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Determination of aflatoxins B1, G1, B2 and G2 in medicinal herbs by liquid chromatography–tandem mass spectrometry☆

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Abstract

An easy method for the determination of aflatoxins B1, G1, B2 and G2 in *Rhammus purshiana* by LC coupled to mass spectrometry has been developed. Aflatoxins were extracted with a mixture of methanol and water and then it was purified by solid-phase clean-up using a polymeric sorbent, not described previously, for the determination of these toxins. The eluted extract was injected into the chromatographic system using a reversed-phase C_{18} short column with an isocratic mobile phase composed of methanol–water (30:70). A single-quadruple mass spectrometry using an electrospray ionization source operating in the positive ion mode was used to detect aflatoxins due to derivatization presenting several disadvantages. Recoveries of the full analytical procedure were 110% for aflatoxin B1, 89% for aflatoxin B2, 81% for aflatoxin G1 and 77% for aflatoxin G2. Detection limit (S/N = 3) was 10 ng and quantification limit (S/N = 10) was 25 ng, calculated as amount in medicinal herb.

Keywords: Aflatoxins; Mycotoxins; *Rhammus purshiana*; SPE; LC; MS

1. Introduction

Aflatoxins B1, G1, B2 and G2 (AFB1, AFG1, AFB2 and AFG2) (Fig. 1) are fungal secondary toxic metabolites produced by *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus nomius* [1]. Aflatoxins are the strongest natural carcinogens and their main target organ is the liver. The International Agency for Research on Cancer (IARC) has classified aflatoxin B1 in the group 1 as a human carcinogen and aflatoxins G1, B2 and G2 in the group 2B as possibles carcinogens to humans [2].

These mycotoxins occur widely in vegetable products like cereals, specially corn, nuts, peanuts, coconut, fruits, dried fruits, beer and meat products of animals fed with contaminated feed [3–7]. They have also been found in medicinal herbs [8–11]. Some years ago, it was thought that most of the medicinal herbs contain substances that inhibit the growth of fungi and the subsequent mycotoxin formation. Recent studies published a list of medicinal herbs that can be contaminated by fungi and detected mycotoxins [12–14].

Aflatoxins are probably the most common mycotoxins to which humans are exposed by ingestion. The determination of these toxins is not easy due to their being found in complex matrixes and should be detected in low concentrations. Most of the analytical methods currently published for the determination of aflatoxins employ solvent extraction with harmful solvents or immunoaffinity columns and multifunctional columns that are very expensive and matrix dependant [15–18]. Chromatographic determination with fluorescence detector is the most commonly used. In order to improve detection limits for AFG1 and AFB1 pre-column formation of hemiacetal derivatives with trifluoroacetic acid

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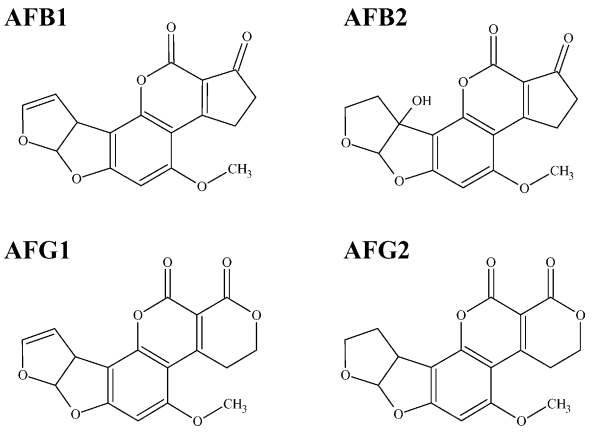

Fig. 1. Structure of alfatoxins.

or by post-column derivatization with bromine or iodine must be done [19–22]. The purposes of the study were to develop a method for the determination of aflatoxins in *Rhammus purshiana* testing a polymeric solid-phase clean-up sorbent in the sample purification step. Similar polymeric sorbents have been described for the determination of these mycotoxins [23,24]. Aflatoxins would be analyzed by liquid chromatography and detected by mass spectrometry single–quadruple using an electrospray ionization source (LC–MS) in order to avoid derivatization due to it presenting several disadvantages [25–26]. In order to evaluate if *R. purshiana* is a good substrate for aflatoxin production, herbs samples were inoculated with *Aspergillus parasiticus* and after the incubation period aflatoxin content was determined.

2. Experimental

2.1. Solvents and reagents

Afatoxins standards (B1, G1, B2 and G2) were supplied by Sigma–Aldrich (St. Louis, MO, USA). The stock solution of 200 mg/l was prepared in LC grade methanol (Merck, Darmstadt, Germany) and stored at 4 °C in the darkness. All other standard solutions were prepared immediately before use by diluting the stock solution with mobile phase.

Milli-Q quality water (Millipore, Bedford, USA) and other chemicals of analytical grade or LC quality needed for determination were obtained from Merck.

2.2. Fungi

A. parasiticus CECT 2681 was used to innoculate medicinal herbs. The culture was maintained on potato dextrose agar (PDA) medium and stored at 4 °C in the darkness.

2.3. Solid-phase clean-up

Purification was performed with Oasis HLB (3 ml/60 mg) cartridges (Waters, Milford, MA, USA) on a vacuum manifold (IST, Hengoed, Mid Glamorgan, UK) connected to a Vacum-Sel 3001001 vacuum membrane pump (J.P. Selecta, Barcelona, Spain) to allow a flow rate of 1 ml/min.

2.4. LC determination

The LC system consisted of an Alliance 2690 (Waters). Chromatographic separations were performed on a LiChrocart C_{18} short column (30 mm × 4 mm, 3 μm) (Merck) at 30 °C, with an isocratic mobile phase of methanol–water (30:70) at a flow rate of 1 ml/min. The injection volume was 10 μl.

2.5. Mass spectrometry (ESI–MS)

Mass spectrometry was performed on a ZMD (Waters) single–quadruple equipped with an electrospray ionization (ESI) source and operating in the positive ion mode. The parameters used for the mass spectrometer in all experiments were: capillary voltage 3.0 kV, source block temperature 100 °C, evaporation temperature 350 °C, solvent gas 475 l/h, cone gas 50 l/h, low mass resolution 15, high mass resolution 15, ion energy 0.5, extractor 7, Rf lens 0.5 and electron multiplier voltage 650. Cone voltage for aflatoxins B1, G1 and G2 was 20 V and for B2 it was 40 V. The ions monitored in single ion recording were the protonated molecule $[M + H]^+$ at *m*/*z* 313.2 for B1, 329.2 for G1, 315.2 for B2 and 331.2 for G2. Sodium adducts $[M + Na]^+$ at *m*/*z* 335.2, 351.2, 337.2 and 353.2 were also monitored.

2.6. Extraction of aflatoxins from Rhammus purshiana

A portion of 5 g of grinded *R. purshiana* was weighed in an erlenmeyer flask. Aflatoxins were extracted with 50 ml of a mixture of methanol–water (80:20) with magnetic stirring for 30 min. The extract was filtered through Whatman filter paper (No. 3) and an aliquot of 4 ml was added 2.6 ml of milli-Q water. It was then passed through the Oasis HLB cartridge at a flow rate of 1 ml/min, previously conditioned with 2 ml of methanol and equilibrated with 2 ml of Milli-Q water. The cartridge was washed with 2 ml of a mixture of methanol–water (30:70). Mycotoxins were eluted with 2 ml methanol and collected in a clean vial. The eluted extract was injected into the LC–MS.

2.7. Inoculation of Rhammus purshiana with fungi

With the purpose of studying aflatoxins production in *R. purshiana* samples, approximately 5 g of medicinal herb was sterilized with UV light treatment during 30 min and then 5 ml of sterile water (121 °C, 20 min) was added. Samples were inoculated with 1 ml of a homogeneous suspension (10^7 conidia/ml) of *A. parasiticus* incubated in PDA for 7 days at 27 °C. Samples of medicinal herbs were incubated at 27 °C for 7 and 14 days in the darkness.

As a positive control for toxins production, 5 g of rice with 5 ml of water were sterilized in autoclave (121 °C, 20 min) and then it was inoculated with 1 ml of a homogeneous suspension (10^7 conidia/ml) of *A. parasiticus* incubated in PDA for 7 days at 27 °C. Samples of rice were incubated at 27 °C for 7 and 14 days in the darkness.

2.8. Linearity, recovery, repeatability, intermediate precision and detection limit

Linearity was done by injecting duplicate aflatoxin standards of 10, 20, 50, 100, 200, 500, 1000, 2000 and 5000 ng of each aflatoxin. Recovery was obtained by addition method. *R. purshiana* samples were spiked with 500, 750 and 1000 pg of aflatoxin standard. Repeatability was estimated by assaying three replicate samples spiked with 500 pg of each aflatoxin standard on the same day by the same analyst. Intermediate precision was estimated from the determination of three samples spiked with 500 pg of each aflatoxin, stored at 4 °C in darkness, by the same analyst on different days.

Quantification and detection limit were determined by spiked samples based on signal to noise ratio of 10:1 for quantification limit and 3:1 for detection limit.

2.9. Calibration curve and quantification

Standard solutions for the calibration curves were prepared daily. Calibration curves were constructed by plotting the observed peak area against the aflatoxin amount injected (10, 50, 200, 400 and 600 ng). Quantification of medicinal herbs was performed by external standard by measuring peak area at each retention time and calculated with the calibration curve. Calibration curves were also fitted by linear least-squares regression and showed correlation coefficients greater than 0.999.

3. Results and discussion

As it has been mentioned before, at the moment there is no official method available for the determination of aflatoxins in medicinal herbs and all the studies consulted employ chloroform in the extraction step. Due to the high toxicity of chlorate solvents, we propose a method that uses a mixture of methanol and water in the extraction step. The purification was made with solid-phase extraction cartridges that contain a polymeric sorbent with reversed-phase properties. The results described below show that it is a good alternative.

3.1. Optimization of the cartridge load step

The first experiment consists of studying the cartridge load step working with aflatoxin standard dissolved in different methanol percentages. Replicate cartridges were conditioned with 2 ml of methanol and equilibrated with 2 ml of water. Cartridges were loaded with 4 ml replicate aflatoxin standards 20 μg/l (80 ng AFS) dissolved in the following proportions of methanol–water (70:30, 50:50, 30:70, 10:90 and 5:95). Toxins were eluted with 2 ml of methanol. With the purpose of assuring the total aflatoxin elution, cartridges were washed secondly with 2 ml methanol. Eluates were injected into the LC–MS. Standards with methanol content lower than 50% gave recoveries greater than 90%. In order to ensure this value, 5 g of *R. purshiana* were spiked with 0.5 ng of each aflatoxin standard. It was extracted with 50 ml of methanol–water (70:30) during 30 min in a magnetic stirrer and then filtered. An aliquot of 4 ml was added with 1.6 ml of water to have 50% methanol in the cartridge sample load step. The sample was applied to the cartridge using the procedure described above. Recoveries calculated by external standard were 33% for AFG2, 54% for AFG1, 45% for AFB2 and 73% for AFB1.

3.2. Optimization of the cartridge washing step

In order to increase recovery results, a washing step before eluting toxins was applied. Different columns were loaded with 4 ml of 20 μg/l of aflatoxins standard (80 ng AFS) dissolved in methanol–water (50:50) previously conditioned with 2 ml methanol and 2 ml water. Then different washing steps, increasing the methanol percentage (5:95, 10:90, 20:80, 30:70 and 50:50) were tested. Aflatoxins were eluted with 2 ml methanol and injected into the LC–MS. The condition that gave the greatest recoveries was methanol–water (30:70). Then, selected conditions were applied to *R. purshiana* samples. A portion of 5 g of *R. purshiana* was spiked with 0.5 ng of each aflatoxin. It was extracted with 50 ml of methanol–water (70:30) during 30 min in a magnetic stirrer and then filtered. An aliquot of 4 ml was added with 1.6 ml of water to have 50% methanol ratio in the sample load step. The sample was applied to the cartridge. It was washed with 2 ml of mixture of methanol–water (30:70). Toxins were eluted with 2 ml methanol and then injected into the LC–MS. Recovery results were 51% for AFG2, 76% for AFG1, 66% for AFB2 and 58% for AFB1.

3.3. Solvent extraction evaluation

In order to evaluate the efficiency of the extraction solvent, different extraction solvents were tried. Samples of 5 g of *R. purshiana* were spiked with 0.5 ng of each aflatoxin and extracted with 50 ml methanol–water (70:30), (80:20) and (90:10). Aliquots of 4 ml of the filtrate were added with water to have 50% methanol content and then applied to the cartridge following the process described above. Greatest recoveries results were obtained with 80% methanol content in the extraction solvent.

3.4. Optimization of the mass spectrometric detection

Mass spectrometric detection was optimized by injecting standards of 20 mg/l of aflatoxins in the positive and nega-

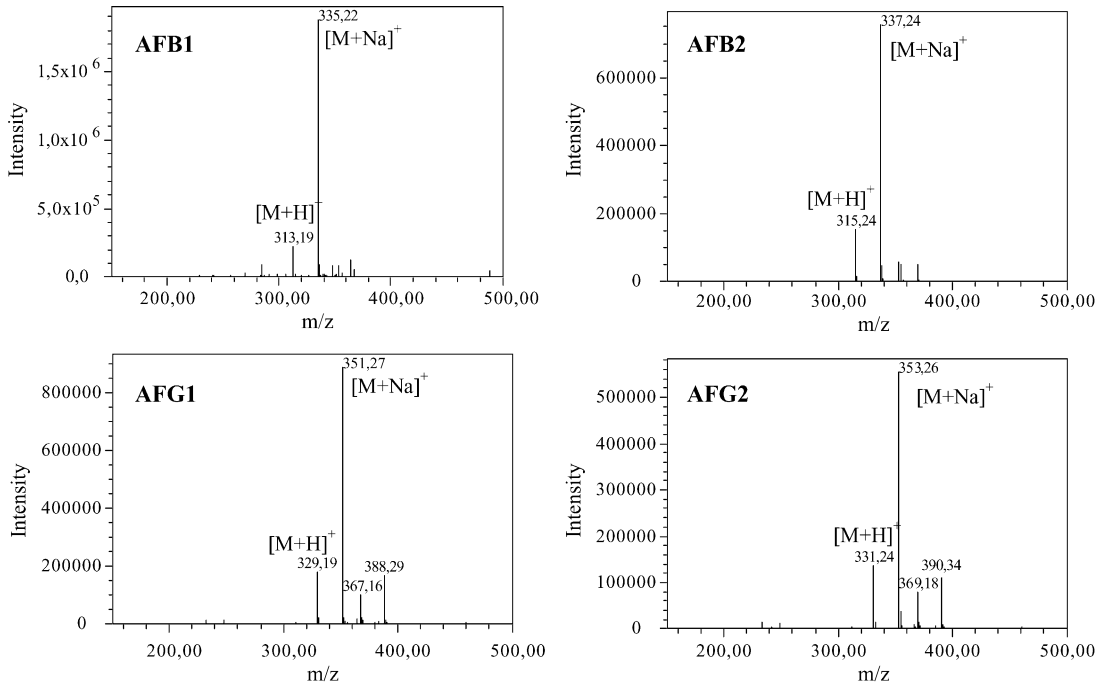

Fig. 2. Aflatoxins mass spectra.

tive ionization mode with different cone voltages (20, 40 and 60 V). Full scan mass spectra were recorded in order to select the most abundant *m*/*z* value. The relative intensity for the most abundant *m*/*z* was used to evaluate the performance of each ionization and the cone voltage value. We have selected positive ionization mode. In negative ionization mode signals are very low. The most abundant fragments are adducts sodium ions at *m*/*z* 335.2 for AFB1, 351.2 for AFG1, 337.2 for AFB2 and 353.2 for AFG2. Protonated molecules, potassium and acetic adducts are also observed. In Fig. 2, aflatoxins mass spectra are shown. Aflatoxins standards were injected with metahol:sodium acetate (5 mM) as a mobile phase to contribute the adduct formation. Signal-to-noise ratios made worse.

A MS chromatogram of *R. purshiana* spiked with aflatoxin standard (3 ng/g) is shown in Fig. 3. Peaks eluting at 3.1, 4.3, 5.3 and 7.7 min were identified as aflatoxin G2, G1, B2 and B1, respectively, from its retention time and from the characteristic *m*/*z* sodium adduct ion.

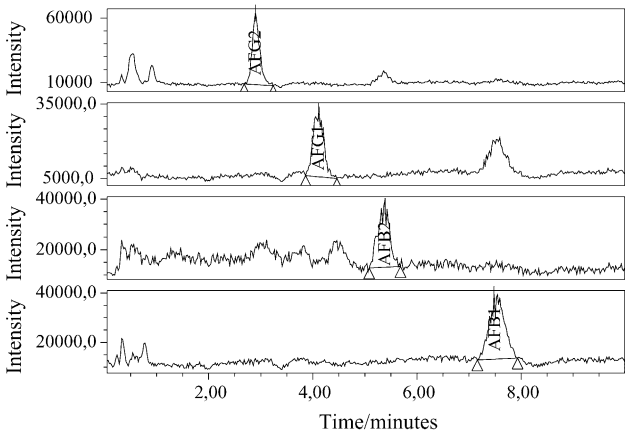

Fig. 3. Mass chromatogram of *R. purshiana* spiked with aflatoxin standard (100 μg/kg).

3.5. Performance of the method

Results of linearity, recovery, repeatability and intermediate precision are shown in Table 1. Calibration curves were linear in the range studied, showing correlation coefficients greater than 0.999. Recovery results for all aflatoxins were greater than 77%.

3.6. Aflatoxin content in samples and samples inoculated with fungi

The method has been applied to five samples of *R. purshiana* obtained from Spanish market. Aflatoxin B1, B2, G1 and G2 were not detected in the samples at a concentration level of 5 μg/kg.

Same samples were also inoculated with fungi in order to evaluate if this medicinal herb is a good substrate for mycotoxin production. Results of aflatoxin production at 7 and 14 days incubation are shown in Table 2. Aflatoxins B1 and G1 were detected in all samples inoculated.

Table 1
Results for linearity, recovery, repeatability and intermediate precision experiments

Aflatoxin	Calibration curve	R^2	Recovery (%)	Repeatability (μg/kg) ± R.S.D. (%)	Intermediate precision (μg/kg) ± R.S.D. (%)
AFB1	$y = 176.6x - 780$	0.999	110.4	101 ± 5.0	102 ± 6.8
AFB2	$y = 174.5x - 3098$	0.999	89.9	83 ± 7.2	83 ± 13.1
AFG1	$y = 157.2x + 3286$	0.999	81.0	81 ± 6.9	90 ± 9.7
AFG2	$y = 195.2x + 4572$	0.999	77.6	82 ± 7.1	70 ± 9.8

Table 2
Aflatoxin detected at *R. purshiana* samples inoculated with *A. parasiticus*

	7 days Incubation		14 days Incubation	
	Sample 1	Sample 2	Sample 1	Sample 2
AFB1	60.5	77.5	15.9	16.1
AFB2	ND	ND	ND	ND
AFG1	32.5	37.0	14.8	15.3
AFG2	ND	ND	ND	ND

4. Conclusions

The method proposed is useful for the simultaneous determination of aflatoxins B1, B2, G1 and G2 in *R. purshiana*. Chromatographic separation was performed using a short column that allows rapid determination obtaining sharp chromatographic peaks and minimising consumption of mobile phase. Extraction and purification steps were made using low quantities of methanol, and we have avoided the use of chlorate solvents that are harmful. The polymeric sorbent tested is as easy to apply as immunoaffinity columns but is cheaper. We employ mass spectrometric detection in order to avoid derivatization due to it presenting several disadvantages.

We can conclude that *R. purshiana* is a good substrate for aflatoxin production by the fungi and it is important to control these mycotoxins.

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