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Determination of Ochratoxin A traces in foodstuffs: Comparison of an automated on-line two-dimensional high-performance liquid chromatography and off-line immunoaffinity-high-performance liquid chromatography system

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ABSTRACT

Automated on-line two-dimensional high-performance liquid chromatography method (2D-HPLC) is proposed to determine Ochratoxin A (OTA) in food samples as an alternative to OTA immunoaffinity column (IAC). An on-line 2D-HPLC system is designed for the analysis of OTA using an affinity-based monolithic column in the first dimension and reversed-phase C18 column in the second dimension. Initially, optimal OTA separation efficiency is determined through traditional HPLC system consisting of a P(HEMAPA) monolithic column coupled with HPLC system. Secondly, after providing optimum conditions, OTA determination was investigated through the 2D-HPLC system. According to results, 2D-HPLC system showed good linearity in the range 0.5 to 20 ng/mL with limit of detection (LOD) and limit of quantification (LOQ) values of 21.2 pg/mL and 64.3 pg/mL, respectively. The P(HEMAPA)-4 monolithic column displayed good recovery of OTA ranging from 104.34% to 107.33%. Relative standard deviations (RSD) varied in the range 0.21% to 1.31% thus indicating the efficiency of P(HEMAPA)-4 monolithic column developed for OTA.

1. Introduction

Ochratoxin A is a mycotoxin and the most toxic of the group of ochratoxins, mainly produced by *Aspergillus* and *Penicillium* [1]. OTA contamination has been reported to occur in cereals and derivatives (wheat, corn, barley), cocoa, coffee, baby formula, spices, beer, grape juice, and wine [2]. Among the mycotoxins in food and feed, OTA is considered as the most nephrotoxic, carcinogenic, teratogenic, hepatotoxic, and immunosuppressive [3,4]. The International Agency for Research on Cancer (IARC) classified OTA as a group 2B possible human carcinogenetic while the European Food Safety Authority (EFSA) suggested tolerable weekly intake (TWI) of 120 ng OTA per kg body weight in 2006 [5].

Several techniques have been proposed for determining OTA in food and foodstuffs, including enzyme-linked immune sorbent assays (ELISA), thin-layer chromatography (TLC), liquid chromatography-mass spectrometry (LC–MS), high-performance liquid chromatography with fluorescence detection (HPLC-FLD), and diverse sensors [6–10]. HPLC-FLD is the most commonly used analytical technique for OTA determination in food due to allowing a very low detection limit [6,11,12]. On the other hand, HPLC-FLD is time-consuming, difficult to automate and require pretreatment procedures prior to analytical determination [13,14]. In this respect, two-dimensional HPLC (2D-HPLC) provides several advantages over one-dimensional HPLC due to automation of the entire system, precise, easy to use and user-friendly [15–18].

2D-HPLC is a liquid chromatography technique that combines two different chromatographic columns through different modes of separations such as ion-exchange, reversed-phase, hydrophobic, hydrophilic and affinity columns. The most important step is the selection of chromatographic column with respect to characteristics of samples. Furthermore, the choice of a variety of LC columns is a critical point to achieve a proper retention time and resolve specific separation problems [19,20]. 2D-HPLC could be performed as off-line and on-line modes, the on-line mode is superior to the off-line version due to its feasibility to automation, better reproducibility, elimination of expert user requirements, and reduction of sample loss [21–23]. Moreover, 2D-HPLC could be utilized in many multidimensional approaches, which allow easily generation

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of high peak capacity, high resolution, high sampling rate, and short analysis time [16,22,24,25].

Currently, the sample preparation processes including extraction, purification, and preconcentration should be applied to eliminate major interfering components while preconcentrating the analytes to enhance sensitivity prior to determination of OTA by analytical techniques. The most frequent sample preparation process for OTA is solid-phase extraction (SPE) with immunoaffinity columns (IACs) [11,26]. As IACs-SPE technique utilizing antibodies as molecular recognition probe for OTA has some drawbacks such as single-use, high cost and antibodies denaturation. Monolithic columns present promising alternative materials to IAC having the advantages as continuous unitary structure, interconnected porous network, simple preparation procedure, cost-effectiveness, and reusability [22,27–32].

The goal of the present study is to develop a 2D-HPLC system for on-line analysis of OTA using two different columns including monolithic HPLC columns based on amino acid-based hydrophobic affinity column and reversed-phase C18 columns. The monolithic column was prepared using a functional monomer derived from L-phenylalanine amino acid for the affinity interactions with OTA. L-phenylalanine amino acid-based functional monomer was chosen due to OTAs chemical structure which is composed of dihydroisocoumarin linked to L-β-phenylalanine [33]. The hydrophobic L-phenyl alanine-containing column was utilized as the first column while the reversed-phase C18 column was used as the second column; they were coupled to an HPLC system for selective and rapid OTA determination in foodstuffs.

2. Experimental

2.1. Chemicals

2-Hydroxyethyl methacrylate (HEMA), ethylene glycol dimethacrylate (EGDMA), L-phenylalanine, methacryloyl chloride, and acetic acid were obtained from Sigma-Aldrich (St. Louis, Missouri, USA). Potassium persulfate (KPS), 2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethane sulfonic acid (HEPES), methanol (LC Grade), and acetonitrile (LC Grade) were purchased from Merck (Darmstadt, Germany). Ochrastandart and Ochraprep® immunoaffinity column (used for comparison) were obtained from R-Biopharm Rhone (Darmstadt, Germany). Deionized water used in all experiments was purified using a Barnstead ion-exchange ultra-pure water system (Dubuque, IA) which had an ROpure LP reverse osmosis high-flow cellulose acetate membrane (Barnstead D2731) and Barnstead D3804 NANOpure organic/colloid removal filters.

2.2. Instrumentation

The prominence 2D-HPLC system (Shimadzu Corp., Kyoto, Japan) for OTA determination is a column-switching HPLC system equipped with two LC-20AT pumps, a CBM-20 A Lite system controller, a CTO-10ASvp column oven, an FCV-20AH$_2$ 6-port 2-position switching valves, an SIL 20 AHT automatic injection unit, and an RF-20 A fluorescence detector system. Reversed phase HPLC column (Inertsil ODS-3 150 × 4.6 mm, 5 μm) used in this study was obtained from GL-Sciences Inc.

2.3. Columns

Monolithic column was prepared in the laboratory [28] according to the procedure as follows: firstly, the synthesis of *N*-methacryloyl-(L)-phenylalanine (MAPA) as a functional monomer was adapted from the procedure reported in our previous study [34]. In the process of preparing poly(2-hydroxyethyl methacrylate-*N*-methacryloyl-(L)-phenylalanine), [P(HEMAPA)] monolithic column, in situ bulk polymerization was performed in a stainless steel HPLC column (150 x 4.6 mm id) according to procedure as follows: HEMA, (1.0 mL) and EGDMA (0.25 mL) were used in the polymerization recipe as monomer phase and cross-linker, respectively. Different amounts of MAPA monomer (column code: 62.5 μmol, 125 μmol, 187.5 μmol, and 250 μmol for P(HEMAPA)-1, P(HEMAPA)-2, P(HEMAPA)-3, and P(HEMAPA)-4, respectively) were dissolved in HEPES buffer (0.5 mL, pH: 4.5). Toluene (0.5 mL) was added to the polymerization solution as a pore former, and this solution was stirred at room temperature. KPS was added to initiate polymerization and this solution was mixed for 10 min before nitrogen gas was flowed through the solution to remove dissolved oxygen. The prepared mixture was poured into an empty HPLC column (150 x 4.6 mm id) and was sealed at both ends with stoppers. In situ bulk polymerization was performed at 75 °C for 3.5 h. After polymerization, unreacted monomers and other components were removed from the monolithic column by washing with deionized water and ethanol. P(HEMA) monolithic column was synthesized using the same polymerization solution without MAPA monomer. In the second dimension, reversed phase C18 column (Inertsil ODS-3 150 x 4.6 mm, 5 m) was used.

2.4. Solvents

In the one-dimensional HPLC system (1D-HPLC), multiple solvent systems were coded as solvent A [acetonitrile (ACN, 100%, v/v)] and solvent B [water/acetic acid (H_2O/HAC; 98:2, v/v)].

In the two-dimensional HPLC system (2D-HPLC), the prepared solvents were for Pump A: solvent A (H_2O/HAC; 98:2, v/v) and solvent B [ACN (100%, v)]; for Pump B, solvent A [ACN:H_2O (25:75 including 2% HAC), v/v)], solvent B [ACN:H_2O (30:70 including 2% HAC, v/v)], and solvent C [ACN:H_2O (60:40 including 2% HAC, v/v)].

2.5. Chromatographic conditions for 1D- and 2D-HPLC systems

In the one-dimension, high-performance liquid chromatography was performed on an LC-20 HPLC system. The flow rate was adjusted to 0.5 mL/min while the temperature was kept constant at 40 °C and the injection volume was 100 μL. The gradient condition was given in Table 1 (left side). All of the mobile phases were filtered through 0.45 μm membranes before used.

An on-line 2D-HPLC system, including two LC-20AT pumps (Pump A and Pump B) and a 6-port 2-position switching valves system, was used differently from 1D-HPLC system. The mobile phases consisted of solvents A and B for pump A and solvents A, B, and C for pump B. The gradient condition was given in Table 1 (right side). 2D-HPLC analyses were performed at a constant temperature of 40 °C. The excitation and emission wavelengths of fluorescence detection, respectively, 333 nm and 443 nm throughout all experiments were the same in both dimensions. Data acquisition was ensured with LabSolution software (Version 5.86, Shimadzu Corp).

2.6. Optimization of OTA determination with 1D-HPLC system

Prepared P(HEMAPA) monolithic columns (150 × 4.6 mm id) were used in 1D-HPLC analyses to evaluate the performance of the columns as pretreatment column. OTA standard solutions were prepared by dilution of the Ochrastandard (Ochratoxin A standard solution at a concentration of 1000 ng/mL in methanol was obtained from R-Biopharm Rhone) in 51% ACN in water (2% HAC, v/v). OTA adsorption dynamics were investigated on P(HEMAPA) monolithic column using several factors including initial OTA concentration, the composition of mobile phase, temperature, injection volume, flow rate, and the amount of MAPA monomer in the monolithic column to get optimum conditions. The affecting factors including OTA concentration, mobile phase composition, flow

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