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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

**Table 1**
Parameters of MS/MS detection method.

| Analyte | $t_R$ (min) | Precursor ion (*m/z*) | Product quantifier/qualifier ions (*m/z*) | Declustering potential (V) | Collision energy (V) | Cell exit potential (V) | Dwell time (ms) |
|---|---|---|---|---|---|---|---|
| DON | 2.17 | 355.1 $[M+CH_3COO]^-$ | 59.1/295.1 | −30 | −45/−13 | −9/−11 | 100/100 |
| AfB1 | 3.37 | 312.9 $[M+H]^+$ | 285.1/241.1 | 101 | 33/53 | 8/8 | 20/20 |
| AfB2 | 3.22 | 314.9 $[M+H]^+$ | 286.8/259.1 | 106 | 37/41 | 10/10 | 20/20 |
| AfG1 | 3.00 | 328.9 $[M+H]^+$ | 242.9/200.1 | 76 | 37/55 | 8/6 | 20/20 |
| AfG2 | 2.84 | 330.9 $[M+H]^+$ | 313.1/189.1 | 81 | 35/57 | 10/8 | 20/20 |
| FB1 | 3.33 | 722.3 $[M+H]^+$ | 704.4/334.2 | 61 | 41/55 | 18/12 | 50/50 |
| FB2 | 4.60 | 706.3 $[M+H]^+$ | 336.2/688.3 | 71 | 51/41 | 12/18 | 25/25 |
| FB3 | 4.03 | 706.3 $[M+H]^+$ | 336.2/688.2 | 96 | 49/41 | 12/16 | 25/25 |
| OTA | 3.83 | 403.9 $[M+H]^+$ | 238.9/357.9 | 71 | 33/21 | 8/12 | 20/20 |
| HT2 | 4.11 | 442.0 $[M+NH_4]^+$ | 263.1/215.1 | 30 | 19/19 | 10/6 | 50/50 |
| T2 | 4.68 | 484.0 $[M+NH_4]^+$ | 305.0/215.0 | 30 | 19/23 | 10/6 | 20/20 |
| ZON | 5.12 | 319.0 $[M+H]^+$ | 301.0/283.1 | 71 | 13/17 | 10/10 | 20/20 |

gasses were 55 au for both periods. The compound-dependent parameters for the MS/MS detection of analytes are presented in Table 1.

### 2.5. Optimization and validation of the method

The efficiency and utility of both sample clean-up procedures were determined through the analysis of a series of blank samples which were spiked at four concentrations of 2 (only for peanuts due to low concentrations of aflatoxins that can be commonly detected in this matrix), 10, 50 and 200 ng/mL. After spiking at the desired concentration, each sample was left overnight under ambient conditions before extraction. The quantification of mycotoxins was performed by external calibration with matrix-matched standards at concentrations ranging from 0.01 to 500 ng/mL. Limits of detection (LOD) and limits of quantitation (LOQ) were estimated as the lowest matrix-matched calibration standards providing signal-to-noise ratios greater than 3 and 10, respectively, at both quantitative and qualitative transitions and matching the intensity ratio observed for the particular compound in the standard solution.

In addition to evaluating the sample clean-up, sample quantification using a variety of calibration standards was investigated. In total, three series of calibration standards were prepared. Firstly, solvent-only calibration standards were prepared by dilution of the mixed stock solution in MeOH:$H_2O$ (1:1, v/v) containing 0.2% of AcA. Secondly, matrix-matched standards were prepared by fortifying extracts of matrices (barley, peanuts, and maize-breakfast cereals) which did not contain mycotoxins. Lastly, "IAC calibration standards" were prepared by eluting solvent, without matrix, through the IACs and subsequently fortifying with mycotoxins stock solution. Linear curve fits were suitable for all analytes with correlation coefficients ($R^2$) of greater than 0.995. These three types of calibration standards were used for the determination of matrix effects from the applied matrices as well as from the immunoaffinity columns alone. Matrix effects were evaluated by computing a signal suppression and enhancement (SSE) factor and using a *t*-test (significance alpha level of 0.05 and 6 degrees of freedom), which allows for the statistical evaluation of the difference between slopes of matrix-matched and solvent-only/IAC linear regression calibration curves. Values of SSE (%) were calculated as the ratio between the slopes of matrix-matched calibration curves and solvent-only calibration curves, as well as with the slopes of IAC calibration curve. Results of *t*-test are expressed as TRUE (T) or FALSE (F) values, when TRUE indicates no statistical difference between slopes; FALSE (F) indicates that slopes are statistically different.

The following certified reference materials were used as quality control samples to check the trueness of the method: Peanut butter BCR-385R for determination of aflatoxins (EU Joint Research Centre (JRC)), Peanut meal BCR-236R for aflatoxins (JCR), Corn for FB1 (ID 26536-1, Romer Labs), Wheat for DON (ID 26429-1, Romer Labs). The certified concentrations of mycotoxins in these materials are shown in Table 4.

## 3. Results and discussion

### 3.1. Development and optimization of UHPLC–MS/MS method

Due to the diversity of the analytes, the development and optimization of a simple and rapid UHPLC–MS/MS method for the simultaneous determination of 12 regulated mycotoxins in 3 matrices presents a number of significant analytical challenges. Due to regulatory requirements for confirmatory methods [15], MS/MS instrumentation was selected in order to fulfill the rule of identification points. The 12 analytes have a broad range of solubilities, span a large concentration range [from μg/kg (ppb) to mg/kg (ppm)] and require different ESI polarities during analysis. Additionally, the need to detect these 12 mycotoxins from a variety of matrices presents challenges in assessing the matrix impact on sensitivity and accuracy. Bearing in mind all of these requirements, the following conditions were developed for the UHPLC–MS/MS method. Due to optimal ionization parameters for the test mycotoxins, both ESI+ and ESI− modes had to be applied for their detection and analyses. Unfortunately, the speed of polarity switching is a major drawback of low resolution (LR) MS instruments that can significantly decrease the sensitivity for some analytes. The only analyte for which the negative ESI− ionization mode is generally required is DON, which is also the target analyte which elutes first. It was possible to develop a method, working with two periods within a single analytical run with each period operated in a different polarity mode. After the optimization of the UHPLC gradient, the polarity switch was established at 2.7 min. Regarding the overall length of the analytical run, due to the employment of immunoaffinity sample clean-up and the usage of a UHPLC system, it was possible to develop a UHPLC method with a total run time of only 10 min. The retention time of the last toxin (ZON) is 5.16 min, allowing time for washing and reconditioning of the column for the next injection within a 10 min run (Fig. 1).

Based on previous work, initial method development conditions included buffer concentrations of 10 mM ammonium acetate and 0.2% AcA in mobile phases A and B and an ion spray voltage at the maximum setting of −4500 V in ESI− mode. However, arcing was observed in the source when operating in negative ion mode, leading to low responses and lack of reproducibility for DON. After

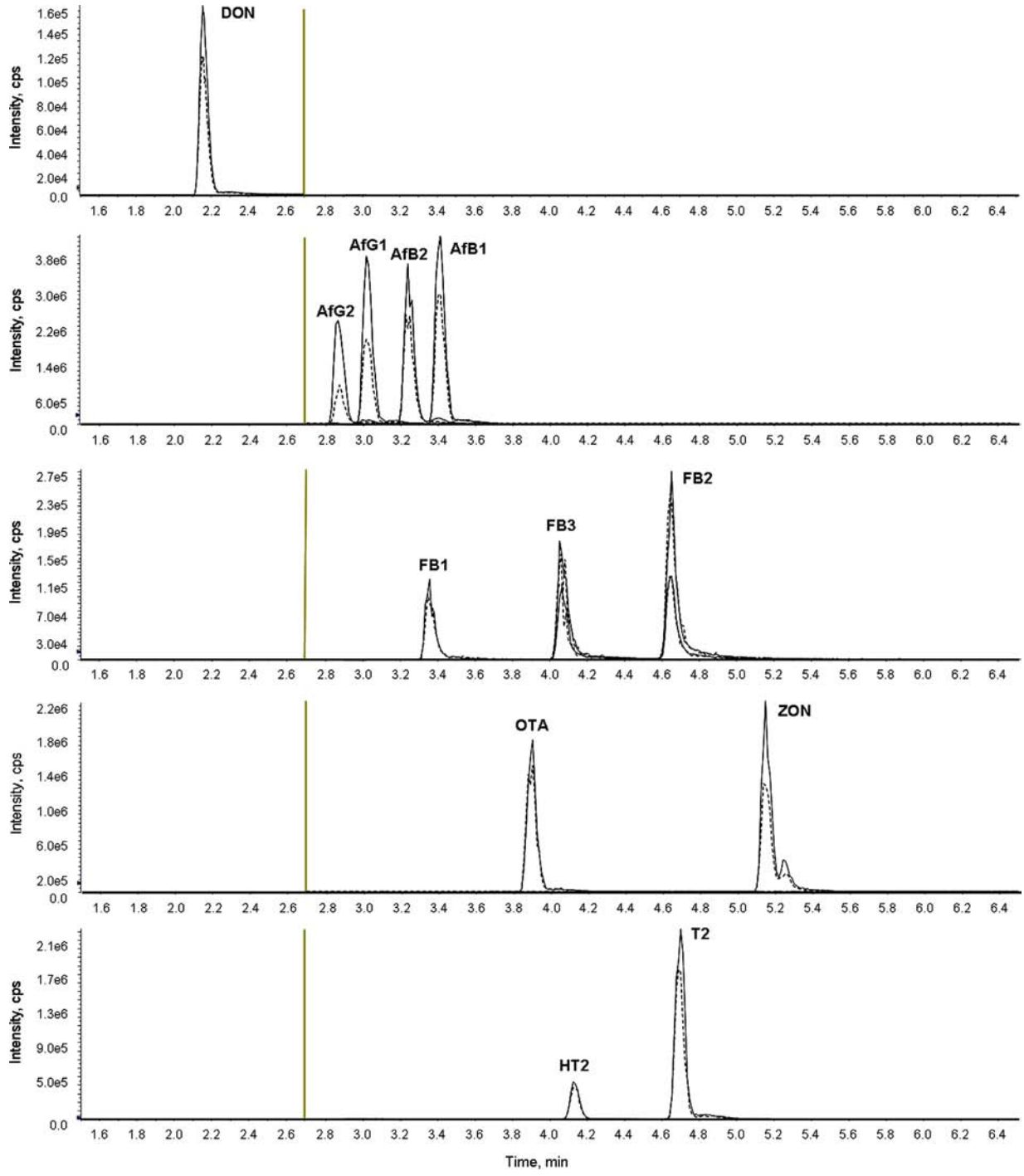


**Fig. 1.** UHPLC–MS/MS chromatograms of mycotoxins.

extensive experimentation, a combination of lowering the ion spray voltage to −4000 V, decreasing the ammonium acetate concentration in both mobile phase to 5 mM, and removing AcA from the mobile phase eliminated any arcing and improved DON sensitivity and repeatability. In addition, the diverter valve was not used as arcing was seen in ESI− mode when the UHPLC flow was diverted to waste.

### 3.2. Extraction and immunoaffinity clean-up optimization

For the analysis of mycotoxins in solid matrices, the extraction is undoubtedly the most crucial step in the entire sample preparation procedure. As mentioned above, the application of IACs is a preferred and selective tool for sample clean-up, but the sample extraction and handling procedure recommended by the manufacturer is very laborious, time-consuming, and produces large volumes of solvent waste. It is not suitable for the environmentally friendly rapid analysis desired by most laboratories. Briefly, this procedure involves a double extraction with phosphate-buffered solution (PBS) in the first step. The PBS is then removed and the same sample is subsequently extracted by a methanol/water extraction solvent. The crude methanol extract is then diluted in order not to exceed the limit of 5% organic solvent that can be applied to the IAC, producing a final volume of up to 55 mL before the extracts are loaded onto the IAC. This is followed by passing 40 mL of wash solvents through the column before eluting the

mycotoxin analytes. The main goal of the present study was to develop and evaluate a new, less laborious sample preparation procedure.

Numerous previously published studies have shown that an 80:20 (v/v) mixture of acetonitrile and water containing either formic or acetic acid is the most effective and widely used extraction solvent for multi-mycotoxins methods [16,17]. Because this extraction mixture is suitable for general multi-mycotoxins analysis, it was expected to fit the purpose for the extraction of target mycotoxins tested in this study. The other question was the compatibility of such a mixture with the functionality of the IACs. For this purpose, the efficacy of IAC antibodies to analytical standards dissolved in given solvents and mixture of solvents was tested. Analytical standards dissolved in PBS, MeOH, $H_2O$, MeCN and in solutions of MeCN with $H_2O$ acidified with both formic and acetic acids were tested; the results are summarized in Table 2. Out of all tested individual solvents, as expected, PBS and $H_2O$ gave the highest values of cross-reactivities. Low recoveries obtained for aflatoxins G1 and G2 were quite surprising since there was no previous extraction and analytical standards were only dissolved in solvents and passed through the columns. Insufficient capacity of columns for these analytes could be another explanation of these observations, unfortunately these data are not provided by producers. Low recoveries were also observed for all three fumonisins when using $H_2O$, MeOH and MeCN. Recoveries for other target compounds were sufficient and ranged from 74% to 111%. Next to pure and diluted solvents, the suggested extraction mixtures of MeCN:$H_2O$:FoA/AcA (79:20:1, v/v/v) were also tested. The results proved that FoA gave lower recoveries for the majority of analytes in comparison to AcA, but recoveries for several analytes in both mixtures were lower than for pure MeCN. Clearly, acidic conditions significantly influenced the functionality of the columns even though samples were diluted with the recommended PBS solution. On the other hand, cross-reactivities of aflatoxins G1 and G2 increased significantly and the recoveries were above 70% for all tested acidic solvent mixtures. Considering these results, only AcA was used in further experiments with spiked samples of maize-based breakfast cereals (matrix-matched analytical standards were used for the calculation of results). Results using MeCN:$H_2O$:AcA (79:20:0.5(1), v/v/v) demonstrate that while the majority of the analytes are not significantly influenced by varying the concentration of AcA in the extraction solvents, recoveries of T-2 and HT-2 toxins were below 20% and the recovery of OTA decreased with increased AcA concentration. These results demonstrate that the suggested Procedure 1, which involves the application of acetic acid to the IAC, would not work properly and thus cannot be commonly applied for the clean-up of samples. Very similar results were obtained by Lattanzio et al. [13] and Soleimany et al. [14], who used the manufacturer recommended double extraction for sample preparation.

Comparing all solvent/IAC results, it was clear that acidic conditions decrease the efficiency of IACs to some extent. On the other hand, acidic conditions are necessary for the extraction of fumonisins. Based on these results, the extraction and clean-up strategy described in Procedure 2 was tested. The same extraction solution, consisting of MeCN:$H_2O$:AcA (79.5:20:0.5, v/v/v) was applied, but the extraction was followed by complete solvent exchange by evaporating 2 mL of crude extract to dryness with a stream of nitrogen and reconstituting the residue in only 10 mL of PBS solution. This processed sample extract was then passed through the IAC. By employing Procedure 2, it was possible to obtain very good recoveries for all tested analytes. Another benefit of this strategy is the significant decrease of solvent volume, which significantly decreases the time necessary for sample preparation as well as minimizing waste. Procedure 2 was used for the validation study and the results are summarized in the following section on performance characteristics of the method.

It is worth noting that a very crucial step in the immunoaffinity clean-up procedure is the speed by which extracts or solvents are passed through the columns. After the extracts are loaded onto the cartridge, the elution rate must be no higher than 1 drop per second. Washing of columns with $H_2O$ can be faster, approximately 2–3 drops per second, but the highest attention should be paid to analytes elution from the cartridges by pure methanol. This issue has also been encountered in the studies by Lattanzio et al. [13] and Soleymani et al. [14]. Since only 3 mL of solvent is used for complete elution of analytes, it is highly recommended to apply the entire volume of methanol onto the columns but pass only about one third of the MeOH volume into the column, close the valves, wait for at least 5 min, and then slowly pass (1 drop per second) another third of MeOH before repeating the process with the final one third of the MeOH. Although such a procedure is quite lengthy, it can significantly increase the recoveries of the analytes because some time is needed for denaturation of the bonds between analytes and antibodies.

### 3.3. Performance characteristics of the method

Method performance characteristics such as recovery, repeatability, linearity, LOD and LOQ were evaluated for all the tested mycotoxins. Optimized Procedure 2 was used for the validation process in barley, peanuts and maize breakfast cereals matrices. The performance data are summarized in Table 3. Corresponding matrix-matched calibration standards were used for the calculation of results for all three matrices. Although many compromises had to be accepted during the development of the sample extraction and

**Table 2**
Recoveries of mycotoxins in IACs for tested solvents and spiked samples (presented results were obtained using a solvent-only standards external calibration).

| Samples (solvents) | Mixture (%) | Concentration AfB1 (ng/mL; μg/kg) | | Recovery (%) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | AfB2 | AfG1 | AfG2 | FB1 | FB2 | FB3 | DON | ZON | HT-2 | T-2 | OTA |
| Solvent (PBS) | 100 | 50 | 91 | 85 | 33 | 23 | 78 | 84 | 82 | 102 | 101 | 99 | 75 | 84 |
| Solvent ($H_2O$) | 100 | 50 | 74 | 82 | 46 | 56 | 60 | 54 | 59 | 83 | 95 | 93 | 96 | 111 |
| Solvent (MeOH) | Dissolved in PBS (5:95, v/v) | 50 | 84 | 85 | 36 | 28 | 65 | 69 | 76 | 92 | 91 | 90 | 81 | 74 |
| Solvent (MeCN) | Dissolved in PBS (5:95, v/v) | 50 | 85 | 78 | 26 | 19 | 53 | 46 | 52 | 105 | 98 | 97 | 75 | 80 |
| Solvent (MeCN:$H_2O$: FoA; 79:20:1, v/v/v) | Dissolved in PBS (5:95, v/v) | 50 | 74 | 68 | 77 | 59 | 73 | 74 | 76 | 84 | 58 | 25 | 40 | 52 |
| Solvent (MeCN:$H_2O$: AcA; 79:20:1, v/v/v) | Dissolved in PBS (5:95, v/v) | 50 | 80 | 77 | 96 | 95 | 74 | 81 | 79 | 86 | 75 | 50 | 95 | 47 |
| Spike Procedure 1 | MeCN:H2O:AcA (79.5:20:0.5, v/v/v) | 50 | 92 | 96 | 112 | 88 | 84 | 86 | 80 | 104 | 84 | 12 | 25 | 97 |
| Spike Procedure 1 | MeCN:H2O:AcA (79:20:1, v/v/v) | 50 | 94 | 101 | 115 | 105 | 88 | 87 | 85 | 88 | 85 | 21 | 36 | 65 |
| Spike Procedure 2 | MeCN:H2O:AcA (79.5:20:0.5, v/v/v) | 50 | 85 | 98 | 82 | 80 | 78 | 80 | 81 | 75 | 86 | 98 | 105 | 85 |

**Table 3**
Performance characteristics of the method (n=5).

| Matrix | Spiking concentrations (μg/kg) | Recovery (RSD, %) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | AfB1 | AfB2 | AfG1 | AfG2 | FB1 | FB2 | FB3 | DON | ZON | HT-2 | T-2 | OTA |
| **Barley** | 200 | 74 (8) | 76 (8) | 73 (7) | 74 (6) | 82 (4) | 79 (6) | 89 (6) | 83 (3) | 80 (7) | 95 (6) | 91 (7) | 83 (6) |
| | 50 | 78 (7) | 82 (9) | 76 (7) | 77 (4) | 79 (5) | 72 (9) | 90 (5) | 75 (5) | 75 (4) | 93 (5) | 89 (8) | 84 (8) |
| | 10 | 74 (7) | 73 (6) | 71 (4) | 73 (8) | n.d. | n.d. | n.d. | 93 (9) | 89 (10) | 99 (5) | 82 (9) | 93 (7) |
| **Maize breakfast cereals** | 200 | 68 (6) | 73 (8) | 63 (12) | 65 (12) | 85 (8) | 80 (7) | 83 (6) | 78 (4) | 79 (5) | 89 (2) | 105 (4) | 80 (3) |
| | 50 | 86 (3) | 99 (2) | 85 (3) | 82 (3) | 82 (7) | 77 (4) | 76 (6) | 92 (5) | 94 (6) | 97 (4) | 112 (8) | 95 (2) |
| | 10 | 88 (2) | 79 (10) | 78 (3) | 73 (5) | n.d. | n.d. | n.d. | 90 (5) | 79 (8) | 92 (8) | 103 (3) | 99 (6) |
| **Peanuts** | 50 | 71 (5) | 82 (6) | 75 (5) | 84 (6) | 75 (10) | 72 (9) | 78 (10) | 83 (5) | 79 (4) | 93 (3) | 95 (5) | 77 (6) |
| | 10 | 74 (7) | 75 (6) | 73 (8) | 72 (8) | n.d. | n.d. | n.d. | 78 (5) | 80 (6) | 90 (3) | 99 (4) | 82 (4) |
| | 2 | 74 (4) | 79 (3) | 78 (4) | 91 (8) | n.d. | n.d. | n.d. | 74 (7) | 75 (6) | 91 (3) | 88 (5) | 103 (3) |
| **All matrices** | LOD | 0.05 | 0.05 | 0.05 | 0.05 | 5 | 5 | 5 | 1 | 1 | 0.5 | 0.5 | 0.1 |
| | LOQ | 0.1 | 0.1 | 0.1 | 0.1 | 10 | 10 | 10 | 5 | 5 | 1 | 1 | 0.25 |
| | Linearity | 0.1–100 | 0.1–100 | 0.1–100 | 0.1–100 | 10–500 | 10–500 | 10–500 | 5–500 | 5–500 | 1–500 | 1–500 | 0.1–250 |

n.d. spiking concentrations were below the limits of quantification for particular analytes.

**Table 4**
Concentrations of mycotoxins detected in reference materials.

| Reference material | Analyte | Certified value (μg/kg) | Analyzed concentration (μg/kg) | |
|---|---|---|---|---|
| | | | Matrix-matched standards quantification | IAC calibration standards quantification |
| Peanut butter (BCR-385R) | AfB1 | 1.77 ± 0.3 | 1.90 | 1.85 |
| | AfB2 | 0.48 ± 0.08 | 0.55 | 0.42 |
| | AfG1 | 0.9 ± 0.4 | 1.20 | 1.18 |
| | AfG2 | 0.3 ± 0.12 | 0.40 | 0.39 |
| Peanut meal (BCR-236R) | AfB1 | 17.1 ± 2.4 | 18.0 | 16.3 |
| | AfB2 | 3.0 ± 0.4 | 2.90 | 3.2 |
| | AfG1 | 3.0 ± 0.5 | 3.20 | 3.3 |
| Wheat | DON | 5000 | 5150 | 5086 |
| Corn | FB1 | 5000 | 4813 | 4750 |

clean-up procedure, recoveries obtained for all tested mycotoxins were in the range from 70% to 120%, as described in Ref. [15]. Recoveries with values below 70% were obtained only in the case of aflatoxins at spiked concentrations of 200 μg/kg in breakfast cereals, but these low values are likely caused by insufficient capacity of IAC antibodies against aflatoxins. Column capacities for particular mycotoxins were not provided by the manufacturer. Three different spiking concentrations were tested for all matrices. In the case of both cereal-based matrices, concentrations up to 200 μg/kg were tested due to the need to confirm performance characteristics at concentrations corresponding to the occurrence of fumonisins in cereals. On the other hand, the lower concentrations of spikes (2, 10 and 50 μg/kg) were tested to account for the occurrence of aflatoxins in peanuts. Method repeatability, expressed as relative standard deviation, was sufficiently low for all tested mycotoxins and did not exceed 12%. Due to a pre-concentration of sample extracts, very low limits of detection and quantification were achieved as shown in Table 3. The trueness of the data obtained by this newly developed method was tested by the analysis of certified reference materials which are available for some representative mycotoxins; results are listed in Table 4. For quantification of analytes both matrix-matched and IAC-solvent calibration standards (discussed below) were used. In the case of the reference material for DON, very low results (recovery of approximately 50%) were obtained when the sample was processed exactly according to Procedure 2. But because the capacity of the IACs for high concentrations was questionable, an aliquot of 0.4 mL of crude sample extract (instead of 2 mL) was taken for the sample preparation. In the case of the reference material for fumonisins, an aliquot of 0.2 mL of crude sample extract (instead of 2 mL) was taken for the sample preparation. These changes in volume resulted in improvement of DON recoveries from the reference material and confirmed our doubts about the capacity of the IACs. Column capacity needs to be tested more thoroughly or should be provided by the manufacturer to avoid inaccurately low concentrations for highly contaminated samples.

### 3.4. Matrix effects of IACs columns

The evaluation of matrix effects was the final component of this study. In LC–MS multi-target analysis, problems related to the ionization of analytes and suppression or enhancement effects are commonly solved through the use of matrix-matched calibration standards. Since our method is primarily intended for everyday usage in routine laboratories where a wide range of matrices need to be analyzed, preparation of particular matrix-matched standards is inconvenient and tedious. For this reason, a study of matrix effects was conducted by the comparison of calibration standards prepared in neat solvent to matrix-matched standard curves. Experiments previously conducted by Lattanzio et al. [13], observed suppression of some analytes (aflatoxins and OTA) and resulted in matrix-matched calibration standards usage for analyses and calculation of results in that study. Our results confirm this observation, with large signal suppression observed in the case of aflatoxins G1/G2 and OTA. In addition, signal enhancement was seen in matrix standards for the fumonisins when slopes were compared to a neat solvent calibration curve.

Although these results indicate the necessity of using matrix-matched standard curves, the general principle of IACs (matrix co-extractives should be almost completely removed from cartridges due to washing) led us to examine the suppression or enhancement effects of analytes eluted from the cartridges alone. For this purpose, “IAC-solvent calibration standards” prepared in IAC method blanks were analyzed as well and compared to five point matrix-matched calibration curves prepared in all matrices (barley, breakfast cereals, and peanuts). Matrix effects were evaluated using SSE calculation and *t*-test results. The values of SSE and results of *t*-test are summarized in Table 5. From these data it is clear that, as previously discussed, when a solvent only calibration was used for comparison, several analytes (fumonisins, aflatoxins, OTA and ZON) experience the strong matrix effects. Use of a calibration curve of IAC-solvent standards helped reduce the suppression/enhancement of some of these analytes. This strategy

**Table 5**
Signal suppression and enhancement (SSE, %) of analytes calculated using slopes of solvent-only and IAC calibration curves. Results of *t*-test express the statistical difference between slopes of matrix-matched and solvent-only/IAC calibration curves. TRUE (T): no statistical difference between slopes values, FALSE (F): slopes are statistically different.

| Analyte | Matrix | SSE (%), *t*-test | |
|---|---|---|---|
| | | Solvent-only calibration | IAC calibration |
| AfB1 | Barley | 79 (F) | 103 (T) |
| | Breakfast Cereals | 83 (F) | 108 (T) |
| | Nuts | 80 (F) | 106 (T) |
| AfB2 | Barley | 96 (T) | 85 (T) |
| | Breakfast Cereals | 97 (T) | 86 (T) |
| | Nuts | 101 (T) | 93 (T) |
| AfG1 | Barley | 75 (F) | 113 (T) |
| | Breakfast Cereals | 67 (F) | 97 (T) |
| | Nuts | 81 (F) | 112 (T) |
| AfG2 | Barley | 82 (F) | 96 (T) |
| | Breakfast Cereals | 68 (F) | 82 (F) |
| | Nuts | 88 (T) | 107 (T) |
| FB1 | Barley | 131 (F) | 118 (F) |
| | Breakfast Cereals | 126 (F) | 92 (F) |
| | Nuts | 114 (F) | 115 (F) |
| FB2 | Barley | 132 (F) | 115 (T) |
| | Breakfast Cereals | 135 (F) | 118 (F) |
| | Nuts | 149 (F) | 110 (T) |
| FB3 | Barley | 124 (F) | 115 (T) |
| | Breakfast Cereals | 117 (F) | 108 (T) |
| | Nuts | 118 (F) | 110 (T) |
| DON | Barley | 95 (T) | 105 (T) |
| | Breakfast Cereals | 83 (F) | 92 (T) |
| | Nuts | 96 (T) | 106 (T) |
| ZON | Barley | 78 (F) | 86 (F) |
| | Breakfast Cereals | 82 (F) | 90 (T) |
| | Nuts | 84 (F) | 93 (T) |
| HT-2 | Barley | 80 (T) | 88 (T) |
| | Breakfast Cereals | 85 (T) | 94 (T) |
| | Nuts | 86 (T) | 94 (T) |
| T-2 | Barley | 93 (T) | 109 (T) |
| | Breakfast Cereals | 86 (T) | 94 (T) |
| | Nuts | 95 (T) | 113 (T) |
| OTA | Barley | 60 (F) | 115 (T) |
| | Breakfast Cereals | 61 (F) | 114 (T) |
| | Nuts | 52 (F) | 101 (T) |

was tested to explore the possibility of using only one set of calibration standards that would be applicable for any matrix. It is clear that the use of the IAC calibration curve is suitable for quantitation of tested mycotoxins in peanuts, barley and maize-based cereals, eliminating the time and effort required to produce matrix-matched calibration curves for each matrix. Research is ongoing to confirm whether this approach is suitable for quantitation of regulated mycotoxins in an even broader range of food matrices.

## 4. Conclusions

The trend in multi-mycotoxins analysis has been to develop multi-target methods covering as many analytes as possible with a focus on easy and fast sample preparations. Although regulated mycotoxins are often included in such methods, there remain only a limited number of methods that can be easily used for the analysis of these mycotoxins in food. The present study was conducted in order to fulfill this gap, producing a sensitive, rapid and accurate multi-target method combining a single immunoaffinity column clean-up with fast UHPLC–MS/MS separation and detection. The analytical strategy optimized in this study represents a reliable tool for rapid screening of mycotoxins in cereals and nuts, because the performance characteristics are in agreement with criteria established in Ref. [18]. Research in this field will continue with evaluation and validation of more matrices (various nuts and cereals, bakery products, dried fruits, etc.) and utilization of this method for the monitoring of mycotoxins in foods.

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