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Rapid and label-free detection of ochratoxin A and aflatoxin B_1 using an optical portable instrument

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ABSTRACT

In this study, we report a novel assay for the combined on site detection of aflatoxin B_1 (AFB_1) and ochratoxin A (OTA), through a colorimetric biosensing system for AFB_1 and a fluorimetric detection for OTA, exploiting the capability of the portable fibre optic spectrometer to perform both analyses. AFB_1 was detected using the acetylcholinesterase (AChE) enzyme that is inhibited by this toxin, and the degree of inhibition was quantified by the Ellman's spectrophotometric method, obtaining a detection limit of 10 μg L^{-1}. OTA quantification was performed by monitoring its intrinsic fluorescence in methanol, reaching a detection limit of 0.1 μg L^{-1}. In order to successfully apply the analytical tool in the food analysis, immunoaffinity columns were used. Clean-up and quantification of both AFB_1 and OTA in millet samples was obtained by HPLC-dedicated AflaOchra-Test HPLC™ (Vicam™) and Afla-OtaCLEAN™ (LC-Tech) immunoaffinity columns, followed by absorption/fluorescence detection. Millet samples which were fortified with both OTA (50 μg kg^{-1}) and AFB_1 (20 μg kg^{-1}), gave recovery values of 100 ± 6% for OTA, and 110 ± 10% for AFB_1, using AflaOchra-Test HPLC™. Single OTA clean-up and quantification in wine samples was obtained, using an OchraTest immunoaffinity column (Vicam™), reaching a detection limit of 0.3 μg L^{-1} and recovery values between 80% and 120%.

These results demonstrated the possibility of employing a single clean-up and a cost-effective, and easy to use analytical system for both AFB_1 and OTA detection at μg kg^{-1} (ppb) level. Furthermore, in the case of positive samples, they could be analysed further, using standard chromatographic procedures, without any additional clean-up step, since the same extraction procedure of standard method is proposed in our method.

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1. Introduction

Mycotoxins are secondary metabolites, which are produced by filamentous fungi that causes a toxic response, when ingested by animals and humans. Mycotoxin infection occurs in crops, during harvesting, storage, processing, or feeding. It is generally accepted that *Aspergillus, Fusarium and Penicillium* are among the most important molds, in producing mycotoxins detrimental to food. The mycotoxins of greatest concern include aflatoxin, which is generally produced by *Aspergillus* molds; deoxynivalenol, zearalenone, T-2 Toxin, and fumonisin, which are produced by *Fusarium* molds; and ochratoxin and PR toxin produced by *Penicillium* molds.

Aflatoxins are a family of extremely toxic, mutagenic, and carcinogenic compounds produced by *Aspergillus flavus* and *Aspergillus parasiticus*. Toxigenic *A. flavus* isolates the produced aflatoxins B_1, and B_2, and toxigenic *A. parasiticus* isolates the produced aflatoxins B_1, B_2, G_1, and G_2 [1–4].

The complete elimination of aflatoxins in human and animal food, though desirable, is extremely unlikely, as they have the potential to arise in a wide range of agricultural products. Risk assessments have been carried out for aflatoxins, and several European regulations have been stated, over the last years, to set the Maximum Residue Levels (MRLs) for mycotoxins in food for

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human consumption [5–8].

Current maximum levels admissible, vary between 2 and 8 μg kg^{-1} for AFB_1, and between 4 and 15 μg kg^{-1} for total aflatoxin (AFB_1+AFB_2+AFG_1+AFG_2), depending on different foods (peanuts, fruits, dried fruit and derived products, cereal and derived products) exploited for direct human consumption or as ingredients in other food products [8].

Ochratoxins are a group of structurally similar metabolites (ochratoxin A (OTA), B (OTB) and C (OTC)), produced by fungi of the genus, *Aspergillus* and *Penicillium*, and in particular, by *Aspergillus ochraceus* and *Penicillium viridicatum*. Among ochratoxins, OTA provides the highest toxic effect and it has been found mainly in cereal and cereal products [9,10].

Legal limits for ochratoxin A have been set by the European Commission, under EC regulations 466/2001 [11], 472/2002 [12,13] and 123/2005 [14], ranging from 0.5 to 10 μg kg^{-1} in a number of food commodities, including cereals and cereal products, raisin, roasted and soluble coffee, wine, grape juice, and foods for infants and children. Human exposure to ochratoxin A also occurs, through the consumption of altered food derived from animals and exposed to contaminated feedstuffs; in these regards, the European Commission, has set the limits to be from 50 μg kg^{-1} to 250 μg kg^{-1} also for animal feed [15].

In this overall scenario, an increasing awareness has occurred, regarding the risks of mycotoxins, on human and animal health. Several analytical methods have been proposed for mycotoxins control, including Thin-layer Chromatography (TLC) [16–18], Gas Chromatography (GC), High Performance Liquid Chromatography (HPLC) [19,20], immunosorbent assay (ELISA) and fluorescence [21,22].

The requirement of fast, cost-effective and in field analyses, is boosting the research efforts towards the development of effective methods to screen mycotoxin occurrence in crop cultures, as well as along the entire food production and distribution chain. Furthermore, the large variability in food matrices and the growing demand for a single method to accurately screen multiple mycotoxins, outlines new challenges for analytical research.

In this present work, we report the development of a portable optical sensing system for the detection of both aflatoxin B_1 (AFB_1) and ochratoxin A (OTA) in food samples, based on a single step, immunoaffinity purification/extraction in conjunction with absorption and fluorescence detection techniques.

Particularly, OTA was extracted from wine samples by means of OchraTest™ immunoaffinity column (Vicam) and was analysed immediately, by using a fibre optic spectrometer, and monitoring OTA intrinsic fluorescence. The use of a dedicated immunoaffinity column for clean-up and fluorescence detection, was adopted to demonstrate the suitability of the fibre optic spectrometer. After that, the extraction of OTA and AFB_1 from millet samples was performed, using the HPLC dedicated immunoaffinity columns, available for multi-toxin extraction, in order to obtain a simultaneous purification of both mycotoxins. Once extracted, OTA was detected by fluorescence measurements, while AFB_1 was determined, evaluating the acetylcholinesterase inhibition by spectrophotometric Ellman's method.

The novelty of the method resides on the simultaneous extraction of both OTA and AFB_1, by means of the same methodology, and the consequent mycotoxin screening on raw materials from feed product, performed by the same portable instrument. Furthermore, the positive samples could be analysed with standard procedures, including chromatographic methods, without further clean-up steps, since the extraction procedure is the same as one of the standard methods.

2. Materials and methods

2.1. Materials

All chemicals from commercial sources were of analytical grade. Acetylcholinesterase (AChE) from *Electric eel* (EC 3.1.1.7) was purchased from Sigma Chemical Co. (St. Louis, MO) through refrigerated transport. Acetylthiocholine chloride (ACTh), methanol, Polyethylene Glycol (PEG), $NaHCO_3$, NaCl, KCl, Na_2HPO_4, KH_2PO_4 and 5,5′-dithiobis-(2-nitrobenzoic acid (DTNB) were obtained from Sigma Chemical Co. (St. Louis, MO). Aflatoxins B_1 (AFB_1) and Ochratoxin A (OTA) were purchased from Vinci-Biochem. Stock solutions of AFB_1 and OTA, were prepared in methanol. Ocean Optics USB2000-FL fibre optic spectrometer supported by SpectraSuite software, was used for spectrophotometric and fluorescence measurements. A quartz cell (46×12.5 mm^2) provided by Hellman (Germany) was employed for fluorescence measurements. The immunoaffinity columns Ochra-Test™ (VICAM) were adopted, for the analysis of wine samples, while for the analysis of AFB_1 and OTA in millet, two types of HPLC dedicated immunoaffinity columns were used: AflaOchra-Test HPLC™ (VICAM) and Afla-OtaCLEAN™ (LC-Tech). A Reacti-ThermTM III Heating Modules was used for the clean-up of millet samples. Phosphate buffer 0.1 M, pH=8 was used for AFB_1 spectrophotometric determination.

2.2. Determination of OTA

The instrument Ocean Optics USB2000-FL fibre optic spectrometer (SpectraSuite), has the advantage of being small enough to be portable. It is constituted by a source of Deuterium lamp and Tungsten lamp, by a first optical fibre that transmits the excitation radiation from the lamp to the sample compartment, and finally by a second optical fibre that transmits the emission radiation from the test solution to the detector signal. The latter consists of an array able to record the received photons.

In the case of OTA measurements where the mycotoxin is excited at 333 nm, the Deuterium lamp was used to provide a light source, emitting between 210 nm and 400 nm, and the optical fibres were placed at an angle of 90° with respect to the light source. The fluorescence emission signals at 450 nm were measured in methanol, in treated wine and in millet samples. Blank fluorescence measurements were provided, analysing 1 mL of methanol; at a later stage, standard concentrations of OTA were added and calibration curves were carried out, from which it was possible to determine the Limit Of Detection (LOD), and linear range.

2.3. Determination of AFB_1

AFB_1 determination was based on acetylcholinesterase (AChE) inhibition. The AChE activity was evaluated by measuring the product of the enzymatic reaction. The ACTh was chosen as substrate, and the thiocholine, produced by the AChE reaction, was evaluated using the spectrophotometric Ellman's method. The thiocholine was measured using DTNB, which reacts with thiocholine to give 2-nitro-5-thiobenzoic acid (TNB), a yellow product, with a maximum absorbance intensity at 412 nm. In this case, the Tungsten lamp was set up to provide a light source emitting between 360 nm and 1700 nm and the optical fibres were placed at 180°, with respect to the light source. An internal filter centred at 405 nm, was allocated between the cuvette and the first optical fibre that transmits the excitation radiation from the lamp to the solution in the cuvette.

The enzymatic reaction was spectrophotometrically monitored analysing a mixture of 0.1 M phosphate buffer solution (pH 8),

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