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Determination of aflatoxins M1, M2, B1, B2, G1 and G2 in peanut by modified QuEChERS method and ultra-high performance liquid chromatography-tandem mass spectrometry

Determinação de aflatoxinas M1, M2, B1, B2, G1 e G2 em amendoim utilizando um método QuEChERS modificado e cromatografia líquida de ultraeficiência com detecção por espectrometria de massas sequencial

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ABSTRACT

A suitable method for routine analysis of aflatoxins M1, M2, B1, B2, G1, G2 in peanut by ultra-high performance liquid chromatography-tandem mass spectrometry was developed and validated. The sample preparation was performed using a triple partitioning (water/acetonitrile/hexane) modified Quick Easy Cheap Effective Rugged and Safe (QuEChERS) method. For the first time, this method is reportedly used for aflatoxins analysis in peanut. Satisfactory recoveries ranged from 71 to 101%, with relative standard deviation lower than 15% were obtained for the target aflatoxins. The determination coefficients were ≥ 0.99 which showed good linearity. The LOD and LOQ varied from 0.03 to 0.26 ng g^{-1} and 0.1 to 0.88 ng g^{-1}, respectively. The validated method was successfully applied to for the determination of aflatoxins in ten peanut samples. Total aflatoxin concentration exceeded the maximum level permitted by the Brazilian regulation in one sample of roasted peanut, while aflatoxins M1 and M2 were detected respectively in three and in one of the samples. The results strongly suggest that peanuts and peanut products should be continuously monitored for the aflatoxins investigated in this work.

KEYWORDS: Aflatoxins; Peanut; QuEChERS; UHPLC-MS/MS

RESUMO

Um método adequado para a análise de rotina de aflatoxinas M1, M2, B1, B2, G1, G2 em amendoim por cromatografia líquida de ultraeficiência com espectrometria de massas foi desenvolvido e validado. A preparação da amostra foi realizada utilizando um método QuEChERS (*Quick Easy Cheap Effective Rugged and Safe*) modificado, empregando partição tripla (água/acetonitrila/hexano). Pela primeira vez este método foi utilizado para análise de aflatoxinas em amendoim. Recuperações satisfatórias, entre 71 e 101%, com coeficientes de variação inferiores a 15%, foram obtidas para as aflatoxinas estudadas. Os coeficientes de determinação foram ≥ 0,99, demostrando boa linearidade. Os limites de detecção e de quantificação variaram de 0,03 a 0,26 ng g^{-1} e de 0,1 a 0,88 ng g^{-1}, respectivamente. O método validado foi aplicado com sucesso na determinação de aflatoxinas em dez amostras de amendoim. Para uma amostra de amendoim torrado foi encontrado valor de concentração de aflatoxinas totais acima do limite máximo permitido pela regulamentação brasileira. As aflatoxinas M1 e M2 foram detectadas, respectivamente, em três e em uma das amostras das analisadas. Os resultados obtidos sugerem fortemente a necessidade de se realizar um contínuo monitoramento da contaminação do amendoim e de seus produtos pelas aflatoxinas investigadas nesse estudo.

PALAVRAS-CHAVE: Aflatoxinas; Amendoim; QuEChERS; CLUE-EM/EM

INTRODUCTION

Mycotoxins are secondary metabolites produced by filamentous fungi and are classified into several groups[1]. Among these, aflatoxins have been considered of special concern to food safety in many countries due to the high occurrence of these compounds as food contaminants and to their toxic effects on human health[2,3,4]. The aflatoxins are classified as being carcinogenic to humans by International Agency for Research on Cancer[5].

The aflatoxins B1, B2, G1, G2 are the major aflatoxin found as contaminants in peanuts and peanut products. The worldwide regulations set the maximum limits for the aflatoxins B1 and/or sum of the aflatoxins B1, B2, G1, G2 (total aflatoxin) in peanut and several other foods. The Brazilian regulation only set the maximum limit (20 ng g^{-1}) for total aflatoxin[6,7,8,9].

The aflatoxins M1 and M2 are the major metabolites of aflatoxins B1 and B2, repectively, produced in the metabolism of mammals and therefore several studies have been conducted to demonstrate the contamination of milk and dairy products by these compounds[10,11]. The aflatoxins M1 and M2 may also be produced by fungi cultures in minor amounts[12,13].

The contamination of peanut butter, natural peanut and peanut cake by aflatoxins M1 and/or M2 has been demonstrated recently[14,15,16]. Nevertheless, the contamination source has not been elucidated so far. The presence of aflatoxin M1 has also been observed in corn[17,18] and in traditional Chinese medicines[19].

Although M1 and M2 aflatoxins have a less toxic potential than B1 and B2, the contamination of peanut and their derivative products should be monitored. Thus, reliable analytical methods are required.

Several analytical techniques and sample treatment methods have been used to determine mycotoxins in food matrices[20,21,22,23]. In the last decade, high performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) and ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) have been widely used for the determination of mycotoxins in food[20], including peanut and their derivative products[15,24,25,26]. Regarding the methods used for sample treatment, the QuEChERS method (quick, easy, cheap, effective, rugged and safe), developed for analysis of pesticides in fruits and vegetables[27], has been widely used for the determination of mycotoxins in food [28,29,30].

For the simultaneous determination of the six aflatoxins in peanut and their derivative products, only the method utilizing clean-up with a home-made mixed cartridge and analysis by UHPLC-MS/MS was published[14].

The aim of this study was thus to develop and validate a method to be used in routine analysis of aflatoxins M1, M2, B1, B2, G1, G2 in peanuts and peanuts products. Briefly, the method involves a simultaneous sample extraction and clean-up step, based on modified QuEChERS method, using hexane to remove the lipids and UHPLC-MS/MS for determination of the target compounds.

To the best of our knowledge, this is the first report describing the use of a triple partitioning modified QuEChERS method for aflatoxins analysis in peanut.

MATERIAL AND METHOD

Reagents and chemicals

Acetonitrile, methanol (HPLC grade) and sodium chloride (> 99%) were purchased from J T Baker (Phillipsburg, NJ, USA). Acetic acid (HPLC grade) was supplied by Tedia (Fairfield, Ohio, USA). Anhydrous magnesium sulfate (> 95%) was supplied by Caledon Laboratory (Georgetown, Ont., Canada). Hexane (> 96%) was obtained from Merck (Darmstadt, Germany). Ultrapure water was obtained from Milli-Q Gradient water system (Millipore, Bedford, MA, USA). The standards (aflatoxins B1, B2, G1, G2, M1 and M2) and ammonium formate (> 99%) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Standard solutions preparation

Individual stock solutions of the aflatoxins were prepared in acetonitrile. The concentrations of the prepared stock solutions (10 µg mL^{-1}) were checked by UV spectrophotometry[31]. Aliquots of the stock solutions were combined to obtain an intermediate solution in acetonitrile (100 ng mL^{-1}) for all target aflatoxins. The working solutions were also prepared in acetonitrile by serial dilutions of the intermediate solution. The solutions were stored at -20ºC until used. To prepare the matrix-matched calibration curves, working solution aliquots were evaporated to dryness and then resuspended with the same volume of uncontaminated samples extracts (blank matrices).

Sample treatment

To 5 g of the sample, weighed in a 50 mL centrifuge tube, were added 10 mL of ultrapure water, 10 mL of hexane and 15 mL of acetonitrile; the tube was then shaken for 30 s; a mixture of 4 g of magnesium sulphate and 1.5 g of sodium chloride was added, the tube was immediately shaken vigorously using a vortex for 1 min and then centrifuged at 3000 rpm for 7 min. An aliquot of 5 mL of the acetonitrile phase was evaporated to dryness under a gentle flow of nitrogen at 45ºC and then the residue was dissolved with 2 mL of methanol/water (1:1, v/v). The solution thus obtained was filtered through a 0.22 mm polyethylene filter before injection.

UHPLC-MS/MS analysis

Liquid chromatography was performed using an ACQUITY UPLC™ system (Waters). A BEH C18 column (100 mm x 2.1 mm i.d., 1.7 µm particle size; Waters, Milford, MA, USA) was used as the stationary phase. The column temperature was maintained at 35ºC. Methanol (Phase B) and aqueous solution of ammonium formate (5 mM) (Phase A) were used as mobile phases. Mobile phase B increased linearly from 10% to 100% in 4 min and then was held

constant for 1.5 min. The system was then re-equilibrated for 2 min at the initial mobile phase composition. The flow rate was set at 0.3 mL min^{-1}. The injection volume was 5 µL.

The detection was performed in positive mode using a tandem mass spectrometry (Waters, Quattro Premier XE) with electrospray ionization interface. The source parameters were capillary voltage 3.5 kV, extractor voltage 3 V, rf lens 0.1 V, multiplier 750 V, desolvation temperature 350ºC, source temperature 120ºC. Nitrogen was used as cone and desolvation gas at a flow of 50 L h^{-1} and 750 L h^{-1}, respectively. Argon was used as collision gas at a pressure of 4 x 10^{-3} mbar. The two ion transitions selected for each aflatoxin and acquisition conditions performed are show in Table 1. The analytes were allocated in one acquisition time windows. For all aflatoxins the dwell time was 200 ms. The interchannel delay and interscan delay were both 5 ms.

Peanut Samples

Eight samples of roasted peanut and two samples of raw peanuts were purchased from local supermarkets in Rio de Janeiro, RJ, Brazil.

RESULTS AND DISCUSSION

UHPLC-MS/MS optimization

The MS/MS conditions were optimized by continuous infusion of individual working standard solutions (1000 ng mL^{-1}) in ESI positive mode. The cone voltage was optimized and the protonated precursor ion $[M+H]^+$ were selected for all compounds. Collision energies were applied to obtain the fragment ions. Then, the collision energies were optimized for each transition and the two more intense product ions were selected in the MS acquisition method (quantification and confirmation transitions ions). The capillary voltage was optimized by injection of the compounds in the chromatography system. The capillary voltage selected was 3.5 kV, which provide satisfactory sensibility for all compounds. Dwell times were optimized in order to obtain satisfactory reproducibility and sensibility.

The sensibility and peak separation of the mycotoxins were evaluated using acetonitrile and methanol as the mobile phases. Greater sensibility and good peak shape for target compounds were achieved using methanol as mobile phase. Aflatoxins M1 and G2 peaks could not be separated in the optimized chromatographic conditions. However, these substances could be detected due to the high selectivity of mass spectrometry.

The effect on the sensitivity of substances due to addition of ammonium formate and ammonium acetate in the mobile phase, which avoid stable adducts formation, was also evaluated. In all cases the sensitivity was higher using ammonium formate in the mobile phase.

Optimization of the sample preparation procedure

The sample preparation method used in this study is based on a procedure reported by Przybylski and Segard[32], on analyzing pesticides in meat based baby-food. The mentioned authors described a modification of the QuEChERS mehod in which it was introduced a triple water, acetonitrile and hexane partitioning extraction step, to reduce liphophile components of the samples.

The method optimization was performed using peanut samples (5 g) fortified with target aflatoxins in the intermediate concentration level used in the validation step (5 ng g^{-1}). The efficiency of extraction for two solvents (15 mL of acetonitrile and 1% of acetic acid in acetonitrile) was evaluated. Acetonitrile was selected as the extraction solvent because no co-elution of matrix-interfering compounds was observed and the obtained recoveries were considered satisfactory for all analytes studied (70 to 120%). The co-elution of matrix-interfering compounds, mainly for aflatoxin M2, and unsatisfactory recoveries (< 70% for aflatoxins B1 and G1; > 120% for aflatoxins M1 and G2) were obtained using 1% acetic acid in acetonitrile. To remove the lipids globules, the addition of different volumes of hexane (5 and 10 mL) was evaluated. The addition of 10 mL of hexane was selected because it was more efficient to remove the lipids globules in the acetonitrile without losing the analytes. To increase the method sensitivity, an aliquot of 5 mL of acetonitrile extract was concentrated to dryness. The residue thus obtained was then dissolved by using a 50/50 (v/v) methanol/water mixture, a mixture of solvents which was found to affect favorably the shape of the peaks and to avoid loss of the target compounds associated with matrix precipitation and filtration.

Validation of the analytical method

Single laboratory validation was performed by evaluating of the following analytical performance parameters: selectivity, linearity, trueness (recovery), precision (repeatability and

Table 1. The selected ion transitions and acquisition MS/MS parameters.

	Quantification transitions	Confirmation transitions	Collision energy* (eV)	Cone voltage (V)
Aflatoxin M2	331.3 > 273.3	331.3 > 285.2	25 / 20	45
Aflatoxin M1	329.2 > 259.2	329.2 > 273.2	25 / 20	50
Aflatoxin G2	331.3 > 313.3	331.3 > 245.3	25 / 30	40
Aflatoxin G1	329.2 > 243.2	329.2 > 311.2	25 / 20	45
Aflatoxin B2	315.2 > 259.0	315.2 > 287.0	30 / 25	50
Aflatoxin B1	313.0 > 269.2	313.0 > 285.2	35 / 25	40

*Values are given in the order: quantification transition ion/confirmation transition ion.

intermediate precision), limit of detection (LOD), and limit of quantification (LOQ).

The selectivity of the method was evaluated by the application in blank matrices (peanut, peanut skinless and peanut roasted). The absence of interference signals eluting at the same analytes retention time was verified in all matrices. The identification of the aflatoxins was performed by comparison of retention time and signal intensity ratios of the two ion transitions monitored. The Figure 1 shows the chromatograms of the studied aflatoxins in roasted peanut.

Liquid chromatography with tandem mass spectrometry detection is a powerful analytical technique for detection and quantification of analytes in complex matrices. However, it is known that using this technique the analyte signals are highly susceptible to matrix effect (signal suppression or enhancement) in the presence of co-eluting matrix components. To investigate the matrix effect, the matrices peanut, peanut skinless and peanut roasted were selected. The calibration curves of each matrix selected and calibration curve in solvent (methanol/water (1:1 v/v) were prepared in duplicate at concentration ranges from 0.15 to 15 ng mL^{-1}. The solutions were analysed in triplicate and ordinary least squares regression was applied for the elaboration of the calibration curve.

The calibration curve slopes were compared by analysis of covariance (ANCOVA), considering a significance level of 5%[33,34,35]. As premise for comparison of calibration curves by ANCOVA, it is necessary to check if the residual variances of the calibration curves are homogeneous. The residual variances were checked by Levene`s test, considering a significant level of 5%. The homogeneity of all the calibration curves was confirmed (p-values > 0.05)[36]. Then, the slopes of the calibration curves of each matrix studied were compared with the slopes of the calibration curves in solvent. There were significant differences between the slopes of the calibration curves prepared in solvent and matrices for the majority of the target compounds, except for the aflatoxins M1 and M2 in peanut and peanut roasted (p-value > 0.05). The slopes of the calibration curves prepared in matrices were also compared in order to find out a possible representative matrix-matched calibration. There were significant differences between the calibration curves for the target aflatoxins (p-value < 0.05). Therefore, matrix-matched calibration was used for quantification of the aflatoxins in routine analyses.

Table 2 shows the values of matrix effects for aflatoxins in the three matrices studies. The numerical values were calculated by Equation 1, where $Slope_{solvent}$ is the slope of the calibration curve in solvent and $Slope_{matrix}$ is the slope of the matrix-matched calibration (positive values indicate signal enhancement and negative values indicate signal suppression).

$$Matrix\ Effect\ (\%) = (Slope_{solvent} - Slope_{matrix} / Slope_{solvent}) \times 100 \quad \text{Equation 1}$$

Due to the presence of significant matrix effects for some mycotoxins, linearity was evaluated using matrix-matched calibration

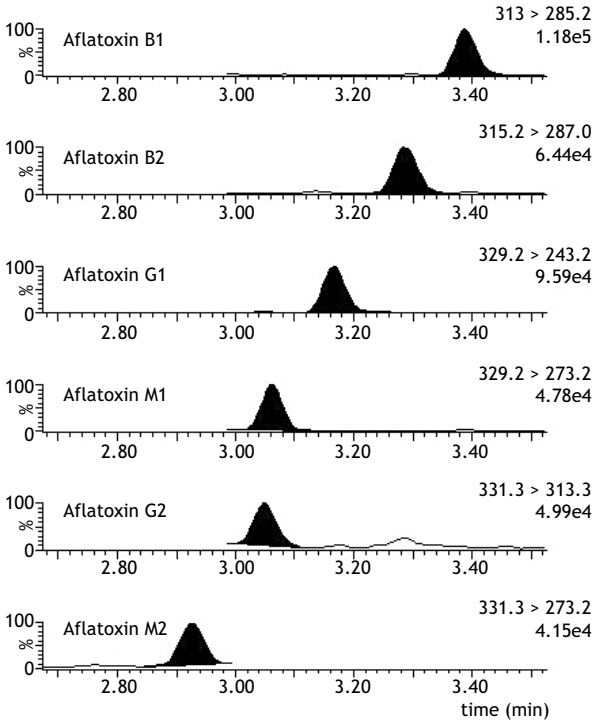

Figure 1. Chromatogram (quantification transitions) of the aflatoxins in the fortified roasted peanut (2.5 ng g^{-1}).

Table 2. Matrix effects (%) for aflatoxins in evaluated matrices.

Aflatoxins	Matrix effects (%)[a]		
	Peanut	Peanut (skinless)	Peanut (roasted)
M2	-7	16	-6
M1	2	15	6
G2	-26	-29	-42
G1	-39	-34	-33
B2	-36	-24	-42
B1	-23	-14	-26

[a]Signal enhancement (+); Signal suppression (-)

curves with three triplicates of six calibration levels between 0.15 and 15 ng mL^{-1}. Ordinary least squares regression was applied for the elaboration of the calibration curve. The resulting linear coefficients were always greater than 0.99. The recovery and precision (repeatability) were evaluated using peanut (blank matrix) spiked with the aflatoxins at 3 levels (ng g^{-1}) with four replicates for each level. The results are reported in Table 2, in which the precision is expressed by the relative standard deviation (RSD) and trueness by the recovery values. The recovery values ranged from 71 to 101%, with RSD lower than 13% for all mycotoxins and in the concentration levels evaluated, showed good trueness and precision of the developed method[37]. The intermediary precision (evaluated in three different days) was evaluated using the intermediate concentration level (5 ng g^{-1}) and satisfactory results were obtained for all target analytes with RSD in the range of 4.8 and 15.1%.

Table 3. Limits of detection, limits of quantification, recovery and precision values obtained in the validation.

Aflatoxins	LOD	LOQ	[a]RSD	1 ng g^{-1} (n = 4)		5 ng g^{-1} (n = 4)		10 ng g^{-1} (n = 4)	
				Rec	[b]RSD	Rec	[b]RSD	Rec	[b]RSD
M2	0.06	0.19	5.4	100.3	12.4	91.3	6.9	82.6	10.7
M1	0.14	0.48	11.9	87.2	9.9	76.4	4.8	74.9	4.1
G2	0.26	0.88	6.0	98.9	10.7	93.8	3.9	85.6	4.5
G1	0.03	0.10	15.1	98.5	1.6	83.1	2.7	79.3	3.8
B2	0.21	0.69	7.3	86.8	8.0	77.7	5.2	71.3	2.1
B1	0.13	0.43	4.8	89.8	4.9	84.9	1.5	83.5	2.0

0.5, 2.5 and 5 ng g^{-1} for the aflatoxins M1 and M2; Rec: recovery; [a]RSD: relative standard deviation (intermediate precision); [b]RSD: relative standard deviation (repeatability); LOD: limit of detection (ng g^{-1}); LOQ: limit of quantitation (ng g^{-1})

Samples spiked with all the target aflatoxins in lower concentration level were used to calculate the limits of detection (LOD) and limits of quantification (LOQ), considering signal-to-noise ratios of 3 and 10, respectively. The summary results are shown in Table 3.

Sample analyses

The validated method was applied to the determination of aflatoxins in commercial peanut samples. The results are shown in Table 4. Total aflatoxins concentration value was found to exceed the Brazilian maximum permitted level (20 ng g^{-1}) in one sample of roasted peanuts.

The aflatoxins M1 and M2 were detected respectively in three and one of the samples, highlighting the importance of the multiresidue method delevoped. A chromatogram for a sample

Table 4. Aflatoxin concentration in peanut samples.

Analyzed samples	Aflatoxins (ng g^{-1})					
	M2	M1	G2	G1	B2	B1
Peanut (roasted)#1	nd	nd	nd	nd	nd	nd
Peanut (roasted)#2	nd	nd	nd	1.03	> LOD	2.34
Peanut (roasted)#3	0.98	0.53	nd	nd	2.78	10.20
Peanut (roasted)#4	nd	0.55	nd	nd	4.08	23.59
Peanut (roasted)#5	nd	nd	nd	nd	nd	0.44
Peanut (roasted)#6	nd	> LOD	nd	> LOD	0.70	2.82
Peanut (roasted)#7	nd	nd	nd	nd	> LOD	1.54
Peanut (roasted)#8	nd	nd	nd	nd	nd	> LOD
Peanut (raw)#1	nd	nd	nd	nd	nd	nd
Peanut (raw)#2	nd	nd	nd	nd	nd	nd

nd: not detected; LOD: limit of detection

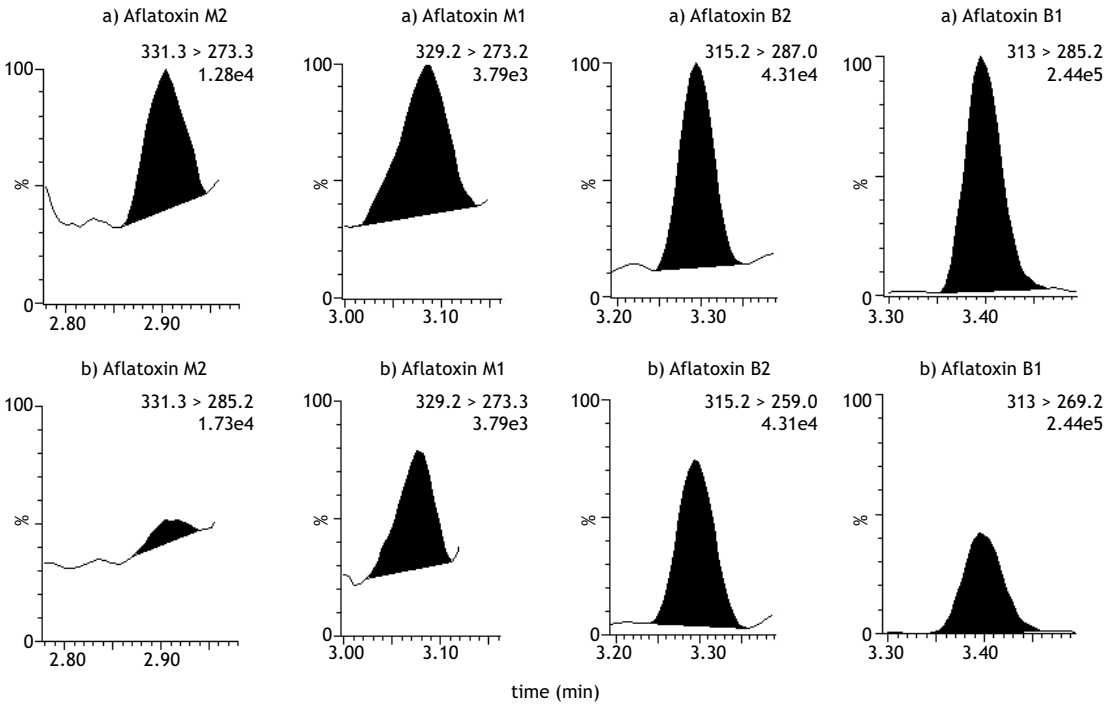

Figure 2. Chromatogram of a roasted peanut sample naturally contaminated by aflatoxins M2 (0.98 ng g^{-1}), M1 (0.53 ng g^{-1}), B2 (2.78 ng g^{-1}) and B1 (10.20 ng g^{-1}) corresponding to: a) quantification transitions, b) confirmation transitions.

naturally contaminated with aflatoxins M2, M1, B2 and B1 is shown in Figure 2.

CONCLUSION

A suitable modified QuEChERS method for analysis of aflatoxins M1, M2, B1, B2, G1, G2 in peanut by UHPLC-MS/MS was developed and validated. The method is be very useful for the quality control of peanut and peanut products, since it consistes of a very simple simultaneous extraction and clean-up step, followed by concentration of the extract. The validated method was successfully applied for the determination of aflatoxins in ten peanut samples. The results strongly suggest that peanuts as well as peanut products should be continuously monitored for the aflatoxins investigated in this work. The sample treatment method here proposed might be a powerful approach for the determination of aflatoxins in other food matrices.

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