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# Rapid simultaneous extraction and magnetic particle-based enzyme immunoassay for the parallel determination of ochratoxin A, fumonisin B1 and deoxynivalenol mycotoxins in cereal samples

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This work describes the development of a rapid method for the extraction and the immunochemical determination of three mycotoxins, deoxynivalenol, fumonisin B1, and ochratoxin A, which are frequently found together naturally in cereal samples (wheat and corn flours). The 3 mycotoxins were extracted simultaneously in dichloromethane, and then three parallel spectrophotometric enzyme immunoassays were carried out in conventional microtiter plates using magnetic beads. Three specific monoclonal mouse antibodies for the three mycotoxins were immobilized in an oriented way using protein G functionalized magnetic particles. The magnetic immunoassay schemes decrease the incubation times, improve greatly the efficiency of the separation and washing steps, and also allow the easy elimination of interfering matrix components from the extracted samples. The method has limits of detection of 5.0 ± 1.4 ng mL$^{-1}$ for FB1, 4.3 ± 1.8 ng mL$^{-1}$ for DON, and 0.1 ± 0.05 ng mL$^{-1}$ for OTA ($n$ = 5), and the relative standard deviations of the determinations of the three mycotoxins are less than about 10% RSD. The method allows us to measure concentrations of these mycotoxins well below the limits of the European Union legislation in cereal samples. The developed multiplex magnetic particle-based enzyme immunoassay (mpEIA) method was validated with certified reference materials (wheat and maize flour samples) and three official AOAC (Association of Analytical Chemists) chromatographic analytical methods for each of the three mycotoxins. This method is high-throughput and accurate for the rapid determination of FB1, DON, and OTA in commercial cereal and feedstuff samples.

## 1. Introduction

Mycotoxins are produced by various fungi species, essentially belonging to the *Aspergillus*, *Penicillium* and *Fusarium* genera. Although hundreds of mycotoxins have already been identified, the most important in terms of natural occurrence and toxicity are aflatoxins, ochratoxins, trichothecenes (fumonisins and deoxynivalenol), zearalenone, and their metabolites.[1,2] These mycotoxins occur in a variety of foodstuffs, and due to their toxicity at very low concentrations the European Union and most countries have very strict regulatory limits.[3,4]

In this work, we present the simultaneous extraction of ochratoxin A (OTA), fumonisin B1 (FB1), and deoxynivalenol (DON) mycotoxins from cereal samples and their quantification using parallel specific magnetic particle-based enzyme immunoassays (mpEIA). FB1 and DON are the most prevalent of about 150 related mycotoxins known as trichothecenes that are formed by a number of species of *Fusarium*. Since *Fusarium* fungi invade crops, their toxic compounds are often found in cereals.[2] FB1 is frequently associated with corn.[2] Ochratoxin A is a mycotoxin from *Aspergillus ochraceus* and *Penicillium verrucosum*, and is the most abundant mycotoxin found as a contaminant in food worldwide (particularly in cereals), and it is dreaded due to its carcinogenic and nephrotoxic activity.[5]

The natural co-occurrence of these three mycotoxins is frequently reported in cereals,[2] since one kind of crop can be infected by different toxigenic molds producing different toxins (*i.e.* trichothecenes and ochratoxins), and also each mold can produce several kinds of mycotoxins simultaneously (*e.g. Fusarium* producing trichothecenes). Processed cereal samples, which can be mixtures of different raw materials, more often contain various types of mycotoxins.[2]

The determination of multi-mycotoxins in cereals and their commodities is challenging as they are present usually at low concentration levels (a few ng mL$^{-1}$) and matrices are very complex.[1,6–8] Liquid chromatography coupled to mass spectrometry (LC-MS) or tandem mass spectrometry (LC-MS/MS) methods are the most used alternatives for multi-mycotoxin

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analysis, since they provide wide linear dynamic ranges, identification, and quantification of a large number of mycotoxins. However, they are laborious and time-consuming methods, requiring extended cleanup and pre-concentration steps after extraction, in addition to a high level of expertise and costly equipment.[1,7]

Antibody-based methods are rapid, simple, selective and sensitive methods extensively used in the screening analysis of mycotoxins.[5,6] A multitude of immunochemical methods have been developed in a variety of different assay formats such as enzyme-linked immunosorbent assays (ELISAs),[6,9] Immunoaffinity column (IAC) based assays,[6] fluorescence polarization immunoassays,[10] or electrochemical immunoassays.[5] ELISA is one of the most commonly used techniques, although most ELISA kits commercialized in large numbers are used for the determination of a single mycotoxin. The use of immunochemical methods focusing on parallel analysis of several mycotoxins is currently a tendency highly desirable to keep analysis time and costs low.[9,11] Some approaches have been proposed in this regard, such as rapid determination of aflatoxin B1, OTA and zearalenone using a variation of an ELISA in kinetic mode,[12] or the use of multiplexed biosensors based on many formats, including multiplexed immunoassays, suspension arrays, membrane-based devices (flow-through and immunochromatographic), and planar microarrays.[9]

On the other hand, antibody-functionalized magnetic beads (MBs) can be coupled to ELISA immunoassays for improving washing and separation steps. Higher sensitivity and selectivity of mpEIA than with conventional ELISA are obtained due to the magnetic separation and concentration effects of the MBs.[5,13,14] In addition, antigen–antibody incubation times in mpEIA are reduced rather than in conventional ELISA because incubations are carried out in dispersion instead of using immobilized antibodies on the solid surface of the wells.[15]

Prior to analysis, mycotoxins have to be conveniently removed from solid food samples, which is usually done by extraction methods into suitable solvents.[7] Higher extraction yields are desired together with minimum extraction of the components of the matrix. This requires compromise to establish optimal conditions during extraction, cleanup, and sample preparations. As a large number of interfering compounds can be extracted with the mycotoxins, the primary sample extracts must be cleaned-up by chromatographic methods to get more accurate and precise results.[7] Solid phase extraction and immunoaffinity columns (IACs) are the most significant methods to clean up sample extracts before using chromatographic methods. Rapid methods for mycotoxins based on antibodies have an advantage over chromatographic techniques in that they usually do not require extensive cleanup or analyte enrichment steps after extraction, owing to their appropriate selectivity.[5] In many cases, only dilutions of the extracts are necessary.[6]

Extraction methods are highly dependent on the mycotoxins, as their solubility is quite different, and the matrix compounds.[16] Mixtures of water/acetonitrile are the most widely used solvents in chromatographic analysis of multiple mycotoxins in cereal commodities, giving good yields for most of the analytes.[7] However, small percentages of acetonitrile cause easy denaturation of the antibodies and enzymes, being a solvent rarely used in immunochemical methods. For instance, FB1 is only partially soluble in water, but highly soluble in methanol (10 mg mL$^{-1}$ MeOH) and acetonitrile.[1,2] Acids also increase the extraction efficiency for polar analytes like fumonisins.[1] DON is a polar molecule highly soluble in water and polar solvents like methanol, ethyl acetate, and acetonitrile.[1,16–18] OTA is more hydrophobic, hardly soluble in water (1 mg mL$^{-1}$), and requires organic solvents for dissolution like dimethyl sulfoxide (100 mg mL$^{-1}$), methanol and ethanol (10–50 mg mL$^{-1}$), or acetone (50–100 mg mL$^{-1}$).[1,18]

The aim of the present study was to optimize an extraction and a multiplex mpEIA immunoassay method for the rapid, effective and reliable parallel determination of FB1, DON, and OTA mycotoxins in cereal commodities. While both FB1 and DON are readily extractable together with aqueous mixtures of methanol, in the case of OTA the recovery percentages decrease considerably with this solvent, which is an especially important problem considering its low concentrations compared to the other two naturally coexisting mycotoxins. We have optimized and compared two extraction procedures for the three mycotoxins from cereal samples, using methanol and dichloromethane extraction solvents respectively, obtaining high recoveries in both cases. The determination of mycotoxins was carried out with magnetic particle-based enzyme immunoassays, which improves sensitivity and selectivity.[14,19] The results were validated using certified materials and official AOAC (Association of Official Analytical Chemists) IAC-HPLC chromatographic methods with fluorescence[8,20] and UV-Visible[21] detection. The developed method of analysis complies with the analytical performance required for the official control of the levels of mycotoxin contents in foodstuffs as recommended by the European Union.[22]

## 2. Experimental

### 2.1. Apparatus

Spectrophotometric measurements were made with a Bio-Rad Model 680 Microplate reader (Bio-Rad, Hercules, CA USA). Standard 96-well polystyrene microplates (ref. 82.1581, Iwaki, Japan) were supplied by Sarstedt (Nümbrecht, Germany). A magnetic 96-well separator (Life-technologies, Oslow, Norway) was used to separate the MBs from the supernatant to make the removal of the solvent easier in the standard ELISA plates. Vortex REAX-Top and Orbital shaker Rotamax-120 were from Heidolph (Schwabach, Germany). A centrifuge Heraeus Multifuge X1R was provided by Thermo Scientific (Rockford, IL USA). A magnetic separation stand for Eppendorf vials (Z5342, 12 positions, 1.5 mL volume) was purchased from Promega (Madison, WI USA). The incubation process for the immobilization of the antibody to the MBs was carried out using an Eppendorf-Thermomixer (Eppendorf, Hamburg, Germany), and a Stainless Steel temperature incubator from Memmert (Nurnberg, Germany) was used in the incubation antibody–antigen process. The immunoassay absorbances were processed with software GraphPad Prism version 6 (GraphPad Software Inc., La Jolla, USA). A high-performance liquid chromatograph Waters

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2796 Bioseparation module was coupled to UV-Vis absorbance UV/Vis Waters 996 and molecular fluorescence (FLD) Waters 2476 detectors (Waters, Hertfordshire, UK) for official AOAC mycotoxin detection procedures.

### 2.2. Chemicals

Monoclonal mouse antibodies for FB1 (mAb-FB1, IC9, IgG1 type), DON (mAb-DON, IB12, IgG1 type), and OTA (mAb-OTA, DE4, IgG1 type) were obtained from R-Biopharm (Darmstadt, Germany). Antibodies were diluted in PBS1 buffer, aliquoted in 20 μL and stored at −20 °C before use. Horseradish peroxidase conjugates of the mycotoxins (FB1-HRP: ref. 5121-FUMCO/NN5666; DON-HRP: ref. 5121-DONCO/NN6994; and OTA-HRP: ref. 5121-OTACO/NN5947) were from Europroxima (Amhem, Holland). Fumonisin B1 (>98%), deoxynivalenol (>98%), and ochratoxin A (>99.5%), and Bovine serum albumin (BSA), Tween 20, and 3,3′,5,5′-tetramethylbenzidine (TMB) were from Sigma-Aldrich (Madrid, Spain). Functionalized magnetic microbeads (MBs), 2.8 μm diameter, were from Invitrogen-Thermo Fisher Scientific (Madrid, Spain), namely Protein G functionalized Dynabeads (MBs-prG, 30 mg $mL^{-1}$ MBs), and M-280 sheep anti-mouse IgG functionalized Dynabeads ($(MBs\text{-}aIgG)_{mouse}$, 10 mg $mL^{-1}$ MBs). Other tested MBs were: Dynabeads Dynal functionalized with protein A (MBs-PrA), and Dynabeads M-280 sheep anti-rabbit IgG ($(MBs\text{-}aIgG)_{rabbit}$, 10 mg $mL^{-1}$ MBs).

Commercial wheat flour Super and Haricaman cornmeal (both from "Harinas de Castilla La Mancha", Toledo, Spain) were purchased at a local supermarket. Immunoaffinity columns (IACs) Fumonitest (ref. G1008), DONTest (ref. G1005), and OchraTest (ref. 13012) used for extraction and cleanup of the samples before HPLC determinations were from Vicam (Milford, USA).

The following buffers were used: (i) phosphate saline buffer (PBS1): 0.1 M sodium phosphate dibasic anhydrous (Sigma Aldrich, Steinheim, Germany), 0.1 M sodium phosphate monobasic monohydrate (Backer, Phillipsburg, NJ USA) and 0.138 M sodium chloride (Scharlab, Sentmenat, Spain), pH = 7.4; (ii) PBS2:PBS1 buffer, pH = 6.5. PBS3: 0.01 M $HPO_4^{-2}$ + 0.001 M $H_2PO_4^-$ + 0.003 M KCl; (iii) PBS3: 0.001 M phosphate buffer + 0.138 M NaCl + 0.003 M KCl, pH = 7.4. All solutions were prepared in MilliQ water.

### 2.3. Simultaneous extraction of FB1 + DON + OTA from cereals

Two procedures were optimized and compared, using methanol and dichloromethane extraction solvents.

**2.3.1 Procedure A.** 2 g (±0.1 mg) of grinded grains or flour sample were weighed in a centrifuge tube of 50 mL. 10 mL of $CH_3OH$ was then added by weighing, which was mixed for 60 minutes using a tumble stirrer. The mixture was centrifuged for 5 minutes at 5000 rpm. After filtration through filter paper, the liquid fraction was collected in a vial. For DON and FB1 determinations, 1 mL of $CH_3OH$ (by weighing) was taken and poured into a glass vial of 5 mL. The remaining weighed fraction was used for determining OTA. Each of the two solutions was evaporated to dryness under a stream of $N_2$ at 50 °C, or alternatively under a stream of $N_2$ by immersing the vial in a water bath at 50 °C. Once the solvents in both vials were evaporated, the solid extract was resuspended in 2 mL of buffer PBS1 (DON and FB1) and in 400 μL of 5% (v/v) ethanol in PBS 0.1 M, pH = 7.4 (OTA). In the case where turbidity is observed in the OTA extract, it is poured into an Eppendorf tube (1.5 mL) and centrifuged for 5 minutes at 10 000 rpm. A volume of 25 μL of these extracts was used for immunoassays.

**2.3.2 Procedure B.** 2 g (±0.1 mg) of the cereal sample (flour powder or grinded grains) was weighed in a centrifuge tube of 50 mL. 5 mL of 1 M HCl was added and then mixed for 5 minutes with a tumbling agitator. The mixture was centrifuged for 5 minutes at 5000 rpm. To determine FB1 and DON, 0.5 mL of the supernatant was taken, and PBS1 was added to a final volume of 5 mL in a 15 mL falcon tube. If turbidity appears, additional centrifugation was performed for 5 minutes at 5000 rpm. To determine OTA, 10 mL of $CH_2Cl_2$ was added by weighing, and mixed for 15 minutes by using a tumble stirrer, and the mixture was centrifuged for 10 minutes at 5000 rpm. Three layers are formed, where the upper layer is the water phase (HCl), the intermediate is formed by wheat flour, and the lower layer is $CH_2Cl_2$. The top layer was removed, and the mixture was stirred in the vortex mixer for 30 seconds and centrifuged for 5 minutes at 5000 rpm. The entire dichloromethane phase was taken, filtered through a filter paper, and collected in a centrifuge tube of 15 mL. 1 mL of 0.13 M $NaHCO_3$ pH 8.1 was added and OTA was extracted into the aqueous phase for 15 minutes at 350 rpm with an orbital shaker. The mixture was centrifuged for 10 minutes at 5000 rpm. The mixture was centrifuged for 5 minutes at 10 000 rpm. A volume of 25 μL of these extracts was used for immunoassays.

### 2.4. Immobilization of mycotoxin antibodies on PrG functionalized magnetic microbeads and mpEIA competitive immunoassays

The three antibodies were immobilized on the surface of protein G modified magnetic microparticles (MBs-prG) (for mAb-DON and mAb-FB1 antibodies), and on $(MBs\text{-}aIgG)_{mouse}$ (for mAb-OTA antibody), as explained before.[19] Briefly, the MBs were homogenously re-suspended in a dispersion by vortexing for 1 min. An appropriate amount of magnetic beads was washed three times with 500 μL of PBS1 buffer by stirring in an Eppendorf vial at 1300 rpm and removing the supernatants. A desired amount of antibody, dissolved in 1 mL of PBS1 buffer, was added and incubated for 10 minutes at 1300 rpm. The suspension was washed three times with 500 μL of PBS1 buffer using magnetic separations, re-suspended in a volume of PBS1 buffer and stored at 4 °C until use. When properly stored (4 °C), the antibody modified magnetic beads were stable for at least 4 weeks. It is not recommended to freeze (−18 °C) the antibody modified MBs. The amount of immobilized monoclonal antibodies was the same in all cases, using an excess of mAb from the binding capacity of MBs-prG (8–10 μg antibody per each 1.5 mg of MBs).

All the immunoassay reactions were made on standard polystyrene flat-bottom microtiter plates coupled with a magnetic support with 96× individual magnets to entrap the MBs on each of

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the microplate wells. Competitive reactions, performed in different wells, contain: (i) 25 μL of mycotoxin (Myc) standards (or samples) to obtain a final concentration in the range from 0.01 to 100 ng $mL^{-1}$ in the wells; (ii) 25 μL of the diluted Myc-HRP conjugates (FB1-HRP 1/100, DON-HRP 1/3 and OTA-HRP 1/2 dilutions (v/v) respectively); (iii) 50 μL of the antibody modified MBs to obtain 5 μg MBs-PrG-mAb-FB1, 25 μg MBs-PrG-mAb-DON, and 5 μg MBs-PrG-mAb-OTA. After incubating for 30 min (FB1, OTA) or 60 min (DON) at room temperature, the wells were washed three times with 300 μL of PBS1 buffer and the solvent was removed under a magnetic field for retaining the modified MBs.

For the mpEIA spectrophotometric measurements of Myc-HRP, 50 μL of TMB reagent (containing $H_2O_2$ as the co-substrate) was added to each well and left to react for 20 min at 25 °C (orbital shaking at 220 rpm.) and, after quenching the reaction with 0.5 M $H_2SO_4$, the absorbance was measured at 450 nm. Standard and samples were measured in replicate (3 times in separate wells).

The sigmoidal relationship between the absorbance and concentration values from the calibration curves of mAb-Myc binding assays was fitted to a 4-parameter logistic model by nonlinear fitting using the software GraphPad Prism v.6, thus optimizing accuracy and precision over the maximum usable calibration range.

### 2.5. Validation of mycotoxin determinations with certified reference materials and HPLC methods

Certified reference materials (CRMs) used were from Trilogy Reference Material (Washington, USA): (i) wheat flour TR-0100 (lot O-W-813) with [OTA] = 23.3 ± 3.2 ng $kg^{-1}$; (ii) cornmeal MT-C-9990: [aflatoxin B1] = 30.3 ± 4.9 μg $kg^{-1}$, [DON] = 1900 ± 100 μg $kg^{-1}$, [FB1] = 1500 ± 100 μg $kg^{-1}$. They were doped in different concentrations by adding 20 μL of a solution from the corresponding mycotoxins to the solid samples and left to evaporate for at least 2 h.

For validating the immunoassay determinations of the three mycotoxins the following AOAC (Association of Official Analytical Chemists) HPLC methods were used for FB1 (AOAC official method 2001.04),[8] OTA (AOAC official method 2000.03),[20] and DON[20] in cereal samples. Briefly, test portion samples of the cereal samples were extracted by blending with acetonitrile–water solvent mixtures, the filtered extracts were cleaned up by passing through an immunoaffinity column (IAC), and the mycotoxins were eluted with methanol. The eluates were evaporated just to dryness, and the residue was dissolved in acetonitrile–water and analyzed by reversed-phase HPLC. DON was detected by HPLC-UV-Vis (absorbance detection). Ochratoxin A and FB1, previously derivatized with *o*-phthaldialdehyde and 2-mercaptoethanol, were determined by fluorescence detection (HPLC-FLD).

## 3. Results and discussion

### 3.1. Optimization of the multiplex spectrophotometric mpEIA immunoassay method

The most important thing in an immunoassay scheme is the correct optimization of experimental variables that provide adequate sensitivity and selectivity. Based on our previous experience with electrochemical immunosensors we have developed,[5,14,15,19] we optimized first in this study the experimental variables influencing the determination of the three mycotoxins by magnetic particle-based enzyme immunoassay (mpEIA) analysis. While extraction of the three mycotoxins was carried out jointly, immunoassays were performed in parallel from the same extract into separate wells of conventional ELISA microplates, thus avoiding cross-reactivities between the three specific antibodies and the conjugates with peroxidase enzyme of each mycotoxin.

Immunoassays were carried out using magnetic particles as the solid surface for immobilization of the antibodies, thereby improving washing and separation steps with 100% efficiency under an external magnetic field. The total volume for the competitive incubations was 100 μL, as smaller volumes hindered incubations and made it difficult to measure absorbances due to the dispersion of light produced by the dispersed MBs in suspension.

The magnetic particles chosen to be studied were functionalized with aIgG (for mAb-OTA) and protein G (for mAb-FB1 and mAb-DON), which bind in both cases to the Fc part of the antibodies, leaving the Fab portion outward for a greater affinity towards antigen molecules. Four types of MBs were assayed preliminarily: MBs-$aIgG_{mouse}$, MBs-PrA, MBs-PrG, and MBs-$aIgG_{rabbit}$. In all of these studies, the same amount of antibody was immobilized onto the MBs (5 μg of mAb each 1 mg MBs). The higher antigen–antibody affinities were assayed by incubating the MBs-mAb-Myc (Myc: FB1, DON, and OTA) with the corresponding Myc-HRP conjugates of the mycotoxins (FB1-HRP, DON-HRP, and OTA-HRP, respectively). By comparing the analytical efficiency of the MBs, the maximum absorbance signals of competitive assays in the absence of mycotoxins were obtained with the MBs-PrG (mAb-FB1, mAb-DON) and with the MBs-$aIgG_{mouse}$ (mAb-$OTA_{mouse}$). In the case of OTA-HRP, the MBs-$aIgG_{mouse}$ showed much more affinity than MBs-$aIgG_{rabbit}$ in binding the mAb-OTA *via* a mouse primary antibody, as expected. The non-specific adsorption of each Myc-HRP on the corresponding unmodified MBs-PrG and MBs-$aIgG_{mouse}$ magnetic beads in Eppendorf vials was negligible, due to the hydrophilic surface (polystyrene) of these kinds of MBs. For the same reason, it was not necessary to use blocking agents (*e.g.* BSA was tested) onto the surface of the polystyrene wells from the microplate to prevent non-specific adsorption of the Myc-HRP conjugates. All incubations of these assays were carried out following the experimental procedure of Section 2.3. The revealing of the peroxidase enzyme HRP (spectrophotometric transduction) was as previously optimized in our laboratory.[15]

The most important immunoassay variables to be optimized are the amount of antibodies (micrograms of antibody-modified MBs), dilution of the Myc-HRP conjugates, and temperature and time of incubations. These parameters were studied to obtain the minimum $EC_{50}$ values (greater sensitivity), and the highest absorbance values at low competitive conditions of the mycotoxin (very small concentrations of the analytes). The absorbance signals from calibrations were fitted by nonlinear

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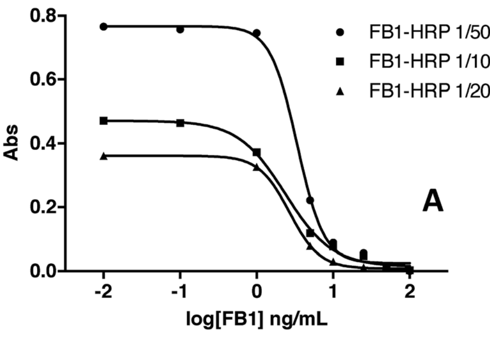


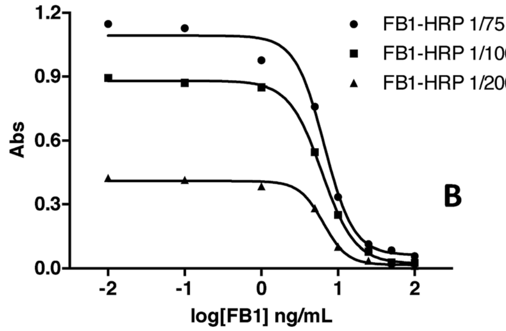




Fig. 1 Calibration plots of FB1 with various dilutions of the conjugate: (A) 5 μg of MBs-prG-mAb-FB1 (16 μg of mAb-FB1 with 16 mg of MBs-prG); (B) 10 μg of MBs-prG-mAb-FB1 (16 μg of mAb-FB1 with 16 mg of MBs-prG).

regression to a 4-parameter logistic model (4 PL) concentration dependent equation.[19]

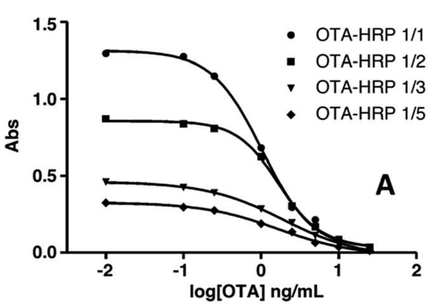
No significant differences were found in incubations carried out at room temperature or at 37 °C. Optimal incubation times were 30 min (OTA, FB1) and 60 min (DON). All types of antibody-modified MBs tested produced very low signals due to non-specific adsorption of conjugates Myc-HRP. Very low absorbances ($A \approx 0.004$) in solutions with 100 ng mL$^{-1}$ concentration of the mycotoxins indicate a high competitive binding of the three mycotoxins to the immobilized antibodies with respect to their corresponding Myc-HRP conjugates.

Higher dilutions of the Myc-HRP conjugates produce large decreases in absorbance and small decreases in the $EC_{50}$ values. Conversely, higher amounts of antibody increase the absorbance values. It is necessary to combine the amounts of antibody (*i.e.* the quantity of antibody-modified MBs) and enzyme conjugate to obtain calibration curves with the greatest variations between the maximum and minimum absorbance values, and to be able to determine the three mycotoxins below the range of concentrations restricted by the EU legislation (*i.e.* to obtain the required sensitivity). For example, this behavior can be seen in Fig. 1 for the FB1 mpEIA immunoassay. An increase of 5 to 10 μg of antibody-modified MBs produces an increase in absorbance of 0.78 to about 1.1 for the lowest FB1 concentrations (FB1-HRP dilution 1/50 (v/v)). The absorbances also decrease with more diluted FB1-HRP conjugate solutions, as expected. A similar behavior occurs for DON and OTA with more diluted Myc-HRP conjugates, as shown in Fig. 2.

To study the possible interference of MBs in the spectrophotometric determinations, increasing amounts of MBs-aIgG$_{mouse}$ (5, 10, 15, 20, and 25 μg) in calibration solutions at the wells of the ELISA microplate [50 μL-5 μg MBs-mAb-OTA, mAb-OTA 10 μg per mg of MBs + 25 μL OTA-HRP 1/2 (v/v) + 25 μL OTA, 0.04, 0.4, 1, 4, 10, 20, 40, and 100 ng mL$^{-1}$] were prepared. By comparing the parameters of the resulting calibration plots with software GraphPad v.6, it was found that there were no significant differences (95% confidence level) among the calibrations obtained, indicating that up to 25 μg the dispersión of MBs did not interfere with the absorbance measurements. The reproducibility of eight replicated measurements (10 ng mL$^{-1}$ OTA) increased from % RSD = 7.7% (5 μg of MBs) to % RSD = 9.2% (25 μg of MBs), but an ANOVA calculation did not show variations of the absorbances of the mpEIA determinations of OTA due to dispersions of MBs until a total amount of 25 μg (95% confidence level).

The optimal immunoassay conditions for the determination of the three mycotoxins are summarized in Table 1.

The analytical performance of the multiplex mpEIA immunoassays was evaluated following the IUPAC guidelines for single-laboratory validation of methods of analysis.[23] The results are summarized in Table 2. All the mycotoxin concentrations of Table 2 refer to those of the microtiter wells. The reproducibility (between days) was in all cases below 11% RSD for the three mycotoxins in concentrations in the wells close to the $EC_{50}$ values ($n = 8$ independent determinations in 4 consecutive days). In all cases, the $EC_{50}$ values are below the



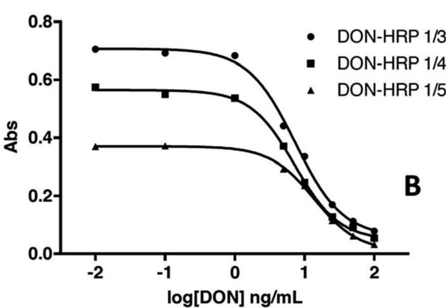




Fig. 2 Calibration plots at various Myc-HRP dilutions of: (A) ochratoxin A (5 μg of MBs-prG-mAb-OTA, 10 μg mAb-OTA each 1 mg MBs-aIgG); (B) DON (25 μg of MBs-prG-mAb-DON, 8 μg mAb-DON each 1 mg MBs-prG).

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Table 1 Optimal conditions for the mpEIA determination of the three mycotoxins. Competitive incubations were carried out at 25 °C

| Mycotoxin | Amount of immobilized antibodies | Amount of modified MBs | Dilution of Myc-HRP[a] (v/v) | Incubation time |
|---|---|---|---|---|
| FB1 | 16 μg mAb-FB1/1 mg MBs-PrG | 10 μg MBs-PrG-mAb-FB1 | FB1-HRP 1/100 (v/v) | 30 min |
| DON | 8 μg mAb-DON/1 mg MBs-PrG | 25 μg MBs-PrG-mAb-DON | DON-HRP 1/3 (v/v) | 60 min |
| OTA | 10 μg mAb-DON/1 mg MBs-aIgG$_{mouse}$ | 5 μg MBs-PrG-mAb-DON | OTA-HRP 1/2 (v/v) | 30 min |

[a] Dilutions of the Myc-HRP conjugates were performed with PBS1 buffer.

Table 2 Analytical performance of the multiplex mpEIA. The reproducibility was calculated for $n = 8$ replicate measurements of the mycotoxin concentration close to the $EC_{50}$ concentration level

| Mycotoxins | $EC_{50}$[a], ng mL$^{-1}$ | LODs[b], ng mL$^{-1}$ | Reproducibility[c] (% RSD) | Detection range[d], ng mL$^{-1}$ |
|---|---|---|---|---|
| FB1 | 5.41 ± 0.23 | 5.0 ± 1.4 | 8.2% | 9.3–58.5 |
| DON | 7.30 ± 0.32 | 4.3 ± 1.8 | 10.6% | 8.8–62.2 |
| OTA | 1.75 ± 0.08 | 0.10 ± 0.05 | 9.44% | 0.4–15.5 |

[a] Mean ± SD of $n = 4$ independent calibrations carried out on the same day (under reproducibility conditions). [b] LOD was calculated as 3 × SD of 8 replicate blank solutions. [c] The % RSD corresponding to the concentration of the mycotoxin close to the $EC_{50}$ of the calibration curves ($n = 8$). [d] Concentrations from $EC_{20}$ to $EC_{80}$ values in the calibration curves.

maximum concentrations of these mycotoxins permitted by the European Union legislation.[3,4]

The specificity was evaluated by assessing the recognition of the three specific antibodies toward the other mycotoxins, and warfarin (WAR) and L-phenylalanine (L-Phen) molecules for the OTA study. The cross-reactivity percentages (% CR) were calculated as the percentage of the OTA concentration obtained from an absorbance signal corresponding to a concentration of 100 ng mL$^{-1}$ of the interfering molecule, interpolating this absorbance in a calibration curve of the mycotoxin corresponding to the antibody being tested. The calculated % CR was respectively: (1) 5.6% (DON), 7.7% (OTA) and 8.8% (OTB) for the mAb-FB1 antibody; (2) 7% (FB1), 12.5% (OTA), and 14.1% (OTB) for the mAb-DON; and (3) 102.8% (OTB), 1.7% (FB1), 1.3% (DON), 0.7% (WAR), and 0.4% (L-Phen) for the mAb-OTA antibody.

### 3.2. Simultaneous extraction of the three mycotoxins before the immunoassay

Mycotoxins must be extracted from the food solid sample before their immunochemical determinations. Ideally, the leaching solvent must be able to remove as much as possible of the mycotoxins of interest in a minimum volume while removing as little as possible of interfering compounds from the matrix.[1]

Cereals are frequently contaminated simultaneously with DON and FB1, since they come from the same species of *fusarium* fungi. FB1 is associated with maize, while DON is most closely associated with durum wheat and oat cereals. Also very frequent is the natural occurrence of OTA in all kinds of unprocessed cereals, in addition to other foodstuffs, simultaneously with DON and FB1. From this starting point, the challenge in extracting simultaneously the three mycotoxins from cereal samples is the incomplete extraction of themselves, owing to their very different chemical structures and properties, and the extraction of different matrix components that can seriously interfere with the immunochemical techniques. The solvents used in extractions can also interfere with the immunoassay.

DON is a very polar molecule with three –OH substituents, and very soluble in aqueous solutions. Phosphate buffer solutions are usually used for extracting DON from the food samples.[1,16] In contrast, FB1 is a linear molecule containing an amino group, and several methyl and –COOH groups, requiring an organic solvent for extraction. Its extraction is usually carried out with methanol: water mixtures in proportions greater than 70/1 (v/v), and in some cases with a small proportion of acetic acid to ensure the protonated (extractable) form of both molecules.[17] An OTA molecule consists of an isocoumarin moiety and a phenylalanine moiety linked by an amide bond; it is a chlorinated hydrophobic molecule, and requires selected organic solvents for appropriate liquid extraction.[1,18]

The multi-mycotoxin extractions for the subsequent chromatographic determinations, *e.g.* in HPLC-MS/MS, are usually made with aqueous mixtures of different percentages of methanol and acetonitrile.[7] Nevertheless, the presence of small percentages of acetonitrile produced partial denaturation and loss of antibody affinity and decreased the catalytic ability of the HRP enzyme used as a label.[24] For these reasons, acetonitrile is not recommended for solvent extraction of mycotoxins in immunochemical methods.[2,6,18] Another problem found in our preliminary tests using acetonitrile was extraction of matrix components from the cereal samples that produced turbidity and a yellow color to the extract, which led to spectrophotometric interference. Acetonitrile is also more difficult to evaporate (b.p. 82 °C) than chloroform (b.p. 61.2 °C) or dichloromethane (b.p. 39.6 °C), in the case of concentrating the extract by evaporation. This problem is especially important in the case of OTA, where the concentrations are much lower than in the case of the FB1 and DON and more prone to interference

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from the matrix. In any case, it was found that percentages of the methanol and acetonitrile exceeding 5% (v/v) and 10% (v/v) respectively in the microtiter wells produced a loss of antibody affinities, mainly in the OTA immunoassay but also with FB1 and DON antibodies.

The major problems in extracting mycotoxins from cereal samples are the incomplete extraction of the mycotoxins themselves and the extraction of matrix components that can seriously interfere with immunochemical techniques. We have optimized both variables by using two extraction procedures with methanol and dichloromethane solvents, procedures A and B respectively (see Section 2.3).

The extraction recoveries were studied with commercial wheat flour (Super) and cornmeal (Haricaman) matrices. The results of doped and undoped cereal samples were validated with chromatographic AOAC official IAC-HPLC-FLD (FB1, OTA)[8,20] and IAC-HPLC-UV-Vis (DON)[21] methods, using different extraction procedures, cleanup/concentration of the extracts with immunoaffinity columns (IACs), and chromatographic conditions.

Of the three mycotoxins, the most difficult to extract is OTA, given its low concentrations with respect to the other two mycotoxins and matrix interference. For these reasons, a different procedure after extraction into the organic solvent is proposed for OTA with respect to FB1 + DON.

We have found extraction yields greater than 90% for FB1 and DON using methanol : water percentages more than 70 : 30 (v/v), but OTA requires 100% methanol for obtaining high recoveries.[16] DON recovery percentages were the same with methanol : water 70 : 30 (v/v) as with PBS 0.1 M (pH = 7.4) (ANOVA, 95% level of confidence). But aqueous PBS is not suitable for extracting FB1 [19] or OTA. The evaporation of the organic solvent was necessary, since a percentage of methanol greater than 5% (v/v) in the extract causes significant interference in the immunoassay of OTA due to the negative influence on the OTA antibody and on the enzymatic detection with the enzyme peroxidase.[15] Similar interference at the same percentage of methanol was shown for FB1 and DON antibodies.

The two kinds of flours (wheat and maize) were doped with about 1500 ng $g^{-1}$ (FB1, DON), and with 5 and 20 ng $g^{-1}$ (OTA). The fortified samples were analyzed by the mpEIA immunoassay procedure to calculate recovery percentages from extraction at the given concentrations. The results are summarized in Table 3. These results are the mean ± SD of three replicate extractions followed by the immunochemical or chromatographic measurements.

Extraction of 1 g of these samples with 10, 0 mL methanol following the procedure A but using 30 min of extraction time, resulted in the following recoveries after determinations by the mpEIA immunoassays: about 92.3% (FB1), 89.8% (DON), and 53.1% (OTA) in the Super wheat flour matrix, and 90.9% (FB1), 103.1% (DON), and 65.4% (OTA) in the Haricanam cornmeal matrix (Table 3). These first low recovery percentages obtained with OTA were improved with 60 min of stirring in the extraction step to 73.6% in Super wheat flour, and 77.6% in Haricanam cornmeal, and also by taking 2 g of the sample instead of 1 g for the extraction with 10 mL of the solvent: 86.5% in Super wheat flour, and 90.3% in Haricanam cornmeal. Attempts to extract with 10 mL of HCl : methanol 1 : 9 (v/v) produced worse recovery results and a yellow turbidity in the organic phase with both types of flours, indicating a major extraction of matrix components which resulted in major matrix interference in the immunoassay (the relative errors due to an excess of OTA concentration were of the order 35%).

Table 3 Recovery percentages of spiked commercial flour samples which were obtained with the two extraction procedures

| Sample (matrix) | Mycotoxin | Concentrations, μg $kg^{-1}$ Spiked | Found | Recovery[a], % |
|---|---|---|---|---|
| Haricanam (maize) | FB1 | 1481.0 ± 2.45 | 1391.8 ± 2.43[b] | 92.3 |
| | | | 1437.0[c] | 95.3 |
| | DON | 1556.2 ± 32 | 1465.9 ± 21.8[b] | 89.8 |
| | | | 1578.4[c] | 96.8 |
| | OTA | 4.94 | 4.23[b] | 84.5 |
| | | | 4.76[c] | 94.8 |
| | | 19.76 | 17.16 ± 1.04[b] | 86.5 |
| | | | 18.25[c] | 92.0 |
| Super (wheat) | FB1 | 1429.3 ± 6.1 | 1308.2 ± 191.4[b] | 90.9 |
| | | | 1470.9[c] | 102.2 |
| | DON | 1475.6 ± 6.1 | 1569.1 ± 151.4[b] | 103.1 |
| | | | 1433.6[c] | 94.2 |
| | OTA | 4.77 | 4.54[b] | 92.4 |
| | | | 4.74[c] | 96.4 |
| | | 19.06 | 17.35 ± 2.41[b] | 90.3 |
| | | | 18.98[c] | 98.8 |

[a] The recovery was calculated taking into account the concentration of the naturally contaminated mycotoxin measured by the corresponding official AOAC IAC-HPLC methods. [b] Methanol extraction for 1 min. [c] Dichloromethane extraction.

The extraction with dichloromethane (procedure B) sought to improve the extraction yield of OTA with respect to procedure A using methanol. The optimized procedure includes pretreatment of the sample with HCl 1 M, extraction into dichloromethane, and re-extraction of OTA into $NaHCO_3$ 0.13 M (pH = 8.1). This procedure provided good extraction yields for DON and FB1 above 90% with Super and Haricaman flours in all cases, while the performance greatly improved for OTA (recoveries greater than 85%) with respect to the procedure A for both wheat and maize samples.

The first attempts to improve the extraction of OTA (corn, wheat) with dichloromethane (1 g in 10 mL $CH_2Cl_2$) were carried out with the subsequent evaporation of the organic solvent at 50 °C with a nitrogen stream and the subsequent dissolution of the solid extract with several aqueous solutions. Different re-extraction aqueous solvents were tested to improve the extraction yield in the case of OTA, namely: (a) 0.13 M $NaHCO_3$ (pH = 8.1); (b) PBS (pH = 7.5); (c) PBS (pH = 8.1); (d) 5% MeOH in 0.1 M PBS (pH = 7.4); and (e) 5% (v/v) MeOH in 0.1 M PBS (pH = 3.5). Both FB1 and DON were successfully dissolved into aqueous PBS1 buffer solution owing to their greater polarity than OTA. The best recoveries were obtained by dissolving the solid extracts of OTA with (c) PBS (pH = 8.1),

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but in the first attempts the extraction recovery was of the order 54%.

For biosensor applications, it has been reported that addition of small amounts of water will wet the matrix substrate and can offer higher extraction efficiencies of mycotoxins, by increasing penetration of the solvent (mixture) into the hydrophilic material.[1] An acid solution as the aqueous phase also can help the extraction process by breaking interactions between the toxins and sample constituents such as proteins or carbohydrates.[2] In some cases, the extracts are also partitioned with other solvents, such as the organic nonpolar *n*-hexane or cyclohexane solvents for partial clean up, to remove excess lipophilic components from the biological matrix.[2,16] We tested by adding 5 mL of 0.5 M $H_3PO_4$ or 5 mL of 1 M HCl to 2 g of the cereal sample prior to extraction with dichloromethane, which improved the extraction efficiency of OTA to about 78–82% (depending of the type of matrix) with the optimum (d) 5% MeOH in 0.1 M PBS (pH = 7.4) solvent for dissolving the solid extract.

Some authors have successfully used liquid re-extraction of OTA from dichloromethane with 0.13 M $NaHCO_3$ instead of evaporation of the organic solvent.[25] Using this concept, we tested acidification of the sample (1 M HCl), extraction of OTA in dichloromethane, and the subsequent liquid–liquid re-extraction of OTA from the organic phase into an aqueous solution (0.13 M $NaHCO_3$, pH = 8.1). This process has the advantage of being faster and less laborious, since it does not require the step of evaporation of dichloromethane. Interference was initially obtained due to the excessive extraction of components of the matrix (maize and cornmeal) into the organic phase, which presented a turbid yellow appearance (2 g of samples in 10 mL dichloromethane). These matrix interference effects could not be completely resolved using an intermediate cleaning with hexane. This produced errors in excess of concentrations of the order of 10–22% at doping levels of 1, 2.5, and 5 ng $g^{-1}$ of OTA. However, decreasing the extraction time with dichloromethane from 30 to 15 min avoided that problem. Other important factors of this extraction procedure that were optimized were the time taken for the acidification of the sample and for the re-extraction of OTA from the dichloromethane phase.

Table 3 summarizes the recovery percentages obtained by the optimum extraction procedures with (a) methanol (procedure A), and (b) dichloromethane (procedure B) respectively. Although both procedures permit the joint extraction of the three mycotoxins from the doped samples with good recovery percentages, the extraction procedure with dichloromethane provided better analytical results for the OTA. In addition, the second procedure B has the advantage of not requiring the evaporation of the organic solvent used in the extraction, so it is quicker and easier to carry it out than procedure A.

### 3.3. Validation of the proposed method and analytical performance

Official AOAC chromatographic analytical methods for mycotoxins constitute a good form of the immunoassay validations, since different types of extraction and quantification procedures are used individually for each of the three mycotoxins.[8,20,21] All these HPLC procedures include cleaning and preconcentration of the extracts using immunoaffinity columns, subsequent evaporation of the eluate, and reconstitution of the solid residue in aqueous mixtures with acetonitrile prior to the chromatographic injection. FB1 is extracted from the cereal sample (2 g) with 5 mL of ACN : $CH_3OH$ : $H_2O$ (25 : 25 : 50) (v/v/v);[8] DON (sample 5 g) is extracted with 20 mL of $H_2O$;[21] and OTA (sample 2 g) is extracted with 10 mL of $CH_3OH$ : $H_2O$ (80/20) (v/v).[20] Elution of the mycotoxins from the IACs is performed with 1–1.5 mL of methanol (HPLC grade).

The sensitivity of the developed immunochemical method allows the determination of the FB1 and DON below the regulatory limits of the European Union legislation for mycotoxins: from 200 μg $kg^{-1}$ (processed cereal-based foods and baby foods) to 1750 μg $kg^{-1}$ (DON) in unprocessed durum wheat, maize and oats and to 4000 μg $kg^{-1}$ (FB1) in unprocessed maize.[3] The minimum quantifiable concentration of OTA in the wells is 0.25 ng $mL^{-1}$. Taking into account the extraction of 2 g sample in dichloromethane, re-extraction in 1 mL of aqueous solution (1 mL of 0.13 M $NaHCO_3$, pH 8.1), and subsequent dilution 1/4 in the wells, the minimum amount of quantifiable OTA in the cereal sample is 0.5 μg $kg^{-1}$. The maximum levels for OTA in foodstuffs of the EU legislation are 3–5 μg $kg^{-1}$ in unprocessed cereals and products derived from unprocessed cereals intended for human consumption.[4] As an exception, the limit is 0.5 μg $kg^{-1}$ OTA in processed cereal-based and dietary foods intended specifically for infants and young children.[4] In this case, close to the quantification limit of our method, sensitivity can be improved taking slightly more quantity of the sample to be extracted (we have tested up to 2.8 g obtaining good analytical results without matrix interference).

For studying accuracy, the proposed extraction procedure B was applied to the certified reference materials and samples shown in Table 4, which summarizes and compares the concentration results with the two types of matrices (corn and wheat) and also compares with those obtained by the chromatographic AOAC official analytical methods for the three mycotoxins. The mycotoxin determinations after extractions were carried out following the optimized mpEIA procedure shown in Section 2.3. While procedures A and B are good for FB1 and DON analytical performances, procedure B is more adequate for the low levels of concentration of OTA extraction without matrix interference and the immunoassay determination, so it was the method proposed as the most advantageous.

All the results of Table 4 meet the requirements of the analytical performance of the analysis methods for the official control of the levels of mycotoxins of the legislation of the European Union:[22] OTA (1–10 μg $kg^{-1}$) % RSD < 20% and recovery percentages of 70–110%; DON and FB1 (>500 μg $kg^{-1}$) % RSD < 20% and recovery percentages of 70–110% (FB1) and 70–120% (DON), respectively.[22]

The proposed method has no significant differences (*t*-test, *P* = 0.05) with the mean concentrations obtained with the IAC-HPLC-FLD and IAC-HPLC-IN-Vis AOAC official methods for the three mycotoxins in the samples shown in Table 4. There are

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Table 4 Comparison between the mycotoxin levels of the cereal samples determined by the proposed extraction (procedure B using dichloromethane) and multiplex mpELISA procedure, and the concentrations of the mycotoxins certified or determined by the IAC-HPLC-UV-Vis (DON) and IAC-HPLC-FLD (FB1, OTA) official AOAC methods

| | Multiplex mpEIA, μg kg$^{-1}$ (mean ± SD) | | | IAC-HPLC-FLD or IAC-HPLC-UV-Vis, μg kg$^{-1}$ (mean ± SD) | | |
|---|---|---|---|---|---|---|
| Sample (matrix) | FB1 | DON | OTA | FB1 | DON | OTA |
| Haricanam (maize) | 29.3 ± 2.1 | 72.1 ± 7.1 | 0.18 ± 0.02 | 26.9 ± 2.5 | 74.4 ± 32.0 | <0.076 |
| Super (wheat) | 12.5 ± 1.1 | 39.83 ± 2.3 | 0.25 ± 0.02 | 9.89 ± 6.1 | 46.3 ± 5.5 | <0.149 |
| TR-F100 (maize) | 1609.0 ± 174.1 | 355.8 ± 31.1 | 0.33 ± 0.05 | 1500 ± 400[a] | 399.7 ± 23.2 | <0.28 |
| TR-D100 (wheat) | 39.4 ± 2.6 | 1352.7 ± 108.5 | 0.27 ± 0.04 | 40.6 ± 1.06 | 1400 ± 200[a] | <0.22 |
| TR-O100 (wheat) | 64.48 ± 4.1 | 40.7 ± 7.8 | 25.89 ± 4.21 | 57.2 ± 4.6 | 30.1 ± 18.7 | 23.3 ± 3.2[a] |
| MT-C-9990[b] (maize) | 1604.2 ± 51 | 2000.8 ± 139.1 | 0.65 ± 0.30 | 1500 ± 100[a] | 1900 ± 100[a] | <0.073 |

[a] Certified content of the CRM material. [b] Aflatoxins certified content of 30.3 ± 4.9 μg kg$^{-1}$.

also no significant differences with the certified concentration values of the CRMs. The relative errors obtained between the mpEIA and the CRMs or the three AOAC official methods are in the range of about less than ±16%.

## 4. Conclusions

We have optimized a simple method for the joint extraction of OTA, FB1 and DON mycotoxins from cereal samples and determination by an mpEIA immunochemical assay, obtaining high recoveries and accurate results at the required ng mL$^{-1}$ concentration levels. The immobilization of the three monoclonal antibodies of the 3 mycotoxins on G protein-functionalized magnetic particles improves the efficiency of washing and separation steps of the immunoassay, thus avoiding the spectrophotometric interference of extracted components from the matrix and the organic solvent used in the extraction.

It was demonstrated that extraction influences matrix interference from wheat and corn samples. The concentrations of the 3 mycotoxins obtained with the validated method proposed in the two types of matrices studied (wheat and corn) did not differ significantly ($t$-test, $P = 0.05$) from the certified values of CRMs. There are also no significant differences ($P = 0.05$) between the concentrations of each of the three mycotoxins analyzed by the proposed method and the concentrations of the mycotoxins from the same samples obtained individually by three official AOAC methods, using in all of them extraction with aqueous acetonitrile mixtures, cleaning with a specific IAC, and determination by HPLC with UV-Vis or FLD detection. The proposed method has the advantages of a very simple sample pre-treatment, rapidity and cost-effectiveness. And the method also allows the determination of the 3 mycotoxins in cereals below the levels permitted by the EU legislation, has good reproducibility (% RSD about 10%), and each of the 3 antibodies has a low affinity for other mycotoxins that have a significant natural co-occurrence in wheat and maize samples.

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## References

1 A. Rahmani, S. Jinap and F. Soleimany, Qualitative and Quantitative Analysis of Mycotoxins, *Compr. Rev. Food Sci. Food Saf.*, 2009, **8**, 202–251.

2 V. L. Pereira, J. O. Fernandes and S. C. Cunha, Mycotoxins in cereals and related foodstuffs: A review on occurrence and recent methods of analysis, *Trends Food Sci. Technol.*, 2014, **36**, 96–136.

3 Commission Regulation (EC) No 1126/2007 of 28 September 2007, amending Regulation (EC) No 1881/2006 setting maximum levels for certain contaminants in foodstuffs as regards Fusarium toxins in maize and maize products, The Commission of the European Communities, Official J. of the European Union, 2007, L-255, 14–17.

4 Commission Regulation (EC) No 1881/2006 of 18 December 2006, setting maximum levels for certain contaminants in foodstuffs, The Commission of the European Communities, Official Journal of the European Union, 2006, L-364, 5–24.

5 J. C. Vidal, L. Bonel, A. Ezquerra, S. Hernández, J. R. Bertolín, C. Cubel and J. R. Castillo, Electrochemical affinity biosensors for detection of mycotoxins: A review, *Biosens. Bioelectron.*, 2013, **49**, 146–158.

6 E. P. Meulenberg, Immunochemical Methods for Ochratoxin A Detection: A Review, *Toxins*, 2012, **4**, 244–266.

7 A. De Girolamo, M. Solfrizzo, V. M. T. Lattanzio, J. Stroka, A. Alldrick, H. P. van Egmond and A. Visconti, Critical evaluation of LC-MS-based methods for simultaneous determination of deoxynivalenol, ochratoxin A, zearalenone, aflatoxins, fumonisins and T-2/HT-2 toxins in maize, *World Mycotoxin J.*, 2013, **6**, 317–334.

8 A. Visconti, M. Solfrizzo and A. D. Girolamo, Determination of Fumonisins B1 and B2 in Corn and Corn Flakes by Liquid

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Chromatography with Immunoaffinity Column Cleanup: Collaborative Study, AOAC method 2001.04-2001, *J. AOAC Int.*, 2001, **84**, 1828–1837.
9 C. M. Maragos, Multiplexed Biosensors for Mycotoxins, *J. AOAC Int.*, 2016, **99**, 849–860.
10 V. Lippolis and C. M. Maragos, Fluorescence polarization immunoassays for rapid, accurate and sensitive determination of mycotoxins, *World Mycotoxin J.*, 2014, **7**, 479–489.
11 I. Y. Goryacheva, S. De Saeger, S. A. Eremin and C. Van Peteghem, Immunochemical methods for rapid mycotoxin detection: Evolution from single to multiple analyte screening: A review, *Food Addit. Contam.*, 2007, **24**, 1169–1183.
12 A. E. Urusov, A. V. Zherdev, A. V. Petrakova, E. G. Sadykhov, O. V. Koroleva and B. B. Dzantiev, Rapid Multiple Immunoenzyme Assay of Mycotoxins, *Toxins*, 2015, **7**, 238–254.
13 R. Eivazzadeh-Keihan, P. Pashazadeh, M. Hejazi, M. De La Guardia and A. Mokhtarzadeh, Recent advances in Nanomaterial-mediated Bio and immune sensors for detection of aflatoxin in food products, *Trends in Anal. Chem.*, 2017, **87**, 112–128.
14 J. C. Vidal, J. R. Bertolín, L. Bonel, L. Asturias, M. J. Arcos-Martínez and J. R. Castillo, Rapid determination of recent cocaine use with magnetic particles-based enzyme immunoassays in serum, saliva, and urine fluids, *J. Pharm. Biomed. Anal.*, 2016, **125**, 54–61.
15 J. C. Vidal, L. Bonel, A. Ezquerra, P. Duato and J. R. Castillo, An electrochemical immunosensor for ochratoxin A determination in wines based on a monoclonal antibody and paramagnetic microbeads, *Anal. Bioanal. Chem.*, 2012, **403**, 1585–1593.
16 R. Krska, E. Welzig and H. Boudra, Analysis of Fusarium toxins in feed, *Anim. Feed Sci. Technol.*, 2007, **137**, 241–264.
17 A. Hallier, F. Celette and C. David, Effects of sampling and extraction on deoxynivalenol quantification, *Food Chem.*, 2011, **127**, 303–307.
18 N. W. Turner, S. Subrahmanyam and S. A. Piletsky, Analytical methods for determination of mycotoxins: A review, *Anal. Chim. Acta*, 2009, **632**, 168–180.
19 A. Ezquerra, J. C. Vidal, L. Bonel and J. R. Castillo, A validated multi-channel electrochemical immunoassay for rapid fumonisin B1 determination in cereal samples, *Anal. Methods*, 2015, **7**, 3742–3749.
20 C. A. Entwisle, A. C. Williams, P. J. Mann, P. T. Slack and J. Gilbert, Liquid Chromatographic Method with Immunoaffinity Column Cleanup for Determination of Ochratoxin A in Barley: Collaborative Study, *J. AOAC Int.*, 2000, **83**, 1377–1383.
21 S. J. McDonald, D. Chan, P. Brereton, A. Damant and R. Wood, Determination of Deoxynivalenol in Cereals and Cereal Products by Immunoaffinity Column Cleanup with Liquid Chromatography: Interlaboratory Study, *J. AOAC Int.*, 2005, **88**, 1197–1204.
22 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, The Commission of the European Communities, Official Journal of the European Union, 2006, L-70, 12–34.
23 J. Gilbert and E. Anklam, Validation of analytical methods for determining mycotoxins in foodstuffs, *Trends Environ. Anal. Chem.*, 2002, **21**, 468–486.
24 J. C. Vidal, L. Bonel, P. Duato and J. R. Castillo, Improved electrochemical competitive immunosensor for ochratoxin A with a biotinylated monoclonal antibody capture probe and colloidal gold nanostructuring, *Anal. Methods*, 2011, **3**, 977–984.
25 M. P. Monge, A. M. Dalcero, C. E. Magnoli and S. M. Chiacchiera, Natural co-occurrence of fungi and mycotoxins in poultry feeds from Entre Rios, Argentina, *Food Addit. Contam., Part B*, 2013, **6**, 168–174.