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Analysis of Aflatoxins in Peeled Peanuts by Liquid Chromatography and Fluorescence Detection

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Aflatoxins are the important mycotoxins, the very important aflatoxins are named B1 (AFB1), B2 (AFB2), G1 (AFG1) and G2 (AFG2), produced by moulds *Aspergillus flavus* and *Aspergillus parasiticus* and infect food and feed crops before and after harvest (Ellis et al. 1991). The International Agency for Research on Cancer classified the AFB1 as a carcinogen of group I (IARC, 2002). Aflatoxins have been found in several foods (Ellis et al. 1991) being peanut and their derivative products (Ali et al. 1999; Blesa et al. 2003; Candlish et al. 2001; Selim et al. 1996; Sobolev and Dorner, 2002; Whitaker et al. 1999) the main commodities to have high aflatoxin level. To protect the health of the consumers, the European Union established legal directives to control their levels in peanuts through the maximum tolerated levels that are 2 and 4 ng/g for AFB1 and total aflatoxins, respectively (Anonymous, 2001).

The extraction methods for aflatoxins are based on the solubility of these toxins in organic solvents The most frequently employed solvents are chloroform (Stroka et al. 2000), methanol (Akiyama et al. 2001), and acetonitrile (Drummer et al. 1999). Furthermore, several authors have used liquid-liquid partitioning (Vinitkektumnuen et al. 1997), immunoaffinity columns (Stroka et al. 2000) or solid-phase extraction (SPE) (Akiyama et al. 2001) cartridges or matrix solid phase dispersion (MSPD) (Blesa et al. 2003; 2004) as purification procedure. On the other hand, the chromatographic method most employed is the liquid chromatography coupled with fluorescence or mass spectrometry detection (Jaimez et al. 2000; Papp et al. 2002).
The purpose of this work is to develop a method based on the extraction and clean-up steps for the determination of AFB1, AFB2, AFG1 and AFG2 in peeled peanuts and its application to real samples from Valencian port (Spain).

MATERIALS AND METHODS

Methanol, acetonitrile, hexane and chloroform were obtained from Merck (Darmstadt, Germany) and sodium chloride and anhydrous sodium sulphate from Panreac (Madrid, Spain) and trifluoroacetic acid (TFA) from Sigma (St. Louis,

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MO, USA). Deionised water (<8Ω cm resistivity) was obtained from a Milli-Q water purification system (Waters-Millipore, Milford, MA, USA). Solvents and water were degassed for 20 min using a Branson 5200 (Branson Ultrasonic Corp., CT, USA) ultrasonic bath.

The aflatoxins B1 (AFB1), B2 (AFB2), G1 (AFG1) and G2 (AFG2) crystalline materials were purchased from Sigma. 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 most 3 months. Working solutions were diluted in acetonitrile and stored at –20°C.

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

Random food samples were obtained from the port of Valencia (Spain) typically as a simple 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.
A modified method of AOAC (2000) was used to extract aflatoxins in peanuts. Briefly, a portion of sample (50g) was mixed with 100 ml of methanol-water (60:40, v/v) containing 4% of NaCl and blended thoroughly using a food processor. The homogenous mixture was filtered through a Whatman No. 5 filter paper, the filtrate transferred to a separation flask and washed with 20 ml hexane. Then, mycotoxins were extracted from the aqueous phase with 30 ml of chloroform and shaken for 1 min. The chloroform fractions were dried over anhydrous sodium sulphate and combined in a round-bottom flask and evaporated (50°C) till dry in a flash evaporator. The sample residue was dissolved in 5 ml of acetonitrile and evaporated to dryness on a Multiblock (60°C) under a stream of nitrogen before derivatization.

The derivatization procedure was prepared by adding 100 μl TFA to the evaporated solution of extract. The solution was homogenised, evaporated to dryness at 45°C and reconstituted in 1 ml of acetonitrile-water (1:1, v/v).

A Shimadzu (Kyoto, Japan) SCL-GA system LC equipped with two LC-GA pumps, a Rheodyne Model 7125 injector (20μl loop) and a SRF-535 fluorescence detector. A LC column Kromasil SC-18 (5μm) (150 x 4.6mm. i.d.) (Scharlau, Barcelona, Spain) was used with mobile phase consisting of a mixture of water-acetonitrile (75:25, 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 (Blesa et al. 2003, 2004).

For confirmation of aflatoxins, a Hewlett-Packard (Palo Alto, CA,USA) HP-110 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. The LC-ESI-MS interface in positive ion mode operated with these 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. Finally, 20 µl were injected in each equipment (Blesa et al. 2003, 2004).

RESULTS AND DISCUSSION

The response linearity was obtained in triplicate with seven concentrations (0.4, 0.8, 1, 2.5, 5, 10 and 20 ng/g). The regression coefficients were all >0.996. The values 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 for each mycotoxin calculated as relative standard deviation (RSD) ranged from 7 and 10% at the lower level and from 5 to 9% at the higher level. The accuracy was expressed as the percentage recovery obtained by the addition of 10 ng/g of the standard levels in triplicate to peanut samples, example of chromatogram with 10 ng/g, for each AF, standard solution is shown in Figure 1a. The average recovery was 81±7, 85±8, 92±9 and 93±7 % for AFB1, AFB2, AFG1 and AFG2, respectively. The limits of quantification (LOQ), (S/N 10:1), were 0.4, 0.7, 2 and 4 ng/g for AFB1, AFB2, AFG1 and AFG2, respectively.

Table 1. Aflatoxin concentrations for imported peanuts from positive samples.

Aflatoxin (ng/g)	Sample		
	1	2	3
AFB1	1.6	5.4	1.2
AFB2	-	5.8	-
AFG1	-	9.9	-
AFG2	-	38.3	-
Total aflatoxins	1.6	59.4	1.2

This study was carried out in cooperation with the Port Health Authorities from Valencia (Spain) which is authorized by the European Union to receive peanuts from other countries (Anonymous, 2003). In total 120 peanut samples were examined and concentrations of aflatoxins in positive samples are shown in Table 1. Three out of 120 samples were positive for these mycotoxins, for AFB1 in ranges of 1.2-5.4 ng/g, the sample with mayor contamination of AFB1 presented values for AFB2, AFG1 and AFG2 of 5.8, 9.9 and 38.3 ng/g, respectively, the chromatogram of this sample are depicted in Figure 1b, the Figure 1c shows a blank of peanut. All positive samples were confirmed by LC-ESI-MS. The

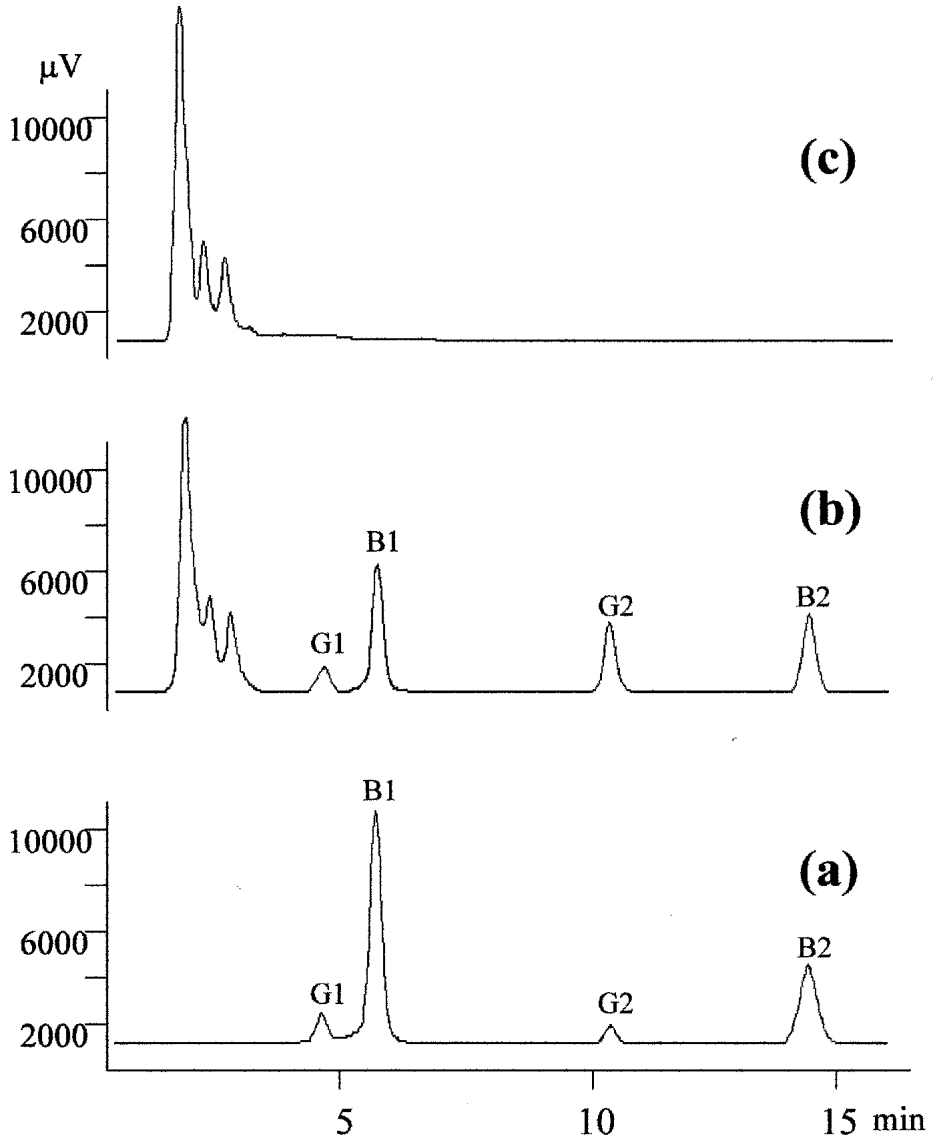

Figure 1. LC-fluorescence chromatograms obtained of (a) aflatoxins standard solution (10 ng/g for each aflatoxin), (b) peeled peanuts containing 5.4, 5.8, 9.9 and 38.3 ng/g of AFB1, AFB2, AFG1 and AFG2, respectively, and (c) blank of peeled peanuts.

obtained results show that 1 out of 3 positive samples contained aflatoxins at levels higher that the European legislated MRLs which are 2 and 4 ng/g from AFB1 and total aflatoxins levels in peanut products (Anonymous, 2001).

The sample which fail the EU legislation was destroyed under the supervision of the competent authority and the other contaminated samples entered in the food chain due to that physical treatments are possible to reduce the contamination (Rustom, 1997). This method is used due to the establishment of monitoring and surveillance programs for mycotoxins that requires suitably equipped laboratories, well-trained staff for both analytical and inspection activities, reliable analysis and sampling methods and application of analytical quality assurance programmes (Park et al. 1999, Sashidhar et al. 1992).

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