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Simple method for the simultaneous isolation and determination of fumonisin B_1 and its metabolite aminopentol-1 in swine liver by liquid chromatography–fluorescence detection

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Abstract

An analytical method based on high-performance liquid chromatography (HPLC) combined with fluorescence detection (FL) has been developed for the simultaneous determination of fumonisin B_1 (FB_1) and its totally hydrolized metabolite aminopentol-1 (AP_1) in pig liver. The sample preparation is based on a single solid phase extraction (SPE). *o*-Phthalaldehyde (OPA) was used for pre-column derivatization before the programmed reversed-phase analysis on phenylhexyl column. The developed method shows good repeatibility for inter- and intra-day precision as well as adequate linearity of calibration curves (r^2 was 0.9855 for FB_1 and 0.9831 for AP_1). Average recoveries from the matrix were 93.6% for FB_1 and 95.3% for AP_1. The limit of quantification (LOQ) in swine liver was 75 μg/kg for FB_1 and 42 μg/kg for AP_1.
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Keywords: Fumonisin B_1; Aminopentol-1; High-performance liquid chromatography (HPLC); Fluorescence detection (FL); Solid phase extraction (SPE); Swine liver

1. Introduction

Mycotoxins are an eterogeneous group of chemical compounds, with low molecular weight, synthetized by the secondary metabolism of several generi of fungi.

These products do not have any biochemical significance in the fungus development but, on the opposite, they cause severe biological damages on animals and humans [1].

Fumonisins are a group of mycotoxins that have been linked with toxicity in several species and that occur worldwide, primarily in corn and corn-based products contaminated by *Fusarium moniliforme* [2].

More than a half of the corn and corn-based products worldwide have been estimated to be contaminated with variable amounts of fumonisin B_1 (FB_1), the most prevalent between the fumonisin subspecies [3,4].

Fumonisins cause various diseases: liver and kidney toxicity and carcinogenicity, pulmonary edema [5], immunosuppression, neurotoxicity [3]. Most of all of the

Abbreviations: FB_1, fumonisin B_1; AP_1, aminopentol-1; SPE, solid phase extraction; OPA, *ortho*-phtalaldehyde; HPLC–FL, high-performance liquid chromatography–fluorescence detection; HLB, hydrophilic–lipophilic balance; SAX, strong anion exchange

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toxicities resulting from exposure to these compounds can be explained by their ability to alter sphingolipid metabolism [6] by inhibiting ceramide synthase [3]. Human consumption of fumonisin-contaminated corn has been linked to the esophageal cancer in different area of South Africa (Transkey) [7] and China (Linxian) [8].

These mycotoxins can reach the human also indirectly through the consumption of products derived from animal fed with contaminated feed.

The International Agency for Research on Cancer (IARC) evaluated the carcinogenic potential of different mycotoxins and classified fumonisin B_1 (FB_1) as “probable human carcinogen” (class 2B) [9].

FB_1 (diester of propane-1,2,3-tricarboxylic acid and 2-amino-12,16-dimethyl-3,5,10,14,15-pentahydroxyeicosane) includes a long chain aminopentol backbone (AP_1) with two ester-linked tricarballylic acids (Fig. 1).

AP_1 originates from FB_1 by hydrolysis of the tricarballylic acid side chains at carbon 14 and 15, which are replaced by hydroxyl groups (Fig. 1).

In particular, AP_1 is produced during nixtamalization [10], a traditional treatment of corn with calcium hydroxide and heat. AP_1 has been detected in commercial masa, tortilla chips and canned sweet corn. In vivo studies demonstrated that AP_1 exhibits the same toxicological effects of FB_1 [11,12].

Some in vivo studies, where rats received diets supplemented with FB_1 or AP_1, show that the latter can be more toxic than the parent compound, and induces fumonisin-like liver and kidney lesions [11]. AP_1 shows also cancer-promoting activity in liver [10]. Moreover, little is known about a possible endogenous hydrolysis of FB_1 by mammal metabolism, even if a study led on primates revealed that the ester moiety of FB_1 was shown to be hydrolyzed in the intestine of vervet monkeys [13].

In recent years, growing interest arose in the study of fumonisin. There are currently several analytical methods for determination of the fumonisin mycotoxins in vegetable matrices, from which the extraction with organic solvent is followed by a clean-up with SPE [14] before HPLC analysis.

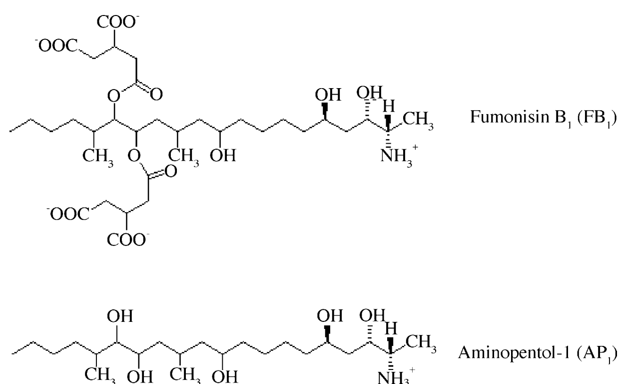

Fig. 1. Chemical structures of FB_1 and AP_1.

The majority of the current methods uses the technique of pre-column derivatization with *ortho*-phthalaldehyde (OPA) under fluorescence detection because of its sensitivity. Moreover, liquid chromatography coupled with mass spectrometry (LC–MS) have been proven to be effective method with high selectivity and sensitivity. Many of them are validated for fumonisins detection in corn and corn-based products [15–17].

LC–MS was recently used to detect FB_1 in pig liver after oral administration [18]. The uptake and distribution of radioactive material-derived residues were also determined in tissues of pigs fed a diet contaminated with ^{14}C-labelled FB_1 [19].

Furthermore, to optimise the instrumental analytical capabilities several clean-up steps have been proposed. The most widespread SPE methods for FB_1 isolation are based on strong anion-exchange (SAX). Other clean-up possibilities are offered by immunoaffinity columns [16,20] or C18 cartridges [21].

For AP_1 isolation, a further step with C18 cartridge [22] or XAD-2 (non-ionic macroreticular resin) column [23] or NH_2 cartridge [21] was necessary.

A simultaneous isolation of FB_1 and AP_1 from corn products was achieved optimising the extraction condition on C18 cartridge [17].

Few authors dealt with the determination of FB_1 alone [18,24] in the more complex animal matrices. Others proposed a separate clean-up procedure for the isolation of FB_1 and its hydrolysis products [23,25] with a similar pattern to that described for vegetable matrices. Instead, for animal matrices, no validated methods were available for the simultaneous isolation and determination of both FB_1 and AP_1, which was the aim of the present work.

2. Experimental

2.1. Chemicals and reagents

Fumonisin B_1 standard was purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Aminopentol-1 was prepared by basic hydrolysis of FB_1 as described in paragraph 2.2. The standards were individually dissolved in methanol to give stock concentrations of 10 mg/L for FB_1 and 5.6 mg/L for AP_1. The solutions were stored at −20 °C. Standard working solutions were prepared by diluting each stock solution with methanol.

The solution of *o*-phthalaldehyde (OPA), purchased from Sigma Chemical Co. (St. Louis, MO, USA), was weekly prepared according to Solfrizzo et al. [26] and stored at 4 °C.

Solid phase extraction (SPE) was performed with Oasis® HLB 3 cc (60 mg) cartridges from Waters Corp. (Milford, MA, USA).

All the solvents were of analytical or HPLC gradient grade (LiChrosolv) and were obtained from Merck (Darmstadt, Germany).

2.2. AP_1 preparation

AP_1 was prepared by basic hydrolysis of FB_1 modifying the method proposed by Caloni et al. [12] as follows: 20 mL of 1 M KOH were added to a 20 mL methanol solution, containing 1 mg of FB_1 and the mixture was incubated for 1 h at 70 °C. After cooling till room temperature, the pH was set to 4.5 with 2 M HCl. The completely hydrolized reaction product was used to prepare the AP_1 stock solution diluting up to 100 mL with methanol, to reach the final concentration of 5.6 mg/L.

2.3. HPLC–FL equipment

All analyses were carried out using a Varian 9012 ternary liquid chromatograph equipped with a 20 μL loop, combined with a Varian 9070 fluorescence detector with excitation (Ex) and emission (Em) wavelenght of 334 and 440 nm, respectively.

A Luna Phenylhexyl 5 μm column (250 mm × 4.6 mm I.D.) (Phenomenex, Torrance, CA, USA) was used.

As mobile phase two solvent systems were employed: 3.4 pH aqueous buffer with 2% of glacial acetic acid and 0.1% of triethylamine (A) and acetonitrile (B). The separation was operated at a flow rate of 1 mL/min with the following linear gradient from A:B (30:70) to A:B (50:50) in 50 min.

The column activity was regenerated washing for 15 min at a flow rate of 1 mL/min with acetonitrile.

2.4. Analytical procedures

2.4.1. Extraction and clean-up of FB_1 and AP_1 from swine liver

Ten grams of swine liver were fortified before extraction with both analytes: FB_1 and AP_1, as described in Section 2.4.2. These samples, cut into small pieces, were homogenized with an Ultraturrax apparatus and then extracted by stirring for 30 min with 80 mL of methanol–water (80:20). One tenth of this suspension was transferred into a tube and centrifuged at 3000 rpm for 5 min.

Four milliliters of the surnatant were removed and washed twice with 4 mL of *n*-hexane [18]. To obtain a faster separation of the two phases, another centrifugation at 3000 rpm for 3 min was carried on. The aqueous phase was evaporated off by vacuum concentrator centrifuge (Hetovac VR1-Heto). At the end, the residue was reconstituted with 2 mL of HPLC mobile phase A.

Oasis® HLB cartridges were conditioned with 2 mL of methanol and equilibrated with 2 mL of water. The sample was then loaded on the cartridge. Two washes were performed: the first with 1 mL of 95:5 (v/v) water–methanol and the second with 1 mL of 94:5:1 (v/v/v) water–methanol–glacial acetic acid. During this step the cartridge was evacuated continuously to dryness for 30 s under vacuum at 5 mm Hg. Finally, the analytes were eluted into a test tube with 2 mL of methanol at a flow rate of 1 mL/min under vacuum.

Table 1
FB_1 and AP_1 fortification levels

Analyte	Final fortification level (μg/kg)	Standard solution (mg/L)	Standard solution added (μL)
	75		75
FB_1	150	10	150
	225		225
	300		300
	42		75
AP_1	84	5.6	150
	168		300
	336		600

The eluate was brought to dryness under a stream of nitrogen. Then, the residue was redissolved in 150 μL of methanol and vortexed for 3 min before the derivatization for HPLC–FL analysis. Finally, 70 μL of OPA reagent were added to 70 μL of sample, vortexed and then injected in HPLC exactly after 1 min.

2.4.2. Preparation of calibration curves and method validation

Matrix-matched calibration standard curves were utilized in this study for the quantification of analytes in pig liver.

Ten grams of swine blank liver were weighed and divided into four different batches. In each batch a required amount of the standard stock solutions of FB_1 (10 mg/L) and AP_1 (5.6 mg/L) was added in order to obtain the fortification levels described in Table 1.

These fortified samples were processed in order to determine the absolute recovery of FB_1 and AP_1 as a part of the method validation procedure.

Twenty samples of unfortified liver were analyzed as negative controls to measure the baseline noise and to confirm that no interferences were present in the region where FB_1 and AP_1 are eluted.

The method was tested for its intra- and inter-day assay repeatability, to determine its accuracy and precision. Swine liver samples were fortified and processed on three different days and within the same day.

2.5. Safety handling procedure

Since FB_1 and AP_1 are hazardous substances, safety precautions were adopted during analytical manipulations. All the procedures were performed with protective clothing and all the glassware, solvents and matrix residues used were decontaminated by soaking them overnight in a sodium hypochlorite (bleach) solution (10% v/v) followed by addition of acetone (5% by volume) for an additional night.

3. Results and discussion

Liver is one of the main target organ of disposition for FB_1 or its metabolites [19]. Because of the intense metabolic activity occurring in liver, many substances, such as amino acids and peptides, could interfere in FB_1 and AP_1 isolation.

Method development for the simultaneous isolation and determination of FB_1 and AP_1 in swine liver required specific optimization of clean-up, derivatization and chromatographic conditions.

3.1. Clean-up procedure development

Fumonisin B_1 is amphipathic zwitterion, which is soluble in polar solvents as well as its metabolite aminopentol-1, which shows an hydrophilic chain in his structure. For this reason, it was chosen 80:20 (v/v) methanol–water as solvent of extraction, according to Scott and Lawrence [22].

In order to optimize the extraction procedure, the amount of sample loaded onto cartridges was only one tenth of the whole, to avoid plugging the Oasis® HLB cartridge and to make the following centrifuge step easier. The sample was centrifuged to eliminate suspended particles; furthermore a double liquid partition with hexane was carried out according to Meyer [18] in order to eliminate the interferences due to fats.

In order to isolate FB_1 and AP_1 simultaneously, it was necessary to modify the most common SPE approach based on strong anionic exchange cartridges (SAX) packed with a quaternary amine used by many authors for the isolation of fumonisins [14,18,23].

Since SAX is a strong anion-exchanger, it should not be used for the retention of very strong anions, which would be difficult to elute from the sorbent. SAX cartridges are indicated for such an ionic compound as fumonisin but not for its hydrolysis product AP_1, because of the lack of anionic carboxylate groups, needed for the SAX clean-up procedure. So, if a SAX procedure for FB_1 isolation were hold, an additional extraction procedure for AP_1 would have been requested. In order to simplify the SPE technique, an alternative approach with Oasis® HLB (hydrophilic–lipophilic Balance) cartridge was chosen. Its sorbent is a macroporous copolymer [poly(divinylbenzene-co-*N*-vinylpyrrolidone)] and exhibits both hydrophilic and lipophilic retention characteristics retaining both the more polar FB_1 and the less polar AP_1. The compounds have been processed in one batch, resulting in a significant increase in throughput.

Preliminary studies with C18 SPE cartridges [17,21,24] did not allow to purify from interferents the analytes and to calculate FB_1 and AP_1 recovery. Other several trials with different cartridges gave satisfactory results only for FB_1 recovery but not for its hydrolysis product. For instance, the strong anion exchanger Isolute® SAX (IST, Mid Glamorgan, UK) exhibited a recovery of 92% for FB_1 and 0% for AP_1; Oasis® MAX (Waters. Milford, MA, USA) that has an anion exchanger mixed with a reversed-phase, showed a recovery of 81% for FB_1 and 8% for AP_1; immunoaffinity columns Fumoniprep® (R-Biopharm, Glasgow, UK), consisting of antibodies reactive with fumonisins [20], indicated a recovery of 68% for FB_1 and 0% for AP_1.

The best result was achieved with Oasis® HLB. These cartridges showed a high reliability, reproducibility and good average recoveries (93.6% for FB_1 and 95.3% for AP_1) using small volumes of solvents, providing samples with a low presence of interferences.

The main feature of our SPE procedure is the simultaneous isolation of both analytes. FB_1 has two carboxylic acid groups that are unionized in an acid medium; to enhance interactions of analyte with adsorbent an acid aqueous buffer was chosen as loading phase.

A preliminary washing step with 1 mL of 95:5 (v/v) water–methanol was carried out. An additional acid washing step with 1 mL of 94:5:1 (v/v/v) water–methanol–glacial acetic acid was necessary to elute other compounds, while still leaving the analytes retained on the SPE adsorbent, before the final elution with methanol.

3.2. Optimization of derivatization

Since fumonisins and its hydrolysis products lack a significant UV chromophore and are not inherently fluorescent, sensitive detection at low levels, necessary for the analysis of sample, requires derivatization of the extract for the fluorometric detection.

The most common derivatizing reagent used for fluorescence determination is *ortho*-phthalaldehyde (OPA) coupled with 2-mercaptoethanol as reaction partner. This method is officially accepted by the Association of Official Analytical Chemists International [27] for the analysis of FB_1 in corn.

o-Phthalaldehyde is an amine detection reagent that reacts with primary amine groups to generate a fluorescent product in the presence of 2-mercaptoethanol.

Derivatization of the primary amine of FB_1 with OPA yielded a highly fluorescent product that was easily separated by reversed-phase chromatography. Because of the instability of OPA derivatives within few minutes [14,28], it was necessary to prepare the conjugates immediately prior to injection and to inject them within 1 min of mixing the reagents [29].

The time-dependent instability of the fluorescent adduct formed between the primary amine and OPA results in fluctuation of the fluorescent signal. The decay of fluorescence is not linearly time dependent, according to Thakur and Smith [23]. As it is shown in Fig. 2, injecting the OPA-FB_1 derivative at different injection times was verified that the intensity signal begins to decrease after one minute. Since the intensity decreases in a time-depending manner although not linear, it is very important for precision and reproducibility that the derivative samples were injected in the same time frame, and however, within 1 min. The immediacy of analysis avoids any problem inherent to the stability of OPA derivatives that are time and temperature depending.

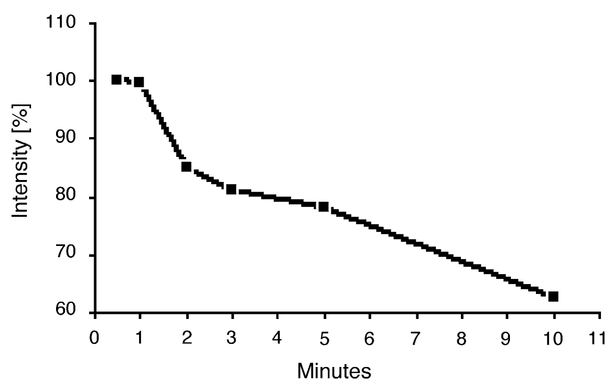

Fig. 2. Fluorescence emitted by the OPA-FB_1 derivative at different HPLC injection times.

Many Authors discussed the variability of the ratio analytes:OPA [23,24] suggesting a 1:2 ratio as the optimal one for the analysis. A 1:1 ratio was instead chosen according to the fact that the maximum signal to noise was given when OPA concentration was 50% of sample, according to Caloni et al. [12].

3.3. HPLC method development

To determine both the analyte clearly and as sensitive as possible, the chromatographic conditions were investigated.

Since FB_1 and AP_1 are polar molecules soluble in water and in polar solvents, they are ideally suitable for the determination by reversed-phase HPLC.

According to the study of Thakur and Smith [23] on meat extracts, a first attempt for the determination of analytes was carried out with a C18 reversed-phase column, using as mobile phase a mixture of acetonitrile and acid buffered water solution. The results achieved on liver samples were not satisfying in terms of selectivity and peak shape.

On the opposite, a phenylhexyl reversed-phase column together with the chosen mobile phase programmed conditions, had provided an effective separation, as shown in Fig. 3; as a matter of fact the retention for such polar and amine compounds as FB_1 and AP_1 was increased, and the elution order of the analytes was reversed. The mobile phase run to a maximum percentage of acetonitrile of 50% to reach the best analytical separation, avoiding the π electrons of the "CN" bond in acetonitrile to compete for all the phenyl "binding sites", making the stationary phase more hydrophobic. Higher percentage in acetonitrile gave unsatisfying separation but were worth to wash the analytical column after each run. For this reason, 100% acetonitrile was used to wash the column as suggested by Thakur and Smith [23].

The presence of interferent peaks in the first part of the chromatogram underlines the importance of longer retention time for FB_1 and AP_1. These interferents are due to substances with a primary amine group that are mainly amino acids and peptides, available to react with OPA, which are present in the liver in considerable amounts.

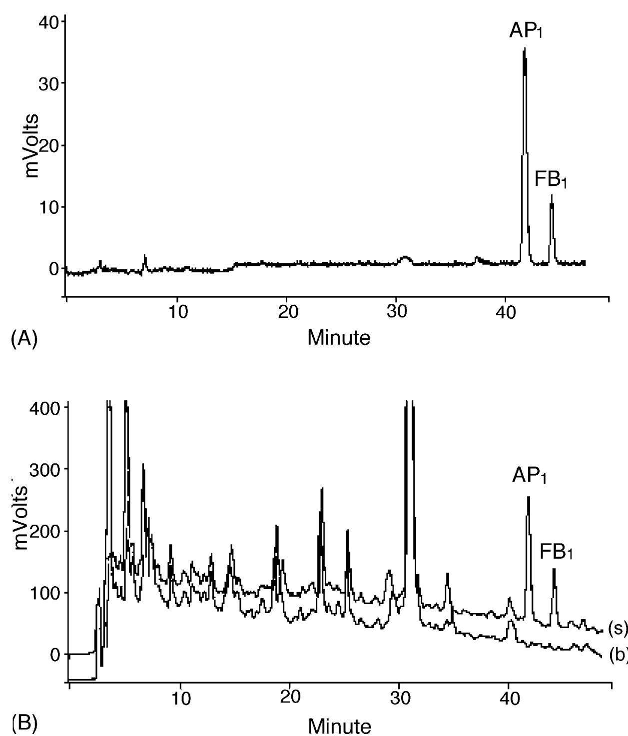

Fig. 3. Spectrofluorometric chromatogram of (A) standard of AP_1 and FB_1 in methanol (1 mg/L) and (B) of AP_1 and FB_1 fortified swine liver(s) with a contamination level of 1 μg/kg compared to a blank samples (b).

3.4. Analytical method validation

The average recovery for FB_1 and AP_1 were 93.6 and 95.3%, respectively. Recoveries at each level of contamination are given in Tables 2 and 3, and were determined comparing the peak area of analytes extracted from spiked samples to the peak area generated by the standards injected directly.

All matrix-matched calibration standard curves were prepared with spiked liver, and the correlation of coefficient values (r^2) were satisfying (0.9855 for FB_1 and 0.9831 for AP_1).

The HPLC–FL method was tested for its intra- and inter-day assay repeatability to determine its accuracy and precision, and results are shown in Table 4.

Table 2
Recovery of FB_1 in fortified liver samples

Level of fortification for FB_1 (μg/kg)	Recovery % ± S.D.
75	88.3 ± 15.6
150	94.2 ± 12.4
225	94.5 ± 3.4
300	97.6 ± 3.9

Table 3
Recovery of AP_1 in fortified liver samples

Level of fortification for AP_1 (μg/kg)	Recovery % ± S.D.
42	89.6 ± 5.6
84	95.5 ± 4.8
168	104.3 ± 5.2
336	91.5 ± 4.3

Table 4
Repeatibility of the method for the determination of FB_1 and AP_1 in spiked liver

Analyte	Fortification level (μg/kg)	Intra-day assay		Inter-day assay	
		Recovery (%)	R.S.D.	Recovery (%)	R.S.D.
FB_1	75	92.1	14.3	84.4	15.5
	150	99.8	8.3	93.2	13.7
	225	93.6	3.5	95.9	2.8
	300	96.4	3.1	99.5	3.9
AP_1	42	92.5	5.6	86.5	2.2
	84	95.7	6.0	97.6	4.1
	168	104.6	5.5	106.0	6.2
	336	91.0	5.0	93.6	3.9

The recoveries and Relative Standard Deviations (R.S.D.s) show good accuracy and precision of the method in both analysis intra- and inter-day, providing an useful tool to screen and quantify FB_1 and its major metabolite AP_1 in liver.

The HPLC–FL method was able to quantify the FB_1 in liver in a range of 75–300 μg/kg and AP_1 in a range of 42–336 μg/kg.

The limit of quantification (LOQ), defined as the smallest analyte content for which the method was validated with the specific accuracy and precision, was 75 μg/kg for FB_1 and 42 μg/kg for AP_1; the limit of detection (LOD), defined as the concentration that yields a signal-to-noise (S/N) ratio of at least 3:1, was 20 μg/kg for FB_1 and 10 μg/kg for AP_1 [30].

The few data available in literature regard the presence of FB_1 in liver, after oral administration; they evidence a contamination level of 231 ± 163 μg/kg [18]. Moreover, a concentration of about 160 μg/kg of FB_1-derived residues was found in liver after oral administration of ^{14}C-labelled FB_1 [19]. These contamination levels are included in the quantification range of the method developed.

Furthermore, we obtained some preliminary data from liver of weaned piglets fed a diet with or without FB_1 at 30 mg/kg feed for 6 weeks. The contaminated animals averaged FB_1 liver concentrations at 135 μg/kg ranging from 90 to 165 μg/kg and AP_1 at 82 μg/kg ranging from 42 to 121 μg/kg. These early results indicated that the method is reliable. Our animals did not received in the diet AP_1, and neither FB_1 nor AP_1 were found in the liver of animals fed the control non-contaminated diet. As such, the appearance of AP_1 in the liver of contaminated animals clearly originates from the breakdown of FB_1 from dietary origin. Further investigation to confirm these data are needed.

4. Conclusions

As demonstrated in the analytical procedure described herein, HPLC–FL coupled with Oasis® HLB extraction allows the simultaneous, rapid and sensitive isolation and detection of fumonisin B_1 (FB_1) and its major metabolite aminopentol-1 (AP_1). The method evaluated in swine liver was sensitive, selective and reliable for the levels founds in the study and it could also be used in the future for monitoring fumonisin and its metabolite in liver. The validated method could be an useful tool for the correct evaluation in animal tissues and in food of animal origin, not only for FB_1 but, also for AP_1 introduced with the diet or originating from endogenous hydrolysis. This could lead to a more complete safety assessment, allowing to prevent the exposure to these dangerous compounds.

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