ORIGINAL ARTICLE

Validation of a liquid chromatography/tandem mass spectrometry method for the detection of aflatoxin B_1 residues in broiler liver

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Abstract Aflatoxin B_1 is a carcinogenic and mutagenic mycotoxin produced mainly by *Aspergillus flavus* and *Aspergillus parasiticus*. It is the predominant mycotoxin found in raw materials used for the manufacture of broiler feeds. The aim of the present study was to develop a new and optimized method for the detection and quantification of aflatoxin B_1 (AFB_1) residues in broiler liver using solid phase extraction (SPE) clean-up and liquid chromatography-electrospray ionization/tandem mass spectrometry (LC–ESI-MS/MS) detection. The method was validated for linearity, accuracy, precision, limit of detection (LOD) and limit of quantification (LOQ). The validation parameters indicated satisfactory linearity ($r^2 > 0.99$), accuracy and precision (4.57% intra-day RSD; 14.65% inter-day RSD) a very high recovery (99 ± 13%) and high sensitivity achieved for AFB_1 in animal samples (LOD = 0.017 and LOQ = 0.050 ng/g). The method was effective for the detection and quantification of AFB_1 residues in broiler liver and could also be potentially used for detecting AFB_1 in other edible animal tissues after natural or experimental AFB_1 exposure with high sensitivity and precision.
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Validación de una técnica para detectar aflatoxina B$_1$ en hígado de pollo por cromatografía líquida de alta performance acoplada a espectrómetro de masas en tándem

Resumen   La aflatoxina B$_1$ (AFB$_1$) es una micotoxina carcinogénica y mutagénica producida principalmente por *Aspergillus flavus* y *Aspergillus parasiticus*. Es la principal toxina que contamina las materias primas utilizadas para la elaboración de alimentos balanceados destinados a la alimentación de pollos parrilleros. El objetivo de este trabajo fue desarrollar un método nuevo y optimizado para detectar y cuantificar bajos niveles de AFB$_1$ en hígado de pollo, usando limpieza por extracción en fase sólida (SPE) y cromatografía líquida acoplada a detección por espectrometría de masa en tándem con ionización por electrospray (LC-ESI-MS/MS). Se validaron la linealidad, la exactitud, la precisión, el límite de detección (LOD) y el límite de cuantificación (LOQ). El método resultó tener linealidad ($r^2 > 0,99$), exactitud y precisión muy satisfactorias (4,57% RSD intradía; 14,65% RSD interdía), un alto porcentaje de recupero (99 ± 13%) y la sensibilidad más alta lograda para la detección de AFB$_1$ en muestras de origen animal (LOQ = 0,050 ng/g y LOD = 0,017). El método fue muy efectivo para detectar y cuantificar bajos niveles de AFB$_1$ en hígados de pollos parrilleros. Este método podría potencialmente utilizarse para la detección de esta toxina en otros tejidos y subproductos de origen animal luego de su exposición a AFB$_1$ con una mayor sensibilidad y precisión.

Introduction

Mycotoxins are toxic secondary metabolites produced by fungi. They are unavoidable contaminants in foods and feeds, exerting harmful effects upon animal and human health[39]. The most important mycotoxins in naturally contaminated foods and feeds are aflatoxins (AFs), ochratoxins, zearalenone, T-2 toxin, deoxynivalenol and fumonisins[7,32]. Aflatoxin contaminated feeds are a severe threat to both livestock productivity and human health and cause significant worldwide economic losses every year[3]. Aflatoxins produced by toxigenic strains of *Aspergillus flavus* and *Aspergillus parasiticus* are the most hazardous naturally occurring toxins for both human and animals. Among the four major AFs – B$_1$, B$_2$, G$_1$ and G$_2$ – aflatoxin B$_1$ (AFB$_1$) is the most toxic and carcinogenic. The manifestation of chronic or acute toxicosis as well as carcinogenicity depends on the dose, duration of exposure, and rate of metabolism to less toxic metabolites[3]. Aflatoxin B$_1$ was classified as a Group 1 carcinogen (carcinogenic to humans) by the International Agency for Research on Cancer[19]. The toxicity of AFB$_1$ in birds has been widely investigated, being the liver target organ for the toxin. Biochemical, hematological, immunological, and pathological effects of AFB$_1$ have also been well-described[38,23–25,27]. The liver is also the main organ where AFB$_1$ is stored, metabolized and/or conjugated to nucleic acids and proteins. Aflatoxin B$_1$ is activated by cytochrome P450 enzymes (CYP), including CYP1A2, CYP3A4 and CYP2A6 and converted to epoxides (AFB$_1$-8,9-exo-epoxide and AFB$_1$-8,9-endo-epoxide), aflatoxin M$_1$ (AFM$_1$), aflatoxin P$_1$ (AFP$_1$), aflatoxin Q$_1$ (AFQ$_1$), or its reduced form aflatoxicol (AFL)[38]. Recent studies have shown that CYP2A6 and, to a lesser extent, CYP1A1 are the

enzymes responsible for bio-activation of AFB$_1$ into epoxide forms in chicken and quail liver[8]. Residues of AFB$_1$ and some of its metabolites have been detected in eggs, liver, gizzard and kidney, as a consequence of the direct intake of contaminated feed[28,2,29,26].

The poultry industry has evolved over the past 30 years to be a major source of animal protein in the human diet. In Argentina and other Latin American countries, the poultry industry is one of the most important animal production systems and has acquired great economic significance, mainly in the meat production sector[21]. Aflatoxin contamination in broiler feeds represents a potential risk for broilers' health and cause economic losses associated to acute and chronic aflatoxicosis. The presence of AFB$_1$ residues in edible broiler tissues leads to the toxin carryover in the food chain. Since AFB$_1$ may remain unchanged in the liver even when exposure levels are relatively low, the development of simple and precise methods for the quantification of mycotoxins in different animal products and edible tissues is mandatory. The methods for AFs detection in the liver described to date use less sensitive techniques or are multi-toxin methods that are not optimized for AFB$_1$. The aim of the present study was to develop a new and optimized method for the detection and quantification of AFB$_1$ residues in broiler liver using SPE clean-up and LC–ESI-MS/MS detection.

Materials and methods

Chemicals and materials

Aflatoxin B$_1$ standard was purchased from Romer Labs® (Tulln, Austria). HPLC-grade organic solvents such as acetonitrile and methanol were purchased from Sintorgan

(Buenos Aires, Argentina). HPLC-grade water was obtained from a Labconco WaterPro Mobile purification system (model 90901-01). Clean-up OASIS®, HLB, 6 cc (200 mg) SPE cartridges were purchased from Waters Corporation (Milford, MA, USA). Pro-analysis grade solvents such as dichloromethane, methanol, acetonitrile and hexane were purchased from Cicarelli (Santa Fe, Argentina).

Samples

Aflatoxin-contaminated liver samples (n = 20) were collected from broilers (*Gallus gallus domesticus*, purchased from Cobb) fed a 50 ng/g AFB_1 contaminated corn-soybean meal standard basal diet, in a previous *in vivo* experiment[23] performed in accordance with international sanitary and ethical guidelines (the protocol was approved by the Committee of Ethics and Biosecurity of the Universidad Nacional de Río Cuarto). In that study, one-day-old male chicks (Cobb) were obtained from a commercial hatchery and placed under continuous fluorescent lighting with feed and water available *ad libitum* until they were 18 day old. On day 18, a total of 160 birds (4 replicates/treatment, with 5 chicks/replicate) were individually weighed and randomly selected. Birds were fed the dietary treatments for 28 days (day 18–46). During the experimental period, broilers received the diet corresponding to each treatment. A grower diet was given from day 1 to 35, and a finisher diet was provided from day 36 until the end of the experiment. A standard corn-soybean meal diet (basal diet) meeting NRC (1994) requirements was fed from day 1 until the end of the experiment. The basal diet contained 2 μg of AFB_1/kg of diet, as determined by the method described by Trucksess et al.[37] Broilers were monitored daily for signs of morbidity and mortality. At the end of the assay, productivity parameters (i.e., body weight gain, feed consumption and feed conversion ratio), biochemical parameters, macroscopic and microscopic changes in the liver of broilers were evaluated. Non-contaminated liver samples (n = 5) used for the recovery assay were obtained from broilers fed an AFB_1-free diet.

Spiking and recovery assay

Five blank liver samples (50 g each) were spiked with 50 μl of a 1 mg/ml AFB_1 solution to obtain a concentration of 1 ng AFB_1 per g of tissue. These samples were processed and analyzed in the same way as naturally contaminated samples and the recovery percentage was calculated.

Extraction

Aflatoxin B_1 from liver tissue was extracted according to the method described by Tavčar-Kalcher et al.[35] with some modifications. Five ml of citric acid (20%) and 10 g of diatomaceous earth were thoroughly mixed with 50 g of ground liver tissue. The toxin was extracted with 100 ml dichloromethane by shaking for 30 min at room temperature. The organic phase was filtered through Whatman N°4 filter paper (Whatman International Ltd., Maidstone, UK). Water was eliminated by adding 5 g anhydrous sodium sulfate and the extract

was filtered for a second time. Twenty ml of the filtrate were evaporated to dryness at 60 °C. The residue was resuspended in 20 ml acetonitrile:H_2O (75:25, v/v) and extracted with 10 ml hexane for fat separation. The mixture was thoroughly mixed, centrifuged and then let to settle until two phases were formed. An aliquot of 10 ml of the aqueous phase (bottom) was evaporated to dryness in a rotary evaporator. For clean-up, the dried extract was resuspended in 10 ml methanol:H_2O (80:20, v/v), diluted by adding 90 ml distilled water and passed through an OASIS®, HLB, 6 cc (200 mg) SPE cartridge (Waters Corporation, Milford, MA, USA) previously conditioned with 5 ml methanol and 5 ml water according to the methodology described by Sørensen and Elbæk[30]. Solid phase extractions were performed in a Vac Elut 20 position Manifold SPE extractor (Agilent Technologies Inc., Santa Clara, CA, USA). The toxin was eluted with 7 ml methanol, evaporated to dryness and stored at −20 °C until analysis. Before LC–MS/MS analysis, the extracts were resuspended in 500 μl methanol:H_2O (20:80, v/v).

LC–MS/MS instrumental conditions

Detection and quantification were performed in a Waters Alliance 2695 HPLC system (Waters Corporation, Milford, MA, USA) equipped with a Waters Alliance 2685 pump, a Waters Alliance 2695 autosampler and a Waters 2996 Photo Diode Array (PDA) detector, interphased to a Quattro Ultima Platinum Tandem quadrupole Micro Mass Spectrometer with electrospray ionization (ESI) source. Chromatographic separation was performed at 25 °C in an X-Bridge™ C18, 150 × 2.1 mm i.d., 3.5 μm particle size column equipped with a 2.1 × 10 mm i.d. security guard cartridge (all from Waters Corporation, Milford, MA, USA). The mobile phase of the chromatographic procedure was a gradient of aqueous 0.1% formic acid (solvent A) and 0.1% formic acid–methanol (solvent B). At the initial time, the eluent was 20% solvent B. This composition was kept over 3 min, after which a linear gradient to 100% of solvent B was performed in 12 min, and then the eluent was kept at 100% of solvent B to clean the column. The initial conditions were stabilized over 10 min before the next injection.

The flow rate was 0.2 ml/min and the injection volume was 50 μl. ESI-MS/MS was performed in multiple reaction monitoring (MRM) mode in positive polarity. The most abundant trace in the MRM mode was used for quantitative analysis. Nitrogen gas was used for both nebulization and desolvation heated to 150 and 200 °C, respectively. The capillary voltage was 3.00 kV. The nitrogen flow was adjusted to 100 and 725 l/h for cone and desolvation gases, respectively. Dwell time was set at 200 ms for all transitions. Cell exit potential was 50 V and cone voltage was 35 V. Data acquisition and processing were performed using Mass Lynx V.4.1 (Waters Corporation, Milford, MA, USA) software. The MRM transitions and experimental conditions are shown in Table 1. Aflatoxin B_1 retention time and the MRM-transitions of the precursor ion [M+H]$^+$ (*m/z* 313) to two product ions (*m/z* 241 and 285) were met to identify AFB_1 in liver tissue extracts. A calibration curve was created by injecting 50 μl of different AFB_1 standard solutions concentration (1–10 ng/ml).

Table 1   Transitions and instrument parameter settings used for data collection in positive ion mode

Compound	Precursor ion [M+H]$^+$ (m/z)	RT (min)	Product ions (m/z)	CV (V)	CE (V)
AFB$_1$	313	11.53	285	50	23
			241		37

RT: retention time; CV: cone voltage; CE: collision energy.

Validation

The method was validated for linearity, accuracy, precision, LOD and LOQ. Linearity of the method was tested by inject- ing three replicates (50 μl) of four levels of AFB$_1$ standard solutions (1—10 ng/ml). The accuracy of the method was determined by a recovery assay as described above and the average content of AFB$_1$ obtained was used to calculate the recovery percentage. Precision was determined by intra-day and inter-day repeatability experiments making three injec- tions of each spiked liver extract per day during four days. The extracts used for inter-day injections were the same as those used in the first day, and were properly kept at −20 °C in the darkness to avoid degradation of the toxin. The mean AFB$_1$ concentrations, intra-day and inter-day rel- ative standard deviation (RSD) values were calculated. The limit of detection (LOD) and limit of quantification (LOQ)

for AFB$_1$ were calculated based on signal-to-noise (S/N) ratios of 3:1 and 10:1, respectively, by were experimentally obtained injecting standard dilutions with the corresponding S/N ratio.

Results

The average concentration of AFB$_1$ found in liver of broil- ers fed the 50 ng/g AFB$_1$ diet was 0.4 ± 0.1 ng/g. Figure 1 shows the LC/ESI(+)-MS/MS MRM chromatograms evidenc- ing the presence of AFB$_1$ (RT 11.5 min) in liver of broilers fed AFB$_1$. The precursor ion of AFB$_1$ obtained from ESI was [M+H]$^+$ m/z 313. When fragmented in the collision cell, the most sensitive transition ions were m/z 241 and 285.

The minimal amounts of AFB$_1$ detectable and quantifi- able with this method were 0.025 and 0.083 ng, respectively. The LOD and LOQ for this method were 0.017 and
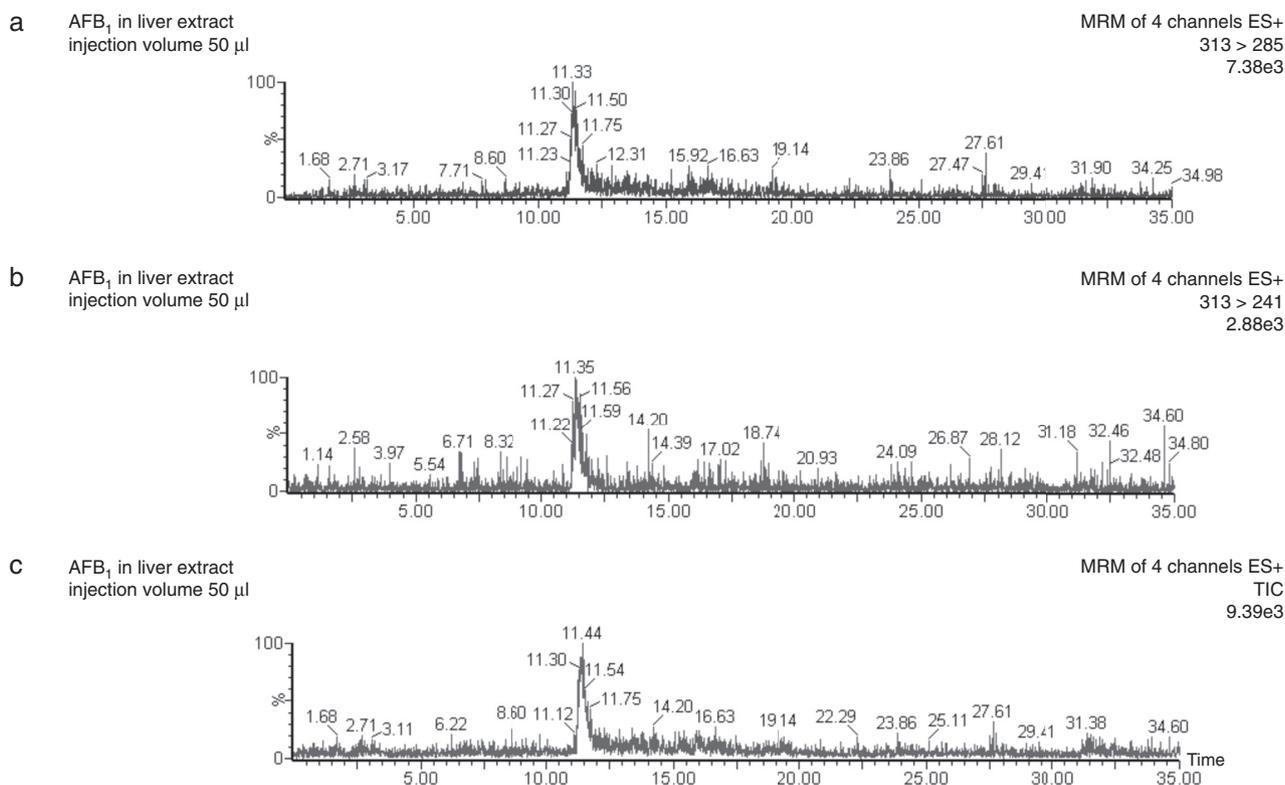

Figure 1   LC-ESI-MS/MS chromatograms obtained in positive ion mode showing the total ion current and selective AFB$_1$ transitions in liver extracts obtained from broilers fed 50 μg/kg AFB$_1$ diet.
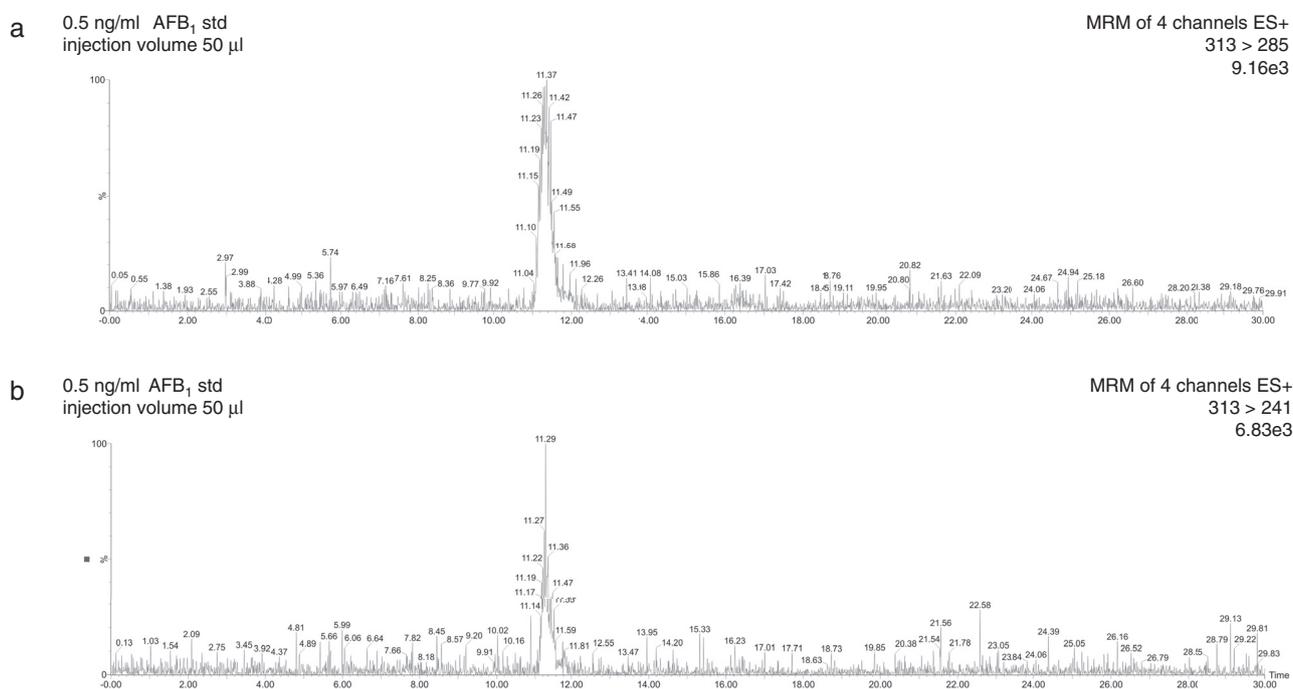
Figure 2 Total ion current and selective reaction monitoring transitions of AFB$_1$ in solvent-only standard.

Table 2 Linearity, limit of detection (LOQ), limit of quantification (LOQ) and recovery percentage for aflatoxin B$_1$ in LC–MS/MS

Linearity			LOD		LOQ		Recovery (%)
Linear range (injected ng)	Linear equation	r^2	(ng)[a]	(ng/g)[b]	(ng)[a]	(ng/g)[b]	
0.05–5	$y = 1 \times 10^8 x - 24057$	0.993	0.008	0.016	0.025	0.050	99 ± 13

[a] Minimal amount of AFB$_1$ mass detected by the instrumental method.
[b] Minimal concentration of AFB$_1$ detected per gram of liver.

0.05 ng AFB$_1$/g of sample, respectively (Fig. 2). Linear correlation ($r^2 > 0.993$) was obtained between peak area and concentration of AFB$_1$ standard solutions ranging from 0.05 to 0.50 ng. The average AFB$_1$ recovery was 99 ± 13% (Table 2). Intra-day RSD was 4.57% and inter-day RSD was 14.65%.

Discussion

In the present study, a sensitive, optimized and precise method for the analysis of AFB$_1$ residues in broiler chicken liver using organic solvent extraction, SPE clean-up and LC–MS/MS detection and quantification was developed. Other methods for the detection of AFB$_1$ in tissues and animal products developed since the 1980s involved less sensitive detection techniques such as ELISA, TLC or HPLC[1,36,17,31,16,6,13,9]. The use of LC–MS/MS improves detection and accurate quantification of AFB$_1$ residues in tissue of animals fed mycotoxin-contaminated feeds. Many multi-mycotoxin LC–MS methods have been developed to detect fungal toxins and metabolites in various types of samples, mainly foods and feeds[9,33,34,4,20,18]. The advantages of

LC–MS techniques rely in the improved detection limits, the confirmation provided by mass spectral fragmentation and the ability to filter out by mass any impurities interfering in spectrophotometric detectors. This study evaluates AFB$_1$ residues in broiler liver, but the method could also be applied to muscle and other kinds of meat for human consumption. Chen et al.[5] developed a similar method using SPE clean-up and LC–ESI-MS/MS for the detection of AFs and ochratoxin A in various animal-derived foods (swine, bovine and sheep muscle, liver, kidney and fat, chicken muscle and liver, fish muscle and skin, hen eggs and dairy milk) obtaining LODs that varied from 0.07 ng/g to 0.59 ng/g and recoveries that ranged from 68.3% to 105.7% with RSDs of less than 17.6%. Sørensen and Elbæk[30] applied another method using SPE clean-up and LC–ESI-MS/MS to quantify 18 mycotoxins and metabolites in bovine milk obtaining a LOD of 20 ng/l for aflatoxin M$_1$. Zhao et al.[40] developed the latest method for the quantification of 30 micotoxins in swine and poultry feeds and derived foods (meat, edible tissues and milk), obtaining LODs and LOQs of 0.1 and 0.5 ng/g AFB$_1$, in pork meat and of 0.02 and 0.01 ng/g in swine feed and dairy milk, respectively. All these methods have the advantage of detecting multiple mycotoxins and derived metabolites in

a single run. However, the conditions of method described in the present study were optimized for extraction and detection of AFB_1, the most hazardous of mycotoxins due to its carcinogenic and genotoxic effects. Therefore, the LOD and LOQ obtained for meat products were improved compared to the multi-toxin methods described by other authors. The importance of AFB_1 in broiler feed and tissues is higher than that of other toxins since AFB_1 constitutes a major public health concern, especially in Latin American countries where there is no regulation for the toxin in matrices such as animal meat or eggs.

When applying the method, AFB_1 residues were found in the liver of broilers fed a 50 ng/g AFB_1 diet showing clear LC/ESI(+)-MS/MS MRM chromatograms showing a well defined peak at 11.5 min. The precursor ion of AFB_1 obtained from ESI was the adduct $[M+H]^+$, m/z 313. When fragmented in the collision cell, the most sensitive transition ions were m/z 241 and 285. Multiple reaction monitoring transitions exhibited high signal intensity in positive ESI mode. The second product ion was monitored for confirmation of the compound identity. Four identification points (retention time, molecular mass of the precursor ion and two characteristic product ions) are included in the method as required for the confirmation of substances listed in group B of Annex I of Directive 96/23/EC (European Commission)[11]. Quantification was performed using the most abundant signal by comparing the areas under integrated peaks of the 313 > 285 reaction monitoring transition in samples with those obtained with AFB_1 standards.

The method was evaluated for linearity, accuracy, precision, LOD and LOQ. A high repeatability in the retention time (11.5 min) was obtained for standards as well as for sample extracts. Lower LOD and LOQ values were achieved (0.017 and 0.05 ng/g, respectively) improving sensitivity compared to other existing methods. A linear correlation ($r^2 > 0.993$) was observed between peak area and concentration of AFB_1 standard injections (50 μl) in the range of 0.05–5.00 ng of AFB_1 injected to the column (1–10 ng/ml solutions), confirming the linearity of the method. The high mean recovery value obtained ($99 \pm 13\%$) was in the range between 50% and 120%, required by commission regulation No. 401/2006/EC (European Commission)[10], yielding very satisfactory accuracy. Intra-day and inter-day RSD values were 4.57% and 14.65%, respectively, indicating the high repeatability and precision of the developed method.

Many countries have established AFB_1 tolerance levels in food in order to reduce toxin exposure. Feeding animals with mycotoxin-contaminated grains that are not suitable for human consumption (diluted with clean grain or feed to reduce mycotoxin levels) is a common practice in developing countries where contaminated material cannot be thrown away. The European Union has established maximum limits ranging from 5 to 20 ng/g for AFB_1 in animal feeds, depending on the type of product and animal species (European Commission)[12]. An action level of 100 ng/g has been established by the Food and Drug Administration for aflatoxins in corn and peanut based feeds intended to mature poultry (FDA)[15]. In Latin America, Brazil has established a limit of 50 ng/g for aflatoxins B_1, G_1, B_2 and G_2, while MERCOSUR, the Latin American common trade market, accepts 20 ng/g

of aflatoxins B_1, G_1, B_2 and G_2 (FAO)[14]. In Argentina, the existing regulation concerning AFs in animal products for human consumption only refers AFM_1 in milk establishing tolerance levels of 0.5 μg/kg (fluid) and 5 μg/kg (powdered) (FAO)[14]. Hence, it is important to control AF residues in meat and other animal products even when there is still no regulation established for them since AFs, when consumed in low levels for long periods of time, have been associated to chronic aflatoxicosis and liver cancer[22].

Conclusion

In conclusion, the developed method proved to be effective for the detection and quantification of AFB_1 residues in broiler liver with great sensitivity. The validation parameters showed satisfactory linearity, accuracy and precision and very high recovery. Therefore, the method could be used for detecting AFB_1 residues in broiler liver and eventually other edible animal tissues after natural or experimental AFB_1 exposure.

Funding

Conflict of interest

Alejandra P. Magnoli, María L. González Pereyra, María P. Monge, Lilia R. Cavaglieri and Stella M. Chiacchiera declare they have no conflict of interest.

Acknowledgements

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