Analysis of ochratoxin A in pig kidney and rye flour using liquid chromatography tandem mass spectrometry (LC/MS/MS)

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A liquid chromatography electrospray tandem mass spectrometric (LC/MS/MS) method is described for analysis and confirmation of ochratoxin A in pig kidney and rye flour using derivatization of ochratoxin A to the methyl ester. Ochratoxin A methyl(d_3)ester is used as internal standard. The method works well, the detection limit is 0.02 μg/kg and the repeatability (coefficient of variation) is between 6% and 16% in the contamination range 0.5 to 8 μg/kg.

Keywords: ochratoxin A, liquid chromatography, tandem mass spectrometry, pig kidney, rye flour

Introduction

Until today, surprisingly there have been few attempts to use mass spectrometric confirmation of positive results in relation to the control of mycotoxin contamination of foods (Gilbert 1996). This might become more important in the future.

Very few studies have been published on the analysis of ochratoxin A using liquid chromatography coupled with mass spectrometric detection (LC/MS). Abraham (1987) used LC/DLI-MS (direct liquid introduction mass spectrometry) for the determination of ochratoxin A in barley extract with selected ion monitoring (SIM) of *m/z* 403. A limit of detection of about 3 μg/kg was achievable, however the eluting ochratoxin A peak had excessive tailing and no data on reproducibility were given. Rayakylä *et al.* (1987) used LC/Thermospray-MS for the determination of ochratoxin A in wheat samples but data on reproducibility and estimate of detection limit were not given. Ominski *et al.* (1996) used LC/DLI-MS for the confirmation of ochratoxin A in pig serum but the ochratoxin A peak showed significant tailing. Overall, these methods were not considered particularly useful for routine analysis and confirmation of ochratoxin A.

As confirmation of the presence of ochratoxin A in two pig kidney reference materials produced in-house was essential for an EU Standard, Measurement and Testing project (Entwisle *et al.* 1996, 1997), this work was initiated. We report for the first time a routine liquid chromatography tandem mass spectrometric method (LC/MS/MS) for the analysis and confirmation of ochratoxin A in foods, utilizing derivatization of ochratoxin A to the methyl ester and employing ochratoxin A methyl(d_3)ester as internal standard. The possible use of ochratoxin B as internal standard was initially investigated, but the results were not satisfactory with the instrumentation and clean-up method used. The employment of an isotope-labelled ochratoxin internal standard was necessary, in agreement with our experiences with quantification of aflatoxins by LC/MS/MS in various foods (Vahl and Jørgensen 1998). Isotope-labelled ochratoxin A is not commercially available and therefore analysis after derivatization of ochratoxin to the methyl ester was chosen as an alternative as the labelled ochratoxin methyl(d_3)ester is easy to synthesize.

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Materials and methods

Chemicals

All solvents and reagents were analytical or HPLC-grade. Boron trifluoride methanol-complex in 20% methanol was from Merck (Darmstadt, Germany), boron trifluoride diethyl etherate, deuterated methanol (CD_3OD, 99.8% D) and ochratoxin A were from Sigma (St Louis, MO, USA). A stock standard solution of ochratoxin A was made in toluene: acetic acid (99:1) and kept at −18°C. The concentration was measured by spectrophotometry ($\varepsilon_{333} = 5550\,cm^{-1}\,M^{-1}$) (AOAC 1995), and diluted with acetonitrile to a working standard solution (250 ng/ml).

Extraction and clean-up

The extraction and clean-up procedure was almost the same as described for cereals and pig kidney (Entwisle *et al.* 1996, 1997, Jørgensen *et al.* 1996). A 50 g sample was extracted with 250 ml dichloromethane: ethanol (4:1) and 25 ml 0.1 M phosphoric acid for 30 min on a shaking device. The extract was filtered through dried sodium sulphate using a water pump. A 50 ml volume of the extract (one-fifth of the sample amount) was transferred to a separatory funnel and extracted three times with 35 ml sodium hydrogen carbonate solution, 4 g in water: methanol (5:2). The collected water phases were washed with 25 ml dichloromethane. Fifteen ml 2 M phosphoric acid was added to the water phase (pH< 3) in a separatory funnel and extracted twice with dichloromethane (2 × 25 ml) The dichloromethane phases were filtered through dried sodium hydrogen carbonate, the dichloromethane was evaporated almost to dryness on a water bath, and the residue was then transferred with dichloromethane: acetic acid (99:1) to a small flask. The solvent was evaporated with nitrogen and the residue immediately derivatized, as described below.

Internal standard

The internal standard was made by using deuterated boron trifluoride methanol(d_4)-complex, which was prepared by mixing boron trifluoride diethyl etherate and deuterated methanol (1:4). One ml of the stock standard solution of ochratoxin A (66 μg/ml) was evaporated to dryness with nitrogen and the residue was dissolved in 1 ml deuterated methanol followed by addition of 0.25 ml boron trifluoride diethyl etherate. The solution was kept at 60°C for 60 min, evaporated to dryness with nitrogen and the residue dissolved in 2 ml of acetonitrile: acetic acid (99:1). The ochratoxin A methyl(d_3)ester was kept at −18°C. The internal standard in LC eluent was made from this solution by evaporation to dryness and dissolution in LC eluent to a concentration of 50 ng/ml.

Derivatization

Ochratoxin A standards and samples were derivatized to the ochratoxin A methyl ester using 14% boron trifluoride methanol-complex in methanol as derivatization agent. Ochratoxin A methyl ester standards with the concentrations of 0, 25, 50, 100, 250 and 500 ng/ml were prepared by evaporation of the solvent from portions of the working standard solution of ochratoxin A with nitrogen, dissolution in 50 μl 14% boron trifluoride methanol-complex and derivatization for 30 min at 60°C, followed by evaporation of solvent and dissolution in 200 μl LC eluent with the internal standard, ochratoxin A methyl(d_3)ester. The final sample extract in dichloromethane was evaporated with nitrogen, and derivatization was done as described for the standards.

LC/MS/MS conditions

The HPLC system consisted of two Jasco PU980 pumps, a HP 1050 autosampler, and a 250 × 2 mm i.d. Spherisorb S5 ODS1 column at ambient temperature. The mobile phase was acetonitrile: water: methanol: acetic acid (50:30:20:0.5). The column was washed with acetonitrile: methanol: acetic acid (71:29:0.5) for 8 min after each sample. The injection volume was 20 μl, the flow rate was 250 μl/min and the total analysis time was 30 min including conditioning of the column after the washing step.

Mass spectrometry was performed using a Micromass Quattro II triple quadrupole mass spectrometer and MassLynx software for control and data processing

(Micromass, UK). Electrospray ionization in the positive mode was used. The electrospray capillary was set at 3.5 kV, the counter electrode at 0.5 kV and the cone at 30 V. The ion source temperature was set at 150°C and the flow rates for nitrogen bath and spray were 300 l/h and 20 l/h, respectively. The pressure of the argon used for collision induced dissociation (CID) was 1.5×10^{-3} mbar. Data were acquired in the mass reaction monitoring (MRM) mode. The ions monitored were *m/z* 239, *m/z* 221 and *m/z* 193, daughter ions of the ochratoxin A methyl ester (*m/z* 418), and *m/z* 239, daughter ion of the ochratoxin A methyl(d_3)ester (*m/z* 421). The collision energy was 30 eV, 40 eV and 50 eV when monitoring the daughter

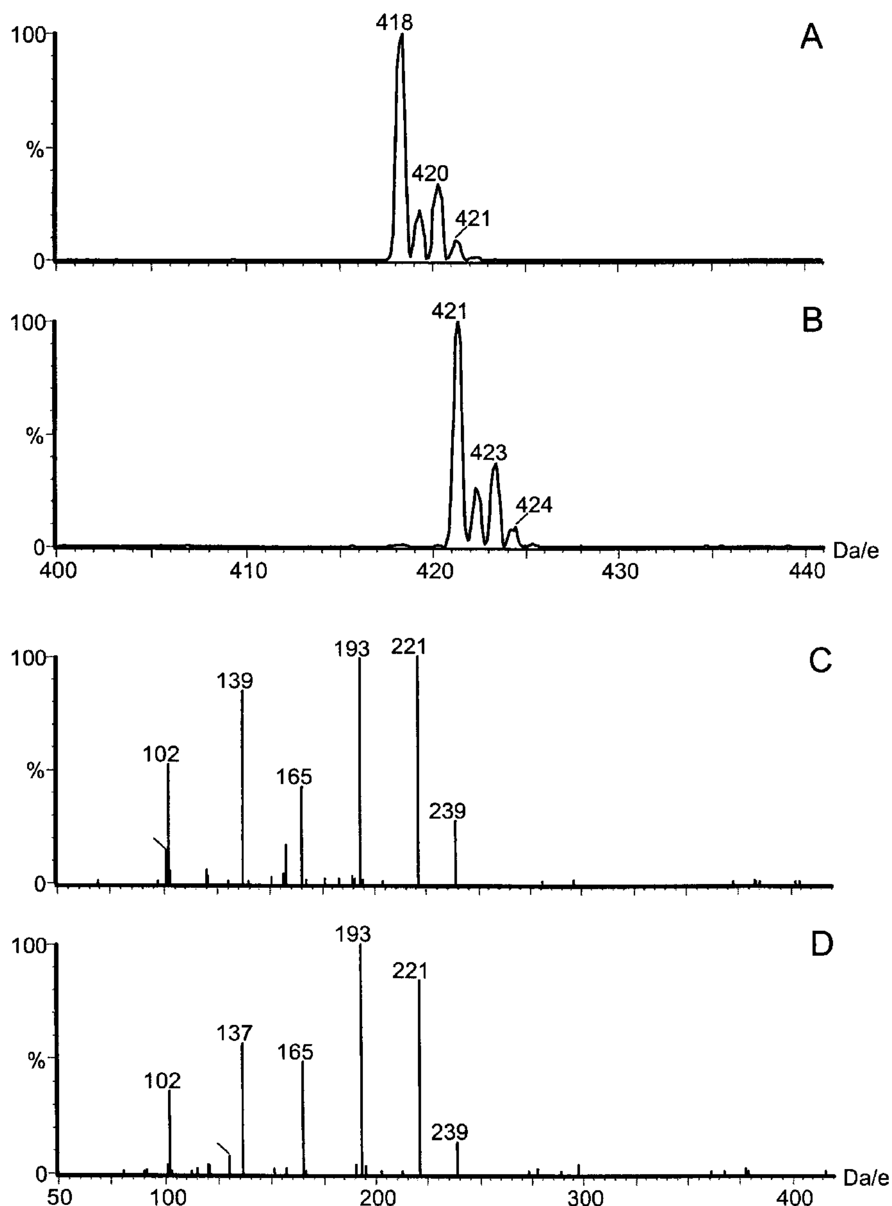

Figure 1. Electrospray positive ion profile mass spectra ochratoxin A ethyl ester (A) and ochratoxin A methyl(d_3)ester (B), and electrospray MS/MS daughter ion centroid mass spectra, recorded at 50 eV collison energy, of the ochratoxin A methyl ester pseudo molecular ion m/z 418 (C) and the ochratoxin A methyl(d_3)ester pseudo molecular ion m/z 421 (D).

ions at *m/z* 239, *m/z* 221, and *m/z* 193, respectively. Quantification of ochratoxin A was done by the internal standard method using the ratios of the areas of the daughter ions of unlabelled ochratoxin A methyl ester to the area of the daughter ion of labelled ochratoxin A methyl ester in the mass chromatograms. Linear calibration curves were calculated for all three daughter ions of unlabelled ochratoxin A methyl ester by the least squares method with the area ratios as the dependent and the ochratoxin A concentration (0, 25, 50, 100, 250, 500 ng/ml) as the independent variable.

The method of analysis was evaluated using samples of freeze-dried pig kidney from an EU Standard, Measurement and Testing project (Entwisle *et al.* 1996, 1997) at two contamination levels (0.5 and 8.2 μg/kg) and one spiking level (5 μg/kg), and samples of blank rye flour at two spiking levels (1 and 5 μg/kg). The blank sample of rye flour was analysed in duplicate on two of the four days of analysis giving a mean level of 0.05 μg ochratoxin A per kg (below the determination limit).

Results and discussion

Electrospray positive ion profile mass spectra of ochratoxin A methyl ester and ochratoxin A methyl-(d_3)ester are shown in figure 1. As the spectra contained no significant mass peaks below the *m/z* of the major pseudo molecular ions the *m/z* range below *m/z* 400 is not shown. Also shown in figure 1 are electrospray MS/MS daughter ion mass spectra, recorded at 50 eV collision energy, of the ochratoxin A methyl ester pseudo molecular ion *m/z* 418 and the ochratoxin A methyl(d_3)ester pseudo molecular ion *m/z* 421. The two daughter ion spectra have no significant differences.

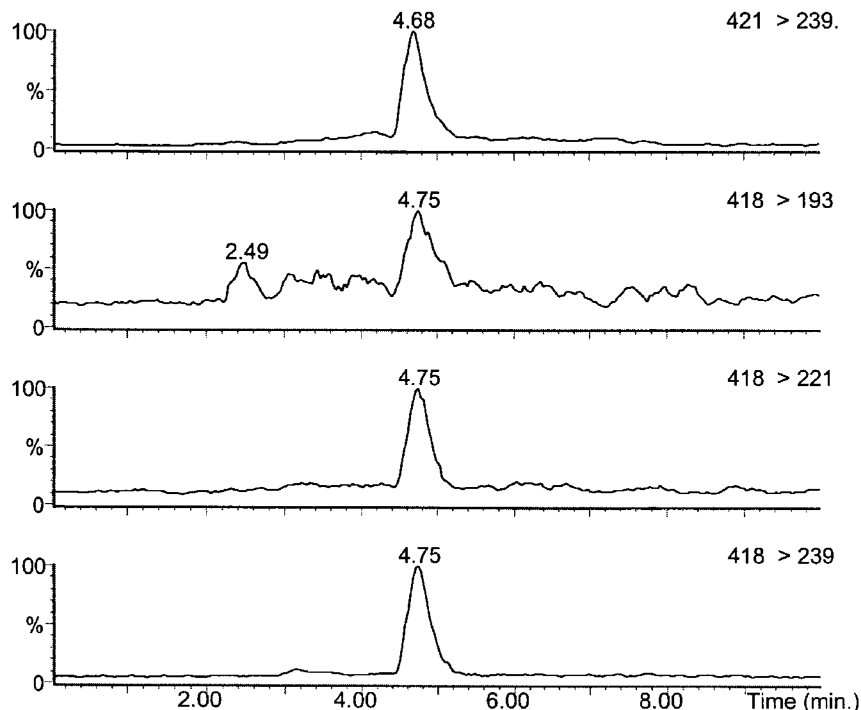

Figure 2. Chromatograms of a pig kidney sample naturally contaminated with 0.5μg/kg ochratoxin A, the absolute amount injected is 500 pg. The MRM (mass reaction monitoring) chromatograms show the trace of the daughter ion m/z 239 of the ochratoxin A methyl(d_3)ester pseudo molecular ion m/z 421 and the traces of the daughter ions m/z 193, 221 and 239 of the ochratoxin A methyl ester pseudo molecular ion m/z 418.

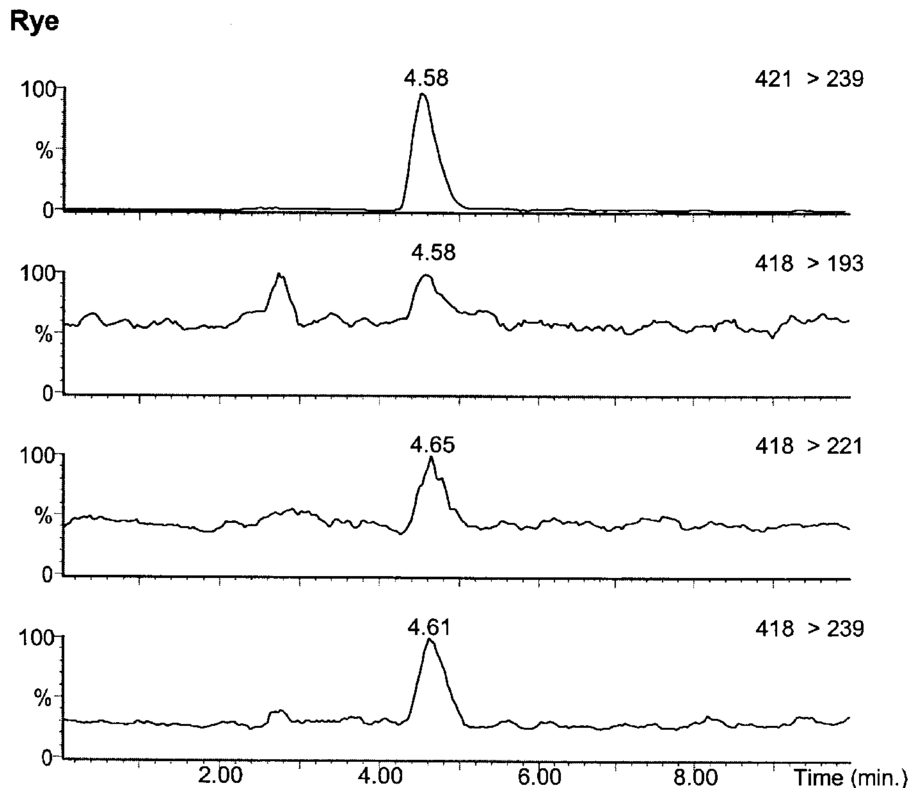

Figure 3. Mass chromatograms of a rye flour sample naturally contaminated with 0.05 µg/kg ochratoxin A, the absolute amount injected is 50 pg. The MRM (mass reaction monitoring) chromatograms show the trace of the daughter ion m/z 239 of the ochratoxin A methyl(d_3)ester pseudo molecular ion m/z 421 and the traces of the daughter ions m/z 193, 221 and 239 of the ochratoxin A methyl ester pseudo molecular ion m/z 418.

Chromatograms of a naturally-contaminated pig kidney sample and of a naturally-contaminated rye flour sample are shown in figures 2 and 3, respectively. The specificity of the MS/MS detection is illustrated by the absence of interfering peaks. The detection limit of the analytical method calculated using three times the standard variation of ten determinations of the reagent blank value was 0.02 µg/kg for 50 g samples.

Table 1 summarizes the results of the analytical method by showing the recoveries of ochratoxin A from both spiked samples of pig kidney and rye flour and samples of pig kidney reference material. The mean values of the recoveries were above 100%. Analytical results of the method must therefore be corrected for recovery. The overall coefficients of variation are satisfactory. The relative standard deviation of the mass spectrometric measurement step was 6% calculated by using measurements of the same 10 extracts on two consecutive days.

The presence of ochratoxin A in the two pig kidney reference materials at the earlier measured levels has been confirmed by the results obtained by the LC/MS/MS method developed.

The above mentioned quantification data were based on the daughter ion *m/z* 239. Quantification was done also on the basis of the daughter ions *m/z* 221 and 193 for further confirmation. The results were not as good as with the use of the daughter ion *m/z* 239. However, based on the daughter ion 221, 82% of the results were within ±10% and 95% of the results were within ±20% of the results based on the daughter ion 239. Based on the daughter ion 193, 66% of the results were within ±10% and 89% of the results were within ±20% of the results based on the daughter ion 239.

In conclusion, a specific and sensitive mass spectrometric method for the analysis and confirmation of ochratoxin A in pig kidney and cereals has been developed.

Table 1. Results of the analytical method using MS/MS for quantification.

	Day 1		Day 2		Day 3		Day 4		Recovery	
	Recovery (%)		Recovery (%)		Recovery (%)		Recovery (%)		Mean	CV
Type of sample	1.det.	2.det.	1.det.	2.det.	1.det.	2.det.	1.det.	2.det.	(%)	(%)
Pig kidney:										
Contaminated 8.2 μg/kg[a]	115	109	91	108	120	108	99	106	107	8
Contaminated 0.5 μg/kg[a]	96	94	106	96	116	120	88	118	104	12
Spiked at 5.0 μg/kg	86	[b]	91	103	117	115	135	120	110	16
Rye flour:										
Spiked at 1.0 μg/kg	117	131	99	119	129	123	110	112	118	9
Spiked at 5.0 μg/kg	126	111	115	131	118	122	128	114	121	6

[a] Mean levels found in homogeneity and stability studies of the pig kidney reference materials (Entwisle *et al.* 1996, 1997) are used as the true value. The same sample clean-up was used as in this study, a mean recovery of 100% was found, and the detection was by fluorescence.
[b] Sample was lost during clean-up.

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