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Simultaneous non-instrumental detection of aflatoxin B1 and ochratoxin A using a clean-up tandem immunoassay column

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Abstract

A set-up and simple method based on the clean-up tandem immunoassay approach was developed for the visual detection of two analytes. The method was based on a 1 mL column with one clean-up layer and two detection immunolayers. As detection immunolayers CNBr-activated Sepharose 4B with coupled secondary rabbit anti-mouse antibodies was used. Different specific antibodies were coupled to each detection immunolayer. The analysis was realised in a competitive ELISA format with visual detection of the developed colour for each detection immunolayer and took 20 min for six sample extracts. The described method was applied to the simultaneous detection of aflatoxin B1 and ochratoxin A in spices with cut-off levels at 5 and 10 $\mu g\,kg^{-1}$, respectively. Results were confirmed by LC–MS/MS with immunoaffinity column clean-up.

Keywords: Multiple analysis; Immunoassay; Rapid methods; Mycotoxins; Ochratoxin A; Aflatoxin B1; Spices

1. Introduction

One of the tendencies in present-day analytical chemistry is the simultaneous determination of multiple analytes in one analytical cycle for a given sample. Development of such methods is very important in the mycotoxin domain. Mycotoxins are present in different kinds of food and feed, and their presence should be controlled at various stages: before and post harvest, before and post processing. Some mycotoxins could simultaneously be present in the foodstuffs, so application of multi-mycotoxin assays allows to reduce the quantity of assays and, therefore, to lower costs and time for food and feed quality control. Recently some chromatographic methods for multiple mycotoxin determination were developed [1–5]. But these methods only yield results within hours and need expensive equipment and highly skilled personnel. Rapid methods for mycotoxin analysis have become increasingly important. At first for rapid simultaneous detection of several mycotoxins in food matrices a thin layer chromatography (TLC) method was developed as an analytical screening procedure [6,7].

Some rapid tests based on immunoassays with instrumental detection, such as a surface plasmon resonance biosensor [8] and a multiplexed assay [9] were also developed. Within the concept of flexible "on-site" testing, the use of special equipment is prohibitive. But only few attempts have been made to prepare tests for simultaneous detection of several mycotoxins, which can be used without specific laboratory equipment: a membrane-based assay in the format of a dipstick [10], an 8-well immunofiltration test device for parallel simultaneous analysis [11], a line immunoblot assay [12], immunoblot approach – ELISAGRAM – combining separation by TLC and determination by ELISA [13], and an immunofiltration-based assay [14].

The goals of this study were: (i) to develop a clean-up tandem multiassay approach for the non-instrumental determination of at least two analytes in intense coloured foodstuffs; (ii) to optimize the clean-up tandem immunoassay columns for simultaneous OTA and AfB1 determination in spices with cut-off levels of 10 and 5 $\mu g\,kg^{-1}$, respectively; (iii) to screen spice

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samples for AfB1 and OTA contamination with clean-up tandem immunoassay and to confirm with multi-LC–MS/MS.

For spices (*Capsicum* ssp. spices, ginger, nutmeg, black pepper, white pepper and turmeric) the EU established the limit for total aflatoxins (sum of aflatoxins B1, B2, G1, G2) at 10 μg kg^{-1}, for AfB1 as the most toxic and widespread aflatoxin at 5 μg kg^{-1} [15] and discusses a limit for OTA in *Capsicum* ssp. spices, ginger, nutmeg, black pepper and white pepper at 10 μg kg^{-1}.

2. Experimental

2.1. Materials

AfB1, aflatoxin B2 (AfB2), aflatoxin G1 (AfG1), aflatoxin G2 (AfG2) and OTA standards and Tween 20 were purchased from Sigma Chemical Co. (Bornem, Belgium). Rabbit anti-mouse immunoglobulin (IgG) (protein concentration: 2.7 g L^{-1}) was supplied by DakoCytomation (Heverlee, Belgium). CNBr-activated Sepharose 4B was purchased from Amersham Biosciences AB (Uppsala, Sweden). Aminopropyl derived silica, Bondesil NH2 (NH2) (mean particles diameter 0.040 mm), tubes (Bond Elut reservoir, 1 and 3 mL) and polyethylene frits (1/4 and 3/8 in. diameter) were supplied by Varian Belgium NV/SA (Sint-Katelijne-Waver, Belgium).

AfB1-horse radish peroxidase (AfB1-HRP) and OTA-horseradish peroxidase (OTA-HRP) conjugates were prepared by the Diagnostic Laboratory, Agricultural Biotechnology Center, Gödöllö, Hungary. Monoclonal antibodies against AfB1 and OTA were produced and characterised by the same institute. The anti-OTA antibody was an IgG1 with kappa light chains with a 9.3% cross-reaction with ochratoxin B but none at all with ochratoxin α, coumarin, 4-hydroxy-coumarin and D,L-phenylalanine. The anti-AfB1 antibody was IgG2a with 79% cross-reaction with aflatoxin M1, 33% with aflatoxin M2, 76% with AfB2, 55% with AfG1, 6% with AfG2 and none at all with aflatoxin B2a and aflatoxin G2a. Phosphate-buffered saline (PBS) 0.01 M, pH 7.4, was used as assay buffer. PBS with 0.05% Tween was used as wash solution. Proclin 300 (5-chloro-2-methyl-4-isothiazolin-3-one and 2-methyl-4-isothiazolin-3-one) was purchased from Supelco (Bellefonte, PA, USA) and was added to the buffers as an antimicrobial preservative. Methanol was HPLC-grade and water was obtained from a Milli-Q Gradient System (Millipore, Brussels, Belgium). The substrate chromogenic solution used was Colorburst™ Blue TMB/Peroxide (ALerCHEK Inc. USA). Stock solutions of AfB1 and OTA (1 mg mL^{-1}) and working solutions (10, 1.0, 0.10 μg mL^{-1}) were prepared in methanol and stored at −20 °C. Ederol filters No. 15, 110 mm were purchased from Binzer and Munktell Filter GmbH (Battenberg, Germany). AflaOchra HPLC™ immunoaffinity columns were supplied by Vicam (Cereal Tester, Fleurus, Belgium).

2.2. Instrumentation

The Waters HPLC system coupled to a Micromass Quatro micro triple quadrupole mass spectrometer was used (Waters, Milford, MA, USA). The analytical column was an Alltima C_{18}, 5 μm, 150 mm × 3.2 mm (Alltech Associates, Deerfield, IL, USA), the guard column was an Alltima C_{18}, 5 μm, 7.5 mm × 3.2 mm (Alltech Associates, Deerfield, IL, USA).

2.3. Procedures

2.3.1. AfB1 and OTA extraction

For the AfB1 and OTA extraction from spices 2.5 g of sample were extracted with 7.5 mL MeOH/3% $NaHCO_3$ water solution (80/20, v/v). After shaking for 15 min at ±200 rpm with an orbital shaker (Orbital Shaker SO3, Stuart Scientific, UK) the extract was filtered through an Ederol filter. This extract (0.5 mL) was diluted with 1 mL of water or 3% $NaHCO_3$ water solution.

2.3.2. Optimization of clean-up conditions

A polyethylene frit was put in a 1 mL tube, followed by the addition of 200 mg of NH2. A second frit was placed above the clean-up layer. OTA and AfB1 solutions (1.5 mL; 2.0, 4.0 and 6.0 ng mL^{-1}) in MeOH/water (25/75, v/v; pH 5.5) or MeOH/3% $NaHCO_3$ water solution (25/75, v/v; pH 9.0) were applied on this clean-up layer. The recoveries of AfB1 and OTA were calculated as a ratio of the peak areas after and before the clean-up step using an HPLC with fluorescence detection. The Waters Alliance 2695 XE HPLC system coupled to a Waters 474 Scanning Fluorescence detector was used (Waters, Milford, MA, USA). The analytical column was an Alltima C_{18}, 5 μm, 150 mm × 3.2 mm (Alltech Associates, Deerfield, IL, USA), the guard column was an Alltima C_{18}, 5 μm, 7.5 mm × 3.2 mm (Alltech Associates, Deerfield, IL, USA). The injection volume was 50 μL. Mobile phase composition was 50% ACN, 48% H_2O, 2% acetic acid for OTA detection and 50% ACN, 50% H_2O for AfB1 detection. Flow rate was 1 mL min^{-1}. The excitation/emission wavelengths were set at 333/470 nm for OTA and 365/455 nm for AfB1.

2.3.3. Clean-up tandem immunoassay columns

The clean-up sorbent (200 mg of NH2) was placed on the bottom of the 1 mL tube above the polyethylene frit (Fig. 1). The next frit was put over this clean-up layer. The detection immunolayers were placed on top, separated by a frit, and then the last frit. Before placing into the tube the primary mouse anti-AfB1 or anti-OTA antibodies were bound to the secondary rabbit anti-mouse antibodies in the assay gel, as follows: 0.5 mL of coupled gel (CNBr-activated Sepharose 4B with coupled secondary rabbit anti-mouse antibodies, diluted with PBS 1:3) was added to 2.5 mL of blocked gel (CNBr-activated Sepharose 4B with glycine blocked active groups, diluted with PBS 1:3) in a 3 mL tube with a bottom polyethylene frit. PBS excess was removed under gravitation. Then 400 μL of the primary mouse anti-AfB1 or anti-OTA antibody solution were added and carefully mixed. In 5 min this solution was flowed through using a plunger and the gel was washed two times with 3 mL of PBS. Then 3 mL of PBS were added and the assay gel was mixed. PBS (100 μL) and then 200 μL of the prepared assay gel solution with bound anti-OTA antibodies were placed on the second frit into the 1 mL clean-up tandem immunoassay column. Above this anti-OTA detection layer the third frit was placed. Assay gel solution with bound anti-AfB1 antibodies (200 μL) was put on