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Short Communication

Identification of ochratoxin A in food samples by chemical derivatization and gas chromatography–mass spectrometry

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ABSTRACT

The contamination of foods with ochratoxin A can be determined very sensitively by high-performance liquid chromatography (HPLC) with fluorescence detection. A novel procedure is described to confirm OA-positive results quantitatively down to the HPLC detection limit of 0.1 ppb. For this, ochratoxin A in the sample extract is converted into its O-methylochratoxin A methyl ester derivative, which is identified subsequently by gas chromatography–mass spectrometry negative-ion chemical ionization and multiple ion detection modes using the hexadeuterated O-methyl-d_3-ochratoxin A methyl-d_3 ester derivative as internal standard for quantification. In the analysis of more than 60 contaminated samples, the procedure was found to be very accurate.

INTRODUCTION

The contamination of foods and feeding stuffs by the mycotoxin ochratoxin A (OA) {(*R*)-N-[5-chloro-3,4-dihydro-8-hydroxy-3-methyl-1-oxo-1*H*-2-benzopyran-7-yl)carbonyl]-L-phenylalanine, $C_{20}H_{18}ClNO_6$, [303-47-9]} is a problem of international concern [1–3]. It has been reported that OA is implicated in Balkan endemic nephropathy, a chronic renal diseasec in man [4,5]. Recently, the Joint FAO/WHO Expert Committee on Food Additives proposed a provisional tolerable weekly intake (PTWI) of 112 ng/kg body weight [6].

For the determination of OA in foods, high-performance liquid chromatography (HPLC) with fluorescence detection has often been used. Positive results have been confirmed by different techniques, *e.g.*, by changing the fluorescence excitation wavelength [7] or scanning the fluorescence spectrum [8], by thin-layer chromatography [9], direct-inlet mass spectrometry (MS) [10], HPLC–MS [11], enzyme-linked immunoassay [12] or methylation of OA to OA methyl ester (OA-Me) or O-methyl-OA methyl ester (OA-Me_2), followed by identification of these derivatives by HPLC [13–15] or gas chromatography–electron impact MS (GC–EI-MS) [16]. However, only immunoassay was suitable for confirming positive OA results in the detection limit range 0.1–0.3 ppb (μg/kg).

This paper describes a sensitive and accurate procedure for the confirmation of OA in foods by its conversion to OA-Me_2, which is detected subsequently by means of GC–negative-ion chemical ion-

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ization (NICI) MS in the multiple ion detection (MID) mode using hexadeuterated OA-Me_2-d_6 as internal standard.

EXPERIMENTAL

Chemicals

OA was obtained from Sigma. A stock solution (100 μg/ml) was prepared in methanol. For the generation of diazomethane and deuterated diazomethane, Diazald and Deutero-Diazald Prep Set, respectively, from Aldrich were used.

Instrumentation

HPLC was performed using a Varian Model 5000 liquid chromatograph equipped with a Hewlett-Packard Model 1046 A programmable fluorescence detector. The analytical conditions were as follows: column, Merck LiChrospher 100 RP-18, 5 μm (250 mm × 4 mm I.D.); mobile phase, water–acetonitrile–acetic acid (525:450:25); flow-rate, 1.0 ml/min; injection volume, 20 μl; excitation wavelength, 330 nm; and emission wavelength, 460 nm.

The GC–MS equipment consisted of a Finnigan Model 4500 quadrupole mass spectrometer with an Incos data system interfaced by direct coupling with a Hewlett-Packard Model 5890 gas chromatograph with on-column injector. The GC column used for the analysis was a 10 m × 0.25 mm I.D. Supelco PTE-5 fused-silica capillary column (film thickness 0.25 μm) coupled with a 2 m × 0.53 mm I.D. deactivated J&W Scientific retention gap. Initially the column was held at 60°C for 2 min and then programmed at 25°C/min to the final temperature of 310°C with a hydrogen carrier gas flow-rate of *ca.* 70 cm/s. Samples were injected with a Hamilton on-column syringe with a 0.17 mm O.D. fused-silica needle. When after a few injections the GC peak became broad, about 20 cm of the retention gap were cut off.

The MS conditions were as follows: NICI–MID mode, reagent gas, methane; pre-pressure, 0.45 Torr; operating pressure, $3 \cdot 10^{-5}$ Torr; electron energy, 70 eV; emission current, 0.30 mA; multiplier voltage, 1.45 kV; ion source temperature, 120°C; interface temperature, 270°C; scan time for *m/z* 416, 417, 419, 431 and 437 ions, 0.2 s each.

Sample preparation

The described clean-up procedure is a modification of the method of Hadlok *et al.* [17]. A 30-g aliquot of the finely ground sample was mixed with 50 ml of 0.4 *M* magnesium chloride solution, 30 ml of 2 *M* hydrochloric acid and 100 ml of toluene in a 500-ml centrifuge tube. The tube was shaken automatically for 60 min and then clarified by centrifugation at 15 900 *g* (9000 rpm) for 40 min. A 50-ml volume of the supernatant toluene layer was run through a Sep-Pak silica cartridge, prewetted with 5 ml of toluene. The cartridge was washed with 10 ml of *n*-hexane, 20 ml of *n*-hexane–diisopropyl ether (1:1), 20 ml of toluene–acetone (95:5) and 10 ml of toluene. Thereafter OA was eluted with 10 ml of toluene–acetic acid (9:1). The eluate was evaporated to dryness on a rotary evaporator and the residue dissolved in 0.50 ml of methanol. A 20-μl volume of this extract was injected on to the HPLC column. For calculation the external standard method was used.

Preparation of the internal standard OA-Me_2-d_6

An excess of an ethereal solution of deuterated diazomethane, freshly prepared from Diazald-N-methyl-d_3 [18], was added dropwise to a solution of 100 μg of OA in 5 ml of deuterium oxide and 2 ml of 2-(2-ethoxyethoxy)ethan(ol-*d*) in a brown 250-ml flask. The mixture was stirred overnight and then concentrated using a gentle stream of nitrogen. After transferring the residue by means of 2 ml of water–methanol (3:1) to a Sep-Pak C_{18} cartridge, prewetted with 2 ml of methanol and 5 ml of water, the cartridge was washed with 10 ml of water–methanol (3:1) and OA-Me_2-d_6 was eluted with 10 ml of methanol–water (3:1). The eluate was evaporated to dryness and the residue dissolved in 10 ml of acetone. The absence of OA and OA-Me-d_3 in the HPLC trace confirmed the quantitative reaction of OA to its OA-Me_2-d_6 derivative. The standard remains stable for at least 4 months.

Derivatization of the sample extract

To 400 μl of the methanolic sample extract an amount of OA-Me_2-d_6 was added that was comparable to the amount of OA in the sample determined. Subsequently an excess of an ethereal solution of diazomethane, freshly prepared from Diazald [18], was added. After automatic shaking over-

night, the solvents were removed, the residue transferred by means of 0.2 ml of methanol to a Sep-Pak C_{18} cartridge, prewetted with 2 ml of methanol and 5 ml of water, and the cartridge was washed with 5 ml of water–methanol (3:1). Subsequently, OA-Me_2 was eluted with 5 ml of methanol–water (3:1) and the eluate evaporated to dryness. The residue was dissolved in 50–80 μl of methanol and injected on to the HPLC column. The fraction eluting between 22 and 29 min was collected, evaporated to dryness and again cleaned, as described above, using a Sep-Pack C_{18} cartridge. Finally, the OA-Me_2-containing residue was dissolved in 50 or 100 μl of acetone and a 1-μl aliquot was injected into the GC–MS system. Comparing the areas of the base peaks at m/z 416 and 419, the internal standard method was used for calculation. The small amount of m/z 419 ion derived from OA-Me_2 (due to the chlorine isotope abundance, 32% of the area of the m/z 417 peak) was taken into consideration.

RESULTS AND DISCUSSION

Analytical procedure

Over 400 food samples were analysed for contamination with OA; the results will be published elsewhere on completion of the study. The analytical procedure given proved to be very rapid and reliable. On spiking OA-free samples with 0.3–5.0 ppb of OA, the recovery was 74–113%. The detection limit was about 0.1 ppb. To confirm positive results, OA can be determined very sensitively, as described here, at levels down to the HPLC detection limit by its derivatization to OA-Me_2 and subsequent GC–MS analysis in the NICI and MID modes. Hexadeuterated OA-Me_2-d_6 served as an internal standard for quantification.

The NICI mass spectra of OA-Me_2 and OA-Me_2-d_6 are shown in Fig. 1a and b, respectively. The base peaks at m/z 416 and 419 are assigned to the $[M-CH_3]^-$ and $[M-CD_3]^-$ ions, respectively, and the peaks at m/z 431 and 437 to the molecular ions M^-. Separated by 2 mass units and corresponding to the natural abundance, the chlorine isotope peaks are recognizable in each instance. To increase the sensitivity of the GC–MS analysis, the MID mode using the m/z 416, 417, 419, 431 and 437 ions was applied. The fragment ion of m/z 418 was not chosen because of occasional interferences from unknown compounds eluting close to OA-Me_2.

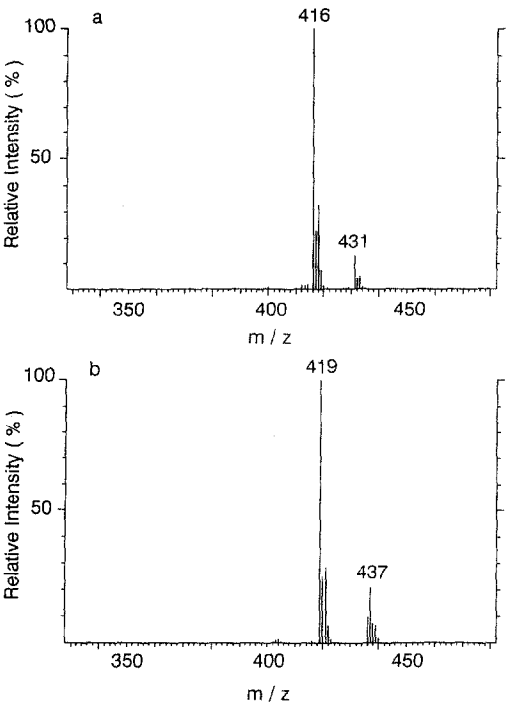

Fig. 1. NICI mass spectra of (a) OA-Me_2 and (b) hexadeuterated OA-Me_2-d_6.

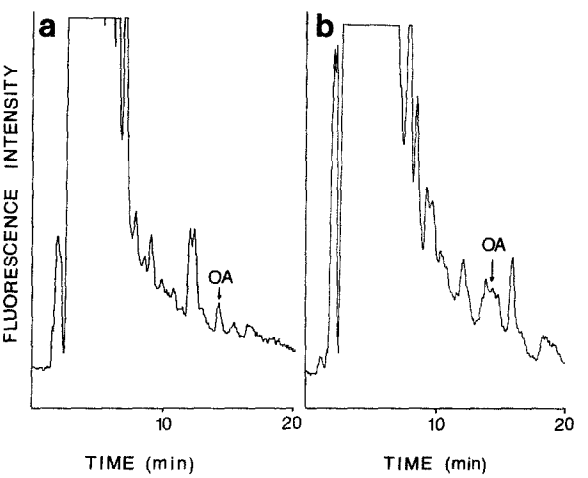

Fig. 2. HPLC determination of OA in (a) a sample of a maize–peanut snack containing 0.13 ppb of OA and (b) a bran sample containing less than 0.17 ppb of OA.

Analysis of samples

Sixty-seven of the samples that according to the HPLC analysis were contaminated with OA were analysed by the GC–MS method. In one sample OA was not detected. Although the HPLC retention time of one peak in this sample was very close to the retention time of OA and there was no shoulder on the HPLC trace after addition of OA, according to the GC–MS data we believe that an unknown compound co-eluted here.

For the other 66 samples, the agreement of the results of HPLC and GC–MS analyses was very good. If the samples were contaminated with more than 0.5 ppb, 99.0% of the value estimated by HPLC was found by GC–MS analysis on average (n = 22, S.D. = 13.1); for less contaminated samples (0.07–0.50 ppb OA) the value was 89.1% (n = 44, S.D. = 33.2).

Fig. 2a and b shows the HPLC of a maize–peanut snack and a wheat bran sample, respectively. The appropriate GC–MS results are shown in Fig. 3a and b. For the snack the contamination with OA was estimated to be 0.13 ppb by both HPLC and GC–MS. Owing to the overlapping peaks, the OA contamination of the bran sample could not be determined accurately by HPLC, but was calculated to be less than 0.17 ppb; using GC–MS, 0.13 ppb of OA was determined.

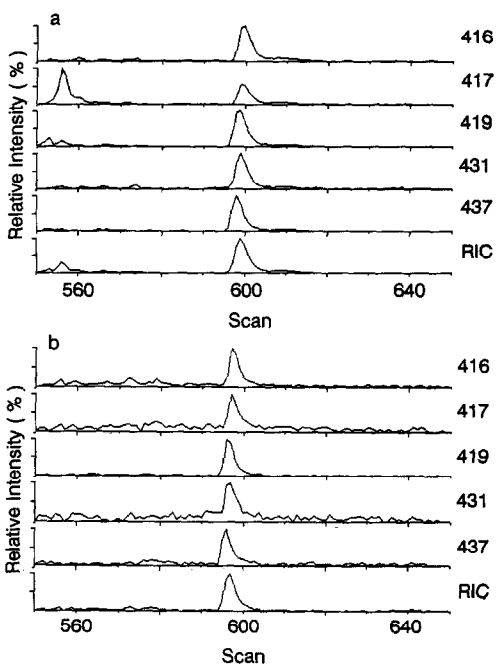

Fig. 3. MID chromatograms of OA-Me_2 and hexadeuterated OA-Me_2-d_6 in the same samples as illustrated in Fig. 2: (a) maize–peanut snack; (b) bran sample.

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