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Short communication

Column liquid chromatography–electrospray ionisation–tandem mass spectrometry for the analysis of ochratoxin

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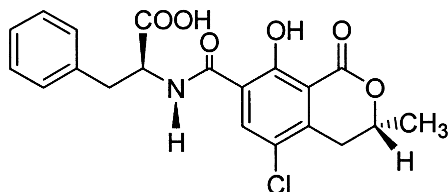
Abstract

A column liquid chromatography–electrospray ionisation–tandem mass spectrometry (LC–ESI-MS–MS) method is described for the analysis of ochratoxin A (OTA) in human food. In combination with selected reaction monitoring (SRM) detection limits of 20 pg (50 fmol) OTA on column were established by the use of model solutions. The present LC–ESI-MS–MS method allows the sensitive and selective monitoring of OTA in complex matrices. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Mass spectrometry; Ochratoxin; Mycotoxins

1. Introduction

Ochratoxin A (OTA, Scheme 1) is a potent nephrotoxic and nephrocarcinogenic mycotoxin produced by several *Aspergillus* and *Penicillium* species. In addition, immunosuppressive, teratogenic and carcinogenic effects have been reported. OTA is suspected as a possible cause of a chronic kidney disease in south-eastern Europe known as 'balkan endemic nephropathy' (BEN), and urinary tract tumors (UTT) [1–5]. Since *Aspergillus* and *Penicillium* species grow well on a variety of substrates and under different conditions of moisture, pH and temperature, the natural occurrence of OTA in human food and animal feed is widespread. Basically, starch-rich food and derived products are contaminated, but OTA has also been found in other products such as coffee, beer, sausage, cow's milk and, more recently, in wine and grape juice [1–3,6–10].



Scheme 1. Ochratoxin A (OTA).

Numerous methods have been described for the determination of ochratoxin A in feed, food, body fluids and animal tissues. Most are based on column liquid chromatography (LC) using reversed-phase columns with fluorescence detection [6–15]. However, analytical problems such as coelution of interfering compounds or retention time shifts can lead to erroneous positive or negative results. This problem can be overcome by the direct coupling of LC and

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mass spectrometry (MS) using soft ionisation techniques. The latter method combines the selectivity and structural information provided by MS with the advantages of LC. The sensitivity of LC–MS depends on the type of interface used and is also affected by the structure of the compound. In the past, Ochratoxin A was determined by LC–MS using a thermospray or a direct liquid introduction interface [16,17]. However, these belong to the first generation of interfaces with several disadvantages in terms of robustness, sensitivity and ease of operation compared to the recently developed soft ionisation techniques such as atmospheric pressure chemical ionisation (APCI) and electrospray ionisation (ESI) [18]. In this paper, we describe the use of LC–ESI-MS–MS for the analysis of ochratoxin A. This combination provides highly structure-specific detection for the determination of ochratoxin A in complex matrices. The sensitivity was enhanced by operating the MS in the selected reaction monitoring (SRM) mode.

2. Experimental

2.1. Materials and reagents

Crystalline Ochratoxin A standard was obtained from Sigma (Deisenhofen, Germany). A stock solution was prepared by dissolving 1 mg OTA in 100 ml of methanol. The stock solution was calibrated spectrophotometrically at 333 nm, using the extinction coefficient 5550 $M^{-1}\,cm^{-1}$ [19]. All other standard solutions were prepared immediately before use by diluting the stock solution with methanol. The stability of the stock solution was checked spectrophotometrically, OTA was stable in the refrigerator over a period of several months. All solvents were of gradient-grade quality and purchased from Merck (Darmstadt, Germany). All other chemicals were of analytical grade and obtained from Fluka (Neu-Ulm, Germany) or Sigma. Food samples were purchased from local markets.

2.2. Instrumentation

ESI–MS was performed on a Finnigan MAT (Bremen, Germany) triple-stage quadrupole TSQ 7000 equipped with the Finnigan electrospray interface. Data acquisition and mass spectrometric evaluation were carried out on a Personal DECstation 5000/33 (Digital Equipment, Unterföhring, Germany) and ICIS 8.1 software (Finnigan MAT). For LC an Applied Biosystems dual-syringe pump model 140B (Bai, Bensheim, Germany) was used. LC of food samples was carried out on a LiChrospher 60-RP select B column (100×2.0 mm I.D., 5 μm; Knauer, Berlin, Germany) using a linear gradient. Solvent A was water with 0.05% trifluoroacetic acid, solvent B was methanol with 0.05% trifluoroacetic acid. For OTA analysis the gradient programme was as follows: 0 min, 40% B; 1 min, 40% B; 7 min, 100% B; 10 min, 100% B. The solvent flow was 200 μl/min. For injection a Spark Triathlon autosampler (SunChrom, Friedrichsdorf, Germany) was used; the injection volume was 5 or 10 μl using the μl pick-up mode.

For the MS the following parameters were used in all experiments: For pneumatically assisted electrospray ionisation, the spray capillary voltage was 3.0 kV and the temperature of the heated inlet capillary, 220°C; the electron multiplier voltage was 1.8 kV. Nitrogen served both as sheath (60 psi) and auxiliary gas (10 units). Full-scan spectra were acquired over the range m/z 200–450 (scan duration, 1.0 s). The product ion spectra were recorded using argon as collision gas at a pressure of 1.8 mTorr and a collision energy of −15 eV and −25 eV in the positive mode and +22 eV in the negative mode, scanning a mass range m/z 200–450 (scan duration, 1.0 s). For measurements in the negative mode OTA solutions in methanol–water (1:1) with 0.05% ammonium acetate were continuously delivered into the ESI interface at 100 $\mu l\,min^{-1}$ by means of a syringe pump system. SRM experiments in the positive mode were performed using the protonated molecule $[M+H]^+$ at m/z 404 and the most abundant product ions at m/z 239 and m/z 358 (collision energy, −25 eV). Quantitation was done using standard solutions of OTA for external calibration. The calibration graph was linear from 0.06 μg to 2.5 μg. The R.S.D. was 3.4% and the correlation coefficient was 0.9882.

2.3. Sample preparation

Sample preparation was performed as described in

[14]. Briefly, samples (20 g or 40 ml) were mixed with a 0.4 *M* magnesium chloride solution, extracted with toluene and purified over a silica cartridge [14]. The final volume of the purified extract was 100 μl (300 μl for coffee); 5 μl (10 μl for coffee) of this solution were injected. Recoveries varied from 95±12% (for beer) to 70±9% (for coffee). All samples were analysed in duplicate.

3. Results and discussion

Under ESI conditions, OTA molecules could effectively be transformed into protonated ions. As shown in Fig. 1A, an abundant protonated molecule $[M+H]^+$ was observed at *m/z* 404 accompanied by a smaller $[M+Na]^+$ adduct at *m/z* 426. Since OTA contains one chlorine atom, the typical isotope pattern with an additional signal for ^{17}Cl–OTA at *m/z* 406 was observed. Almost no fragment ions were obtained under ESI conditions (Fig. 1A). Due to the phenylalanine moiety with the COOH-group,

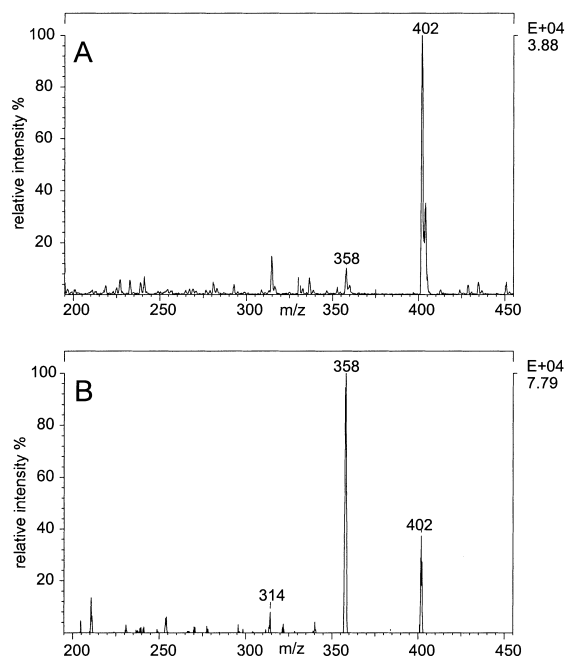

Fig. 2. (A) Negative electrospray mass spectra of Ochratoxin A. (B) Product ion spectra (+22 eV, 1.8 mTorr Ar) of Ochratoxin A, obtained after CID of the precursor ion *m/z* 402 $[M-H]^-$.

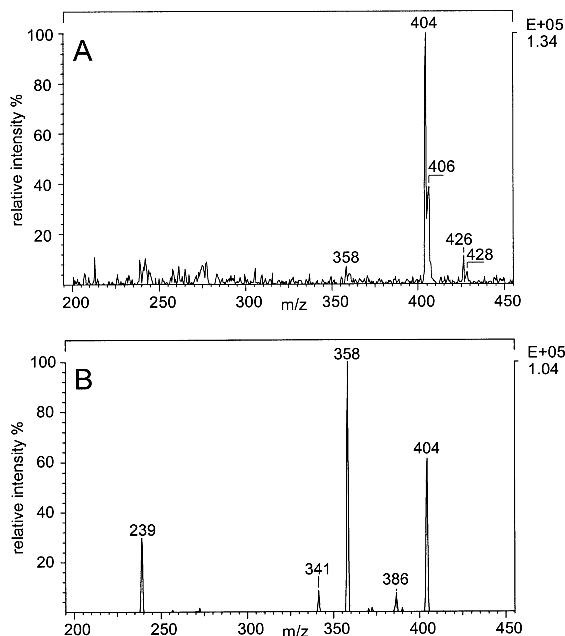

Fig. 1. (A) Positive electrospray mass spectra of Ochratoxin A. (B) Product ion spectra (−15 eV, 1.8 mTorr Ar) of Ochratoxin A, obtained after CID of the precursor ion *m/z* 404 $[M+H]^+$.

ochratoxin could also be analysed in the negative mode. This revealed a strong $[M-H]^-$ ion at *m/z* 402 with the typical chlorine pattern and again no fragmentation was observed (Fig. 2A). Low energy collision induced dissociation (CID) of the protonated $[M+H]^+$ and the deprotonated molecule $[M-H]^-$ with argon serving as collision gas produced characteristic product ion spectra (Fig. 1B, 2B). The fragmentation of $[M+H]^+$ at *m/z* 404 yielded an abundant product ion $[M+H-H_2O-CO]^+$ at *m/z* 358 and smaller product ions $[M+H-H_2O]^+$ at *m/z* 386, $[M+H-\text{phenylalanine}]^+$ at *m/z* 239 and $[M+H-H_2O-CO-NH_3]^+$ at *m/z* 341 (Fig. 1B). The fragment ion at *m/z* 341 is formed by the loss (rearrangement reaction) of a molecule containing N, since Ochratoxin A contains an uneven amount of nitrogen atoms. With ionsource CID (APCID) on (−30 eV) it was shown that this ion originates from the fragment ions *m/z* 386 and *m/z* 358. In the negative mode, the most abundant product ion *m/z* 358 originated from $[M-H]^-$ (*m/z* 402) by the loss

of CO_2 (−44 amu) (Fig. 2B). However, since the loss of CO_2 in the negative product ion spectra is not very characteristic, all other experiments were performed under positive ESI conditions.

Based on the characteristic product ions obtained from ochratoxin after CID, we developed a SRM experiment, resulting in high sensitivity and selectivity as well as effective reduction of chemical noise. Besides their retention time on the LC column, analytes were identified in quadrupole 1 by their protonated molecules $[M+H]^+$ representing a spectral filter on molecular mass information together with their characteristic product ions obtained in quadrupole 3 after CID of the protonated molecules in quadrupole 2. For the analysis of ochratoxin A quadrupole 1 was set to m/z 404 and quadrupole 3 to the characteristic product ions $[M+H-H_2O-CO]^+$ at m/z 358 and $[M+H-\text{phenylalanine}]^+$ at m/z 239. Compared with other methods, the use of LC–ESI-MS–MS in the SRM mode provides highly structure-specific information and is especially helpful to confirm doubtful 'OTA positive' results obtained by LC with fluorescence detection. In order to increase the intensity of the product ions, SRM experiments were performed at a collision energy of −25 eV. The detection limit (S/N>3) for LC–ESI-MS–MS of ochratoxin A applying SRM in the positive mode was 20 pg, or 50 fmol, on column. The quantification limit was 60 pg and the calibration curve was linear from 0.06 μg to 2.5 μg. In Fig. 3 is shown the typical result for a standard (40 ng OTA ml^{-1}, 200 pg OTA on column) with the monitored product ions at m/z 358 and m/z 239.

Subsequently, ochratoxin-containing food samples such as wheat, coffee and beer were analysed by means of LC–ESI-MS–MS. Sample clean-up was performed as described in the Section 2. It was recently shown in an interlaboratory study that the solid-phase extraction procedure used is comparable with other clean-up methods such as immunoaffinity phases [20]. As a representative example, Fig. 4 shows the result of a SRM experiments of a beer sample contaminated with OTA at a concentration of 12 ng l^{-1}. The peak eluting at 5.05 min was identified as OTA from its retention time and from the product ions at m/z 358 and m/z 239. Three more peaks at 4.35, 6.25 and 8.45 min also gave a signal at m/z 358. However, only the OTA peak exhibited the product ion m/z 239. Therefore the product ion m/z 358 is not very characteristic for OTA and the loss of the phenylalanine moiety yielding m/z 239 unambiguously proved the presence of OTA. All other food samples (n=40) analysed were contaminated with 0.01–0.2 μg/kg of OTA and yielded similar LC–ESI-MS–MS chromatograms as shown in Fig. 4 with the characteristic OTA signal at around 5.05 min. The reproducibility of the retention time was very good and only shifted up to 10 s. Quantitation was done using standard solutions of OTA for external calibration (see Section 2).

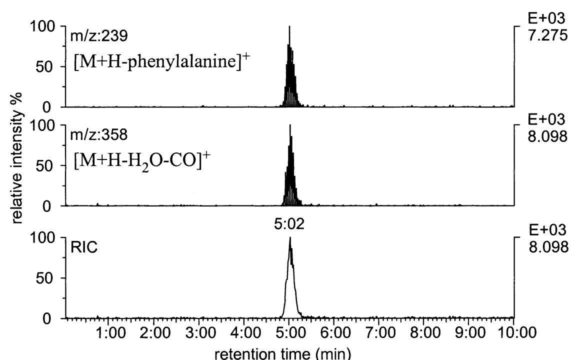

Fig. 3. LC–ESI-MS–MS chromatogram of a OTA reference sample (40 ng OTA ml^{-1}) obtained by SRM experiment (−25 eV, 1.8 mTorr Ar). RIC: reconstructed ion chromatogram.

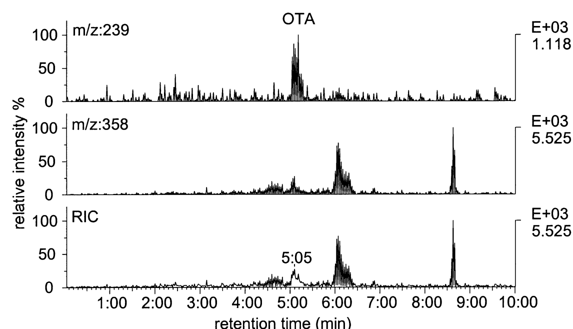

Fig. 4. LC–ESI-MS–MS chromatogram of a OTA containing beer sample (12 ng l^{-1}) obtained by SRM experiment (−25 eV, 1.8 mTorr Ar). RIC: reconstructed ion chromatogram.

4. Conclusion

In summary, LC–ESI-MS–MS in combination with SRM is a very sensitive and useful method for the determination of OTA in foods and feeds and has several advantages compared to established methods. First, the sensitivity (ca. 20 pg) is similar to that obtained with fluorescence detection currently in use. Secondly, the problem of coelution of interfering compounds can be overcome by the structural information provided by tandem mass spectrometry. LC–ESI-MS–MS is especially helpful to confirm doubtful 'OTA positive' results obtained by LC with fluorescence detection. One should add that, since OTA is a potent nephrotoxic and nephrocarcinogenic mycotoxin it is necessary to develop not only the most sensitive method for the analysis of complex mixtures but also to identify and monitor metabolites and degradation products of OTA. These studies are currently under investigation.

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