Macedonian Journal of Chemistry and Chemical Engineering, Vol. **41**, No. 1, pp. xx–xx (2022)

MJCCA9 –

ISSN 1857-5552
e-ISSN 1857-5625

Received: December 30, 2021
Accepted: June 3, 2022

DOI: 10.20450/mjcce.2022.2422
Original scientific paper

STUDY ON THE EFFECTIVENESS OF A MULTI-TOXIN IMMUNOAFFINITY CLEAN-UP FOR RELIABLE COST-EFFECTIVE HPLC-FLD ANALYSIS OF MYCOTOXINS IN CORN-BASED FOODS

Biljana Stojanovska-Dimzoska*, Zehra Hajrulai-Musliu, Risto Uzunov, Aleksandra Angeleska, Katerina Blagoevska, Radmila Crceva Nikolovska, Gordana Ilievska, Elizabeta Dimitrieska-Stojković

Food Institute, Faculty of Veterinary Medicine, Ss. Cyril and Methodius University in Skopje, Lazar Pop Trajkov 5-7, 1000 Skopje, North Macedonia

bsdimzoska@fvm.ukim.edu.mk

A different, reliable, and cost-effective strategy for the analysis of aflatoxins, ochratoxin A, and zearalenone in corn-based foods was proposed, including one multi-toxin immunoaffinity column (IAC) sample preparation and three different high-performance liquid chromatography fluorescence detection methods. The analytical procedures were tested and verified, keeping in mind their occurrence at trace levels in corn-based foods. With the validation of the proposed multi-toxin IAC methodology and comparison of the performance characteristics with methods using a single-toxin IAC, we confirmed the reliability of the multi-toxin IAC procedure versus the single-toxin IAC. The methods were validated by revealing satisfactory performance characteristics; for example, the obtained values of limit of detection were significantly lower than the maximum limits for all mycotoxins of concern. In addition, the recovery values were between 70.9 % and 106.1 % for all mycotoxins of interest, with precision values lower than 10.5 %. The proposed methodology is an excellent candidate for future standardization due to its trustfulness and efficiency for mycotoxin analysis.

Keywords: mycotoxins; multi-toxin IAC; HPLC-FLD; validation

ИСТРАЖУВАЊЕ НА ЕФИКАСНОСТА НА ПРОЧИСТУВАЊЕТО СО МУЛТИТОКСИНСКИ ИМУНОАФИНИТЕТНИ КОЛОНИ ЗА СИГУРНА И ЕВТИНА HPLC-FLD АНАЛИЗА НА МИКОТОКСИНИ ВО ХРАНА БАЗИРАНА НА ПЧЕНКА

Предложена е посебна, сигурна и евтина стратегија која вклучува единично прочистување со мултитоксински имуноафинитетни колони (IAC) и три различни HPLC-FLD методи за анализа на афлатоксини, охратоксин А и зеараленон во храна базирана на пченка. Истражуваните аналитички постапки беа испитани и верифицирани согласно со присуството во траги на испитуваните микотоксини во храната базирана на пченка. Валидацијата на предложената мутитоксинска (IAC) методологија ја потврди сигурноста на оваа методологија, како и споредливоста на добиените параметри со методологијата која користи единично-токсински IAC. При валидацијата на методите беа добиени задоволителни параметри, така што утврдените вредности за границите на откривање беа значително пониски од максимално дозволените граници за присуство на испитуваните микотоксини. Дополнително, вредностите за аналитички принос се движеа помеѓу 70,9 % и 106,1 % за сите микотоксини кои беа предмет на ова истражување, со вредности за прецизноста помали од 10,5 %. Предложената методологија претставува одличен кандидат за идна стандардизација, како резултат на потврдениот доверлив и ефикасен начин за микотоксиколошка анализа.

Клучни зборови: микотоксини; мултитоксински IAC; HPLC-FLD; валидација

1. INTRODUCTION

Mycotoxins, natural compounds found in molds, widely contaminate plant origin products such as crops, food, and feed. Since their recognition as a public health concern because of their toxicity and carcinogenicity, improving the detection of these contaminants has been a subject of constant improvement in the analytical technology.[1] Among the regulated mycotoxins, aflatoxins B_1 (AFB_1), G_1 (AFG_1), B_2 (AFB_2) and G_2 (AFG_2), ochratoxin A (OTA), and zearalenone (ZEA) are the most commonly found in cereals and cereal products.[2] The European Commission has established maximum limits (ML) for the presence of these mycotoxins in raw cereals and derived products intended for human consumption: 2 μg kg^{-1} for AFB_1 and 4 μg kg^{-1} for sum of aflatoxins (AFB_1, AFB_2, AFG_1, and AFG_2); 5 μg kg^{-1} or 3 μg kg^{-1} for OTA (unprocessed or processed cereals); and 100 μg kg^{-1} or 75 μg kg^{-1} for ZEA (unprocessed or processed cereals).[3]

Considering the various physico-chemical characteristics of the different groups of mycotoxins, chromatography-based methods coupled with mass spectrometry are the most favorable techniques because they can analyze a large group of mycotoxins in a single run and can perform sufficiently accurate and precise measurements of multiple mycotoxins in food and feed.[4–10] However, this method has disadvantages, including low sensitivity and specificity due to the so-called "matrix effect" caused by other sample components. This effect causes suppression or enhancement of the signal of the analyte due to the alteration of the ionization efficiency as a result of the presence of co-eluting substances.[5] It leads to high detection limit (LOD) and quantification limit (LOQ) values, which could be a major drawback especially when it comes to aflatoxins regulated with low ML values.[3] In addition, despite those analytical disadvantages, the liquid chromatography with tandem mass spectrometry (LC-MS/MS) method is very expensive technique and requires specific knowledge and training of the personnel. Therefore, high-performance liquid chromatography with fluorescence detection (HPLC-FLD) is still widely used for detection complex matrices due to its great versatility, high sensitivity, and high selectivity.[2,11–16] Moreover, most of the standard methods for determining mycotoxins are still based on this technique.[12] The enzyme-linked immunosorbent assay (ELISA) method is still the method of choice for screening purposes, particularly when the laboratory is dealing with a large number of samples.[17,18]

No matter which method of detection determination is chosen, special attention should be paid to the purification step prior to the instrumental analysis. Thus, a variety of extraction procedures are available in order to obtain a pure extract that is free from interfering substances that affect the fluorescence.[7] Solid-phase extraction (SPE) is the most used technique, and several SPE columns are commercially available with different solid phases ranging from C18 materials to more specific ones such as NH_2 columns and QuEChERS kits.[2,8,12,13,19] However, immunoaffinity columns (IAC) based on specific antibodies, which provide a molecular recognition mechanism, represent a powerful tool for selective extraction.[5] They manifest many advantages, such as maximum elimination of interfering substances, minimal loss of toxins, and improved specificity compared to SPE. The working principle is based on the use of antibodies specific to the toxin molecule. The columns contain a gel suspension (sepharose or agarose gel) of monoclonal antibody (single-toxin IAC) or polyclonal antibodies (multi-toxin IAC) specific to the mycotoxin of interest.[20] After the extraction step (usually with an organic solvent), the sample extract is filtered and diluted. Then, it passes through the IAC. If the toxin is present in the sample, it is retained by the specific antibody in the gel. A washing step is then performed in order to eliminate unbound material. At the end, the toxin is released from the column with a solvent. The eluate is collected prior to the instrumental analysis.[20]

The most developed HPLC-FLD methods for determining mycotoxins used commercially available IAC for the clean-up procedure prior to the instrumental detection. Many authors have reported the use of single-toxin[2,12,13,15] or multi-toxin[4–6,9,11,14,20–23] IAC, which have been successfully applied because of their advantages: simple and rapid sample preparation, effectiveness, and high sensitivity. The main difference between the two types of IAC is the method of extraction. Usually, when the single-toxin IAC are used, it means that the number of extraction procedures that should be performed matches the number of individual mycotoxins to be analyzed. However, when multi-toxin IAC are used, only one extraction procedure is carried out. Although multi-toxin IAC are more expensive than single IAC, the time of analysis is significantly shortened.

Taking into consideration the facts described above with respect to the extraction procedures, types of IAC, and methods of analysis, the aim of

our study was to test, verify, and propose different, reliable, and cost-effective strategies for the analysis of mycotoxins in corn-based foods. The proposed methodology consisted of one multi-toxin IAC sample preparation and three different HPLC-FLD determination methods. With the validation of the proposed multi-toxin IAC methodology and comparison of the performance characteristics with methods using single-toxin IAC for determination of aflatoxins (AFB_1, AFB_2, AFG_1, and AFG_2), OTA, and ZEA in corn-based foods, we confirmed the applicability of multi-toxin IAC versus single-toxin IAC.

In addition, this research study provides data on whether the multi-toxin IAC that are primarily intended for LC-MS/MS methods could be successfully used for routine analysis, applying cost-effective, reliable HPLC-FLD method of analysis, without compromising the sensitivity and quality of the chromatographic separation. Through the examination of the method performances (sensitivity, selectivity, accuracy, recovery, repeatability, and reproducibility), the fulfillment of the EU regulatory criteria for all mycotoxins of concern was checked.[24,25] The study was mainly focused on the capability of multi-toxin IAC, proving their efficiency, and illustrating the high potential of these columns. Finally, this study establishes a reliable quantitative methodology for the analysis of mycotoxins of interest with regard to the method performance requirements. This is of great importance keeping in mind their potential hazard for human health by the consumption of contaminated food.

2. EXPERIMENTAL SECTION

2.1. *Apparatus*

HPLC analysis for aflatoxins, OTA, and ZEA was carried out with a Perkin Elmer (PE) chromatographic system equipped with a binary pump (PE LC-250), manual injector (PE Rheodyne 7125), and fluorescence detector (PE LC-240). For each of the analytes (aflatoxins, OTA, and ZEA), we applied three different HPLC–FLD methods using different mobile phases, different columns, and different conditions of the fluorescence detector. The analytes were isocratically separated at ambient temperature on RP C18 columns purchased from Supelco (Sigma-Aldrich) with the following characteristics: 250 mm × 4.6 mm, 5 μm for aflatoxins, and 150 mm × 4.6 mm, 5 μm for OTA and ZEA. For aflatoxins, the mobile phase consisted of a water : acetonitrile : methanol (600 : 350 : 50, *V/V/V*) mixture with the addition of 119 mg KBr and 350 μl of 4 mol l^{-1} HNO_3. An acetonitrile:water:acetic acid (99 : 99 : 2, *V/V/V*) mixture was used for OTA, and an acetonitrile:water (65 :35, *V/V*) mixture was used for ZEA. The flow rate for all three methods was 1 ml/min, and the injection volume was 100 μl. The detection was performed at the respective excitation and emission wavelengths at which the maximum sensitivity was provided: λ_{ex} = 360 nm and λ_{em} = 440 nm for aflatoxins, λ_{ex} = 333 nm and λ_{em} = 460 nm for OTA, and λ_{ex} = 274 nm and λ_{em} = 440 nm for ZEA. The run time was 30 min for aflatoxins and 10 min for OTA and ZEA. A nitrogen evaporator (OA-Heat, N-Vap 116, Organomation, USA) was used to concentrate the sample extracts for OTA and ZEA. The Kobra® cell applied for electrochemical derivatization of aflatoxins AFB_1 and AFG_1 was purchased by R-Biopharm Rhône.

2.2. *Reagents and standard solutions*

HPLC reagents (water, methanol, acetonitrile) were purchased from Carlo Erba reagents (France). Benzene, KBr, and NaCl were purchased from Sigma-Aldrich (USA), and 65 % HNO_3, glacial acetic acid, and phosphate buffer solution (PBS) were purchased from Merck (Darmstadt, Germany). For the purification step, single-toxin IAC (Aflaprep®, Ochraprep®, and Easi-Extract®Zearalenone) and multi-toxin IAC (AO ZON Prep®) purchased from R-Biopharm Rhône, Glasgow, Scotland, were used. As calibration standards, an aflatoxin mix (AFB_1 1068 ng ml^{-1}, AFB_2 305 ng ml^{-1}, AFG_1 1088 ng ml^{-1}, AFG_2 281 ng ml^{-1}) from Supelco (Sigma-Aldrich, USA), an OTA standard with a concentration of 50 μg ml^{-1} (Supelco, Sigma-Aldrich, USA), and a ZEA standard with a concentration of 50 μg ml^{-1} (Supelco, Sigma-Aldrich, USA) were used.

The aflatoxin intermediate mix solution (AFB_1 100 ng ml^{-1}, AFB_2 31.07 ng ml^{-1}, AFG_1 102.8 ng ml^{-1}, AFG_2 29.36 ng ml^{-1}) was prepared from the aflatoxin mix standard, diluting an aliquot in a volumetric amber flask to 10 ml. Seven working standard solutions (for AFB_1 in the range of 0.25–15 ng ml^{-1}; for AFB_2 in the range of 0.14–4.3 ng ml^{-1}; for AFG_1 in the range of 0.51–15.34 ng ml^{-1}; and for AFG_2 in the range of 0.13–3.96 ng ml^{-1}) were prepared from the stock solution in 5 ml volumetric amber flasks. All working standards were dissolved in a mixture of methanol:water (1:1) and kept in a refrigerator at 2−8 °C.

An aliquot of the OTA standard (50 μg ml^{-1}) was used to prepare the OTA intermediate solution

with a concentration of 5 μg/ml using silica coated glass vials in order to prevent the analyte from binding on the glass wall. Furthermore, it was used for the preparation of the second intermediate solution with a concentration of 1000 ng ml^{-1}. Seven working solutions in the range of 1.0–500 ng ml^{-1} were prepared from the intermediate solution. All working standards were diluted with the filtrated mobile phase and kept in a refrigerator at 2−8 °C.

An aliquot of the ZEA standard (50 μg ml^{-1}) was used to prepare a ZEA stock solution with a concentration of 10 μg/ml in a 5 ml volumetric amber flask diluted with acetonitrile. Next, this solution was used for the preparation of seven working solutions in the range of 10–2000 ng ml^{-1}. All working standard solutions were diluted with acetonitrile and kept in a refrigerator at 2−8 °C.

2.3. *Sample preparation*

2.3.1. *Extraction procedures applying single-toxin IAC*

The extraction and purification of aflatoxins from corn-based samples were performed according to ISO 16050:2003 standard.[26] Briefly, 25 g of test sample was extracted with 70 % methanol and NaCl into a stainless-steel extraction blender. The extract was filtrated through a fluted paper, diluted with water, and afterwards filtrated through a microfiber filter paper. An aliquot of the extract quantitatively passed through the single-toxin IAC followed by a washing step with water, and finally, the elution of the aflatoxins was performed with 1 ml of methanol in an amber vial. The elution step was repeated one more time with 1 ml of water, which passed through the column. The combined eluates were used for the HPLC analysis.

For the extraction and purification of OTA in corn-based samples, we used the Association of Official Analytical Chemists (AOAC) method.[27] Twenty-five grams of the sample was extracted with 65 % acetonitrile in a blender. The solution was filtered and dissolved with PBS buffer. The whole extract was passed through the single-toxin IAC followed by a washing step with water. The elution of OTA into the vial was performed by passing four 1 ml portions of methanol. The eluate was dried at 50 °C under a stream of nitrogen, and the dry residue was re-dissolved in 1 ml of the filtered mobile phase.

The extraction and purification of ZEA from corn-based samples was performed according to the method proposed by Visconti and co-workers.[16] Twenty grams of the sample was extracted with 90 % acetonitrile in a blender. The extract was filtered and diluted with water. An aliquot of the sample solution passed through the single-toxin IAC followed by the process of washing with water. The elution of ZEA into the vial was performed with methanol. The eluate was evaporated at 50 °C under a stream of nitrogen until it was dry. The dry residue was re-dissolved in 250 μl of the filtered mobile phase.

2.3.2. *Extraction procedures applying multi-toxin IAC*

The extraction and purification process for mycotoxins (aflatoxins, OTA, and ZEA) in corn-based samples using multi-toxin IAC was performed according to the manufacturer's instructions.[28] Twenty-five grams of the sample was extracted with 100 ml of the extraction solvent (80 % methanol) in a blender for 2 min. The solution was filtered through a fluted filter. Ten ml of the filtrate was diluted with 40 ml of PBS buffer. Twenty ml of the extract was passed through the multi-toxin IAC at a flow rate of about 1 drop/sec. Then, the column was washed with 20 ml of PBS buffer. The elution of mycotoxins into the vial was performed by passing 1.5 ml of methanol at a flow rate of 1 drop/sec. The elution step was repeated one more time with 1.5 ml of water, which passes through the same column. For the determination of the single toxins, three procedures described in section 2.1. were used.

2.4. *Validation procedure*

The validation procedure was accomplished in compliance with Regulation 401/2006/EC [24], which is specific for mycotoxins, and Decision 2002/657/EC concerning the performance of analytical methods.[25] The linearity was tested using seven working standard solutions for each toxin described in section 2.2.

The limit of detection (LOD) was determined as 3.3 × SD/slope, where the standard deviation (SD) was estimated from the measurement of the background response from 10 blank samples. The slope was calculated from the calibration curve. The limit of quantification (LOQ) was determined as 10 × SD/slope in the same manner as the LOD calculation.

The selectivity of the method was established by adding a known amount of the mycotoxin standard solution to a blank sample at low concentration levels. Then, the samples were extracted, purified, and quantified from the corresponding

peaks in the chromatograms. The method is considered selective when no interfering peaks coexist at the retention times of the mycotoxins of interest.

The determination of trueness (one component of accuracy) was performed by means of a certified reference material (CRM). The procedure included analysis of six replicates of the CRM in accordance with the test instructions for the method. The trueness of a method can also be quantitatively expressed as bias or relative bias. In practice, relative bias is calculated as (observed value/truth value)/truth value × 100 %. For all mycotoxins analyzed in our study, we used CRM (CRM-7 corn, Trilogy, lot. MTC-999D) with known concentrations of the analytes given in Table 2.

Recovery, the second accuracy parameter, was examined by fortifying blank corn samples (previously confirmed as blanks by HPLC-FLD) at selected concentration levels. The following fortified concentration levels were tested: 1, 2, and 5 μg kg^{-1} for AFB_1; 0.31, 0.56, and 1.55 μg kg^{-1} for AFB_2; 1.02, 2.06, and 5.12 μg kg^{-1} for AFG_1; 0.29, 0.66, and 1.46 μg kg^{-1} for AFG_2; 2.5, 5.0, and 7.5 μg kg^{-1} for OTA; 37.5, 50, and 100 μg kg^{-1} for ZEA. The analysis was performed with six replicates at each level of fortification in one day.

The precision of the method was assessed by repeatability and within-laboratory reproducibility experiments. Repeatability (intra-day repeatability) was determined through the standard deviation (SD) and relative standard deviation (RSD_r) using the data from the accuracy experiment and either using the CRM's or blank fortification approach. The within-laboratory reproducibility (RSD_R) of the method was determined in the same fortified concentration levels with six replicates at each level. The analysis was performed in two days by two different analysts.

The combined measurement uncertainty (u) was estimated at the following concentration levels: 2.0 μg kg^{-1}, 5.0 μg kg^{-1}, and 100 μg kg^{-1} for AFB_1, OTA, and ZEA, respectively. The factor that primarily influenced the uncertainty was the within-laboratory reproducibility; however, other factors that affect the measurement process, such as uncertainty of standard solution concentration, pipettes, volumetric flasks, and analytical balance, were also taken into consideration. All factors were presented as a percentage in agreement with the NIST Uncertainty Guideline.[29] The coverage factor of 2 was used to calculate the expanded measurement uncertainty (U), corresponding to a confidence level of approximately 95 %.

3. RESULTS AND DISCUSSION

The different characteristics of mycotoxins make it difficult to find an optimal experimental condition that comprise extraction process, clean-up, and analysis which involves as much as possible analytes. The validation experiments within this study were performed using two types of IAC (single-toxin and multi-toxin), and the total results acquired from the validation procedure are suitable for evaluation according to the reference criteria.[24, 25]

In addition to meeting the required method performance criteria, the clean extracts obtained when multi-toxin IAC were applied meant that there was no matrix and no background effects that might cause interferences in the HPLC-FLD analysis. The chromatograms obtained when the IAC clean-up procedure was performed have sharp symmetrical peak shapes free of any interferences and practically no impurity. Figure 1 shows example chromatograms of aflatoxins, OTA, and ZEA in spiked blank corn with the following concentrations: AFB_1 5 μg kg^{-1}, AFB_2 1.55 μg kg^{-1}, AFG_1 5.12 μg kg^{-1}, AFG_2 1.46 μg kg^{-1} (a), OTA 5 μg kg^{-1} (b), and ZEA 100 μg kg^{-1} (c), when multi-toxin IAC were applied for the clean-up procedure. As seen from the chromatograms, the peaks are well-defined, and the matrix effect is present at the beginning of the run with no interference in the mycotoxin detection. The absence of interfering peaks is additional proof of the selectivity of the methods used for the analysis of aflatoxins, OTA, and ZEA.

The validation characteristics of the studied HPLC–FLD method with regard to the method linearity and sensitivity are shown in Table 1. The linearity of the methods, expressed through seven-point calibration curves, indicates respectable results with high coefficients of correlation ($R^2 > 0.998$) in the examined concentration range for all suggested mycotoxins. The LOD and LOQ shown in Table 1 were agreeable regardless of whether single-toxin or multi-toxin IAC were used for sample purification. The determined LOD and LOQ values for all tested mycotoxins were well below 10 % of the statutory limits set in the EU regulation[3] and comparable to those reported by other authors.[6,4,23] The LOD and LOQ obtained in this study indicated that the sample matrix that could influence the HPLC–FLD determination is equally well-removed from the final extract when both purification methods were applied. Moreover, the multi-toxin IAC provided somewhat lower LOD and LOQ values for the ZEA method (Table 1).

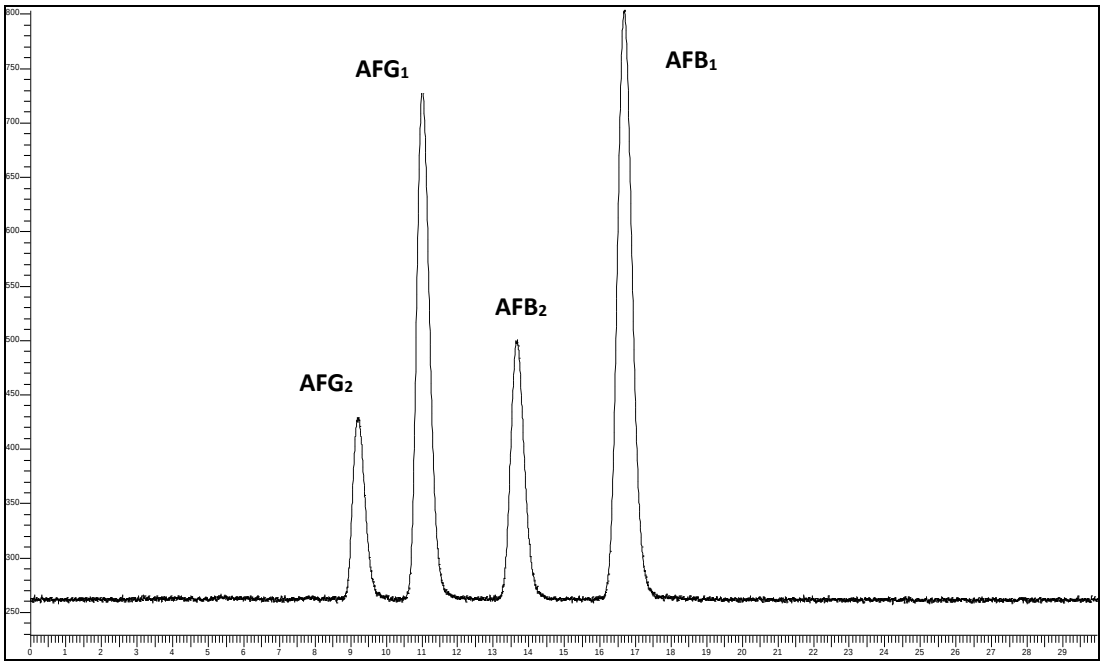

Fig. 1. (a) Chromatogram of fortified corn with the concentration of aflatoxins: AFB_1 5 μg kg^{-1}, AFB_2 1.55 μg kg^{-1}, AFG_1 5.12 μg kg^{-1}, AFG_2 1.46 μg kg^{-1}

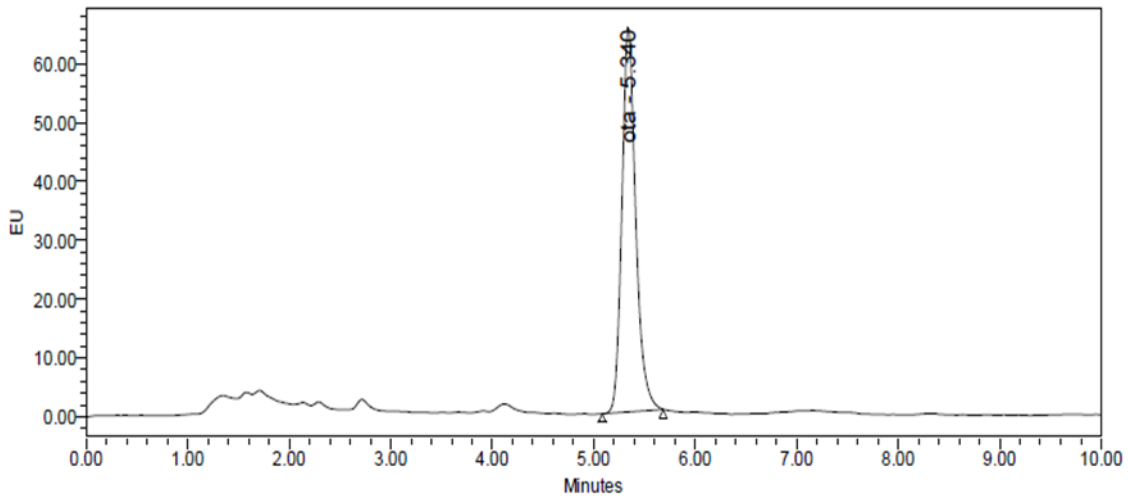

Fig. 1(b) Chromatogram of fortified corn with the concentration of OTA: 5 μg kg^{-1}

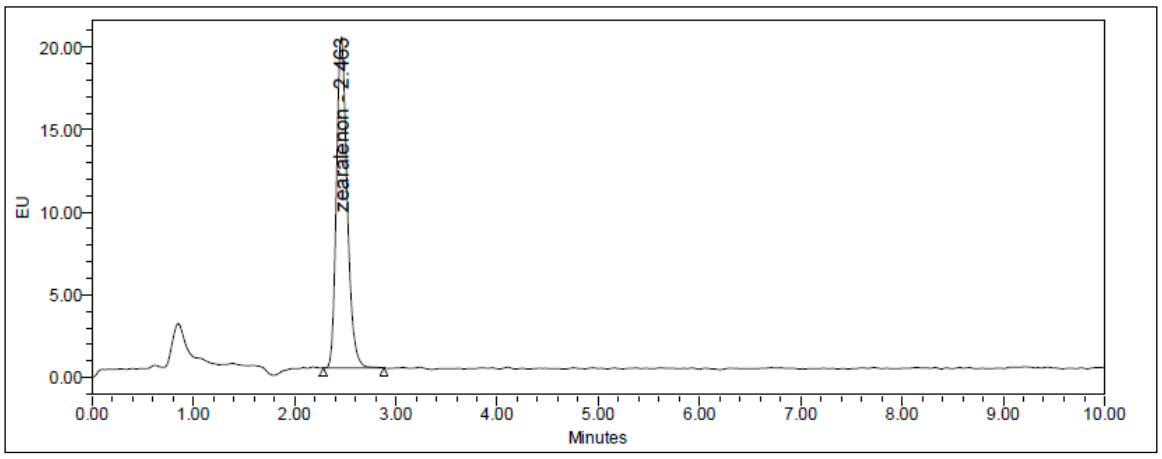

Fig. 1(c) Chromatogram of fortified corn with concentration of ZEA: 100 mg kg^{-1}

T a b l e 1

Calibration curve data, LOD, and LOQ

	Range ng ml^{-1}	R^2	Calibration curve equation	LOD (μg kg^{-1})		LOQ (μg kg^{-1})	
				Single-toxin IAC	Multi-toxin IAC	Single-toxin IAC	Multi-toxin IAC
AFB_1	0.25–15.0	0.999	$y = 947197x + 3025.6$	0.05	0.06	0.15	0.18
AFB_2	0.071–4.26	0.999	$y = 1000000x + 56452$	0.05	0.05	0.15	0.15
AFG_1	0.258–15.51	0.999	$y = 580471x + 72088$	0.03	0.03	0.09	0.09
AFG_2	0.083–4.99	0.999	$y = 596893x + 25717$	0.04	0.05	0.12	0.15
OTA	1.0–500	0.999	$y = 22535x - 53860$	0.04	0.03	0.12	0.09
ZEA	10–2000	0.999	$y = 2121.6x - 34459$	1.34	1.37	4.02	4.11

The results obtained from the determination of trueness and recovery established by means of CRM and by fortifying blank corn samples at three selected concentration levels, respectively, are given in Table 2. In addition, the obtained mean values from six replicates were associated with the corresponding confidence intervals (CI) for 95 % probability. From the results, it can be concluded that there was no difference regardless of whether single-toxin or multi-toxin IAC were used. Thus, the results for the trueness using CRM were in the range of 91.68–117.77 % for all mycotoxins when single-toxin IAC were applied and 97.07–108.88 % when multi-toxin IAC were used. The EU method performance criteria for aflatoxins and OTA have stipulated that the recoveries should be 70–110 % for concentrations from 1.0 to 10.0 μg kg^{-1}. For ZEA, recovery values should be 60–120 % and 70–120 % for levels ≤ 50 μg kg^{-1} and > 50 μg kg^{-1}, respectively.[24,25] All results obtained from the trueness experiment fall within the required range. The data for the relative bias (Table 2) are low with the exception of AFB_1. Keeping in mind that the results for aflatoxins are always expressed as AFB_1 or total aflatoxins, the bias results for AFB_2 can be neglected, and thus, their influence on the final value will be minor. In general, data obtained from the relative bias indicate the closeness between the determined and certified CRM value.

The recovery data determined from six replicate analysis of spiked blank material at three concentration levels for all proposed mycotoxins were also in accordance with the performance criteria set up in Commission Regulation 401/2006.[24] They were in the range of 78.78–106.8 % for all analyzed mycotoxins using single-toxin IAC and 70.96–106.16 % using multi-toxin IAC (Table 2). In addition, the recovery data were also in good agreement with other relevant studies.[11,12,19,21,23]

Table 3 shows the results for precision (RSD_r), and the within-day repeatability values were in the range 1.48–11.35 % using single-toxin IAC and 2.05–10.54 % using multi-toxin IAC for all mycotoxins tested. All RSD_r values are suitable according to the benchmark (mean value for RSD_r shall not exceed 20 %).[24,25]

The results for the between-day repeatability and within-laboratory reproducibility for all mycotoxins are given in Table 4. All RSD_r values were in the range of 1.16–11.87 % when single-toxin IAC were applied and in the range of 2.05–10.81 % when multi-toxin IAC were applied. The within-laboratory reproducibility data (RSD_R values) for all mycotoxins tested exhibited satisfactory agreement between the experiments performed in the two different days.

Regarding the aflatoxins, the maximal accepted repeatability should be calculated according to the Horwitz equation.[24] However, for low concentration levels (< 100 μg kg^{-1}), the obtained values should be as low as possible.[24,25]

The EU method performance criteria for OTA have established that the RSD_R value should be ≤ 30 % for concentrations from 1.0 to 10.0 μg kg^{-1}.[24] For ZEA, the RSD_R value should be ≤ 50 % and ≤ 40 % for levels ≤ 50 μg kg^{-1}, and > 50 μg kg^{-1}, respectively.[24] All results obtained from the within-laboratory reproducibility experiment fall within the required range: 2.3–13.28 % when single-toxin IAC were tested and 3.11–13.41 % when multi-toxin IAC were applied.

The measurement uncertainties determined at the proposed concentration level (2.0 μg kg^{-1} for AFB_1, 5.0 μg kg^{-1} for OTA, and 100 μg kg^{-1} for ZEA) were 18.5 %, 15.6 %, and 16.3 % for AFB_1, OTA, and ZEA, respectively.

T a b l e 2

Determination of mean with confidence interval, trueness, and recovery established by means of certified reference material and spiking at three concentration levels determined by six replicates at each fortification level

			AFB_1			**AFB_2**			**AFG_1**			**AFG_2**		**Total aflatoxins**	**OTA**			**ZEA**	
	Declared concentration (μg kg^{-1})		18.8			0.9			2.4			NP*		22.1	1.0			352	
trueness established by means of CRM	Determined mean concentration ±CI*** (μg kg^{-1})	single-toxin IAC	18.5 ± 0.22			1.1 ± 0.05			2.6 ± 0.1			ND**		22.2 ± 0.8	1.1 ± 0.02			351.5 ± 6.9	
		multi-toxin IAC	18.3 ± 0.33			1.0 ± 0.05			2.4 ± 0.1			ND**		21.6 ± 0.7	1.0 ± 0.02			346 ± 7.8	
	Mean trueness %	single-toxin IAC	91.68			117.77			106.25			ND**		100.22	108.0			99.84	
		multi-toxin IAC	97.07			108.88			99.58			ND**		97.82	98.0			98.32	
	Relative bias %	single-toxin IAC	–1.6			22.2			8.33			/		0.45	10			–0.14	
		multi-toxin IAC	–2.6			11.1			0			/		–2.26	0			–1.7	
recovery established by spiking at three concentration levels	Spiked concentration (μg kg^{-1})		1.0	2.0	5.0	0.31	0.56	1.55	1.02	2.06	5.12	0.29	0.66	1.46	/	2.5	5.0	7.5	37.5	50	100
	Determined mean concentration ± CI*** (μg kg^{-1})	single-toxin IAC	1.03±0.14	1.64±0.1	4.15±0.06	0.25±0.03	0.49±0.05	1.47±0.08	1.06±0.02	2.15±0.09	5.25±0.26	0.233±0.01	0.52±0.11	1.54±0.05	/	2.28±0.05	4.88±0.11	7.34±0.09	37.22±4.3	49.22±4.7	106.8±2.4
		multi-toxin IAC	0.92±0.12	1.77±0.01	4.98±0.04	0.22±0.06	0.52±0.02	1.59±0.09	0.98±0.05	2.00±0.08	5.06±0.19	0.27±0.04	0.59±0.13	1.55±0.07	/	2.32±0.08	4.85±0.13	7.62±0.07	37.61±4.1	50.15±4.9	102.7±2.1
	Mean recovery %	single-toxin IAC	103.0	82.26	82.91	81.62	87.8	95.03	103.9	104.4	102.5	80.03	78.78	105.1	/	91.2	97.6	97.9	99.25	98.44	106.8
		multi-toxin IAC	92.0	88.5	99.6	70.96	92.85	102.58	96.07	97.08	98.82	93.1	89.39	106.16	/	92.8	97.0	101.6	100.3	100.3	102.7

*NP – not present
**ND – not detected
***CI – confidence interval

T a b l e 3

Within-day repeatability data for aflatoxins, OTA, and ZEA obtained from the spiking experiment at three concentration levels determined by six replicates at each fortification level

Compound	Spiked concentration (μg kg^{-1})	Determined concentration (μg kg^{-1})		SD_r (μg kg^{-1})		RSDr (%)	
		Single-toxin IAC	Multi-toxin IAC	Single toxin IAC	Multi-toxin IAC	Single-toxin IAC	Multi-toxin IAC
AFB_1	1.0	1.03	0.92	0.14	0.12	4.0	4.25
	2.0	1.64	1.77	0.10	0.01	6.6	5.58
	5.0	4.15	4.98	0.06	0.04	1.48	2.25
AFB_2	0.31	0.25	0.22	0.03	0.06	13.63	10.15
	0.56	0.49	0.52	0.05	0.02	10.04	9.84
	1.55	1.47	1.59	0.08	0.09	5.49	6.25
AFG_1	1.02	1.06	0.98	0.02	0.05	2.04	3.41
	2.06	2.15	2.00	0.09	0.08	8.35	7.87
	5.12	5.25	5.06	0.26	0.19	4.58	4.96
AFG_2	0.29	0.23	0.27	0.01	0.04	7.75	8.05
	0.66	0.52	0.59	0.11	0.13	5.96	6.09
	1.46	1.54	1.55	0.05	0.07	3.07	3.58
OTA	2.5	2.28	2.32	0.05	0.08	1.76	1.97
	5.0	4.88	4.85	0.11	0.13	2.37	2.05
	7.5	7.34	7.62	0.09	0.07	1.50	1.88
ZEA	37.5	37.22	37.61	4.30	4.11	11.35	10.54
	50	49.22	50.15	4.68	4.87	9.62	8.34
	100	106.81	102.73	2.42	2.08	2.46	2.83

T a b l e 4

Between-day repeatability and within-laboratory reproducibility, expressed by SD_r, RSD_r, and RSD_R for aflatoxins, OTA, and ZEA determined by six replicates at each fortification level

Compound	Spiking level	Repeatability conditions	Determined mean concentration (μg kg^{-1})		SD_r (μg kg^{-1})		RSD_r (%)		RSD_R (%)	
			Single-toxin IAC	Multi-toxin IAC	Single-toxin IAC	Multi-toxin IAC	Single-toxin IAC	Multi-toxin IAC	Single-toxin IAC	Multi-toxin IAC
AFB_1	2 μg kg^{-1}	Day I	1.84	1.78	0.10	0.09	6.60	5.25	8.23	7.25
		Day II	1.81	1.82	0.08	0.07	4.93	5.01		
AFB_2	0.56 μg kg^{-1}	Day I	0.49	0.51	0.05	0.02	10.04	9.24	12.15	12.68
		Day II	0.49	0.53	0.03	0.05	6.86	8.69		
AFG_1	2.06 μg kg^{-1}	Day I	1.55	1.68	0.09	0.1	8.35	8.35	10.5	10.5
		Day II	1.64	1.72	0.07	0.09	6.38	6.38		
AFG_2	0.66 μg kg^{-1}	Day I	0.46	0.51	0.01	0.03	5.96	7.94	13.28	13.41
		Day II	0.55	0.59	0.03	0.04	11.87	10.81		
OTA	5 μg kg^{-1}	Day I	4.87	4.95	0.11	0.08	2.37	3.25	2.63	4.38
		Day II	4.92	5.05	0.05	0.09	1.16	2.94		
ZEA	100 μg kg^{-1}	Day I	111.39	100.54	2.21	1.22	1.99	2.05	2.3	3.11
		Day II	111.12	99.97	1.30	1.64	1.17	2.34		

T a b l e 5

Validation parameters from published methods and our study obtained from use of multi-toxin IAC

Matrix	Analytes	Method	Recovery %	LOD (μg kg^{-1})	LOQ (μg kg^{-1})	RSDr %	Ref.
Grains	Aflatoxins	UHPLC-MS/MS	nr*	0.1–1.3	0.3–3.8	nr*	(1)
	OTA			0.4	1.1		
	ZEA			0.3	0.9		
Feed	Aflatoxins	UHPLC-MS/MS	93.9–102	0.02–0.04	0.06–0.12	2.9–3.4	(4)
	OTA		95.6–99.7	0.12	0.36	2.3	
	ZEA		90.9–95.5	0.25	0.75	2.1	
Cereal feed	Aflatoxins	UHPLC-MS/MS	62–113	0.1–0.4	0.3–1.1	2.1–33.4	(5)
	OTA		53–89	0.7	2.0	1.8–24.6	
	ZEA		46–106	14.7	44.5	6.0–32.3	
Food	Aflatoxins	UHPLC-MS/MS	77–102	0.03–0.05	0.05–0.1	3.8–16.2	(6)
	OTA		60–89	0.2	0.4	6.2–13.6	
	ZEA		72–91	0.5	2.6	4.0–7.9	
Maize	Aflatoxins	UHPLC-MS/MS	71–100	0.3–0.8		4–13	(9)
	OTA		74–82	0.6	nr*	2–5	
	ZEA		50–103	0.7		7–9	
Corn, Corn product	Aflatoxins	UHPLC-MS/MS	86.9–112.1	0.1–0.5	0.4–1.6	6.5–13.8	(10)
	OTA		103.1	0.1	0.3	4.3	
	ZEA		93.5	0.2	0.6	3.8	
Barley	Aflatoxins	HPLC-FLD	71.7–97.2	0.005–0.15	0.04–0.15	1.4–9.9	(11)
	OTA		79.9–83.3	0.013	0.15	6.4–11.5	
	ZEA		89.9–109.3	0.34	6.0	2.6-5.5	
Wheat bran	Aflatoxins	HPLC-DAD-FLD	70.2–105.8	0.12–0.36	0.38–1.08	1.7–8.5	(21)
	OTA		92.0–100.9	0.40	1.20	1.7–4.8	
	ZEA		84.5–97.3	6.74	20.43	3.1–3.5	
Cereals	Aflatoxins	UHPLC-MS/MS	84–129			3–19	(22)
	OTA		97–123	nr*	nr*	3–12	
Maize feed	Aflatoxins	HPLC-FLD	79–110	0.04–0.12	0.12–0.39	nr*	(23)
	OTA		95–100	0.02	0.06		
	ZEA		85–88.8	0.92	2.8		
Corn	Aflatoxins	HPLC-FLD	70.9–106.1	0.03–0.06	0.09–0.18	2.2–10.1	(our study)
	OTA		92.8–101.6	0.03	0.09	1.8–2.05	
	ZEA		100–102.7	1.37	4.11	2.8–10.5	

*Nr – not reported

In Table 5, the performance characteristics of the reported HPLC-FLD and HPLC-MS/MS methods for simultaneous determination of aflatoxins, OTA, and ZEA using multi-toxin sample preparation are presented. The published HPLC-FLD methods in some studies exhibited comparable[23] or lower LOD values[11] than our data; however, another study has reported a higher LOD.[21] Depending on the LOQ calculation approach, some authors obtained significantly higher values for ZEA.[21] The method sensitivity is highly dependent on the matrix type; thus, a matrix containing lower fats such as barley exhibits a lower influence on LOD and LOQ values.[11] The methods that used mass spectrometry for detection of mycotoxins of concern exhibited higher LOD and LOQ values than our method.[1,4–6,9,11,22]

When we compare the obtained recovery and precision values with the ones obtained from other authors (Table 5), although some of the authors reported lower recoveries,[5,9] most of the values still comply within the limits set in the EC Regulation.[24]

In references to the time consumed for chromatographic separation, the published methods reported a single run for the analysis of aflatoxins, OTA, and ZEA.[1,4–6,9–11,21–23] In our study, we used three different chromatographic methods for separate determination of aflatoxins, OTA, and ZEA with the use of one multi-toxin sample preparation IAC. Although the advantage of using the single determination method is unquestionable, there are still some disadvantages for such an approach. When the HPLC-FLD method was applied, it is usually very difficult to analyze aflatoxins, OTA, and ZEA in one run. Thus, this run was at least 50 min long,[23] which offers practically no benefits in terms of using a faster method of analysis when compared to the total run time of all three methods described in this study (50 min). In addi-

tion, there was a likely problem concerning the ZEA determination, keeping in mind that the derivatization of AFB_1 and AFG_1 will turn ZEA into a less fluorescent compound, resulting in a decreased method sensitivity.[21] It is well known that AFB_1 and AFG_1 need derivatization in order to enhance their fluorescence activity. In our study, we applied a low-cost bromine derivatization with Kobra® cell, connected on-line with the HPLC–FLD system. Kobra® is actually an electrochemical cell that generates a reactive form of bromine for derivatization of AFB_1 and AFG_1. It is widely used instead of other derivatization techniques (photochemical reaction, post-column iodine derivatization, use of trifluoroacetic acid, pyridinium bromide perbromide)[11] because it has low input costs and does not require expensive chemical reagents or other additional equipment.[11] In addition, when a single short-time run was applied,[11] the adjustment of the gradient and fluorescence conditions resulted in a high baseline drift and presented matrix interferences in the OTA and ZEA peak surroundings, which could very likely contribute to the lower sensitivity and low peak resolution.

The use of the LC-MS/MS technique could solve the problem due, in part, to the fact that derivatization and sometimes sample pre-treatment are not needed. However, this technique has some drawbacks, such as matrix influence that suppresses or enhances the analyte signals. Consequently, a matrix-matched calibration and use of deuterated internal standards are imperative. In addition, the equipment and maintenance costs, as well as personal training costs, should be taken into consideration. Nonetheless, the reported higher LOD and LOQ when LC–MS/MS was used could not be neglected (Table 5).

Therefore, for obtaining the best separation and unambiguous detection and quantification of the analytes of interest, we decided to use the one sample preparation procedure for all mycotoxins and three different but shorter and undoubtedly highly sensitive, selective, accurate, and precise HPLC–FLD methods for the separation and determination of aflatoxins, OTA, and ZEA. By applying the proposed methodology, the benefit of lowering the sample preparation costs and use of low-cost determination methods for sensitive, selective, and reliable quantification of aflatoxins, OTA, and ZEA was significant in comparison to the single determination methods (Table 5).

4. CONCLUSIONS

When sample purification is sufficiently selective, it may be possible to rely on cheaper and precise techniques such as HPLC–FLD. By using this chromatographic technique, combined with multi-toxin IAC, it is possible to obtain LOD and LOQ values comparable or even lower than those achieved by LC–MC/MC.

This study proved that the proposed methodology – one extraction procedure for aflatoxins, OTA, and ZEA using one multi-toxin IAC sample clean-up and three short HPLC-FLD procedures – is an excellent candidate for future standardization due to its trustfulness and efficiency for mycotoxin analysis. Thus, the occurrence of mycotoxins at trace levels demands the development of very reliable and robust tools for the sample preparation prior to their chromatographic analysis. Multi-toxin IAC that contain several antibodies allow for the simultaneous extraction of toxins from different chemical groups, thus decreasing both the total analytical time and the method cost, as only one cartridge is required. Furthermore, the analyst could choose the proper analytical method, whether it is a single or multiple method of determination.

Our approach offers very simple, sensitive, and accurate HPLC–FLD procedures for the determination of aflatoxins (AFB_1, AFB_2, AFG_1, AFG_2), OTA, and ZEA in corn-based samples using multi-toxin IAC for sample clean-up. We reached our goal of confirming through the validation experiments for linearity, selectivity, accuracy, recoveries, repeatability, and reproducibility that the use of this analytical strategy is a very favorable choice for routine analysis in laboratories dealing with a large number of samples on a daily basis.

REFERENCES

(1) Jung, S. Y.; Choe, B. C.; Shin, G. Y.; Kim, J. H.; Chae, Y. Z., Analysis of roasted and ground grains on the Seoul (Korea) market for their contaminants of aflatoxins, ochratoxin A, and *Fusarium* toxins by LC-MS/MS. *Int J Pharm Pharm Sci* **2012,** *Vol.6* (12), 637–640.

(2) De Saeger, S.; Sibanda, L.; Van Peteghem, C., Analysis of zearalenone and α-zearalenol in animal feed using high-performance liquid chromatography. *Anal. Chim. Acta* **2003**, (487), 137–143.

(3) European Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. *Off. J. Eur. Union* **2006** L364, 5–54.

(4) Hu, X.; Hu, R.; Zhang, Z.; Li, P.; Zhang, Q. and Wang, M., Development of a multiple immunoaffinity column for simultaneous determination of multiple mycotoxins in feed using UPLC-MS/MS. *Anal Bioanal Chem* **2016**, *408* (22), 6027–36. https://doi:10.1007/s00216-016-9626-5.

(5) Solfrizzo, M.; Gambacorta, L.; Bibi, R.; Ciriaci, M.; Paolini, A. and Pecorelli. I., Multimycotoxin analysis by

LC-MS/MS in cereal food and feed: Comparison of different approaches for extraction, purification and calibration. *J. AOAC Int.* **2018,** *Vol. 101* (3), 647–657.

(6) Wilcox, J.; Donnelly, C.; Leeman, D.; Marley, E., 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. *J. Chromatogr. A* **2015,** *1400*, 91–97.

(7) Smaoui, S.; Braïek, O. B.; Hlima H. B., Mycotoxins analysis in cereals and related foodstuffs by liquid chromatography-tandem mass spectrometry techniques. *J. Food Qual* **2020,** *Vol 2020* 23 pages, Article ID 888117. https://doi.org/10.1155/2020/888117.

(8) Rajakylä, E.; Laasasenaho, K.; Sakkers, P. J. Determination of mycotoxins in grain by high-performance liquid chromatography and thermospray liquid chromatography-mass spectrometry. *J. Chromatogr.* **1987**, *384*, 391–402.

(9) Lattanzio, V. M. T.; Solfrizzo, M.; Powers, S.; Visconti, A., Simultaneous determination of aflatoxins, ochratoxin A and *Fusarium* toxins in maize by liquid chromatography/tandem mass spectrometry after multitoxin immunoaffinity clean-up. *Rapid Commun. Mass Spectrom.* **2007,** *21*, 3253–3261. https://doi: 10.1002/rcm.3210.

(10) Park, J.; Kim, D. H.; Moon, J. Y.; An, J. A.; Kim, Y. W.; Chung, S. H.; Lee, C., Distribution analysis of twelve mycotoxins in corn and corn-derived products by LC-MS/MS to evaluate the carry-over ratio during wet-milling. *Toxins* **2018**, *10* (319). https://doi: 10.3390/toxins10080319.

(11) Ibáñez-Vea, M.; Corcuera, L. A.; Remiro, R.; Murillo-Arbizu, M. T.; González-Peñas, E.; Lizarraga, E., Validation of a UHPLC-FLD method for the simultaneous quantification of aflatoxins, ochratoxin A and zearalenone in barley. *Food Chem.* **2011**, *127*, 351–358.

(12) Kong, W. J.; Liu, S. Y.; Qiu, F.; Xiao, X. H.; Yang, M. H., Simultaneous multi-mycotoxin determination in nutmeg by ultrasound-assisted solid-liquid extraction and immunoaffinity column clean-up coupled with liquid chromatography and on-line post-column photochemical derivatization-fluorescence detection. *Analyst* **2013**, *138*, 2729–2739. https://doi: 10.1039/c3an00059a.

(13) Sáez, J. M.; Medina, A.; Gimeno-Adelantado, J. V.; Mateo, R.; Jimenez, M., Comparison of different sample treatments for the analysis of ochratoxin A in must, wine and bear by liquid chromatography. *J. Chromatogr. A* **2004**, *1029*, 125–133.

(14) Asghar, M. A.; Iqbal, J.; Ahmed, A.; Khan, M. A.; Shamsuddin, Z. A.; Jamil, K., Development and validation of a high-performance liquid chromatography method with post-column derivatization for the detection of aflatoxins in cereal and grains. *Toxicol. Ind. health* **2014**, *Vol.32* (6), 1122–1134. https://doi.org/10.1177/0748233714547732.

(15) Trucksess, M. W.; Weaver, C. M.; Oles, C. J.; Fry Jr, F. S.; Noonan, G. O., Determination of aflatoxins B_1, B_2, G_1 and G_2 and ochratoxin A in ginseng and ginger by multitoxin immunoaffinity column clean-up and liquid chromatographic quantitation: collaborative study. *J. AOAC Int.* **2008**, *Vol. 91* (3), 142–1049.

(16) Visconti, A.; Pascale, M., Determination of zearalenone in corn by means of immunoaffinity clean-up and high-performance liquid chromatography with fluorescence detection. *J. Chromatogr. A* **1998**, *815*, 133–140.

(17) Leszczyńska, J.; Maslowska, J.; Owczarek, A.; Kucharska, U., Determination of aflatoxins in food products by the ELISA method. *Czech J. Food Sci.* **2001**, *19*, 8–12.

(18) M. Z. Zheng; J. L. Richard; J. Binder., A review of rapid methods for the analysis of Mycotoxins. *Mycopathologia* **2006,** *161*, 261–273. https://doi 10.1007/sl 1046-006-0215-6.

(19) Muñoz-Solano, B., González-Peñas. E., Mycotoxin determination in animal feed: an LC-FLD method for simultaneous quantification of aflatoxins, ochratoxins and zearalenone in this matrix. *Toxins* **2020**, *12*, 374. https://doi: 10.3390/toxins12060374.

(20) Delaunay, N.; Combès, A.; Pichon, V., Immunoaffinity extraction and alternative approaches for the analysis of toxins in environmental, food or biological matrices. *Toxins* **2020**, *12*, 795. https://doi: 10.3390/toxins12120795.

(21) Irakli, M. N.; Skendi, A.; Papageorgiou, M. D., HPLC-DAD-FLD method for simultaneous determination of mycotoxins in wheat bran. *J. Chromatogr. Sci.* **2017**, 1–7. https://doi: 10.1093/chromsci/bmx022.

(22) Bessaire, T.; Mujahid, C.; Mottier, P.; Desmarchelier, A., Multiple mycotoxins determination in food by LC-MS/MS: an international collaborative study. *Toxins* **2019**, *11*, 658. https://doi: 10.3390/toxins11110658.

(23) Abdallah, M. F.; Girgin, G.; Baydar, T., Mycotoxin detection in maize, commercial feed and raw dairy milk samples from Assiut city, Egypt. *Vet. Sci.* **2019**, *6*, 57. https://doi: 10.3390/vetsci6020057.

(24) European Commission Regulation (EC) No 401/2006 of 23 February 2006 laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs. *Off. J. Eur. Union* L70, **2006**, 12–34.

(25) European Communities, Commission Decision of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results (2002/657/EC). *Off. J. Eur. Union*, **2002**, L 2218–2236.

(26) EN ISO 16050:2003: Foodstuffs – determination of aflatoxin B_1 and the total content of aflatoxins B_1, B_2, G_1 and G_2 in cereals, nuts and derived products – high-performance liquid chromatographic method, European Committee for Standardization 2003.

(27) AOAC Official Method 2000.03 Ochratoxin A in barley *Off. Methods Anal. AOAC Int.* **2005**, Natural Toxins chapter 49, 64–66.

(28) AO ZON PREP, R-biopharm Rhône LTD, Manufacturer's instructions, Production code: P112/P112B

(29) Taylor B.; Kuyatt, B. C., Guidelines for evaluating and expressing the uncertainty of NIST measurement results. In: NIST Editorial Review Boards. *NIST Tech. Note* **1994**, 1297 1–20.