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Validation of a UHPLC-FLD analytical method for the simultaneous quantification of aflatoxin B1 and ochratoxin a in rat plasma, liver and kidney

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ABSTRACT

A rapid and simple method for the simultaneous quantification of AFB1 and OTA in rat plasma, liver and kidney by UHPLC-FLD has been successfully validated according to the following criteria: selectivity, stability, linearity, precision, accuracy, recovery, robustness and limits of quantification and detection. The extraction method, calibration curves and chromatographic conditions are common for the three matrices. Plasma and homogenized tissue samples (100 μL) were extracted with acetonitrile:formic acid mixture (99:1) (300 μL). Chromatographic separation was performed with a mixture of water and acetonitrile:methanol (50:50), both acidified with 0.5% of formic acid using a gradient profile. The method avoids the use of immunoaffinity columns and allows reduction of sample and solvent volumes as well as toxic wastes. The detection is based on a photochemical reaction which enhances the AFB1 response without affecting the OTA signal before reaching the fluorescent detector. The mycotoxin recovery for each matrix was very efficient, between 93% and 96% for AFB1 and between 94% and 96% for OTA. For both mycotoxins the LOQs were 2 μg/L in plasma and 8 μg/kg in liver and kidney. The method has successfully been applied to rat samples after a single oral administration of a mixture of AFB1 and OTA and it could be a useful tool in toxicokinetic and toxicological studies.

1. Introduction

Mycotoxins are small (MW∼700 amu) secondary metabolites produced by different fungal species that can contaminate many agricultural commodities during harvest and/or while in storage. Most of mycotoxins are produced by *Fusarium* (pre-harvest), *Penicillium* (post-harvest) and/or *Aspergillus* (pre/post harvest) fungi. They can reach human beings through contaminated food, as well as via edible products (milk, eggs, meat, blood, etc.) obtained from animals fed with contaminated feeds [1]. Due to the globalization of the trade in agricultural commodities, mycotoxins may currently appear in any developed or developing country in local or imported products.

Aflatoxins and ochratoxin A (OTA) belong to the most frequently occurring mycotoxins [2]. The IARC classified Aflatoxin B1 (AFB1) and OTA as class 1 (human carcinogen) and class 2B (possible human carcinogen), respectively [3–5]. Aflatoxin B1 is genotoxic *in vivo* and *in vitro*; its target organ is liver but it may cause tumors in other organs such as colon and kidney [6,7]. Ochratoxin A is a potent nephrocarcinogenic compound in rodents but despite the controversy with regard to its mechanisms of action, some existing research suggests that it is an indirect genotoxic agent. Moreover, OTA is nephrotoxic, hepatotoxic, teratogenic and immunotoxic, and recent studies have related it to neurodegenerative diseases such as Parkinson and Alzheimer [8,9]. Due to the aforementioned, a Tolerable Weekly Intake (TWI) of 120 ng/kg of body weight has been established for OTA [10] but, as of yet there is no threshold for AFB1; therefore, ALARA (as low as reasonably achievable) principle limit must be applied as it is not possible to identify an intake without risk [11].

Human population is chronically exposed to multiple mycotoxins because of several reasons. First, the same food might be contaminated by more than one mycotoxin as the co-occurrence of AFB1 and OTA in edible products has been demonstrated; some examples are in dried fruits and figs [12], in paprika [13,14], and in breakfast cereals [15]. Moreover, since the human diet is varied, mycotoxins might reach humans from different pathways; and, finally, mycotoxins are thermostable and can remain in food even after the fungus has been removed [1]. Co-exposure to different mycotoxins, could originate synergic or additive toxic

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effects on human or animal health; however, knowledge regarding this aspect or regarding the influence of co-ocurrence on toxicokinetic or toxicological characteristics of the mycotoxins is still scarce.

Reference literature reports numerous methods for individual quantification of AFB1 or OTA, most of which are applied to foodstuffs, but also applied to animal matrices such as poultry [16], fish meat [17] eggs [18], milk [18,19], swine [20], and meat and meat products [18,19]. Nevertheless, very few of these studies are suitable for application in complex biological samples and/or during toxicological studies because they use large volumes of sample, which is not easily available when working with laboratory animals. Moreover, most of these studies do not report validation data, which is very important so as to provide evidence of the reliability of the results [21].

The development and validation of methods for the simultaneous determination of the most important mycotoxins in biological matrices would be very useful because they would be adequate tools for toxicokinetic and toxicology studies that investigate the effects of co-exposure, minimizing the cost of the analysis and the use of laboratory animals.

In this paper, a UHPLC-FLD method with simultaneous extraction and analytical quantification procedures for AFB1 and OTA and in rat plasma, kidney and liver has been validated and successfully applied in biological samples, demonstrating its usefulness for toxicological or toxicokinetic studies.

2. Materials and methods

2.1. Safety precautions

Aflatoxin B1 and ochratoxin A are toxic substances. They were always manipulated in solution, avoiding the formation of dust and aerosols. Nitrile gloves were used for all procedures carried out and during the manipulation of treated animals or contaminated samples FPP3 masks were used.

2.2. Reagents

For the analytical standards, AFB1 was purchased as a solution of 2 mg/L in acetonitrile (ACN) and OTA was purchased as a solution of 10 mg/L in ACN, both from OEKANAL® Fluka (Schnelldorf, Germany) as certified reference materials. For oral administration, mycotoxins were purchased in powder from Sigma (Steinheim, Germany) and they were dissolved in 0.1 M $NaHCO_3$ (Riedel-de Haën, Seelze, Germany), adjusted to pH 7.4 with HCl and maintained at −20 °C until their use. For the tissue homogenates, sodium phosphate buffer (0.05 M, pH 6.50) was prepared by adding 6.90 g of NaH_2PO_4 xH_2O (Merck, Darmstadt, Germany) to 900 mL of type II water. The pH of the dissolution was adjusted to 6.5 with NaOH (Agilent technologies, Waldbronn, Germany) and the volume was adjusted to 1 L. All reagents used for the HPLC analysis were of analytical grade. ACN and methanol (MeOH) HPLC grade and formic acid were obtained from Sigma Aldrich (St. Quentin Fallavier, France). Millipore type I water was obtained daily from a Milli-Q water-purifying system (Millipore, Bedford, MA, USA).

2.3. Standard solutions

Working standard solutions (mixture of AFB1 and OTA) were prepared by appropriate dilution of the commercial standards with ACN and MeOH to a proportion of 50:50 (v/v). Four working solutions were prepared (750, 300, 30 and 3 μg/L of AFB1 and OTA) and stored at −20 °C. The calibration standards were prepared by evaporating volumes of working standard solutions (after being kept at room temperature during 30 min) under a stream of nitrogen and then the residues were dissolved in 200 μL of mobile phase. The calibration standards were kept in the injection tray at 4 °C without illumination until analysis.

2.4. Animals

The *in vivo* experiments were approved by the Ethics Committee on Animal Experimentation of the University of Navarra.

Ten-week-old male and female Fisher 344 (F344) rats, purchased from Harlan (Horst, The Netherlands), were used. On the day of arrival, the animals were weighed (weight variation did not exceed ±20% [22,23]) and then distributed to polycarbonate cages with stainless steel covers for one week in order to allow acclimatization to the environmental conditions: 12 h day/night cycle, temperature 22 ± 2 °C, relative humidity 55 ± 10%, standard diet (Harlan Iberica, Spain) and water *ad libitum*.

Plasma, kidney and liver used as blank samples for the validation of the method were obtained from 12 non-treated animals (6 male and 6 female). In the application of the method, two male rats were administered a single dose of a mixture of AFB1 and OTA (0.5 mg/kg of AFB1 and 0.1 mg/kg b.w. of OTA) by oral gavage. Samples of plasma, liver and kidney were extracted and analyzed.

2.5. Sample obtaining and mycotoxin extraction procedure

Sampling methods were based on those described by Vettorazzi et al. for toxicokinetic studies of OTA in rat [24] with some modifications. Blood from decapitation was collected in BD (Plymouth, UK) Vacutainer tubes (5.4 mg K3E, 3 mL) and centrifuged at 1266 × *g* for 15 min. The obtained plasma was aliquoted and stored at −80 °C until the extraction of mycotoxins was carried out. Liver and kidney were extracted from the animals, washed with water until the external blood was removed, and then blotted on filter paper. Pieces of each organ were cut and weighed. The medium weights of them (15–20% of relative standard deviation (RSD)) were: 1.64 g and 1.02 g of male and female livers respectively and 0.33 g and 0.23 g of male and female kidneys respectively. Afterwards, they were flash-frozen in liquid nitrogen and stored at −80 °C. In order to prevent cross contamination between samples, all the dissection material was cleaned with water and rinsed with ethanol after each animal necropsy.

For mycotoxin quantification, kidney and liver were homogenized for approximately 1 min in a round-bottom plastic tube (sterile PP-tube, from Greiner bio-one GmbH (Frickenhausen, Germany)) with 4 μL of cold sodium phosphate buffer (0.05 M, pH 6.50) per mg of tissue in a Polytron PT 3000 homogenizer with a metal rod (PT-DA 3012/2 TS Kinematic (Littau, Switzerland)). After each use, the homogenizer was cleaned with water, rinsed with ethanol and turned on until ethanol evaporation. The tissue homogenates were aliquoted (between 500 μL and 1 mL) and stored for at least one day at −80 °C until the mycotoxin extraction was carried out.

Before the extraction step, plasma or tissue homogenates were kept at room temperature for 30 min and vortexed. Next, 100 μL of the sample (plasma or homogenates) was mixed with 300 μL of the extractive solution (ACN – 1% HCOOH), vortexed for 2 min and centrifuged at 6200 × *g* for 15 min at 4 °C in order to precipitate proteins. The supernatant (200 μL) was evaporated to dryness under vacuum (in a miVac DUO concentrator, Genevac (Ipswich, UK) during 15 min at 40 °C) and immediately afterwards the dry sample was resuspended in 200 μL of mobile phase and vortexed during 2 min. Plasma samples with expected high concentrations of OTA were diluted 1:20 in mobile phase. Dilution factor for plasma was 4 (80 in the case of high concentrated samples), and 16 for kidney and liver. The pH attained in the extraction step was approximately

2.6. Samples were filtered and placed in vials in the HPLC tray at 4 °C in darkness until injection

2.6. Apparatus and chromatographic conditions

UHPLC analyses were performed with an Agilent Technologies 1200 liquid chromatographic system equipped with a fluorescence detector (G1321A model) controlled by ChemStation B.03.02 software (Hewlett-Packard). Mycotoxins were separated on an Ascentis® Express C18 column (150 mm × 2.1 mm; 2.7 μm) from Supelco (PA, USA). The mobile phase was a mixture of an organic phase (A) (MeOH-ACN, 50:50, v/v) and water (B), both acidified with 0.5% of formic acid. The injection volume was 40 μL and the flow rate was 0.9 mL/min. Chromatography was performed at 60 °C. Proportion of both organic and aqueous phases was switching between isocratic and gradient profiles during the entire analysis procedure. The elution program starts isocratic until minute 2.4 with 30% of A, then from 2.4 to 2.5 min the organic phase increases up to 43%, from 2.5 to 8.3 min another isocratic profile at 43% of A, from 8.3 to 10.0 min there is a last increase up to 65% of A and finally, from minute 10.0 the system returns to 30% of A to restore the starting conditions during 5 min. The retention times under these conditions were 2.5 min for AFB1 and 8.4 min for OTA. Before the sample entered the fluorescence detection cell, a photoderivatization device (AURA Industries, NY, USA) with a mercury lamp (λ = 254 nm) and a knitted reactor coil of 0.25 mL (5 m × 0.25 mm) was included. During the first 4 min of analysis, fluorescence conditions were optimized for AFB1 (excitation 366 nm and emission 433 nm wavelengths), and after that for OTA (excitation 225 nm and emission 461 nm wavelengths).

The chromatographic separation was evaluated for each matrix at the limit of quantification level using the following parameters: retention time (t_R,) retention factor (k'), symmetry, peak width at half height (w_h), number of theoretical plates (N) and resolution (R_s).

2.7. Validation of the UHPLC-FLD method

The analytical method was validated for each mycotoxin according to the following method performance characteristics: selectivity, stability, linearity, precision and accuracy (within- and between-day variability), recovery (in intermediate precision conditions), limits of detection (LOD) and quantification (LOQ), and robustness. Selectivity, LOD, LOQ, recovery and stability were studied for each matrix. For this purpose, a pool of blank samples of plasma, kidney or liver obtained from 12 animals (6 male and 6 female) was used.

Selectivity of the method was improved by using a photochemical reactor (PHRED) before a fluorescence detector with blank plasma and tissue homogenates. However, the ability of the method to distinguish AFB1 and OTA from other endogenous components was evaluated for the 3 matrices by analyzing and comparing blank samples before and after being spiked with the mycotoxins at LOQ levels. Moreover, due to the fact that OTA basal levels were detected in plasma, samples that are naturally contaminated with OTA were analyzed and afterwards spiked with a standard solution in order to see an increase of the area of the corresponding peak. In addition, in both cases (naturally contaminated and spiked) the samples were reanalyzed after changing the % of organic component in the mobile phase in order to delay OTA peak and therefore be able to observe the presence or absence of broadening, shoulders or others interfering peaks.

The stability of mycotoxins was determined in the working solution, during storage of samples at −80 °C and in the chromatograph tray before analysis at 4 °C. Three concentrations of working solutions (1, 10 and 100 μg/L) stored at −20 °C were analyzed during 8 weeks. Mycotoxin stability in the HPLC chromatographic tray was evaluated for the three matrices by analyzing the extracts of spiked samples (40 μg/L plasma and 160 μg/kg liver/kidney) just after preparation and over a period of 15 h. Moreover, the stability of mycotoxins in plasma and in tissue homogenates stored at −80 °C has also been determined by analyzing spiked samples (40 μg/L plasma and 160 μg/kg liver/kidney) 24 h, 1 week, 1 month and 6 months after preparation.

For each mycotoxin, two calibration curves were plotted from 0.5 up to 150 μg/L using calibration standards. The range was split into two: a low calibration curve from 0.5 to 30 μg/L, with eight data points; and a high calibration curve from 30 to 150 μg/L with six data points. Three replicates of each calibration standard were analyzed. Within- and between-day precision and accuracy of the linearity were studied by analyzing three replicate calibrations standards at 0.5, 2, 15, 30, 90 and 150 μg/L on one day (within-day) and on three different days (between-day).

Due to the fact that adequate reference biological spiked materials were not available, fortification was carried out with known concentrations of mycotoxins in order to establish the recovery and precision of the method. AFB1 and OTA were added to blank plasma, liver or kidney homogenate pools in order to obtain 2, 8, 120, 600 μg/L in plasma, and 8, 32, 480, 2400 μg/kg in organs, in triplicate. The corresponding volume of the working standard was evaporated under a stream of nitrogen and resuspended in 100 μL of blank plasma, kidney or liver homogenate by mixing in vortex for 2 min. Mycotoxins were then extracted as described in section 2.4 and then analyzed. In order to assure the quantification of very high levels of OTA (levels in plasma higher than 600 μg/L could be expected), a dilution step was added when analyzing the recovery of 600 μg/L. Just after resuspending the plasma fortification extract in mobile phase (200 μL), 10 μL of this suspension were added to 190 μL of mobile phase, mixed and analyzed. The dilution factor was 20; therefore, it was possible to cover a range of up to 6000 μg/L in plasma. The recovery values (%) for all of the matrices were calculated by dividing the experimental mycotoxin concentration obtained by the nominal mycotoxin level. In the spiked plasma samples, the response was subtracted from the areas obtained in the plasma blank pool. The repeatability and intermediate precision of this process were studied by carrying out the complete recovery experiment for each matrix, for the 4 concentrations on one day and on three different days.

LOQ was determined by analyzing three replicates of fortified plasma (2, 4, 8 μg/L), kidney and liver (8, 16, 32 μg/kg). The lowest concentration for which acceptable data of recovery and precision was obtained was considered to be the LOQ [25].

LOD was calculated theoretically using the method based on the calibration curve extrapolation at zero concentration [25]:

$$\mathrm{LOD} = \frac{[Y_{bl} + (k\,x\,S_{bl})]}{b} \quad (k = 3)$$

where Y_{bl} and b are the intercept and the slope, respectively, of a curve that represents the area of each concentration versus the nominal concentration after analyzing the samples of spiked plasma, kidney or liver for obtaining the LOQ; k is 3; S_{bl} (standard deviation of the blank) is the intercept of the curve obtained by representing the standard deviation for each concentration level versus the nominal concentration.

Robustness: The influence of changes in the pH of the mobile phase (0.1% of formic acid in the aqueous phase), changes in the column batches (either new or in use), and the influence of column temperature (59 °C and 57 °C) on areas and retention times of a working standard of 30 μg/L of AFB1 and OTA were studied. Moreover, it was considered to be important to study the influence of light degradation of AFB1 during sample or standard manipulation (either in darkness or exposed to light). Three replicates

of the working standard were analyzed in each condition and the concentrations obtained were compared with the nominal values (30 μg/L).

The acceptance/rejection criterion used were:

- *Stability*: peak areas ±10% of the peak areas obtained just after sample preparation (0 h).
- *Linearity*: the determination coefficient (r^2) higher than 0.990, the slope interval not having to include zero ($p \leq 0.05$) and the intercept interval having to include zero ($p \leq 0.05$), the representation of residuals versus the estimated values having to rise to a distribution of the points at random and not having to reflect any trend, and the RSD (relative standard deviation) between response factors lower than 10%. ±10% RSD and ±10% standard error of the mean (SE) for all the concentrations excepting the lower one, which is the limit of quantification, and whose accepted accuracy and precision was within ±15%.
- *Recovery*: ±10% RSD in repeatability and intermediate precision conditions.
- *Robustness*: ±10% of standard error of the mean (SE).

3. Results and discussion

Up to now, many OTA determination studies have been performed on different biological samples and animal species using a wide variety of techniques [8,24,26–28]. However, there is a lack of modern techniques (LC/MS and HPLC) for studying the presence of AFB1 in body fluids or tissues, and most of the methods were initially limited to their use in food matrices [18,29], although later they were modified for their application to toxicological studies. Moreover, no validated UHPLC-FLD methods for simultaneous determination of OTA and AFB1 in plasma, kidney and liver have been previously described in laboratory animals, in spite of the fact that these methods would be of great interest for toxicological or toxicokinetic studies of mycotoxins.

3.1. Sample obtaining and mycotoxin extraction and cleanup

For aflatoxins determination, chromatographic methods described by Siraj et al. [30], Gregory [31], Lamplugh [32,33] were not suitable for toxicological studies conducted with small animals. Coulombe and Sharma [34] and Wong [35], among others, used radioactivity quantification methods in order to achieve enough sensitivity and recovery for the tiny samples that are generated during toxicological studies. Plakas et al. combined an HPLC quantification with ^{14}C-labeled AFB1 detection [36]. In the case of OTA. Vettorazzi et al. [24] validated a method successfully applied in a study of OTA toxicokinetics in rats [28].

The sampling method has been inspired in the work described by Vettorazzi et al. [24] for detecting OTA in plasma, liver and kidney of rats. OTA was extracted from the matrix with an organic solvent (ethanol) in very acid conditions using trichloroacetic acid (TCA). Unfortunately, this extraction process was not suitable for the simultaneous extraction of AFB1 and OTA because the pH conditions were too low for the AFB1 stability and because TCA, a trihaloacetic acid such as TFA (used for AFB1 derivatization [32,37]), gives undesired chemical reactions. On the other hand, mycotoxins bind proteins [7,8,38] and the extraction step needs low pH conditions to release OTA and AFB1 and to precipitate the proteins [19]. In addition, ACN obtained clearer extracts than ethanol in preliminary studies. Therefore, proteins from samples were precipitated with acidified ACN (1:3 ratio). Combinations of ACN–HCOOH (10%, pH = 2.62 and 1%, pH = 2.63), ACN–H_3PO_4 (1%, pH = 2.72 and 0.5%, pH = 2.84) and ACN–HCl (0.05%, pH = 2.59) were assayed. After analysis of the resulting extracts, ACN–HCOOH 1% yielded clean enough extracts and recovery values over 90% for both mycotoxins in the three matrices.

With the procedure described, the simultaneous extraction of both mycotoxins has been achieved only starting from 100 μL of plasma or 25 mg of tissue and covering a range of 2–6000 μg/L in rat plasma and 8–2400 μg/L in rat kidney and liver. Only one solvent step extraction is needed, avoiding immunoaffinity column clean up and toxic solvents such as chloroform, ethyl acetate or dichloromethane which cause health and environmental hazards.

3.2. Development of the UHPLC-FLD quantitative analytical method

Chromatographic conditions were investigated in order to achieve the best separation and resolution of peaks so as to allow quantification. The method starts with an isocratic set which permits the elution of the polar components extracted with the mycotoxins. Due to its polar properties, AFB1 appears early in the chromatogram. The front peaks corresponding to liver and kidney matrices are larger than those corresponding to plasma (Fig. 1). OTA needs an increase in the organic proportion of the eluent and it had to be delayed for up to minute 8.4 because several interfering peaks appear in kidney and liver, something that does not occur with plasma samples.

Aflatoxin B1 and ochratoxin A have fluorescent properties that make them good candidates for their detection with the use of fluorescence. However, it is well known than AFB1 suffers quenching in aqueous solvents; therefore, derivatization reactions would be necessary to quantify low levels. Either pre-column or post-column derivatization could be possible [37,39,40]. Pre-column derivatization was initially discarded in order to obtain a simple and quick method. In addition, post-column iodine or bromine (Kobra cell) derivatization was also discarded because both reagents need to add extra pumps and chemical reactors to the UHPLC system. Furthermore, iodine derivatization decreases OTA peak intensity [37]. The derivatization was performed with a photo-chemical reactor made with a knitted coil and a mercury lamp placed in line just after the column [40]. The PHRED makes derivatization fast and easy, and minimizes intervention of the analyst. The AFB1 signal is approximately 15 times more intense with the lamp on while OTA response remains unaffected.

Due to the fact that the same method has been applied for two mycotoxins in three different matrices, the effect of possible interferences of the matrix at the limit of quantification level has been studied for each one of them. There were no substantial differences in the chromatographic parameters for the same mycotoxin in the three matrices (see Table 1).

3.3. Validation of the method

The method was selective for kidney and liver because no interference peaks appeared at the retention time of AFB1 or OTA in blank samples. In the case of plasma, no interference peaks appeared at the retention time of AFB1; however, basal OTA levels were detected in blank plasma. The experiments made in order to assure selectivity for OTA (reanalysis of spiked blank contaminated plasma in different chromatographic conditions) showed that the standard OTA peak appeared at the same retention time as that of the interference. Moreover, after delaying of the peak, no broadening or distortion of the peak shapes was observed.

The working solutions remained stable up to 4 weeks at −20 °C (results not shown). After 6 weeks, the AFB1 concentration was unstable; after 8 weeks, the OTA concentration became unstable. Moreover, after 12 weeks, AFB1 started degrading in the working solution solvent (MeOH:ACN) while OTA remained unaffected. Spiked samples remained stable stored at −80 °C during 6 months

Table 1
Chromatography parameters of AFB1 and OTA obtained at the concentration of the limit of quantification in plasma, liver or kidney.

	AFB1			OTA		
	Plasma	Liver	Kidney	Plasma	Liver	Kidney
Retention time (t_R) (min)	2.54	2.59	2.58	8.38	8.50	8.48
Retention factor (k')	2.45	2.95	3.05	10.38	11.97	12.29
Symmetry	0.77	0.78	0.65	0.94	1.09	0.97
Peak width at half height (w_h)	0.13	0.13	0.14	0.11	0.12	0.11
Number of theoretical plates (N)	2099	2115	1935	29631	28867	31130
Resolution (R_s)*	3.11	7.61	9.26	21.41	20.58	20.58

* Calculated between the mycotoxin peak and its nearest peak in the chromatogram.

(results not shown). This coincides with the stability observed for OTA in biological samples by Vettorazzi et al. [24]. OTA and AFB1 were stable in processed plasma, liver and kidney for 15 h in the HPLC tray at 4 °C without light (results not shown).

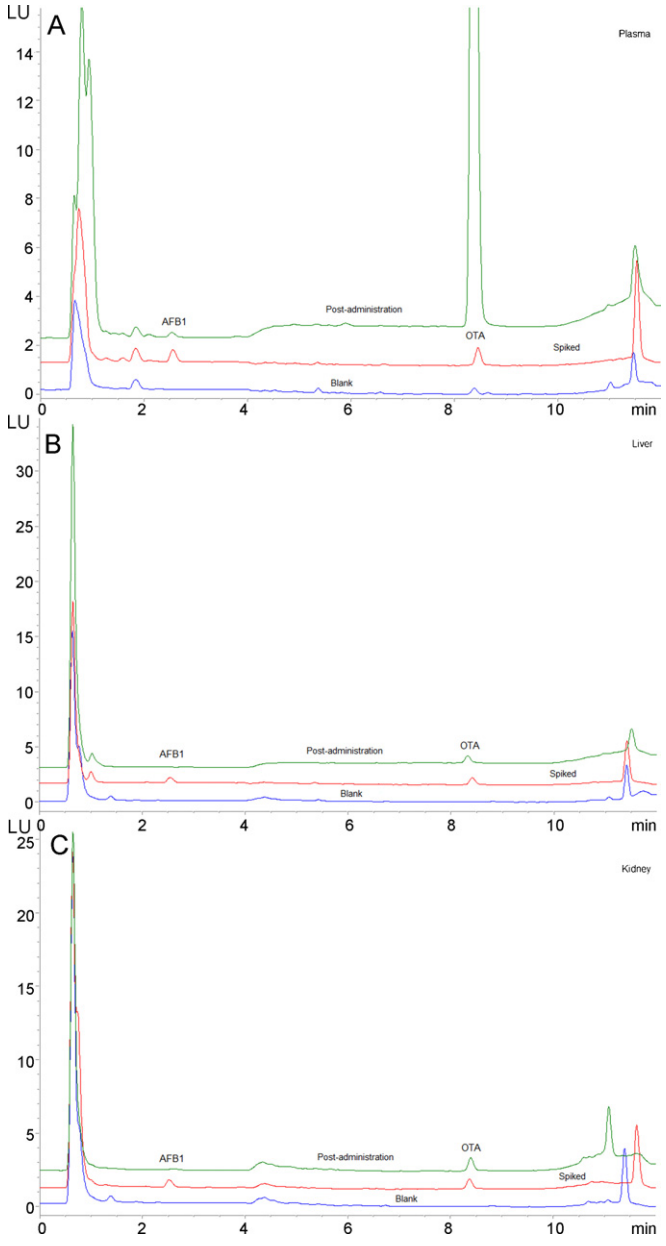

Fig. 1. Superimposed chromatograms of blank, spiked and post-administration extracted sample of (A) plasma, (B) liver and (C) kidney.

Linearity has been assessed in a wide range of concentrations in order to include not only high levels but also levels due to natural exposures that occur in laboratory animals fed with contaminated feed [24,26]. The four calibration curves generated showed a good linear relationship between response (area of the peaks) and the respective AFB1 or OTA concentrations. All of the requirements for linearity have been met for the two concentration intervals of each mycotoxin (see Table 2). Precision (RSD) and accuracy (SE) of the linearity showed adequate values, less than 10% in the whole interval (at the LOQ level less than 15%) (see Table 3).

The recovery for each matrix was very efficient, between 93% and 96% for AFB1 and between 94% and 96% for OTA. Moreover, the RSDs obtained in the within-day and between-day experiments were below 10% in each case (at the LOQ level below 15%), thereby demonstrating the precision of the analytical procedure (see Table 4). The range of the recovery study went up to 600 μg/L in plasma, liver and kidney; however, according to Vettorazzi et al. [24], higher concentrations of OTA might be expected in plasma, so the recovery of the dilution step was evaluated and the RSD was also below 10%. The recovery obtained in this method for AFB1 was successful for every matrix and higher than in other HPLC methods found in the literature [30,32,34]. In terms of OTA, the recovery was comparable to other methods for its analysis in plasma or serum [24,27] or higher in liver and kidney [20,24,27].

For both mycotoxins, the LOQs were 2 μg/L in plasma and 8 μg/kg in liver and kidney and they were considered to be the lowest concentrations in their range of quantification [21,41]. The limits of quantification obtained for AFB1 using the photoderivatization instrument were satisfactory and better than others found in bibliography for HPLC methods [16,31–33]. They were also comparable with radioactivity detection that uses very small amounts of sample [34–38,42]. For OTA, quantification limits are comparable to or better than those of other HPLC methods that use 2–100 times more sample volume [19,20,24]. The calculated LODs for AFB1 were as follows: 0.1 μg/L in plasma and 0.01 μg/kg in kidney and liver; the calculated LODs for OTA were: 0.3 μg/L in plasma and 0.01 μg/kg in kidney and liver.

In the study of robustness (see Table 5), the pH of the mobile phase had a significant impact on the quantification of OTA. An increase in the pH yielded a decrease in the OTA signal, whereas AFB1 signal remains without changes. Ochratoxin A has been described as a weak acid and it is important to maintain a pH below 4.4 in order to assure that OTA molecule is in its protonated form [19]. In addition, different lots of columns were tested and the quantification was satisfactory while the retention times appeared to be affected. On the other hand, peak areas did not change after slight differences in column temperature. Due to the high pressure conditions of the chromatography, slight changes in temperature, column batches or flow affected the pressure; as a result, the retention times were affected but the quantification was considered to be robust. Moreover, it was very important to keep samples in the dark during their obtainment or manipulation due to the fact that AFB1 degrades easily when exposed to light.

Table 2
Linearity data calculated with three replicates of each concentration of the range.

Mycotoxin	Range			Curve equation $y=(b\pm Sb)x+(a\pm Sa)$*	Determination coefficient (r^2)	Slope limits	Intercept limits	RSD (%) response factor
	Standard (μg/L)	Plasma (μg/L)	Liver/kidney (μg/kg)					
AFB1	0.5–30	2–120	8–480	$y=(2.11\pm0.03)x+(0.06\pm0.43)$	0.9988	(2.04; 2.18)	(−0.99; 1.10)	2.5
	30–150	120–600	480–2400	$y=(2.48\pm0.06)x-(7.78\pm5.74)$	0.9975	(2.31; 2.65)	(−23.72; 8.15)	6.1
OTA	0.5–30	2–120	8–480	$y=(2.52\pm0.03)x+(0.19\pm0.49)$	0.9989	(2.43; 2.60)	(−1.01; 1.38)	3.5
	30–150	120–600	480–2400	$y=(2.90\pm0.09)x-(7.45\pm7.96)$	0.9965	(2.66; 3.14)	(−29.56; 14.66)	6.3

* b: slope, *Sb* standard deviation of the slope, a: intercept, *Sa*: standard deviation of the intercept.

Table 3
Precision and accuracy of the instrumental system.

Mycotoxin	Concentration (μg/L)	Repeatability (within-day) ($n=3$)			Intermediate precision (between-day) ($n=9$)		
		Mean	RSD (%)	SE (%)	Mean	RSD (%)	SE (%)
AFB1	0.5	0.44	7.3	13.0	0.44	5.6	12.2
	2	2.1	4.1	5.3	2.0	5.7	1.0
	15	14.9	4.0	0.3	16.1	5.9	7.2
	30	31.7	6.4	5.8	30.0	6.0	0.0
	90	84.7	8.6	5.8	88.9	6.5	1.1
	150	147	2.1	1.7	153	3.8	2.4
OTA	0.5	0.45	8.6	9.9	0.45	7.8	10.8
	2	2.0	2.8	0.6	2.2	6.5	9.2
	15	14.5	3.5	3.6	15.7	6.3	4.6
	30	31.1	6.3	3.7	29.6	5.9	1.2
	90	87.1	8.3	3.2	91.2	6.9	1.4
	150	153	2.0	2.1	158	3.4	5.6

Table 4
Recovery study for AFB1 and OTA in plasma, liver and kidney.

Mycotoxin		Concentration (μg/L)[a] (μg/kg)[b]	Repeatability (within-day)				Intermediate precision (between-day)			
			Recovery (%) $n=3$	RSD (%) $n=3$	Global recovery (%) $n=12$	RSD (%) $n=12$	Recovery (%) $n=9$	RSD (%) $n=9$	Global recovery (%) $n=36$	RSD (%) $n=36$
AFB1	Plasma	2	89.1	1.5	93.8	5.7	85.4	4.1	93.0	8.9
		8	95.1	2.6			97.1	5.5		
		120	101.5	1.6			102.9	2.1		
		600	89.7	1.0			86.7	5.0		
		($d=20$)[c] 30	99.0	5.5			103.4	6.6		
	Liver	8	97.9	4.1	95.8	6.0	92.2	10.4	95.2	8.1
		32	94.8	2.5			97.8	5.2		
		480	101.9	2.9			102.5	2.3		
		2400	88.6	2.9			88.2	2.7		
	Kidney	8	94.2	3.3	97.0	5.8	100.7	6.2	96.0	8.1
		32	102.0	1.9			94.3	9.7		
		480	101.0	2.3			100.0	3.1		
		2400	91.2	6.5			88.7	5.4		
OTA	Plasma	2	97.9	1.5	96.5	7.5	92.8	5.8	96.0	8.9
		8	88.3	3.6			91.5	5.6		
		120	106.6	1.9			108.7	2,1		
		600	93.1	0.8			91.2	4.8		
		($d=20$)[c] 30	96.1	5.8			103.2	7.9		
	Liver	8	82.5	4.9	91.3	10.0	90.3	8.7	94.4	8.8
		32	89.5	4.5			89.7	5.4		
		480	105.4	3.0			105.9	2.4		
		2400	90.3	2.8			91.5	2.8		
	Kidney	8	91.4	4.9	96.9	7.1	85.1	8.5	94.5	9.7
		32	95.4	5.7			91.6	5.6		
		480	105.6	2.0			106.1	2.5		
		2400	95.1	6.6			95.3	4.4		

[a] μg/L for plasma.
[b] μg/kg for liver and kidney.
[c] Recovery study of the dilution step ($d=20$) of plasma.

Table 5
Study of robustness with standards: % of HCOOH in mobile phase, different column batches, changes in column temperatures and exposure to light.

	AFB1				OTA			
	Concentration (μg/L)		Retention time (min)		Concentration (μg/L)		Retention time (min)	
	Mean	SE (%)	Mean	SE (%)	Mean	SE (%)	Mean	SE (%)
Nominal	30		2.55		30		8.43	
0.1% HCOOH in mobile phase	30.0	0.0	2.53	0.7	25.7	14.2	8.33	1.1
Column batch A	29.4	2.0	2.21	13.0	30.6	2.0	7.48	11.3
Column batch B	27.7	7.5	2.38	6.3	30.6	1.9	7.81	7.3
Column at 59 °C	29.0	3.2	2.55	0.0	30.0	0.1	8.33	1.2
Column at 57 °C	29.3	2.2	2.65	4.1	30.1	0.3	8.69	3.1
Day light exposure	Degraded				28.7	4.5	8.38	0.6

Table 6
Concentration of AFB1 and OTA in plasma (μg/L), liver and kidney (μg/kg) from rats administered AFB1 + OTA (0.5 mg/kg b.w.) by oral gavage.

	Sample	Concentration (μg/L)[a] (μg/kg)[b]	
		AFB1	OTA
Plasma	1	9.8	427
	2	3.1	919
	3	0.13[c]	543
	4	<LOD	528
	5	1.7[c]	549
	6	<LOD	422
	7	<LOD	424
	8	<LOD	420
Liver	1	<LOD	35.5
	2	<LOD	31.7
Kidney	1	6.1[c]	42.6
	2	6.6[c]	41.5

[a] μg/L for plasma.
[b] μg/kg for liver and kidney.
[c] <LOQ.

3.4. Application to real samples

The method was successfully applied in real samples obtained from rats that received administration of a mixture of AFB1 and OTA (0.5 mg/kg b.w.) in a single dose by oral gavage (see Table 6). The analytical results have been corrected with the recovery value for each mycotoxin. The highest level found in plasma for OTA was 919 μg/L, the dilution step ($d = 20$) was applied and permitted quantification. On the contrary, low levels of AFB1 have been detected in plasma samples (<LOD). In kidney and liver, comparable levels of OTA have been found in both organs, and AFB1 was at a very low level in both of them.

4. Conclusions

A rapid and simple method for the simultaneous quantification of AFB1 and OTA in rat plasma, liver and kidney by UHPLC-FLD has been validated and successfully applied. The process is economical because only low volumes of solvents are needed, and the use of immunoaffinity columns is not necessary in the purification process. In addition, this method uses a low-volume column that permits working under high pressure conditions, thereby giving high resolution in short time assays. The two most important advantages of this method are that it enables the simultaneous quantification of AFB1 and OTA in three biological matrices, and that only 100 μL of plasma or 25 mg of tissue are sufficient enough for obtaining results in a wide range of concentrations, with adequate recovery and LOD and LOQ values. These characteristics make it very useful for carrying out experimental work in toxicokinetic and toxicological studies.

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