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Rapid determination of ochratoxin A in cereals and cereal products by liquid chromatography☆

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Abstract

A new method based on extraction with octylsilica (C_8) followed by liquid chromatography coupled with fluorescence detection (LC–FLD) was studied to determine ochratoxin A (OTA) from cereals and cereal products. Optimization of different parameters, such as type and amount of solid phase, type and volume of eluent and amount of sample were carried out. Recovery of OTA from rice samples spiked at 10 ng/g level was of 86% with relative standard deviation of 5%. The limits of detection and quantification of the proposed method were 0.25 and 0.75 ng/g, respectively. Furthermore, LC–FLD after of OTA methylation and liquid chromatography coupled to mass spectrometry with an electrospray interface were used for confirmation of OTA in all studied samples. The proposed method was applied to 62 samples of cereals and cereal products and the presence of OTA was found in seven samples.

Keywords: Food analysis; Cereals; Ochratoxin A; Mycotoxins

1. Introduction

Ochratoxin A (OTA) is a mycotoxin produced widely in cereals and cereal products. In temperate climates, OTA is mainly produced by *Penicillium verrucosum* although *Aspergillus ochraceus* and related species also produced OTA in cold and wet climates [1]. Experimentally, OTA has been shown to be a teratogenic, potent renal carcinogen, immunosupressive, enzyme inhibitor and it has effects on lipid peroxidation and has been implicated in Balkan nephropathy in humans. OTA is listed as a possibly carcinogen of group 2B by the International Agency for Research on Cancer (IARC) [2]. In the European Union [3], the OTA level in cereals and cereal products are regulated with maximum residue levels (MRLs) that cannot be greater than 5 and 3 μg/kg, respectively.

Several analytical methods for determining OTA in cereals and cereal products have been reported. These methods generally involve liquid extraction with several solvents, such as mixtures of dichloromethane–citric acid [4], acetonitrile–water [5], methanol–phosphoric acid [6] and methanol–sodium chloride [7]. A clean up procedure is frequently used and it usually employs solid-phase extraction columns such as anion-exchange (SAX) [8], silica [9], C_{18} [10] and immunoaffinity columns (IACs) [5,11–13]. IACs play a predominant role in separation and purification of OTA from crops. Detection and determination is performed by immunochemical methods as enzyme linked immunosorbent assay (ELISA) [4] and chromatographic procedures such as thin layer chromatography with densitometry detection [14], gas chromatography with mass spectrometry detection [15] and especially by liquid chromatography with fluorescence (LC–FLD) [5,9,16], mass spectrometry (LC–MS) [17] or tandem mass spectrometry (LC–MS–MS) detection [18].

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Residue analysis must be simple and rapid to prevent distribution of harmful products, and economic enough to allow developing programmes for monitoring OTA over a wide number of food samples. However, only a few of the methods found in the literature comply with these premises.

The objective of this study is to optimise a simple method based in the use of C_8 solid phase to extract and purify OTA in cereals and cereal products in which determination is carried out by LC–FLD. Furthermore, two methods for confirmation, LC–FLD after methylation of OTA and LC–MS of this mycotoxin in these products were used. Finally, the proposed method was applied to 62 cereals and cereal products.

2. Experimental

2.1. Chemical and reagents

OTA crystalline material was purchased from Sigma (St. Louis, MO, USA). Stock standard solution with concentration of 500 μg/ml was prepared in methanol, kept in security conditions at −20 °C, wrapped in aluminium foil, due to that OTA gradually break down under UV light, and held for less than 3 months. Standard working solutions were prepared by appropriate diluting in the same solvent and stored in glass-stopped tubes at −20 °C.

Dichloromethane, acetonitrile, methanol and chlorhydric acid (37%) were supplied by Merck (Darmstadt, Germany), diethylamine and formic acid (98–100%) by Scharlau (Barcelona, Spain). Deionised water (<8 MΩ cm resistivity) was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Chromatographic solvents and water were degassed for 20 min using a Branson 5200 (Branson Ultrasonic Corp., CT, USA) ultrasonic bath.

Solid phases used were C_8 (50 μm), C_{18} (50 μm), silica (40 μm), phenyl (50 μm) and neutral aluminium oxide from Análisis Vínicos (Tomelloso, Spain), ENVI-Carb from Supelco (Bellefonte, PA, USA) and Lichrolut-EN from Merck.

2.2. Samples

Random food samples were obtained in the cereal packers and supermarkets for cereals and cereal products, respectively, from May 2003 to September 2003, typically as a single bag. All samples were stored in the dark and dry place at room temperature (18–23 °C). The samples were divided with a subsample divider and a 200 g subsample was analysed [19].

2.3. Extraction procedure

Samples (200 g) were prepared using a food processor and mixed thoroughly. An aliquot (2.5 g) of the sample was placed into a mortar (50 ml capacity) and was gently blended with 1.5 g of the solid phase (C_8) for 5 min using a pestle, to obtain a homogeneous mixture. This mixture was introduced into a 100 mm × 9 mm i.d. glass chromatographic column with a coarse frit (no. 2) and covered with a plug of silanized glass wool at the top of the column. OTA was eluted with 20 ml methanol–formic acid (99:1, v/v) with a vacuum manifold. The eluate was evaporated to 3 ml with a gentle stream of N_2 at 45 °C, and then, it was filtered through a nylon acrodisk (0.45 μm) and centrifuged at 5000 rpm for 10 min. The extract was filtered again and evaporated to 0.5 ml with N_2 at 45 °C.

2.4. Liquid chromatography analysis

A Shimadzu (Kyoto, Japan) SCL-6A system LC equipped with two LC-6A pumps, a Rheodyne Model 7125 injector (20 μl loop) and an SRF-535 fluorescence detector was used. A LC column Nova-Pak C_{18} (5 μm) (150 mm × 3.9 mm i.d.) was used with a mobile phase consisting of a mixture of methanol–formic acid 0.1 M (70:30, v/v) at a flow rate of 0.4 ml/min. Detection of OTA was carried out using 334 and 464 nm as wavelengths for excitation and emission, respectively. The limit of detection (LOD) (S/N, 3:1) and limit of quantification (LOQ) (S/N, 10:1) were 0.25 and 0.75 ng/g, respectively.

2.5. Confirmation procedures

2.5.1. LC–FLD after methylation of OTA

The method of Zimmerli and Dick (1995) [12] was used. Briefly, 200 μl of the extract was diluted to 2.5 ml methanol and 0.1 ml concentrated HCl were added. The solution was left standing overnight at room temperature. Thereafter, the methanol was evaporated and the residue was taken up in 200 μl methanol–formic acid 0.1 M (70:30, v/v). 90% of the OTA was methylated with this method. The LC analysis was identical to that described above.

2.5.2. Confirmation by LC–MS

OTA was conducted using a Hewlett-Packard (Palo Alto, CA, USA) HP-1100 Series LC–MS system equipped with a binary solvent pump, an autosampler and a mass detector (MS) coupled with an analytical workstation (HP Chem Software). The MS system consisted of a standard atmospheric pressure ionisation (API) source configured as electrospray interface (ESI). The LC–ESI–MS interface in negative ion mode operated under the following conditions: 350 °C gas temperature, 13.0 l/min drying gas nitrogen flow, 3.5 kg/cm^2 nebulizer gas pressure and 3500 V capillary voltage. Using a fragmentor of 120 V, the ions selected for OTA identification were ($[M–H]^-$) and ($[M–H–CO_2]^-$) at *m/z* 402 and 358, respectively. The sample extract was injected into the LC using the same conditions specified above. The LOD and LOQ were 0.30 and 0.95 ng/g, respectively. According to several authors [17,20], the choice of an acid mobile phase was carried out for confirming OTA by LC–MS in negative ion mode in this study.

3. Results and discussion

3.1. Method performance

The following modifications were made to optimize the analysis from cereals and cereal products: (a) type; (b) volume of eluent; (c) type of solid phase; (d) amount of solid phase and (e) amount of sample used. The optimization was tested in rice.

3.1.1. Type of eluent

Twenty milliliter of dichloromethane, acetonitrile, methanol, methanol–diethylamine (99:1, v/v) and 1% methanol–formic acid (99:1, v/v) were tested ($n = 5$) as elution solvents by using 2.5 g of C_8 solid phase and 1.5 g of rice fortified with 10 ng/g level. The best recoveries and relative standard deviation (86 and 5%, respectively) were found with the methanol–formic acid solution.

3.1.2. Eluent volume

Table 1 shows that the maximum recovery was reached with 20 ml and it was not improved by using higher volumes. For this reason, 20 ml was selected.

3.1.3. Type of solid phase

C_8, C_{18}, silica, phenyl, Lichrolut-EN, ENVI-carb and aluminium oxide were studied ($n = 5$). For ENVI-carb and aluminium oxide, recoveries were lower than 20%. The values obtained for the other solid supports are shown in Table 2. The best recoveries for OTA analysis were obtained using C_8 and methanol–formic acid as elution solvent. The differences between the mean recoveries obtained with C_8, LiChrolut-EN, ENVI-carb and aluminium oxide were of statistical significance, whereas the recoveries obtained with C_8, C_{18}, silica and phenyl were close. The tested C_8 was chosen because their chromatograms are more clean than those generated by other phases.

Table 1
Mean recoveries and relative standard deviations (R.S.D., in parenthesis) obtained with different volumes of methanol–formic acid as eluting solvent for rice samples spiked at 10 ng/g level ($n = 5$)

Eluent volume (ml)	Recovery, % (R.S.D., %)
5	68 (8)
10	72 (6)
15	75 (7)
20	86 (5)
25	84 (5)
30	78 (6)
35	85 (8)
40	73 (10)

Table 2
Mean recoveries and relative standard deviations (R.S.D., in parenthesis) obtained with different solid supports from rice samples spiked at 10 ng/g level ($n = 5$)

Solid support	Recovery, % (R.S.D., %)
C_8	86 (5)
C_{18}	81 (8)
Silica	79 (12)
Phenyl	81 (10)
Lichrolut-EN	22 (9)

Table 3
Mean recoveries and relative standard deviations (R.S.D., in parenthesis) obtained with different amounts of C_8 solid phase and rice samples spiked at 10 ng/g level, using methanol–formic acid (99:1, v/v) as eluting solvent ($n = 5$)

g of sample + g of C_8	Recovery, % (R.S.D., %)
0.5 + 0.5	69 (6)
2 + 1.5	74 (7)
2 + 2	78 (11)
2.5 + 1.5	86 (5)
3 + 1	77 (5)

3.1.4. Amount of solid phase and samples

Results reported in Table 3 shows that the best recoveries are obtained using 1.5 g of C_8 solid phase and 2.5 g of sample.

The Fig. 1 shows the LC–FLD chromatogram obtained following the proposed extraction procedure for rice fortified with OTA at 10 ng/g level. Validation of the method was carried out according to the parameters selected. Precision was calculated in terms of intra-day repeatability ($n = 5$) and

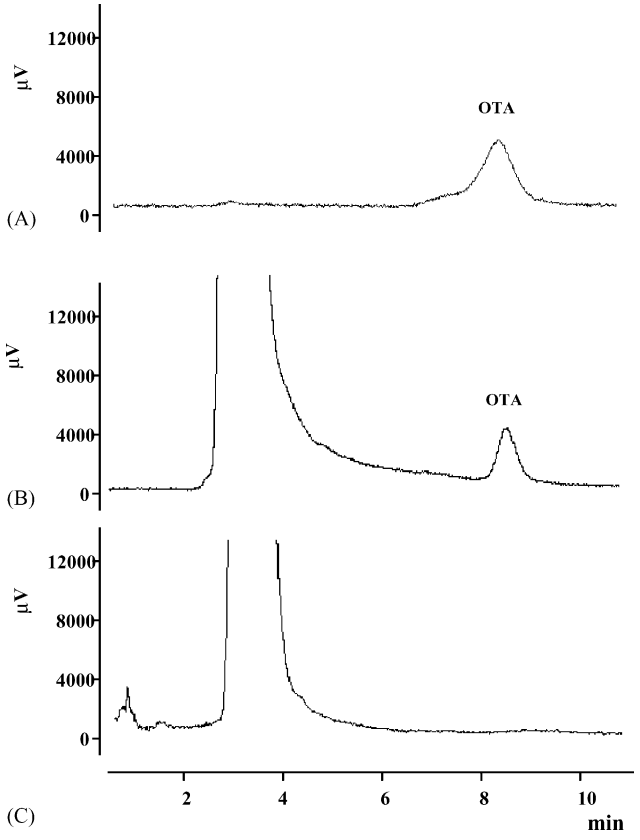

Fig. 1. LC–fluorescence chromatograms obtained from rice with the proposed extraction procedure: (A) standard of OTA; (B) fortified with 10 ng/g of OTA and (C) a rice non-fortified sample extract.

inter-day reproducibility (five different days) at 10 ng/g level. The intra-day repeatability evaluated as R.S.D. ranged from 4 to 7% at 3 ng/g level and from 3 to 6% at the 10 ng/g level. The inter-day reproducibility was lower than 8% for all instances. Linearity was verified ($n = 3$) with seven concentrations (0.25, 0.75, 2.5, 5, 10 and 20 ng/g). The regression coefficients were all >0.998.

In order to investigate if natural constituents interfere with quantification, parallel calibration graphs obtained from matrix-extracted and solvent-based standards were performed. The covariance analysis for OTA according to the calculated F-values were lower than the Snedecor's F-values tabulated ones indicating that both straight lines were not parallel ($P < 0.05$) and matrix effect is not negligible. For this reason, all analyses were conducted with spiked samples that were previously analyzed and did not contain OTA. Recoveries obtained for all cereals and cereal products spiked at 10 ng/g level ($n = 5$) analyzed with the proposed method ranged from 77 to 89%.

The results of the study reflected that the extraction of OTA using 2.5 g of sample and 1.5 g of C_8 solid phase gave clean chromatogram profiles and recoveries that were considered as valid for analyzing residues of OTA in foods according with European specification [19].

3.2. Application to real samples

The proposed method of extraction followed by LC–FLD was applied in 62 cereals and cereal products (Table 4). The selection of these products was done because they are well known for being susceptible to fungal growth and toxin production [1] and are greatly consumed in Spain [21].

Fig. 2 shows the LC–FLD, LC–FLD after methylation procedure and LC–ESI–MS chromatograms obtained by the proposed extraction procedure for a positive cereal product sample.

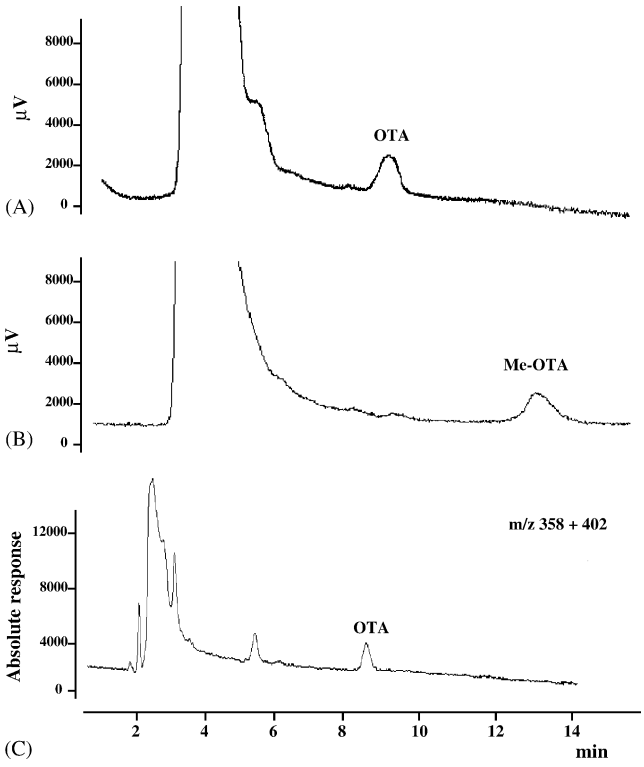

Fig. 2. Chromatograms of a positive sample of breakfast cereal (wheat and oat) containing OTA (5.35 ng/g): (A) LC–FLD; (B) LC–FLD with methylation of OTA and (C) LC–ESI–MS.

For cereal grains, the presence of OTA was not detected in any samples (Table 4). In the literature, several authors did not find this mycotoxin in cereals samples [9,22]. However, concentration of OTA higher than MRLs (5 ng/g) [7,23–25] was found in cereal samples. The occurrence of OTA in cereal grains is considered to depend principally on the condition of the grain at harvest, how carefully the grain is dried and the quality of the storage facilities [1].

Table 4
Incidence of ochratoxin A in different samples analyzed by the proposed method

Sample (cereal)	No. of samples	No. of samples with OTA level			Value of OTA (ng/g)
		<LOD	LOD–LOQ	>LOQ	
Cereals					
Rice	9	9	0	0	–
Wheat	5	5	0	0	–
Barley	5	5	0	0	–
Cereal products					
Pasta (wheat)	6	6	0	0	–
Flour (wheat)	15	15	0	0	–
Flour (corn)	3	3	0	0	–
Cookie (wheat)	4	1	3	0	–
Bread (wheat)	4	4	0	0	–
Breakfast cereals (wheat)	2	1	0	1	1.88
Breakfast cereals (corn)	4	3	1	0	–
Breakfast cereals (wheat and corn)	2	1	0	1	1.51
Breakfast cereals (wheat and oat)	2	1	0	1	5.35
Breakfast cereals (whole wheat)	1	1	0	0	–[a]

[a] False positive.

For cereal products, the obtained results are shown in Table 4. The presence of OTA was detected and confirmed in 7 out of 43 (16.3%) cereal product samples. One sample of breakfast cereals, elaborated with wheat and oat, was found to contain 5.3 ng/g of OTA; this level is higher than the European legislated MRLs (3 ng/g) [3]. According to the Skaug et al. (2001) [26], breakfast cereals could be important contributors to dietary OTA intake.

The presence of problems due to the co-extractive substances in the matrix have been observed by Wood et al. (1995) [11], thus, a confirmation of the positive analysis is necessary. The breakfast cereal of whole wheat (Table 4) shows the presence of an interfering signal by LC–FLD at the same time of retention of OTA. This problem was resolved with the procedures of confirmation, LC–FLD after methylation of OTA and LC–ESI–MS. As a consequence, such breakfast cereal sample (whole wheat) was classified as a false positive.

In conclusion, the proposed method is an inexpensive, rapid, straightforward cleanup procedure for determining OTA in cereals and cereals products being around 10 samples processed per day.

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