legislation toward mycotoxins [2]. In order to establish a balance between importing and exporting countries and to ensure the safety of domestic food products, reliable, fast and cost-effective strategies are being developed for the analysis of mycotoxins in various foodstuffs [2]. Most current research is directed towards the development and validation of analytical methods for the simultaneous determination of the following regulated mycotoxins: aflatoxins, deoxynivalenol (DON), HT-2 and T-2 toxins, zearalenone (ZON), fumonisins, ochratoxin A (OTA), and patulin [3–6].

Numerous procedures, both instrumental and bioanalytical, have been developed for the simultaneous determination of multiple mycotoxins, including non-regulated mycotoxins [7,8]. However, many laboratories still apply individual methods for the separate determination of each mycotoxin for which legislation limits or action levels were established [4]. These methods, which generally rely on non-specific detection techniques [e.g. fluorescence detector (FLD), ultraviolet detector (UV), diode array detector (DAD), or gas chromatography–mass spectrometry (GC–MS) instrumentation], are often set by the Association of Official Analytical Chemists (AOAC) or by the European Committee for Standardization (CEN) [9]. The methodology is often time consuming, less sensitive, and less specific than liquid chromatography–tandem mass spectrometry (LC–MS/MS) methods. LC–MS/MS based instrumentation has become more affordable and is now commonly found in regulatory, industrial, and private laboratories. Moreover, this technique represents a specific, reliable and high-throughput analytical strategy for monitoring mycotoxins in various food matrices [2,3,7,8].

The use of immunoaffinity columns (IACs) in the clean-up and pre-concentration of mycotoxins has been the subject of a large amount of research [10–12]. The great advantage of IACs is the high specifity of imprinted antibodies to target analytes. Unfortunately, the majority of commercially available IACs have antibodies specific to only one or a small group of closely related mycotoxins, e.g. fumonisins or aflatoxins. Multi-functional IACs, in combination

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with LC–MS/MS, have the potential for an effective and unique high-throughput analytical procedure for the single analysis of mycotoxins [11,12]. To the best of our knowledge, there is only one commercially available IAC which was developed for the determination of majority of regulated mycotoxins (DON, ZON, aflatoxins, fumonisins, OTA, T-2 and HT-2 toxins). The great disadvantage of these IACs is a double-extraction step recommended by the column producers (VICAM, Waters, USA). This two-step extraction is extremely time-consuming and inconvenient, because at the end a huge volume of diluted sample extract must pass through the column. Publications in which this procedure has been applied obtained very good recoveries, higher than 79% for all analytes on cereal samples and on a wide range of concentrations of mycotoxins using both MS and traditional FLD and photodiode array (PDA) detectors [13,14].

For regulatory purposes it is highly desirable to have a reliable, precise and fast analytical procedure which is applicable to all above discussed mycotoxins in various food commodities. Cereal-based foodstuffs and nuts represent matrices of the most interest globally for mycotoxin analysis. A simple optimized and validated analytical procedure for the determination of mycotoxins DON, ZON, OTA aflatoxins B1, B2, G1, G2, fumonisins (FB1, FB2), T-2 and HT-2 toxins in maize breakfast-cereals, barley and peanuts is described herein. This method merges the advantages of multi-functional IAC specifity with the speed and sensitivity of ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC–MS/MS) instrumentation. It can be used for the detection of several co-occurring mycotoxins in a single run, resulting in more cost-effective and faster analysis of food samples.

2. Materials and methods

2.1. Analytical standards

Analytical standards of deoxynivalenol (DON), zearalenone (ZON), ochratoxin A (OTA), HT-2 toxin (HT2), T-2 toxin (T2), aflatoxins B1, B2, G1, G2 (AFB1, AFB2, AFG1, AFG2) and fumonisins B1, B2, B3 (FB1, FB2, FB3) were purchased from Romer Labs (Franklin, MO, USA) with declared purities ranging from 95.0% to 98.9%. Solid compounds were dissolved in acetonitrile (MeCN) and further diluted with MeCN to produce individual stock solutions with a concentration of 1 mg/mL; liquid standards were diluted with MeCN to produce individual stock solutions at a concentration of 10 μg/mL. From all of individual stock solutions, one mixed-stock solution at concentration of 1 μg/mL was prepared by diluting with MeCN. All standards and stock solutions were stored at −20 °C in glassware.

2.2. Materials and chemicals

Ultrapure water (18 MOhm) was produced by an Aqua Solutions 2001 BU Water Deionizer (Jasper, GA, USA). IACs Myco6in1™ were purchased from VICAM (Watertown, MA, USA). LC–MS grade acetonitrile (MeCN) and methanol (MeOH) were supplied by Fisher Scientific (Pittsburgh, PA, USA). Acetic acid (≥95%) (AcA), formic acid (≥95%) (FoA), amonium acetate (≥99.9%), were supplied by Fisher Scientific (Pittsburgh, PA, USA). A solution of phosphate buffer (PBS) was prepared by dissolving of PBS saline tablets, purchased from Sigma-Aldrich (St. Louis, MO, USA), in ultrapure water.

2.3. Samples, extraction and immunoaffinity clean-up procedures

Maize-based breakfast cereals, barley and peanuts were obtained from a local retail market and used during the validation of the method. All samples were finely homogenized using a variable-speed laboratory blender LB10, model 38BL54 (Waring Commercials, Torrington, Connecticut, USA). For extraction, 5 g portions of homogenized samples were extracted with 20 mL of an MeCN:H_2O:AcA (79.5:20:0.5, v/v/v) solution for 60 min. Crude sample extracts were then centrifuged for 2 min at 5000 rpm. Two different sample procedures were compared for the immunoaffinity clean-up process.

Procedure 1. 2 mL portion of crude extract was diluted with 33 mL of PBS buffer and the entire solution was passed through the multi-functional IAC at a rate of approximately 1 drop per second and the eluent was discarded. After the diluted sample extract passed through, the IAC was washed with 10 mL of ultrapure H_2O which was subsequently discarded. Mycotoxins were eluted from the column by means of 3 mL of MeOH, evaporated to dryness by a gentle stream of nitrogen at 60 °C, and reconstituted in 0.5 mL of MeOH:H_2O (1:1, v/v) containing 0.2% of AcA by vortex mixing. All samples were filtered prior to UHPLC–MS/MS analysis.

Procedure 2. Complete solvent exchange was applied in this procedure. An aliquot of 10 mL of crude extract was evaporated to dryness by a gentle stream of nitrogen (60 °C) and subsequently dissolved in 10 mL of PBS buffer by vortex mixing (some precipitation of matrix was observed, particularly in maize breakfast cereals, but this did not adversely affect the final results). This solution was passed through the IAC at a rate of approximately 1 drop per second. The remaining procedure was identical to Procedure 1 as columns were washed with 10 mL of ultrapure H_2O, mycotoxins were eluted by 3 mL of pure MeOH, which was evaporated by a stream of nitrogen and reconstituted in 0.5 mL of MeOH:H_2O (1:1, v/v) containing 0.2% of AcA by vortex mixing. All samples were filtered prior to UHPLC–MS/MS analysis.

2.4. UHPLC–MS/MS method

For simultaneous determination of all mycotoxins, an UHPLC–MS/MS method was developed for their separation and detection. Ultra-high performance liquid chromatography (UHPLC) was performed using a Prominence UFLC XR chromatographic system (Shimadzu, Kyoto, Japan), coupled to a ABSciex 4500 QTRAP hybrid triple quadrupole/linear ion trap mass spectrometer equipped with a TurboIon electrospray (ESI) ion source (AB Sciex, Toronto, ON, Canada). The chromatographic separation was carried out using a 100 mm × 2.1 mm i.d., 1.7 μm particle size, Acquity UPLC HSS T3 endcapped reversed phase analytical column (Waters, Milford, MA, USA) maintained at 40 °C. The autosampler temperature was held at 10 °C and the injection volume was 10 μL. Mobile phases consisted of 5 mM ammonium acetate in water (A) and methanol with 5 mM ammonium acetate (B). The mobile phase flow rate was set at 0.4 mL/min. For separation of mycotoxins, the following gradient was applied: the initial composition of mobile phase contained 5% B, its volume was rapidly increased within 1 min to 50% B. The gradient was steadily increased from 50% B at 1 min to 100% of B at 7 min and held until 8 min of analysis. At 8.1 min the B composition was stepped down to its initial conditions (5%) and maintained for another 2 min.

The QTRAP 4500 mass spectrometer was operated in both positive and negative ionization modes which were applied for two periods within a single run of analysis for a total of 10 min. The first period, from the start of the run to 2.7 min was operated in negative ion mode (ESI−) for the determination of DON. At 2.7 min, the polarity was switched to positive ionization mode (ESI+) for the second period where all remaining analytes were detected. The settling time was 50 ms; dwell times varied for different analytes and are shown in Table 1. The ion source temperature was set at 450 °C, ion spray voltage operated in −4000/4500 V in ESI−/ESI+, respectively. Curtain gas was set at 20 arbitrary units (au), nebulizer and Turbo