Determination of Fumonisins in Milled Corn Grains Using HPLC–MS

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Abstract

A new method for determination of fumonisins in corn samples was developed and validated. The mycotoxins were extracted by a mixture of methanol–acetonitrile–water (1:1:2, v/v/v) and determined on a liquid chromatograph with mass spectrometric detection. The separation was performed on Zorbax XDB-C_{18} column (150 × 4.6 mm; 5 µm) with a Metaguard ODS-2 precolumn (30 × 4.6 mm; 5 µm) using gradient elution with mobile phase consisting of acetonitrile and 5 mmol/L ammonium acetate (adjusted by acetic acid to pH 3.0). For detection of ($M+H^+$) ions, a quadrupole mass spectrometer in single ion monitoring mode was applied. Developed method showed very good linearity in a tested range of concentration. Detection limit is 62.0 µg FB_1/kg and 58.5 µg FB_2/kg of maize grains. Because the detection limits lie under the maximum permitted EU levels, the method is suitable for determination of fumonisins in milled corn grains.

Introduction

The fumonisins, a family of food-borne carcinogenic mycotoxins, were first isolated in 1988 from cultures of *Fusarium verticillioides* (previously known as *Fusarium moniliforme*) (1), one of the most common field fungi associated with corn and corn-based foods and feeds worldwide (2). A 19–20 carbon amino-polyhydroxyalkyl chain characterizes this group of mycotoxins, which is diesterified with propane-1,2,3-tricarballylic acid (3). Of the 28 analogues FBs identified to date, the fumonisin B_1 (FB_1), fumonisin B_2 (FB_2), and fumonisin B_3 (FB_3) are the most important (Figure 1). These three compounds have similar toxicity, but FB_1 is the most associated with food contamination (usually constituting about 70% of the total FBs content) (2,4,5).

Fumonisins induce equine leukoencephalomalacia (ELEM) in horses, porcine pulmonary edema (PPE) hydrothorax and hepatic syndrome in pigs, and hepatotoxic and carcinogenic effects and apoptosis in the liver of rats (6–8). Fumonisins frequently co-occur with aflatoxins in corn and have been shown to promote aflatoxin carcinogenicity in trout (9). Consumption of fumonisin-contaminated corn correlated with the high incidences of human esophageal cancer in South Africa and China (10–12). The carcinogenic risk of fumonisins to humans was evaluated by the International Agency for Research on Cancer (IARC) in 1993, and these toxins have been classified as potential carcinogens for humans (class 2B carcinogens). Maximum levels for fumonisins (FB_1 + FB_2) in maize and maize products are setting in Commission Regulation (EC) No. 1126/2007 (for milling fractions of maize with particle size > 500 µm–1,400 µg/kg and for milling fractions of maize with particle size ≤ 500 µm–2,000 µg/kg) (13).

High-performance liquid chromatography (HPLC) with fluorimetric (14–18) or mass spectrometric (19–22) detection (MS) is the most commonly used method for the analysis of fumonisin representatives. The application of HPLC with evaporative light scattering detector was also reported (23). Furthermore, gas and thin-layer chromatography (24) and capillary electrophoresis (25) can be used as well. Competitive enzyme-linked immunosorbent assays (ELISA) (26), which are easy to perform and do not require extensive equipment, may only be used for the quantitative screening of total fumonisin contents due to its non-selectivity of high cross-reactivity of fumonisin B_1, B_2, and B_3. Next, the drawback of ELISA is comparatively poor precision, linearity, etc.

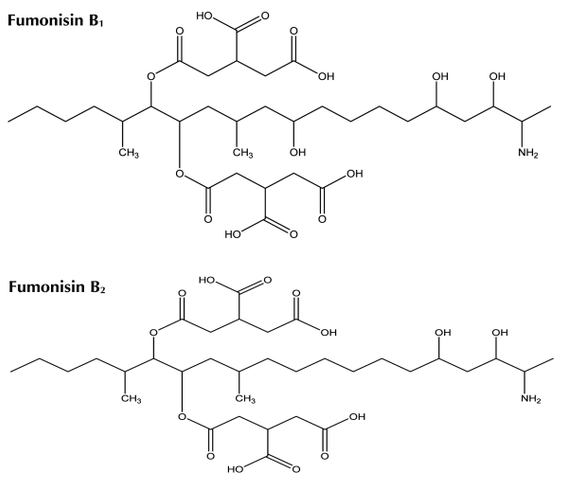

Figure 1. Chemical structures of fumonisin B_1 and fumonisin B_2.

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The extraction of fumonisin compounds from edible matrices has been performed using a mixture of methanol–water, acetonitrile–water, or acetonitrile–methanol–water (27–29). The cleanup, to remove matrix impurities and concentrate the fumonisins, can be done by solid-phase extraction using either reversed-phase (C_{18}) (30) or strong anion-exchange (SAX) cartridges (31,32), or by immunoaffinity columns (18,33). Analytical methods based on HPLC are described previously (21,22,34).

In this work, an innovative procedure for determination of fumonisin B_1 and B_2 in milled corn samples was done. It combines high efficient extraction with methanol–acetonitrile–water using UltraTurrax blending and HPLC–MS.

Experimental

Chemicals and reagents

Analytical-grade purity ammonium acetate, fumonisin B_1 and B_2 standard solution (concentration of FB_1 = 50.1 ± 0.7 µg/mL, FB_2 = 50.2 ± 0.7 µg/mL, in acetonitrile/water = 50/50), and gradient-grade purity solvents acetonitrile and methanol were purchased from Sigma Aldrich (Prague, Czech Republic). All solutions were prepared in deionized water (Demiwa ros, Vatek, Czech Republic) and stored in darkness at 4°C, the standard of pure solid fumonisins at –20°C.

Chromatographic instrumentation

The HPLC system HP 1100 (Agilent Technologies, Palo Alto) consisted of vacuum degasser unit (model G1322A), quaternary pump (G1311A), autosampler (G1313A), and quadrupole mass spectrometer (G1946VL) with electrospray ionization was used. The ChemStation software (Rev. A 10.02) controlled the chromatographic system, and it was used for chromatogram evaluation. The separation was performed on a Zorbax Eclipse XDB-C_{18} column (150 × 4.6 mm; particle size 5 µm) equipped with a Metaguard ODS-2 precolumn (30 × 4.6 mm; 5 µm). Mobile phase consisted of water respective ammonium acetate (1, 2, 5, or 20 mM; pH adjusted by concentrated acetic acid to 3.0) and acetonitrile. Linear gradient elution of fumonisins was applied. The composition of mobile phase at the beginning was 33% acetonitrile, at 8 min was 60%, and at 9 min was again 33%. The column was conditioned prior to each analysis for 6 min. Total time of analysis, including prewash, was 20 min. The flow rate of mobile phase was 0.8 mL/min. The volume of injected sample was 5 µL. All measurements were performed in laboratory thermostated to 20°C.

Extraction

Extraction process is based on modified European Standard EN 114352:2004 "Foodstuffs—Determination of fumonisin B_1 and B_2 in maize based food—HPLC method with immunoaffinity column clean up" (34). Twenty-five milliliters of extraction solution (methanol–acetonitrile–water, 1:1:2, v/v/v) was added to 10 g of milled corn grains and mixed on Heidolph Diax 900 homogenizator for 2 min at speed 3 (Sigma Aldrich). Next, the flask was centrifuged (Universal 32R, Hettich, Germany) at 4000 rpm for 5 min. The supernatant was transferred to a 50-mL volumetric flask. Extraction of solid rest was then repeated with 20 mL and 15 mL of extraction solution. Volume of collected extracts was adjusted in 50 mL. Extracts were filtered through a polytetrafluoroethylene membrane filter (SMI-LabHut Ltd., Gloucestershire, UK) with pore size 0.20 µm prior to HPLC–MS determination.

Preparation of standard solutions and spiked samples

Calibration solutions were prepared fresh from stock solution of fumonisin B_1 and B_2 mixture by dilution with an appropriate amount of mobile phase and stored at 4°C in a dark place until analysis. Spiked samples were prepared by addition of pure mycotoxin standard to maize flour sample.

Results

HPLC–MS method optimization

To obtain the highest sensitivity of the quadrupole mass spectrometric detector, it was necessary to set up all parameters. At the start, the composition of mobile phase was optimized. In screening experiments, there was positive influence of acidic solution on ammonium acetate. Thus, the effect of acidic acetate concentration was tested and compared with ionization in neutral water–acetonitrile mobile phase. Presence of ammonium acetate in mobile phase led to higher ionization. We assumed that the presence of ammonium ions supports the formation of protonated species. The mechanism of this formation process of this species is speculative. For example, the protonated species can be formed via unstable intermediate $(M+NH_4)^+$. The highest ionization was obtained at 5 mmol/L concentration. With a higher concentration of ammonium acetate, there appeared problem with electrospray functionality due to high conductivity of aerosol and discharges in the spray chamber.

The quadrupole mass detector allows setting of the fragmentor (collision) voltage and fragmentation regulation of charged compounds. The trend of abundance of molecular ions as a function of fragmentor voltage has typical shape. At low voltage, adducts of molecular ion with sodium respective to mobile phase constituents are formed on an account of single charged protonated molecular ion. This leads to the molecular signal decreasing. When voltage is increasing, the abundance of molecular ion is increasing, and amount of adducts is lowering. At voltage 300 V, the molecular peak show maximum intensity. Higher voltage leads to higher molecular fragmentation and significant decreasing of signal. The dependence of signal intensity on electrospray capillary voltage is hyperbolic. With increasing voltage, the abundance of molecular ions increases. The highest signal was observed with voltage 6000 V.

Nebulizer pressure did not have a significant influence on ionization. Low nitrogen pressure caused problems with insufficient evaporation of mobile phase under relatively high flow rate (0.8 mL/min). The highest possible pressure (60 psig) was chosen as the optimal value for maximal evaporation.

The ionization obtained at nebulizer gas flow 12 L/min was approximately 20% higher than those under low flow rates. High flow rate supports better mobile phase evaporation and ionization.

Because fumonisins are relatively stable in heat, high temperature of drying gas can be applied. The influence of temperatures at intervals 150–350°C was investigated. Low temperatures (below 200°C) made ionization difficult because the aerosol was wet and caused discharges in electrospray chamber. The best ionization was obtained at 350°C. The problem was caused due to the high flow rate of mobile phase and relatively low content of organic solvent in it. On the other side, the compromise between time analysis and ionization was reached.

The developed method is faster in comparison with methods using fluorescence detection because it was not necessary to perform the derivatization step. The detection limit (LOD) for both detected fumonisins allows method application in food control without the necessity of preconcentration.

The developed method was then applied on determination of fumonisins in various samples of grounded maize corn. The mycotoxin content in this material ranged between > LOD–10.878 mg FB_1/kg and > LOD–2.114 mg FB_2/kg. Higher concentrations of FB_1 than FB_2 were found in all positive samples. These results correspond to those of analysis of *Fusarium verticillioides* naturally contaminated food samples, where the FB_1 forms 70–80% of total fumonisins and FB_2 15–25% (35).

Table I. Quantification of FB1 and FB2

	LOD		LOQ		RSD %		Calibration
	(µg/mL)	(µg/kg)	(µg/mL)	(µg/kg)	(n = 6)	r^2	equation
FB1	0.0124	62.0	0.041	202.7	1.95	0.9997	$y = 43937x - 390.08$
FB2	0.0117	58.5	0.040	199.1	2.04	0.9997	$y = 44927x - 399.95$

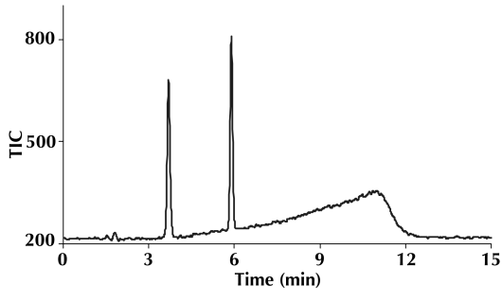

Figure 3. Chromatograms of pure standard solution and real samples obtained under optimal conditions.

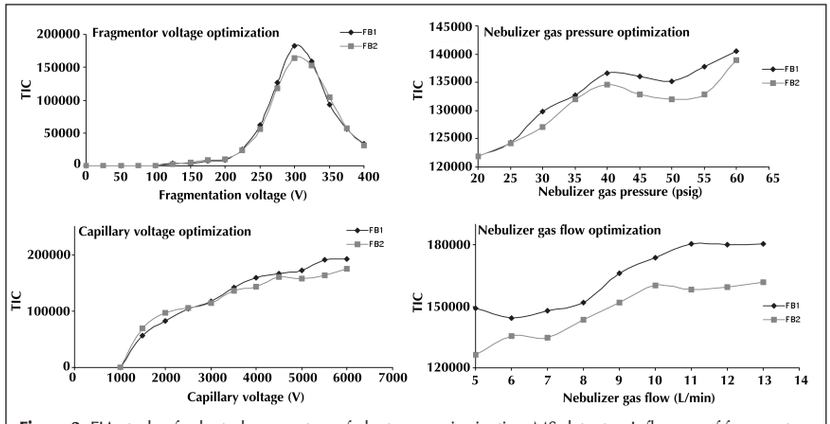

Figure 2. FIA study of selected parameters of electrospray ionization-MS detector. Influence of fragmentor voltage, capillary voltage, nebulizer gas pressure, and nebulizer gas flow were examined.

Chromatographic method development

Primarily, it was necessary to find the best conditions separation and ionization of fumonisins. In the case of chromatographic separation, it was optimized composition of the mobile phase. Operational range of MS parameters were: nebulizer gas flow (5–13 L/min; step 1 L/min), nebulizer pressure (20–60 psig; step 5 psig), gas temperature (150–350°C; step 50°C), capillary voltage (1000–6000 V; step 500 V), and fragmentor voltage (0–400 V; step 25 V). These parameters were optimized in positive ion mode of electrospray ionization-MS detection. In accordance to Commision Decision 2002/657/EC (36), selected fumonisins were detected as ($FB_1 + H^+$) (722.4 *m/z*) or ($FB_2 + H^+$) (706.4 *m/z*) and confirmed using fragments *m/z* 334 and 352 for FB_1 and *m/z* 336 and 318 for FB_2. The optimization was carried out by flow injection analysis (FIA) or several standard solutions varying in concentration (Figure 2). The optimal settings of electrospray ionization-MS detector were found as follow: nebulizer pressure 60 psig, nebulizer gas flow 12 L/min, gas temperature 350°C, capillary voltage 6000 V, and fragmentor voltage 300 V.

Effect of ammonium acetate

We assumed that the content of acidic ammonium acetate in mobile phase could significantly improve ionization of fumonisins. Thus, we have tested five solutions: deionized water and four solutions of different ammonium acetate concentrations (1, 2, 5, and 20 mmol/L) adjusted to pH 3.0 by concentrated acetic acid. The concentration of buffer 5 mmol/L yields the best results. Chromatogram obtained under the best conditions of pure standard solution is presented on Figure 3.

Calibration curve for pure standards—linearity and limit of detection/quantification

The linearity of developed HPLC determination was investigated in the range of concentration between 5–0.005 µg/mL, which corresponds to 25,000–25 µg/kg. Each selected concentration was measured in triple repetition. The ratio between molecular ions and confirmation fragments was also monitored. Developed method showed very good linearity over the whole range of concentrations. LOD in the mean of 3× the noise level was 0.0124 µg/mL (62.0 µg/kg) for FB_1 and 0.0117 µg/mL (58.5 µg/kg) for FB_2. Then, the limit of quantitation (LOQ, 10× the noise level) reached 0.041 µg/mL (202.7 µg/kg) for FB_1 and 0.040 µg/mL (199.1 µg/kg) for FB_2 (Table I).

Extraction process

In this work, we searched for an extraction method with the highest yield of mycotoxins. The method used in European Standard uses a two-step extraction on an orbital shaker for 20 min each time. The recovery reported for this approach is 75.6 and 72.0% for fumonisin B_1 and B_2, respectively. Our developed method is faster and yields higher recovery. It uses

Table II. Compound Recovery of Fumonisin During Extraction

Number of extraction	100 µg/kg		2000 µg/kg (limit EU)		5000 µg/kg	
	FB1 (%)	FB2 (%)	FB1 (%)	FB2 (%)	FB1 (%)	FB2 (%)
1	61.1 ± 2.0	53.5 ± 1.9	62.7 ± 1.5	56.9 ± 0.7	63.2 ± 1.6	56.2 ± 1.7
2	88.1 ± 2.1	83.3 ± 1.8	91.2 ± 1.8	85.0 ± 1.7	91.7 ± 1.7	88.3 ± 1.8
3	94.7 ± 2.2	93.9 ± 2.1	100.0 ± 1.9	95.3 ± 1.8	100.6 ± 1.8	99.6 ± 2.3
4	98.2 ± 2.2	96.4 ± 2.4	102.2 ± 2.2	99.0 ± 2.3	102.9 ± 2.1	101.0 ± 2.3

Table III. Precision and Recovery of Determination of FB1 and FB2 in Maize Samples (*n* = 5)

Compound	Analyte content (µg/kg)		Measured (µg/kg)	Recovery (%)
	Maize	Spike		
Intra-day (n = 5)				
FB1	485.2 ± 17.5	101.2 ± 2.3	594.0 ± 18.9	101.3
		2013.2 ± 62.3	2558.4 ± 59.6	102.4
		5092.3 ± 142.5	5728.1 ± 144.5	102.7
Inter-day (n = 25)				
	492.5 ± 21.4	107.3 ± 3.9	600.5 ± 23.2	100.2
		1995.6 ± 71.3	2518.0 ± 69.2	101.2
		5021.2 ± 159.3	5563.3 ± 159.2	100.9
Intra-day (n = 5)				
FB2	263.6 ± 8.4	102.1 ± 3.2	358.9 ± 15.9	98.3
		2024.0 ± 58.9	2239.6 ± 67.5	97.9
		5008.3 ± 104.2	5177.0 ± 162.3	98.2
Inter-day (n = 25)				
	259.9 ± 11.3	99.7 ± 3.9	352.8 ± 29.2	98.1
		2079.3 ± 67.9	2339.2 ± 79.5	99.3
		5052.4 ± 122.3	5264.5 ± 173.1	99.1

more invasive extraction with an UltraTurrax homogenizer. Each sample was extracted with 25, 20, and 15 mL of extraction solvent for 5 min. The recovery observed in spiked material was 94.7–100.6% for FB_1 and 93.9–99.6% for FB_2. Addition of one more extraction step had no significant influence on recovery. Three extractions were selected as an optimum condition.

Recovery studies were performed on spiked samples at three concentration levels—lower, equal to, and higher than the limit required in EU for maize flour. The recovery was similar in all concentration levels.

In comparison with some other methods validated for fumonisin determination, the recovery is high. Often, yield is in the range from 50 to 105%.

Extraction development

Samples of milled maize spiked with fumonisins standard of three levels, below EU limit, limit, and up to the limit (100, 2000, and 5000 µg/kg), were used for monitoring of fumonisins recovery during single extraction steps. Each determination was done in five repetitions. The recovery of fumonisins during four extraction steps is shown in Table II.

Three extraction steps were selected as sufficient for fumonisins determination in maize samples. In comparison with procedure by European Standard (ES), the precision of developed method is approximately four-times higher. These results can be caused by difference of used instrumentation for extraction. Although ES recommends extraction in an orbital shaker for 20 min, we had applied an UltraTurrax blender for 5 min. This phenomenon was observed earlier (37). During European interlaboratory comparison study for the determination of fumonisins, higher recoveries were obtained using shaking than with blending.

Accuracy, precision, and recovery

Accuracy, precision, and recovery of the fumonisins determination were evaluated with real samples spiked with appropriate amount of standards to give concentrations between 100–5000 mg/kg. Coefficients of variation of inter-day (five days) and intra-day were determined from an analysis of six spiked samples. Each day, calibration curves were measured, and the concentrations of analytes were calculated from these curves. Accuracy was evaluated by comparing measured concentrations with the known concentration of fumonisins (Table III).

Conclusion

In this work, two extraction processes of fumonisin B_1 and B_2 from maize samples were compared. Extraction method using triple extraction with acetonitrile–methanol–water solution in a homogenizer had significantly higher recovery than the one on orbital shaker. The recovery of developed extraction method was 96.4–102.9% in tested range of concentration, which was chosen around the EU limits for maize flour. New HPLC–MS method for fumonisin determination was developed, optimized, and applied for analysis of real samples. Developed methodology allows detection of 62.0 µg FB_1/kg and 58.5 µg FB_2/kg. Method was validated and successfully applied for analysis of reference material and real samples.

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