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Journal of Chromatography A, 1011 (2003) 49–54
JOURNAL OF CHROMATOGRAPHY A
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Determination of aflatoxins in peanuts by matrix solid-phase dispersion and liquid chromatography ☆

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Received 7 March 2003; received in revised form 27 May 2003; accepted 17 June 2003

Abstract

A new method based on matrix solid-phase dispersion (MSPD) extraction was studied to determine aflatoxin B1, B2, G1 and G2 from peanuts. Optimization of different parameters, such as type of solid supports for matrix dispersion and elution solvents were carried out. The method used 2 g of peanut sample, 2 g of C_{18} bonded silica as MSPD sorbent and acetonitrile as eluting solvent. Recoveries of each aflatoxin spiked to peanut samples at 2.5 ng/g (5 ng/g for aflatoxin G2) level were between 78 and 86% with relative standard deviations ranging from 4 to 7%. The limits of quantification ranged from 0.125 to 2.5 ng/g for the four studied aflatoxins using liquid chromatography (LC) with fluorescence detection. In addition, LC coupled to mass spectrometry with an electrospray interface was used for confirmation of aflatoxins present in real samples. Eleven peanut samples from different countries were analyzed by the proposed method and by using an enzyme-linked immunosorbent assay (ELISA). ELISA test is a good screening method for investigation of these mycotoxins in peanut samples.

Keywords: Food analysis; Matrix solid-phase dispersion extraction; Extraction methods; Aflatoxins

1. Introduction

Aflatoxins B1 (AFB1), B2 (AFB2), G1 (AFG1) and G2 (AFG2) are toxic metabolites produced by the food spoilage fungi *Aspergillus* particularly *A. flavus* and *A. parasiticus*. The AFB1 is listed as a carcinogen of group I by the International Agency for Research on Cancer [1]. Aflatoxins have been found as contaminants in agricultural and food products [2] being peanut [3–7] and their derivative products such as peanut butter [8] and oil [9], the main commodities to have high aflatoxin contamination. In the European Union, the aflatoxin B1 and the total aflatoxin level in peanut products are regulated with maximum residue levels (MRLs) that cannot be greater than 2 and 4 ng/g, respectively [10].

The most common solvents used for aflatoxins extraction are mixtures of chloroform–water [11], methanol–water [8] or acetonitrile–water [12]. For

☆ Presented at the 2nd Meeting of the Spanish Society of Chromatography and Related Techniques, Barcelona, 26–29 November 2002.

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0021-9673/03/$ – see front matter
doi:10.1016/S0021-9673(03)01102-6

clean-up, the use of immunoaffinity columns [8] or solid-phase extraction cartridges [13] which replaced the liquid–liquid partition procedures have been reported. Several chromatographic methods [14–18] have been used to analyze aflatoxins in foods being liquid chromatography (LC) with fluorescence detection (FLD) [19] or mass spectrometry detection (MS) [20] the most employed. Niedwetzki and Geschwill [21] developed an automatic work station for determination of aflatoxins.

Matrix solid-phase dispersion (MSPD) is applied to the analysis of several residues [22,23]. However to date, MSPD has not been used for analysis of aflatoxins from foods. The objective of this study was to apply the MSPD to the extraction of aflatoxins from peanuts and quantify the compounds by LC determination. The proposed method is applied to real samples and it is compared with the enzyme linked immunosorbent assay (ELISA) as a tool for routine analysis of aflatoxins in peanuts.

2. Experimental

2.1. Chemical and reagents

Acetonitrile, acetone, ethanol, diethyl ether, hexane, methanol and methylene chloride were supplied by Merck (Darmstadt, Germany). HPLC-grade water was obtained by filtering deionised water through a 0.45-μm filter with a Waters-Millipore (Milford, MA, USA) system. Solvents and water were degassed for 20 min using a Branson 5200 (Branson Ultrasonic Corp., CT, USA) ultrasonic bath and solid-phases used for MSPD were silica (40–60 μm), phenylsilica (50 μm), octylsilica (C_8) (50 μm) and octadecylsilica (C_{18}) (50 μm) bonded silica from Análisis Vínicos (Tomelloso, Spain).

The aflatoxins B1, B2, G1 and G2 crystalline materials were purchased from Sigma (St. Louis, MO, USA). Stock standard solutions of aflatoxins with concentrations of 500 μg/ml were prepared in methanol, kept in security conditions at −20 °C, wrapped in aluminium foil due to that the aflatoxins gradually breaks down under UV light and held for at least 3 months. Working solutions were diluted in acetonitrile and stored at −20 °C.

As safety notes, soak all used laboratory ware, pipette tips and kit components in 10% solution of household bleach before discarding. Accidental spills of aflatoxins must be swabbed with 5% NaOCl bleach.

2.2. Extraction procedure

Samples (200 g) were prepared using a food processor and mixed thoroughly. An aliquot (2 g) of the sample was placed into a mortar (50 ml capacity) and 2 g of the C_{18} sorbent and 0.5 g of sand were added and gently blended for 5 min using a pestle, to obtain a homogeneous mixture. The mixture was introduced with 1 g of silica into a 100×9 mm I.D. glass chromatographic column with a coarse frit (No. 2) and covered with a plug of silanized glass wool in the top of the column. Then, 4 ml hexane followed by 1 ml diethyl ether and 4 ml methylene chloride was passed through and discarded. After that, aflatoxins were eluted with 20 ml acetonitrile. The eluate was evaporated to dryness with gentle stream of N_2 at 45 °C. A volume of 2 ml of methanol was added, thoroughly mixed for 5 min and centrifuged at 5000 rpm for 10 min. The extract was filtered with a nylon acrodisk (0.45 μm), evaporated to dryness with N_2 at 45 °C, redissolved with 100 μl of trifluoracetic acid (TFA) for 3 min, re-evaporated to dryness with N_2 at 45 °C, and reconstituted in 1 ml of acetonitrile–methanol–water (1:1:1, v/v) for LC–FLD and in 0.1 ml of methanol–water (1:1, v/v) for LC–electrospray ionization (ESI) MS.

Recovery studies were carried out by spiking fresh samples (2 g) of raw peanut from local markets with 2.5 ng/g for aflatoxin B1, B2 and G1 and 5 ng/g for AFG2. For this study, samples were previously first analysed by ELISA, LC–FLD and LC–ESI-MS before being spiked and none of them were found of the studied aflatoxins.

The extraction procedure described above is based on the data obtained from different optimisation assays. They involved the study of different solid supports for matrix dispersion (silica, phenylsilica, C_8 and C_{18}) and also different solvents (ethanol, acetone, methanol, acetonitrile–water and acetonitrile) used for elution.

2.3. LC analysis

A Shimadzu (Kyoto, Japan) SCL-GA system LC equipped with two LC-GA pumps, a Rheodyne

Model 7125 injector (20 μl loop) and a SRF-535 fluorescence detector. A LC column Kromasil SC-18 (5 μm) (150×4.6 mm I.D.) (Scharlau, Barcelona, Spain) was used with a mobile phase consisting of a mixture of water–acetonitrile (25:75, v/v) at a flow-rate of 0.7 ml/min. Detection of aflatoxins was carried out using 365 and 435 nm as wavelengths for excitation and emission, respectively.

For confirmation of aflatoxins, a Hewlett-Packard (Palo Alto, CA, USA) HP-1100 Series LC–MS system equipped with a binary solvent pump, an autosampler and a MS coupled with an analytical work station was used. The MS detector consisted of a standard atmospheric pressure ionisation (API) source configured as electrospray. Separations were carried out at room temperature. The LC–ESI-MS interface in positive ion mode operated under the following conditions, 350 °C gas temperature, 13.0 l/min drying gas flow, 40 p.s.i. nebulizer gas pressure and 4000 V capillary voltage. The fragmentor selected was 120 V. Using this interface, the ions obtained for AFB1, AFB2, AFG1 and AFG2 were the protonated molecule $[M+H]^+$ and the sodium adduct $[M+Na]^+$ at m/z 313, 315, 329 and 331, and 335, 337, 351 and 353, respectively. These pairs of m/z ions were, respectively, selected for AFB1, AFB2, AFG1 and AFG2 identification. The mobile phase was a mixture water–methanol (55:45, v/v) at flow-rate of 0.7 ml/min. Finally, 20 μl were injected in each equipment.

2.4. Enzyme linked immunosorbent assay (ELISA) analysis

Samples (10 g) were analysed by the Aflatoxin B–G ELISA kit (TECNA R&D Diagnostics, Trieste, Italy) using the protocol of the manufacturer.

3. Results and discussion

3.1. Method performance

For validation studies, peanuts were selected because they are the most susceptible commodity to high aflatoxin contamination [2] and they are greatly consumed in our country [24]. On the other hand, the elution solvents and the polarity of solid-phases for the MSPD extraction must be adequately selected due to the possibility of interference from matrix components and the need for determining aflatoxins at levels close to the limit of sensitivity of the instruments. For this reason, 20 ml of ethanol, methanol, acetone, acetonitrile and acetonitrile–water (9:1, v/v) were tested as elution solvents. Results are shown in Table 1. Although, the use of the different eluting solvents assayed produced similar recoveries, acetonitrile was considered the best for the extraction because it gave cleanest extracts and chromatograms.

Table 1
Average recoveries (%) and relative standard deviation (RSD, %, in parenthesis) obtained with different elution solvents by using C_{18} MSPD extraction of raw peanuts spiked at 2.5 ng/g level (5 ng/g for AFG2, $n=5$)

Solvent	Aflatoxins			
	B1	B2	G1	G2
Acetone	77 (5)	70 (7)	78 (5)	77 (4)
Acetonitrile	78 (4)	81 (6)	82 (6)	86 (7)
Acetonitrile–water (9:1, v/v)	78 (6)	70 (5)	81 (4)	81 (8)
Ethanol	69 (5)	65 (4)	71 (3)	63 (6)
Methanol	71 (3)	67 (7)	75 (4)	69 (5)

Furthermore, silica, phenylsilica, C_8 and C_{18} were checked as solid supports for MSPD. Recoveries obtained by using these solid-phases are exposed in Table 2, it can be seen that the best recoveries for all aflatoxins obtained using C_{18} (with 20 ml of acetonitrile as elution solvent). The differences between the mean recoveries obtained with C_{18}, phenylsilica and silica were of statistical significance, but not those between C_{18} and C_8. The C_{18} phase proved to be better peanut-dispersant than the other solid supports assessed due to their hydrophobic characteristics which provided high affinity for these compounds, moreover it produced chromatograms more clean

Table 2
Average recoveries (%) and relative standard deviation (RSD, %, in parenthesis) obtained with different solid supports for matrix solid-phase dispersion, using acetonitrile as elution solvent, of raw peanuts spiked at 2.5 ng/g level (5 ng/g for AFG2, $n=5$)

Solid support	Aflatoxins			
	B1	B2	G1	G2
C_{18}	78 (4)	81 (6)	82 (6)	86 (7)
C_8	69 (6)	73 (5)	76 (5)	76 (4)
Phenyl	63 (5)	61 (4)	68 (7)	60 (8)
Silica	28 (9)	37 (7)	36 (10)	29 (9)

than those generated by other phases. The use of silica failed to extract the studied aflatoxins and produced more heterogeneous results.

Validation of the method was carried out according to these preliminary observations. Precision was calculated in terms of intra-day repeatability ($n=5$) and inter-day reproducibility (5 different days) on 2.5 ng/g (5 ng/g for AFG2) and 20 ng/g concentration levels for each analyte. The intra-day repeatability evaluated as RSD ranged from 4 to 7% at the lower level and from 3 to 6% at the higher level. The inter-day reproducibility was lower than 8% for all instances. Linearity was verified in triplicate with seven concentrations (0.1, 0.25, 0.5, 2.5, 5, 10 and 20 ng/g). The regression coefficients were all >0.997. In order to investigate if natural constituents of raw peanuts interfere with quantification, parallel calibration graphs obtained from matrix-extracted and solvent-based standards were performed. The covariance analysis for each aflatoxin showed that the calculated F values were lower than the F Snedecor tabulated ones indicating that both straight lines were parallel ($P<0.05$) and hence matrix effect is negligible. Fig. 1 shows the LC–FLD chromatograms, obtained following the MSPD extraction procedure, for a raw peanut sample (A) and non-fortified (B). Fig. 2 shows mass chromatograms obtained in ESI positive ion mode from raw peanuts spiked (A) and non spiked (B). Table 3 reflected the limits of detection (LODs) (S/N 3:1) and limits of quantification (LOQs) (S/N 10:1) for MSPD–LC coupled with FLD and MS in raw peanuts.

3.2. Application to real samples

The MSPD extraction followed by LC–FLD determination, LC–ESI-MS confirmation and ELISA test were applied to eleven types of peanuts (Table 4) from different countries (Brazil, China and USA) being three samples of unknown origin. All samples were obtained from Spanish supermarkets. In Table 4, the obtained results show that 4 samples (36.4%) contained aflatoxins at levels below the European legislated MRLs [10]. The analyses performed with these four samples by LC–FLD and LC–ESI-MS also showed the presence of aflatoxins. Table 4 shows that in a raw peanut sample from China, ELISA gave a positive result but LC–FLD did not found AFB1, this can be explained because the cross-reactivity of the ELISA test with AFG1 which was present in such sample. According to the manufacturer's data, the ELISA procedure determines the aflatoxin B1 (100%) with a cross-reactivity of about 20, 33 and 2.3% for aflatoxin B2, G1 and G2, respectively. This cross-reactivity is a limitation for the use of the ELISA test for quantification

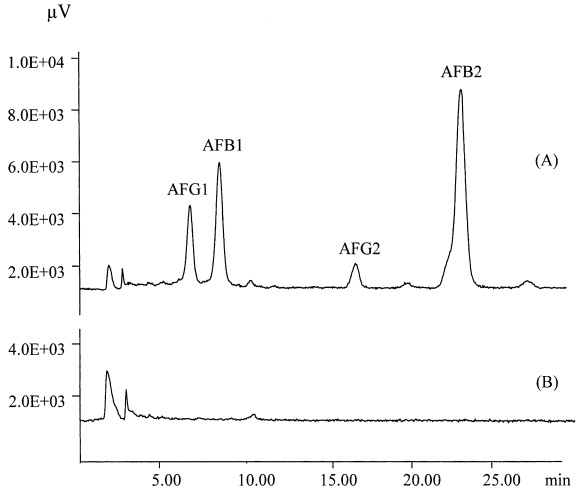

Fig. 1. LC–fluorescence chromatograms obtained after MSPD extraction: (A) raw peanut extract fortified with 1.5, 0.75, 2.5 and 1 ng/g of AFG1, AFB1, AFG2 and AFB2, respectively and, (B) non-fortified extract.

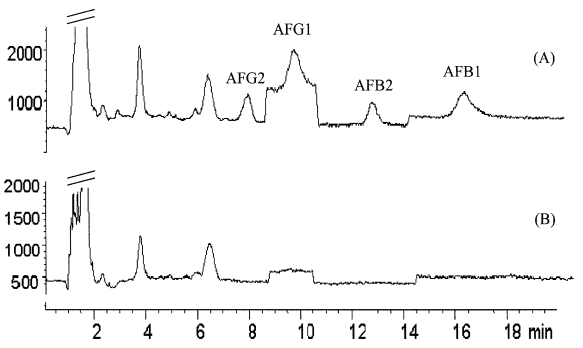

Fig. 2. LC–ESI-MS chromatograms obtained after MSPD extraction: (A) raw peanut extract fortified with 0.2 ng/g of AFG1 and AFB1 and 0.6 ng/g of AFG2 and AFB2 and (B) non-fortified extract.

Table 3
Limits of detection (LOD) and quantification (LOQ) for aflatoxins

	Aflatoxin	Instrumental		MSPD method	
		LOD (ng/ml)	LOQ (ng/ml)	LOD (ng/g)	LOQ (ng/g)
LC–FLD	AFB1	0.08	0.25	0.04	0.13
	AFB2	0.08	0.25	0.04	0.13
	AFG1	0.3	0.95	0.15	0.5
	AFG2	1.45	5	0.75	2.5
LC–ESI-MS	AFB1	1.35	4	0.07	0.2
	AFB2	3.8	12	0.2	0.6
	AFG1	1.35	4	0.07	0.2
	AFG2	3.8	12	0.2	0.6

purposes. On the other hand, no false negatives were found with the ELISA test.

Positive samples had occurrence of AFB1 and AFG1, but neither AFB2 nor AFG2 were detected in the analyzed samples. All positive samples were confirmed by LC–ESI-MS. Fig. 3 shows the LC–FLD and LC–ESI-MS chromatograms obtained by the MSPD procedure for a positive peanut sample.

These results demonstrate that for aflatoxin analysis of peanuts, ELISA and LC can complement each other. ELISA can be employed as the initial test and backed up by MSPD and LC due to that they are an appropriate methodology for routine aflatoxin analysis in peanuts at concentrations below MRLs. This combination can be beneficial in the quantification of these compounds because of the large number of samples that can be analyzed in a cost-effective way. Furthermore, the application of MSPD is easy to handle, time-saving, fewer interferences and requiring less solvent.

Acknowledgements

This work has been supported by the Spanish Ministry of Science and Technology in the framework of the Food Program (CAL 00-066).

Table 4
Incidence of aflatoxins in peanuts analyzed by ELISA and LC–FLD

Sample	Country of origin	ELISA AFB1 (ng/g)[a]	LC–FLD			
			AFB1 (ng/g)	AFB2 (ng/g)	AFG1 (ng/g)	AFG2 (ng/g)
Peeled salted peanuts	China	n.d.	n.d.	n.d.	n.d.	n.d.
	Unknown	0.15	0.15	n.d.	0.63	n.d.
Raw peanuts	China	0.13	n.d.	n.d.	0.61	n.d.
	USA	0.17	0.13	n.d.	n.d.	n.d.
Raw in-shell peanuts	Brazil	n.d.	n.d.	n.d.	n.d.	n.d.
Roasted and salted in-shell peanuts	China	n.d.	n.d.	n.d.	n.d.	n.d.
	Unknown	n.d.	n.d.	n.d.	n.d.	n.d.
Roasted in-shell peanuts	Brazil	n.d.	n.d.	n.d.	n.d.	n.d.
	China	n.d.	n.d.	n.d.	n.d.	n.d.
	Unknown	0.28	0.25	n.d.	1.68	n.d.
Salted peanuts	USA	n.d.	n.d.	n.d.	n.d.	n.d.

n.d. = not detected (below the quantitation limit).
[a] See text for cross-reactivity with AFB2, AFG1 and AFG2.

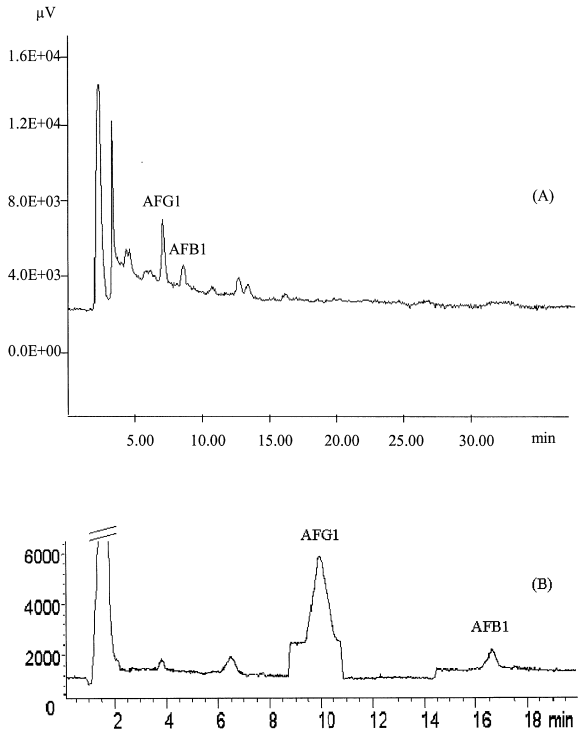

Fig. 3. Chromatograms of a positive peanut sample containing AFB1 (0.25 ng/g) and AFG1 (1.68 ng/g); (A) LC–FLD and (B) LC–ESI-MS.

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