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Short communication

Determination of Fumonisin B_1 in animal tissues with immunoaffinity purification

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ABSTRACT

Immunoaffinity extraction combined with high-performance liquid chromatography (HPLC) with fluorescence detection was developed to determine Fumonisin B_1 (FB_1) in duck tissues. The method was linear over a concentration range of 0.013–0.250 μg of FB_1/g of liver, kidney and muscle. The limit of quantification was 0.013 μg FB_1/g in tissue. The mean percentage of extraction was 75% for liver and kidney and 53% for muscle. This method can be used in duck for the detection of FB_1 contamination after exposure, the liver being the most contaminated tissue.

1. Introduction

Fumonisin B_1 (FB_1) is the major mycotoxin produced by *Fusarium verticillioides* and *Fusarium proliferatum* [1], which are found worldwide in maize and maize products. In animals, the ingestion of contaminated feed leads to encephalomalacia in horses, to pulmonary edema in pigs, and to hepatic and renal toxicity in several species [2]. Moreover, FB_1 is reported to be carcinogenic in rodents [3] and has been linked to esophageal cancer in humans [4]. Taking into account its toxicity, the International Agency of Research on Cancer (IARC) classified FB_1 in 2B group of molecules that are considered as probably carcinogenic for humans [3].

In the face of these problems, several recommendations and regulations have been made by the FDA, JECFA and EU to limit FB_1 in food consumed by humans [5–7]. Quantification of FB_1 in vegetal food is relatively well documented. Most of the analytical techniques are based on fluorescence detection of derivatised FB_1 after its separation by HPLC [8,9] and the lasted sophisticated method for analysis of fumonisins is RP-HPLC/ESI-MS or RP-HPLC/ESI-MS-MS because of the selectivity of these hyphenated techniques [10,11]. By contrast, measurement of FB_1 in tissues is poorly documented. Moreover, most data concern its toxicokinetics in animals and the data were obtained using radio labelled toxins [12,13]. Theses kinetics reveal a poor absorption and a rapid elimination of FB_1, suggesting that the persistence of the toxin in tissues after ingestion is weak. Surprisingly, a recent study conducted in France reveals that the strongest FB_1 concentration in food was observed in avian livers with a mean concentration of 0.0525 μg/g [14]. Finally, only two methods have been described concerning the determination of non-radio-labelled FB_1 in tissues. The first method uses extraction with strong anion exchange (SAX) cartridges and quantification with HPLC connected to a quadrupole mass spectrometer with electrospray ionisation [15]. The second method is based on solid phase extraction followed by HPLC and fluorescence detection [16].

The purpose of this study was to describe a method of quantification of FB_1 in tissues that uses immunoafinity columns for the extraction of the mycotoxin. Derivatised FB_1 was quantified by fluorescence detection after separation by HPLC. The limits of detection were validated for quantification of FB_1 extracted from liver, kidney and muscle. Subsequently, the method was investigated to detect FB_1 contamination of tissue in duck after its oral administration at two different doses.

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2. Experimental

2.1. Preparation of FB_1 calibrants

Standard FB_1 (CAS N° 116355-83-0) was purchased from Biopure (Tulln, Austria). This stock solution has a certified concentration (51.100 mg/l in acetonitrile/water (1:1)), and was diluted with the same solvent to obtain solutions ranging from 0.001 to 2.5 mg/l. To prepare these calibrant solutions, 50 μl of the stock solution was mixed with 972 μl of acetonitrile:water (1:1) to give a solution containing 2.5 mg/l of FB_1 (S1 solution). Then 500 μl of the S1 solution was mixed with 500 μl acetonitrile:water (1:1 to give a solution containing 1.250 mg/l (S2 solution). Then 500 μl of the S2 solution was diluted with 500 μl of the same solvent to give a solution containing 0.625 mg/l (S3 solution). 100 μl of each solution (S1, S2, S3) were diluted with 900 μl acetonitrile:water (1:1) giving 0.25, 0.125 and 0.063 mg/l of FB_1 solutions, respectively. The same dilution (100 μl of diluted standard solutions with 900 μl of solvent) was carried out for the three last diluted solutions giving 0.025, 0.013 and 0.006 mg/l of FB_1 solution, respectively. The last dilution (0.001 mg/l) was obtained by mixing 100 μl of the 0.013 mg/l with the solvent (900 μl). All the solutions were stored in the dark at 4 °C as recommended by the manufacturer. The pure FB_1 used in the duck assays was purchased from Sigma (St. Louis, MO, USA).

2.2. HPLC analysis

The HPLC system comprised a M 2200 pump (Bischoff, Leonberg, Germany) connected to a Prontosil C18 column, 5 μm, 250 mm × 4.6 mm, 120 Å (Bischoff, Leonberg, Germany). Chromatographic conditions comprised a mobile phase composed of CH_3OH/NaH_2PO_4 0.1 M pH 3.35 (75/25 v/v) delivered at a constant flow rate of 1.00 ml/min. The derivatised mixture was injected at a volume of 20 μl. Fluorescence detection was performed by a RF 10A XL detector (Shimadzu, Japan) with an excitation and emission wavelength of 335 and 440 nm, respectively. The chromatograms obtained were exploited using PIC 3 software (ICS, Toulouse, France).

Samples were derivatised in the presence of β-mercaptoethanol with *o*-phtaldialdehyde (OPA, purchased from Sigma, St. Louis, MO, USA), prepared weekly and stored at 4 °C according to Rice et al. [17]: briefly, 50 μl of OPA, 50 μl of 0.1 M borate buffer at pH 8.3, and 50 μl of H_2O were added to 50 μl of standard solution or sample. Then, after one min, 20 μl of the derivatised mixture was injected on the chromatographic system. All theses operations were performed with an automatic injector (718-AL Alcott, Norcross, GA, USA).

The standard solutions were first used to perform a regression study between the area observed and the quantity injected: 10 standard solutions (0, 0.001, 0.006, 0.013, 0.025, 0.063, 0.125, 0.250, 0.650 and 1.250 mg/l) were injected in triplicate to check the linear range between 0.000 and 1.250 mg/l of FB_1. The 1.250 mg/l concentration tested corresponds to the highest spike according to the protocol used for tissue extraction.

Repeatability was assessed with two standard solutions (0.625 and 1.250 mg/l) injected eight times. The same standard solutions were tested over 1 week to assess between-run precision ($n = 6$).

2.3. Extraction

Liver or kidney samples (1 g) were first homogenised with a teflon Potter (500 rpm) in 2 ml of distilled water, and breast muscle samples (1 g) were homogenised in distilled water (2 ml) with an Ultraturrax TP18 (Ika Laboratory and Analytical Equipment, Staufen, Germany) (3000 rpm for 20 s). Protein was precipitated with 2 ml of acetonitrile/methanol (1:1) and 25 mg of NaCl. Samples were first placed on a stir table for 120 min at 300 rpm and then centrifuged for 15 min at 3000 × *g* at room temperature. Three ml of the supernatant fraction was defatted twice with 4 ml of hexane and centrifuged for 15 min at 3000 × *g* at room temperature. Two ml of the aqueous phase were diluted with 8 ml of Phosphate Buffer Saline, pH 7.4 (PBS). This solution was passed through a FUMONIPREP cartridge (R. Biopharm Rhône Ltd., Glasgow, Scotland) as recommended by the manufacturer. The column was then washed with 10 ml of PBS, pH 7.4. Finally, FB_1 was eluted with 1.5 ml of methanol and 1.5 ml of water, respectively. The eluate was evaporated at 40 °C in the dark under a gentle stream of Nitrogen. The dry residue was resuspended with 200 μl of acetonitrile/water (1:1).

2.4. Validation of the whole method

Blank liver, kidney and muscle samples obtained from ducks fed mycotoxin-free feed were fortified with different levels of FB_1 to determine the recovery rate, linearity and limit of detection, limit of quantification of the whole method in tissue.

Fortified samples containing 0.0063, 0.0125, 0.025, 0.0625, 0.125 and 0.250 μg FB_1/g were obtained by adding 100 μl of standard solution of FB_1 (respective concentrations of 0.063, 0.125, 0.250, 0.625, 1.250 and 2.5 mg/l) to 1 g of "blank tissue" before the extraction step.

Ten samples of unfortified liver, kidney and muscle were analyzed to measure the noise ratio baseline in the range of retention time of FB_1. The limit of detection (LOD) was defined as the smallest FB_1 amount that yielded a signal three times higher than the noise ratio obtained with blank tissues.

Fortified samples of liver, kidney and muscle were analyzed in triplicate to measure the recovery rates and to confirm the linearity of the method previously obtained using standard solutions. The limit of quantification (LOQ) was then defined as the smallest amount of compound for which the method was validated with sufficient accuracy (<25% in both intra- and inter-day assay).

The inter-day reproducibility was assayed on the recovery rates of FB_1 obtained from liver, kidney and muscle samples determined in triplicate at two levels of fortification: 0.025 and 0.250 μg FB1/g.

2.5. Animals—experimental protocol

All experimental procedures using birds were in accordance with the French National guidelines for the care and use of animals for research purposes. Fourteen male mule ducklings aged 1.5 weeks, weighing around 250 g (Pygavi, Muret, France) were randomly assigned to three groups. All the animals fasted 8 h before treatment and were weighed before dosing. The control group comprised 10 animals who received 10 ml/kg body weight of NaCl 0.9% per os.

The other groups comprised two animals who received an oral dose of 5 and 40 mg/kg body weight of FB_1 (Sigma, St. Louis, MO, USA), respectively in NaCl 0.9% at an injection volume of 10 ml/kg.

Animal were euthanized 2 h after administration and liver, kidney and breast muscle were immediately removed and frozen at −20 °C until FB_1 analysis.

2.6. Safety handling procedures

Because FB_1 is a hazardous substance, suitable procedures were used during the development of the method and animal experimentation. Protective clothing, including rubber gloves and laboratory coats, was worn throughout the assays. For decontamination following analyses, all used glassware was soaked in sodium hypochlorite (5% w/v) for at least 30 min followed by addition of acetone (5% v/v) for 30 min more. Glassware was then rinsed in dis-

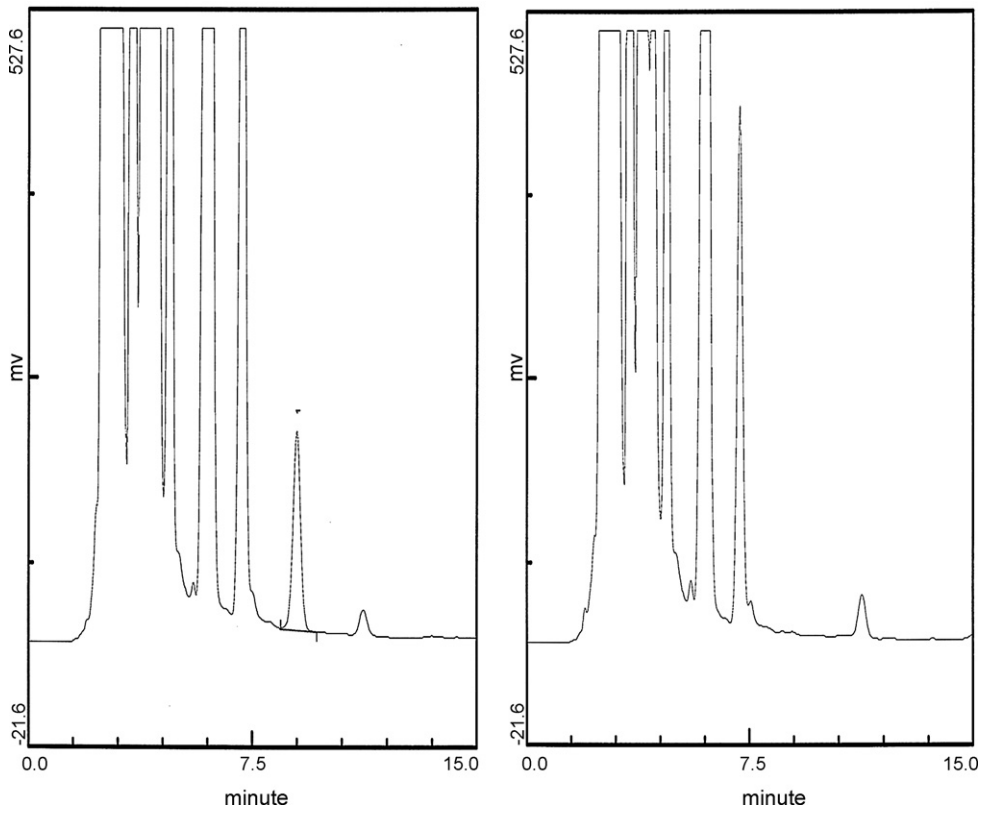

Fig. 1. Typical spectrofluorometric chromatogram obtained for a liver from duck that received 5 mg FB_1/kg BW (left) 2 h before killing and from a duck that not received FB_1 (right).

tilled water. Disposal of immunoaffinity columns, matrix residues and animals respected official biological waste treatment procedures.

3. Results and discussion

3.1. HPLC method: validation

The derivatisation reaction and the injection of the derivatised mixture were performed by an automatic injector because of the instability of OPA derivatives [9].

The response of the detector (area in $\mu v \times s$) to the concentration of FB_1 (in μg/ml) was linear for the whole range of concentrations tested (0.063–1.250 mg/ml) with a correlation coefficient (r^2) of 0.999.

The repeatability of two standard FB_1 solutions (0.625 and 1.250 mg/l) was 4.09 and 4.15%, respectively. The inter-day reproducibility was suitable because the variation coefficients of the same standard solutions obtained over the course of 1 week ($n = 6$) were below 5% (4.70 and 4.79%, respectively).

3.2. Validation of the whole method

Typical chromatograms obtained from a blank liver and from a liver from duck that received an oral dose of 5 mg/kg of FB_1 (2 h before killing) are shown in Fig. 1. Although some peaks were observed in the first part of the chromatogram, they did not interfere with the FB_1 retention time (eluted at around 10 min). These peaks may correspond to substances with a primary amine group that are more polar than FB_1 [16]. The profile of the chromatogram obtained for the kidney and the muscle of duck were quite similar to those obtained for the liver (data not shown).

Recovery rates of FB_1 from tissues at each level of contamination are given in Table 1. The recovery rate was constant for each type of tissue whatever the level of fortification used (ANOVA, $p < 0.05$), except for kidney at the 0.013 μg/g dose. With this method, a mean recovery rate of around 75% was obtained for the dosage of FB_1 extracted from the liver and kidney, whereas a recovery rate of 53% was obtained for the muscle. The recovery rates obtained are in agreement with those obtained by Meyer et al. for swine tissues by using SAX extraction and LC–MS determination of FB_1 [15].

Table 1
Repeatability and inter-day reproductibility on recovery of the whole method on spiked liver, kidney and muscle samples ($n = 3$)

Fortification level (μg/g)	Intra-day recovery (% ± SD)			Inter-day recovery (% ± SD)		
	Liver	Kidney	Muscle	Liver	Kidney	Muscle
0.013	79.4 ± 6.5	42.0 ± 9.7	48.5 ± 8.0			
0.025	72.0 ± 2.3	67.4 ± 10.0	57.9 ± 8.6	68.7 ± 6.1	60.8 ± 8.6	66.1 ± 1.5
0.063	73.4 ± 3.7	80.7 ± 9.6	49.4 ± 5.3			
0.125	76.2 ± 10.2	68.2 ± 9.5	56.4 ± 6.1			
0.25	71.7 ± 7.6	89.0 ± 10.4	53.0 ± 6.6	70.4 ± 3.0	75.9 ± 5.9	66.0 ± 8.3
Mean	74.5	76.3	53.0			

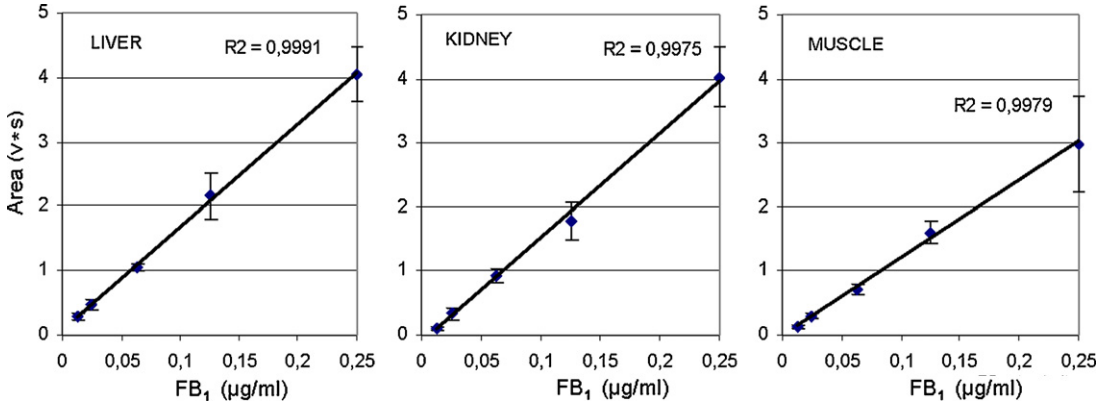

Fig. 2. Calibration for the whole method (mean ± SD, $n = 3$): Response of the detector (area in $\mu v \times s$) as a function of the spiked concentration of FB_1 in tissue (µg/g).

The repeatability of the whole method was allowable for the three types of tissue analyzed: intra-day variation coefficients (defined as standard deviation × 100/mean presented in Table 1) obtained with liver, kidney and muscle ranged from 3.2 to 10.6%, from 11.7 to 14.8% and from 10.7 to 16.5%, respectively. The inter-day variation coefficients obtained for the three tissues were also quite similar (from 2.2 to 12.5%) whatever the FB_1 fortification, confirming the correct reproducibility of the method for FB_1 values comprised between 0.025 and 0.250 µg/g. These results are comparable to those obtained in liver by Pagliuca (3.2–15.5% for repeatability and 4–18.4 for the inter-day assay) by using SPE and HPLC combined with fluorescence detection [16].

As shown in Fig. 2 a good linearity of the whole method was obtained for FB_1 concentrations in tissues ranging from 0.013 to 0.250 µg FB_1/g with an $r^2 = 0.9991$; 0.9975 and 0.9979 for liver, kidney and muscle, respectively. All samples with higher FB_1 concentrations must be diluted and re-analyzed to be within the range of linearity of the method.

The limit of detection (LOD) was estimated at around 0.010 µg of FB_1/g for the liver, kidney and muscle. This LOD was confirmed by fortification assays at the level of 0.006 µg FB_1/g of tissue. The chromatogram obtained for the fortification level of 0.006 µg/g was similar to that obtained with blank tissues (data not shown). The limit of quantification (LOQ) was 0.013 µg FB_1/g of tissue for liver and muscle. For kidney, regarding the weak recovery rate obtained with 0.013 µg FB_1/g, five more blank samples were spiked at this concentration to test the matrix match calibration curve registered for this tissue. The mean ± SD was 0.0113 ± 0.0016. This result confirms that the LOQ for kidney is 0.013 µg FB_1/g of tissue. This was lower than the LOQ obtained using SAX extraction for plasma (0.050 µg/g) [20], and tissue (0.075 µg/g) [16], but higher than the LOQ obtained by mass spectrometry (0.005–0.01 µg/g) [15].

3.3. Determination of FB_1 in tissue after oral administration

The FB_1 concentrations in tissues obtained from ducks that received oral doses of FB_1 2 h before killing are presented in Table 2. Two doses of FB_1 were administrated. The high dose (40 mg FB_1/kg BW) was used to be sure that FB_1 could be detected in tissue. The lowest (5 mg FB_1/kg BW) was used because it can be considered as representative of the maximum exposure of avian species recommended by the FDA. Indeed, the maximum recommended level of fumonisins in feeds in avian species is 50 mg/kg and mean feed consumption of duck is around 10% BW [5]. In both cases, the highest concentrations of FB_1 were obtained for the liver (5.31 and 0.34 µg FB_1/g). This result is in agreement with data obtained in toxicokinetic studies in rat, swine and laying hens using ^{14}C-FB_1 [18–20,13].

Table 2
Determination of FB_1 contents (µg/g of tissue) in liver, kidney and muscle 2 h after an oral dose of 5 and 40 mg FB_1/kg body weight in mule duckling

Oral dose (mg/kg)	Animal	Liver	Kidney	Muscle
40	1	6.696	2.155	0.730
	2	3.927	0.950	0.220
5	1	0.285	0.072	<LOD
	2	0.406	0.110	0.080

LOD: limit of detection: 0.013 µg/g.

It also agrees with the description of the presence of FB_1 in avian livers during a study on food safety in France [14]. The concentrations of FB_1 in kidneys were lower than those obtained in the liver (1.55 and 0.09 µg FB_1/g). This result is in agreement with toxicokinetic data using ^{14}C- FB_1 [18–20,13]. Only one study reported much higher concentrations of FB_1 in the kidney than in the liver following oral administration of the toxin in rat [21]. Interestingly, the ratio of FB_1 concentrations in liver vs kidney was nearly constant whatever the dose administrated to the ducks (3.4 and 3.8 for the 40 and 5 mg FB_1/kg BW treated ducks). Very low concentrations of FB_1 were found in muscles of ducks fed 40 mg FB_1/kg BW whereas FB_1 was below the LOQ in one animal that received 5 mg/kg BW. These results are in agreement with those previously obtained using ^{14}C-FB_1, the muscle being the least contaminated tissue in rat, swine and laying hens [19,20,13]. Only results obtained in monkeys revealed higher concentrations of FB_1 in muscle than in kidney [22,12].

4. Conclusion

The use of immunoaffinity columns for the extraction of FB_1 from tissues followed by quantification of the toxin by fluorescence after HPLC separation was validated for the determination of FB_1 in tissues. A mean percentage of extraction of 75% was obtained for liver, kidney, and of 53% for muscle with a limit of quantification of 0.013 µg FB_1/g for all tested tissues. This method can be used for the detection of FB_1 contamination after animal exposure, the liver being the most contaminated tissue.

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