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Determination of ochratoxins in nuts and grain samples by in-tube solid-phase microextraction coupled with liquid chromatography–mass spectrometry

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ABSTRACT

A simple and sensitive method for the determination of ochratoxins A and B in nuts and grain samples was developed using an automated in-tube solid-phase microextraction (SPME) coupled with liquid chromatography–mass spectrometry (LC–MS). Ochratoxins were separated within 5 min by high-performance liquid chromatography using an Inertsil ODS-3 column with 5 mM anmonium acetate/acetonitrile (65/35, v/v) as the mobile phase. Electrospray ionization conditions in the positive ion mode were optimized for mass spectrometric detection of ochratoxins. The pseudo molecular ion $[M+H]^+$ was used to detect ochratoxins with selected ion monitoring (SIM) mode. The optimum in-tube SPME conditions were 20 draw/eject cycles of 40 μL of sample using a Carboxen-1006 PLOT capillary column as an extraction device. The extracted ochratoxins were easily desorbed from the capillary by passage of the mobile phase, and no carryover was observed. Using the in-tube SPME/LC–MS with SIM method, good linearities of the calibration curves ($r = 0.9993$ for ochratoxin A and $r = 0.9989$ for ochratoxin B) were obtained in the concentration range from 0.5 to 20 ng/mL. The detection limits (S/N = 3) for ochratoxins A and B were 92 and 89 pg/mL, respectively. The in-tube SPME method showed above 15–19-fold greater sensitivity than the direct injection method (10 μL injection). The within-day and between-day precisions (relative standard deviations) were below 5.1% and 7.7% ($n = 6$), respectively. This method was applied successfully to analysis of nuts and grain samples without interference peaks. The recoveries of ochratoxins spiked into extraction solution from nut samples were above 88%. Ochratoxins were detected at 0.7–8.8 ng/g levels in various nuts and grain samples.

1. Introduction

Ochratoxins A (OTA) and B (OTB) (Fig. 1) are naturally occurring mycotoxins produced by several species of the general Aspergillus and Penicillium like *Aspergillus ochraceus* or *Penicillium viridicatum* [1,2]. Ochratoxin is known to occur in commodities such as cereals, coffee, dried fruit and red wine. It has been shown to be hepatotoxic, nephrotoxic, teratogenic and carcinogenic to animals and is classified as a possible human carcinogen (category 2B) by the International Agency for Research on Cancer (IARC) [3]. Moreover, ochratoxin A is suspected to be the causative agent behind Balkan endemic nephropathy (BEN), a kidney disease encountered among the population of Southern Europe [4]. Therefore, ochratoxin contamination is a worldwide problem concerning food and feed safety and several countries have instituted ochratoxin restrictions in nuts and grain. The EC regulations have set a maximum tolerable limit for ochratoxin A at 3 μg/kg for all products derived from unprocessed cereals [5]. In view of the recognized adverse effects caused by ochratoxin and the need for regulatory control, monitoring of its level in nuts and grain samples is important to evaluate health risks due to human consumption of these products. Therefore, a sensitive, selective, and simple method to determine the presence and level of ochratoxin in nuts and grain samples is essential.

Analyses of ochratoxins have been carried out mainly by high performance liquid chromatography (HPLC)-fluorescence detection (FLD) [6–10], liquid chromatography–mass spectrometry (LC–MS) or LC–MS/MS [11–17], and immunological methods [18]. The details of the determination of ochratoxins in food samples have been summarized in some reviews [19–22]. LC–MS methods require derivatization of ochratoxins prior to analysis, although they are highly sensitive. HPLC with FLD detection is most widely used and has been adopted as the Association of Official Analytical Chemists (AOAC) official method [23]. However, HPLC methods reported previously are less sensitive.

LC–MS methods are specific and sensitive, and are becoming increasingly popular. However, most of the above methods require sample preparation steps, such as extraction, concentration, and isolation. Although ethyl acetate extraction and solid-phase extraction have been usually used as sample preparation techniques, most of these techniques are complicated and time-consuming.

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Table 1
Program for in-tube SPME process.

Sequence	Event	Switching valve	Vial	Draw/eject		
				Cycle[a]	Volume (μL)	Speed (μL/min)
1	Conditioning of the capillary	Load	MeOH	D/E (2)	40	200
2	Drawing of air into the capillary	Load	Empty	D (1)	50	200
3	Conditioning of the capillary	Load	Water	D/E (2)	40	200
4	Extraction of analytes into the capillary	Load	Sample	D/E (20)	40	200
5	Needle washing	Load	MeOH	D/E (1)	2	200
6	Desorption of analytes from the capillary	Inject	–	–	–	–
7	HPLC separation of analytes and return to sequence 1	Load	–	–	–	

[a] D, draw; E, ejection.

Complicated pretreatment methods may introduce errors, and the use of large volumes of organic solvent poses a health hazard to those performing the analyses and contributes to environmental pollution. One efficient pretreatment method is fiber solid-phase microextraction (SPME) method, but extraction and desorption of samples are difficult to automate [24]. Therefore, it is important to develop an efficient sample pretreatment method, and automation will reduce labor and cost. A routine analysis method will also facilitate the processing of large numbers of samples.

In-tube SPME, using an open tubular fused-silica capillary with an inner surface coating as the SPME device, is simple and can be easily coupled on-line with HPLC and LC–MS. In-tube SPME allows convenient automation of the extraction process, which not only reduces the analysis time, but also provides better accuracy, precision, and sensitivity than manual off-line techniques. We have developed an in-tube SPME method for the determination of various compounds in food samples by coupling with HPLC [25,26] and LC–MS [27–29]. The details of the in-tube SPME technique and its applications have been summarized in a number of reviews [30–35]. Here, we report an automated on-line in-tube SPME/LC–MS method for the determination of ochratoxins in nuts and grain samples.

2. Experimental

2.1. Materials

OTA and OTB were purchased from Sigma–Aldrich Japan (Tokyo, Japan) and dissolved in methanol to make a stock solution at a concentration of 1 mg/mL. The solutions were stored at 4 °C and diluted to the required concentrations with pure water prior to use. LC–MS grade acetonitrile and water used as mobile phases were purchased from Kanto-kagaku (Tokyo, Japan). All other chemicals were of analytical grade.

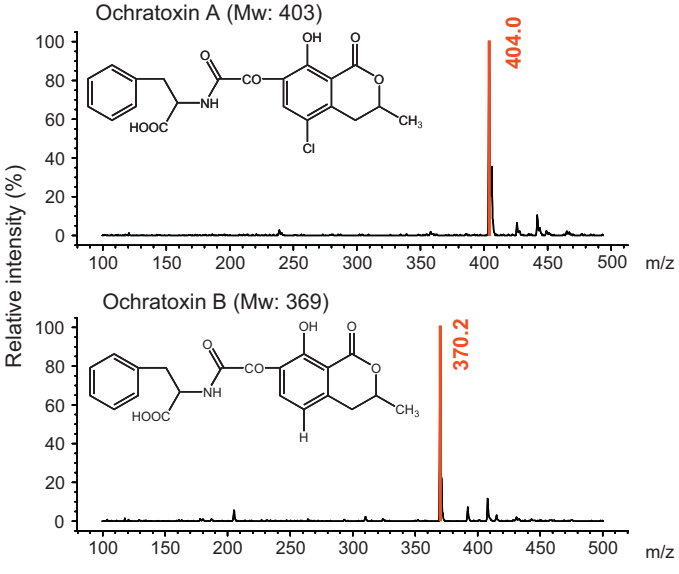

Fig. 1. ESI-mass spectra of OTA and OTB.

2.2. Instrument and analytical conditions

The LC–MS system was a Model 1100 series LC coupled with an atmospheric pressure (AP) electrospray ionization (ESI) MS (Agilent Technologies, Boeblingen, Germany). An Inertsil ODS-3 column (50 mm × 2.1 mm, particle size of 4 μm; GL Science Inc., Tokyo, Japan) was used for LC separation under the following conditions: column temperature, 40 °C; mobile phase, 5 mM anmonium acetate/acetonitrile (65/35, v/v); and flow rate, 0.2 mL/min for 10 min run. ESI-MS conditions were as follows: nebulizer gas N_2 (50 psi); drying gas, N_2 (12 L/min, 350 °C); fragmentor voltage, 110 V; capillary voltage, 2000 V; ionization mode, positive mode; mass scan range, 100–500 amu; selected ion monitoring (SIM), *m/z* 404 (ochratoxin A) and 370 (ochratoxin B); and dwell-times for the ions in SIM, 289 ms. LC–MS data were processed with an HP ChemStation.

2.3. In-tube solid-phase microextraction

A GC capillary column (60 cm × 0.32 mm i.d.) was used as the in-tube SPME device, and placed between the injection loop and injection needle of the autosampler. The injection loop was retained in the system to avoid fouling of the metering pump. Capillary connections were facilitated by use of a 2.5 cm sleeve of 1/16-in polyetheretherketone (PEEK) tubing at each end of the capillary. PEEK tubing with an internal diameter of 330 μm was shown to be suitable to accommodate the capillary used. Standard 1/16-in stainless steel nuts, ferrules, and connectors were used to complete the connections. CP-Sil 5CB, CP-Sil 19CB (Varian Inc., Lake Forest, CA, USA), Supel Q PLOT and Carboxen-1006 PLOT (Supelco, Bellefonte, PA, USA) were tested for comparison of extraction efficiency. The autosampler software was programmed to control the in-tube SPME extraction, desorption, and injection. (A) *Sampling and extraction*: vials (2 mL) were filled with 1 mL of sample for extraction, and set into the autosampler programmed to control the SPME extraction and desorption technique. In addition, 2-mL autosampler vials with a septum, one containing 1.5 mL of methanol and another containing 1.5 mL of water, were set into the autosampler. The capillary column was washed and conditioned by two repeated draw/eject cycles (40 μL each) of these solvents, and then a 50-μL air plug was drawn prior to the extraction step. The extraction of ochratoxin onto the capillary coating was performed by 20 repeated draw/eject cycles of 40 μL of sample at a flow rate of 150 μL/min with the six-port valve in the LOAD position. After extraction, the tip of the injection needle was washed by one draw/eject cycle of 2 μL of methanol. (B) *Desorption and LC–MS analysis*: the extracted