Original Paper

Inter-Laboratory Study of an LC-MS/MS Method for Simultaneous Determination of Fumonisin B₁, B₂ and B₃ in Corn

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To validate an LC-MS/MS method using a strong anion exchange cartridge for simultaneous determination of fumonisin B₁, B₂ and B₃ in corn, an inter-laboratory study was performed in 9 laboratories using one fumonisin-negative corn sample, three spiked corn samples (FB₁: 100–1,000 µg/kg, FB₂ and FB₃: 10–100 µg/kg) and two naturally contaminated corn samples. The recoveries were in the ranges of 79.7–87.2% for FB₁, 78.6–103.2% for FB₂ and 80.1–92.8% for FB₃. The relative standard deviations for repeatability (RSD_r) ranged from 3.7 to 8.0% for FB₁, from 2.6 to 15.3% for FB₂ and from 4.3 to 9.7% for FB₃. The relative standard deviations for reproducibility (RSD_R) for FB₁, FB₂ and FB₃ were in the ranges of 6.3–10.1%, 5.9–18.7% and 9.3–16.0%, respectively. The HorRat values for all analytes ranged from 0.2 to 0.9. The difference of the trueness between the two kinds of commercially available anion exchange cartridges used in this study was not significant ($p > 0.05$). Surveillance for fumonisins in corn grits was performed using the validated method. All of the samples were contaminated with fumonisins and the mean concentrations for FB₁, FB₂ and FB₃ were 118.1, 37.3 and 17.9 µg/kg, respectively. These results indicated that the method for simultaneous determination of FB₁, FB₂ and FB₃ in corn was successfully developed and validated.

Key words: fumonisin; LC-MS/MS; inter-laboratory study; corn

Introduction

Fumonisin is a group of mycotoxins produced by some strains of *Fusarium* spp., such as *F. moniliforme* and *F. proliferatum*^{1), 2)}, and they are frequently detected in corn and corn-based foods³⁾. They are classified into four main types (A, B, C and P), of which the most frequently detected is fumonisin B₁ (FB₁). In a surveillance of retail foods in Japan, FB₁ was detected in not only corn and corn-based foods, such as corn grits, cornflakes, corn soups and corn snacks, but also beer, soybeans, millet and asparagus⁴⁾. Co-contamination of fumonisin B₂

and B₃ (FB₂ and FB₃) was observed in samples containing FB₁, but their amounts were generally lower than that of FB₁. FB₁ causes many diseases in animals, such as leukoencephalomalacia in horses, pulmonary oedema in swine and cancer in rats⁵⁾. Based on epidemiologic studies in a number of countries in various regions of the world, human oesophageal cancer and neural tube defects have been associated with fumonisins^{6), 7)}. These toxic events are considered to be due to alteration of sphingolipid metabolism by inhibition of ceramide synthase⁸⁾. Based on animal studies, the International Agency for Research on Cancer (IARC) has evaluated FB₁ as being possibly carcinogenic to humans (Group 2B)^{9), 10)}. FB₂ and FB₃ have similar toxicity to FB₁, but

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FB₁ is the most toxic among them¹¹). In 2001, the Joint FAO/WHO Expert Committee on Food Additives (JEC-FA) recommended a provisional maximum tolerable daily intake (PMTDI) of 2.0 µg/kg body weight per day for FB₁, FB₂ and FB₃, alone or in combination¹²). To reduce the intake of fumonisins, some countries have established regulatory limits or guidance concentrations for them in foods and feeds. For example, the European Union regulated the maximum levels of fumonisins (as the sum of FB₁ and FB₂) in maize-based products and unprocessed maize in the range of 200 to 2,000 µg/kg¹³). The US FDA Guidelines for fumonisins (as the sum of FB₁, FB₂ and FB₃) recommend 2–4 ppm as a maximum in whole and processed maize products for human consumption¹⁴).

Many methods have been developed for determining fumonisins in corn and corn-based foods, and collaborative studies for validation of the methods using TLC¹⁵, ELISA¹⁶ and HPLC with fluorimetric detection after derivatization¹⁷ have been performed. In these methods, fumonisins were extracted from samples with organic solvents, such as methanol or acetonitrile, and clean-up was mainly performed using a C₁₈ cartridge, a strong anion exchange (SAX) cartridge or an immunoaffinity column. Several methods for simultaneous determination of multiple mycotoxins using LC-MS/MS have been reported recently^{18–20}). Since co-contamination of aflatoxins, trichothecenes and fumonisins is often observed in foods, these LC-MS/MS methods have become mainstream for mycotoxin analysis.

Japan has not yet regulated fumonisins in foods, but a maximum level for them is expected to be set in the near future. Occurrence data collected by a reliable analytical method are essential to assess human exposure to fumonisins in foods. It is desirable that the limit of quantification of a method for surveillance is as low as possible. The sensitivity of a method using LC-MS/MS is expected to be higher than that of the reported validated methods using ELISA or fluorescence HPLC. In this study, we validated an LC-MS/MS method using a SAX cartridge for simultaneous determination of FB₁, FB₂ and FB₃ in corn through an inter-laboratory comparison.

Materials and Methods

Chemicals

Crystalline FB₁ and FB₂ were purchased from Sigma-Aldrich (St. Louis, MO, USA) and FB₃ was purchased from PROMEC, Medical Research Council (Tygerberg, South Africa). The purity was confirmed to be more than 98%. Each compound was dissolved in acetonitrile–water (1 : 1) (500 µg/mL for FB₁ and 50 µg/mL for FB₂ and FB₃) and the solution was stored at –20°C. Portions of the three stock solutions were mixed and diluted with acetonitrile–water (1 : 1) to make a mixed standard solution (10 µg/mL each of FB₁, FB₂ and FB₃) and three mixed spiking solutions (solution 1 : 10 µg/mL of FB₁ and 1 µg/mL each of FB₂ and FB₃; solution 2 : 50 µg/mL of FB₁ and 5 µg/mL each of FB₂ and FB₃; solution 3 : 100 µg/mL of FB₁ and 10 µg/mL each of FB₂ and FB₃).

Acetonitrile and water were of HPLC grade. Methanol and formic acid were of analytical reagent grade. Bond Elut LRC SAX (Agilent Technologies, Palo Alto, CA, USA) and InertSep LSC SAX (GL Sciences, Tokyo, Japan) were used as SAX cartridges.

Preparation of samples

Fumonisin-negative corn sample (<0.1 mg/kg for FB₁) and two naturally contaminated corn samples were purchased from Trilogy Analytical Laboratory Inc. (Washington, MO, USA). The advertised concentrations of FB₁ in naturally contaminated corn I and corn II were 0.8 mg/kg and 1.7 mg/kg, respectively. These samples were packed into bags of 30 g each. Each participant in the inter-laboratory study received the following: (a) fumonisin-negative samples for the fortification test; (b) a pair of each of the two naturally contaminated corn samples; (c) a mixed standard solution and spiking solutions.

Fortification procedure

For evaluating recovery, 200 µL of the mixed spiking solution was added to 20.0 g of fumonisin-negative corn in a 300-mL flask (final concentrations, spike 1 : 0 µg/kg each of FB₁, FB₂ and FB₃, spike 2 : 100 µg/kg of FB₁ and 10 µg/kg each of FB₂ and FB₃, spike 3 : 500 µg/kg of FB₁ and 50 µg/kg each of FB₂ and FB₃, spike 4 : 1,000 µg/kg of FB₁ and 100 µg/kg each of FB₂ and FB₃) and kept at room temperature. After 1 h, fumonisins were extracted from the spiked samples and quantified according to the protocol defined below. The concentrations of the spiked samples were set on the basis of the results of a four-year (2004–2007) surveillance for FB₁, FB₂ and FB₃ in corn-based foods⁴).

Protocol used by participants

The method validated in this study is based on the method of Sydenham *et al.*¹⁷ (Fig. 1). Briefly, a 20.0 g sample was extracted with 100 mL of methanol–water (3 : 1) by shaking for 15 min. The extract was transferred into a 50-mL centrifuge tube and centrifuged at 3,000×g for 5 min. A SAX cartridge was conditioned with 8 mL of methanol and then with 8 mL of methanol–water (3 : 1). Aliquots of 10 mL of the supernatant were applied to the cartridge under gravity flow. The cartridge was washed with 8 mL of methanol–water (3 : 1) and with 8 mL of methanol, and then fumonisins were eluted with 14 mL of methanol–acetic acid (99 : 1). The collected eluate was dried under nitrogen at approximately 40°C. The residue was dissolved in 1 mL of acetonitrile–water (1 : 1). After centrifugation (10,000×g, 5 min), the sample was subjected to LC-MS/MS analysis.

LC-MS/MS conditions

Each laboratory determined the FB₁, FB₂ and FB₃ concentrations by LC-MS/MS. Five microliters of the final solution was loaded on an Inertsil ODS-4 column (3.0×50 mm, 2 µm; GL Sciences) at 40°C. The mobile phase was a binary gradient of solvent A (0.1% formic acid in

Fig. 1. Flow diagram for the determination of fumonisin B₁, B₂ and B₃ using LC-MS/MS coupled to a strong cation-exchange cartridge

water) and solvent B (acetonitrile) programmed as follows: at 0 min, 25% B; at 5 min 50% B; at 8 min, 50% B. The flow rate was set at 0.2 mL/min. Electrospray ionization in the positive mode was used. The selected reaction monitoring mode of LC-MS/MS and all other conditions were set by each laboratory so that the highest signal peak of 5 ng/mL standard solution was more than 10 times larger than the background noise level.

Calibration curve

The mixed standard solution was diluted with acetonitrile–water (1 : 1). The concentration of FB₁, FB₂ and FB₃ and the peak area were plotted for seven standard solutions with different concentrations (2.0, 5.0, 10.0, 20.0, 50.0, 100.0, 200.0 ng/mL). The concentration of fumonisins in the sample solution was calculated from a calibration curve. Limits of detection (LODs) and limits of quantification (LOQs) were calculated based on signal-to-noise ratios of 3 : 1 and 10 : 1, respectively.

Inter-laboratory study design

To validate the method, an inter-laboratory study was carried out using six materials (three spiked samples, two naturally contaminated samples and a fumonisin-negative sample). The study involved 11 laboratories in Japan.

Statistics

The results from participants were initially evaluated for evidence of outliers using statistical Cochran (between duplicates) and Grubbs single and Grubbs pair value tests (between laboratory means)²¹⁾. The relative standard deviations for repeatability (RSD_r) and reproducibility (RSD_R), and the HorRat value, which is the ratio of the RSD_r to the predicted RSD_r, were obtained using an analysis of variance according to the AOAC guideline²²⁾. The predicted RSD_r of the HorRat value was calculated according to the Thompson report²³⁾. The criteria for analytical methods mentioned in Commission Regulation (EC) No. 401/2006²⁴⁾ were used to evaluate these parameters. The mean difference between the two cartridges was compared by the method described in

ISO 5725-6²⁵⁾. The critical difference was calculated with the following formula:

$$\text{Critical difference } (\mu\text{g/kg}) = 2 \times \sqrt{\frac{\{S_{R,A}^2 - (1 - 1/N_A) \times S_{r,A}^2\} / L_A}{+ \{S_{R,B}^2 - (1 - 1/N_B) \times S_{r,B}^2\} / L_B}}$$

Parameters for Bond Elut LRC SAX: L_A =number of laboratories, N_A =number of repetitions, $S_{R,A}$ =standard deviation for reproducibility, $S_{r,A}$ =standard deviation for repeatability. Parameters for InertSep LSC SAX: L_B =number of laboratories, N_B =number of repetitions, $S_{R,B}$ =standard deviation for reproducibility, $S_{r,B}$ =standard deviation for repeatability.

The homogeneity and average concentration of FB₁, FB₂ and FB₃ in the naturally contaminated corn samples supplied in this study were tested before the materials were distributed. *F*-tests at the 95% confidence level showed that two naturally contaminated corn samples could be regarded as homogeneous because the calculated *F*-value was less than the critical value. The mean concentrations calculated from the homogeneity test were as follows: naturally contaminated corn I; FB₁ 633.7±21.3 µg/kg, FB₂ 304.2±12.7 µg/kg, FB₃ 122.7±5.4 µg/kg, naturally contaminated corn II; FB₁ 1288.5±48.1 µg/kg, FB₂ 720.0±31.2 µg/kg, FB₃ 192.8±5.9 µg/kg. In the fumonisin-negative sample, FB₁ (21.8±1.7 µg/kg), FB₂ (10.1±1.1 µg/kg) and FB₃ (4.0±0.3 µg/kg) were detected.

Surveillance for FB₁, FB₂ and FB₃ in corn grits

Corn grits samples were purchased from local supermarkets in major cities such as Tokyo, Osaka, Nagoya, Yokohama and Chiba in Japan between the summer of 2010 and the winter of 2012. The examination was performed at the National Institute of Health Sciences. Samples whose particle diameter was more than 1 mm were ground to a fine powder. The others were directly extracted without further processing. FB₁, FB₂ and FB₃ were quantified according to the protocol used in the in-

Table 1. Measurement conditions of each laboratory

Laboratory		A	B	C	D	E	F	G	H	I
HPLC	Manufacturer	Agilent Technologies	Shimadzu	Agilent Technologies	Shimadzu	Agilent Technologies	Shimadzu	Agilent Technologies	Agilent Technologies	Waters
	Instrument	1100 series	Prominence	1100 series	Prominence	1200 series	Prominence	1100 series	1100 series	2695
	Manufacturer	AB SCIEX	AB SCIEX	AB SCIEX	Thermo Scientific	Agilent Technologies	AB SCIEX	AB SCIEX	AB SCIEX	Waters
LC-MS/MS	Instrument	API 3000	API 4000	API 4000	TSQ Quantum Ultra	6410 Triple Quad LC/MS	API 4000	API 4000 QTRAP	API 4000 QTRAP	Quattro micro API
	0.1% formic acid in water									
	Acetonitrile									
Gradient method	% of solvent B	10% (0 min) → 50% (4–11 min)	25% (0 min) → 50% (5–8 min)	25% (0 min) → 50% (5–8 min)	25% (0 min) → 50% (5–8 min)	25% (0 min) → 50% (5–8 min)	25% (0 min) → 50% (5–8 min)	25% (0 min) → 50% (5–8 min)	25% (0 min) → 50% (5–8 min)	25% (0 min) → 75% (7–10 min)
	Flow rate (mL/min)	0.2								
	Precursor ion (<i>m/z</i>)	FB ₁	722 [M+H] ⁺							
FB ₂		706 [M+H] ⁺								
FB ₃		706 [M+H] ⁺								
Quantifier ion (<i>m/z</i>)	FB ₁	352	334	334	334	334	334	334	334	334
	FB ₂	336	336	336	336	336	336	336	354	336
	FB ₃	336	336	336	336	336	336	336	354	336
LOD (µg/kg)	FB ₁	2	0.03	1	1	1	0.01	0.4	0.1	0.03
	FB ₂	2	0.1	1	1	1	0.01	1	0.1	0.04
	FB ₃	2	0.1	1	1	1	0.01	0.4	0.1	0.04
LOQ (µg/kg)	FB ₁	5	0.1	2	5	2	0.03	1	0.2	0.1
	FB ₂	5	0.2	3	5	2	0.03	2	0.2	0.1
	FB ₃	5	0.2	3	5	2	0.03	2	0.4	0.1

Table 2. Results of the inter-laboratory study on the determination of fumonisin B₁ by LC-MS/MS in corn

Laboratory	Bond Elut LRC-SAX										InertSep LSC SAX									
	Spiked sample					Naturally contaminated corn (µg/kg)					Spiked sample					Naturally contaminated corn (µg/kg)				
	0 µg/kg	100 µg/kg	500 µg/kg	1,000 µg/kg	1,000 µg/kg	I	II	I	II	0 µg/kg	100 µg/kg	500 µg/kg	1,000 µg/kg	1,000 µg/kg	I	II	I	II	I	II
A	23.4	20.9	110.7	104.3	420.9	396.1	827.3	753.1	650.5	569.6	1,128.1	1,168.0	27.1	19.1	112.3	93.1	396.7	342.6	798.3	815.7
B	20.7	26.3	85.0 ^a	100.8	408.0	374.1	701.5	715.9	525.2	506.0	1,061.6	1,202.3	22.1	28.2	104.6	109.8	500.9	429.3	867.0	775.4
C	21.4	25.8	135.5	125.8	544.4	434.6	930.5	987.7	690.0	606.0	1,180.6	1,195.8	19.9	27.5	113.1	130.3	429.3	444.5	878.3	957.3
D	19.5	22.8	102.9	111.1	488.8	416.0	857.5	801.1	689.0	614.5	1,304.7	1,217.3	22.9	22.2	106.3	96.7	466.0	368.2	878.5	825.2
E	25.8	27.1	113.8	104.7	470.5	501.0	935.5	972.8	625.6	622.3	1,237.8	1,301.5	25.6	22.2	108.5	103.6	443.6	460.1	879.6	845.4
F	21.8	24.3	101.6	105.0	409.3	414.4	786.7	795.3	592.6	554.4	1,045.6	1,066.2	22.3	24.9	99.0	109.1	401.4	394.1	672.6	746.3
G	27.5	21.4	106.5	106.6	435.2	426.4	843.5	859.0	593.2	610.0	1,178.9	1,216.9	27.9	21.7	105.0	101.2	422.6	419.6	813.4	820.9
H	19.2	22.5	112.5	110.3	447.6	470.4	895.8	936.9	646.5	645.6	1,283.6	1,214.0	20.0	21.6	109.3	100.6	404.3	435.3	854.7	791.9
I	17.6	21.1	101.2	104.1	452.4	411.7	849.2	896.6	639.0	606.0	1,104.7	1,225.6	15.3	21.0	112.1	110.1	413.9	414.4	905.4	808.9
Mean (µg/kg)	22.7	109.8	440.1		852.6	610.3	1,185.2			22.9	105.1	421.5	829.7	578.1	1,103.7					
Mean recovery (%)	—	87.2	83.5		83.0	—	—			—	82.3	79.7	80.7	—	—					
Outlier (Cochran test)	—	0	0	0	0	0	0			—	0	0	0	0	0	0				
Outlier (single Grubbs test)	—	0	0	0	0	0	0			—	0	0	0	0	0	0				
Outlier (paired Grubbs test)	—	1	0	0	0	0	0			—	1	0	0	0	0	0				
Repeat-ability	—	4.0	8.0	8.0	3.7	5.8	4.6			—	6.3	7.7	5.5	4.8	5.0					
relative SD [RSD _r , %]	—	8.6	9.6	10.1		8.2	6.7			—	6.3	8.7	7.8	9.3	10.1					
Reproducibility	—	0.4	0.5	0.6	0.6	0.5	0.4			—	0.2	0.5	0.5	0.6	0.6					
relative SD [RSD _R , %]	—	0.4	0.5	0.6	0.6	0.5	0.4			—	0.2	0.5	0.5	0.6	0.6					
HorRat	—	0.4	0.5	0.6	0.6	0.5	0.4			—	0.2	0.5	0.5	0.6	0.6					

^a: Underline means an outlier

Table 3. Results of the inter-laboratory study on the determination of fumonisin B₂ by LC-MS/MS in corn

Laboratory	Bond Elut LRC-SAX										InertSep LSC SAX									
	Spiked sample					Naturally contaminated corn (µg/kg)					Spiked sample					Naturally contaminated corn (µg/kg)				
	0 µg/kg	10 µg/kg	50 µg/kg	100 µg/kg	100 µg/kg	I	II	I	II	0 µg/kg	10 µg/kg	50 µg/kg	100 µg/kg	100 µg/kg	I	II	I	II	I	II
A	11.3	9.8	20.6	19.5	54.9	52.9	94.4	94.2	320.5	286.0	612.6	588.0	12.2	8.8	19.4	15.8	49.8	51.0	94.9	84.0
B	9.0	12.8	13.6	17.8	42.1	44.8	71.3	77.1	297.8	307.6	577.1	608.4	10.2	12.9	17.0	19.9	55.0	50.6	82.6	83.0
C	10.2	11.8	24.3	18.7	60.8	52.4	108.3	108.0	371.9	393.2	761.4	763.8	8.7	13.5	25.2	19.9	62.9	56.6	110.9	122.7
D	9.8	10.3	21.6	26.5	63.6	46.5	90.4	89.8	369.7	368.9	751.4	642.6	11.3	10.5	22.7	27.8	66.2	39.3	95.7	88.4
E	12.5	14.0	21.3	17.5	56.1	55.2	91.3	97.3	346.1	341.6	692.1	722.7	12.6	10.1	19.9	18.5	54.3	61.5	96.7	91.4
F	11.5	11.9	21.8	21.9	50.9	55.2	93.4	90.9	383.0	347.5	641.1	641.5	11.4	11.6	21.5	21.5	50.4	50.0	92.5	90.1
G	14.5	11.4	19.1	21.7	56.8	56.0	95.4	95.7	323.6	334.3	660.5	660.5	14.6	11.1	19.4	22.1	56.8	49.4	99.7	95.7
H	10.5	13.2	29.3	23.3	62.4	69.5	79.2	98.6	357.5	353.1	741.1	723.7	11.5	12.4	32.7	22.6	61.0	64.2	85.2	80.2
I	8.7	9.6	18.2	17.9	54.6	43.6	92.8	86.4	354.3	340.5	586.9	632.6	7.4	9.2	21.5	18.5	55.8	46.5	97.9	77.4
Mean	11.3	20.8	54.3	54.3	92.3	92.3	344.3	663.4	344.3	344.3	663.4	663.4	11.1	21.4	54.7	54.7	89.7	89.7	333.3	626.8
Mean recovery (%)	—	95.3	86.1	86.1	81.1	81.1	—	—	—	—	—	—	—	103.2	87.2	87.2	—	—	—	—
Outlier (Cochran test)	—	0	0	0	1	1	0	1	0	0	1	0	—	0	1	0	0	0	0	0
Outlier (single Grubbs test)	—	0	0	0	0	0	0	0	0	0	0	0	—	0	0	0	0	0	0	0
Outlier (paired Grubbs test)	—	13.0	10.3	10.3	2.9	2.9	4.0	2.6	4.0	4.0	2.6	2.6	—	15.3	7.4	7.4	3.9	3.9	4.1	4.1
Repeatability relative SD [RSD _r , %]	—	17.6	13.3	13.3	10.3	10.3	8.6	9.9	8.6	8.6	9.9	9.9	—	18.7	10.1	10.1	5.9	5.9	11.6	11.6
Reproducibility relative SD [RSD _R , %]	—	0.8	0.6	0.6	0.5	0.5	0.5	0.6	0.5	0.5	0.6	0.6	—	0.9	0.5	0.5	0.3	0.3	0.7	0.7

^a: Underline means an outlier

Table 4. Results of the inter-laboratory study on the determination of fumonisin B₃ by LC-MS/MS in corn

Laboratory	Bond Elut LRC-SAX										InertSep LSC SAX													
	Spiked sample					Naturally contaminated corn (µg/kg)					Spiked sample					Naturally contaminated corn (µg/kg)								
	0 µg/kg	10 µg/kg	50 µg/kg	100 µg/kg	100 µg/kg	I	II	0 µg/kg	10 µg/kg	50 µg/kg	100 µg/kg	100 µg/kg	I	II										
A	(3.7) ^a	(3.5) ^a	13.3	12.7	48.7	45.4	92.7	90.1	98.8	88.8	186.0	174.8	4.2	2.7	11.7	10.0	43.5	43.2	87.5	78.7	79.1	76.1	147.1	166.2
B	2.0	2.7	7.9	9.3	39.0	38.1	67.4	73.0	86.4	85.1	163.6	174.0	2.5	3.1	10.5	11.0	47.8	42.9	76.7	81.3	91.9	93.1	168.5	181.5
C	3.7	3.5	14.5	12.1	49.7	46.0	107.8	101.2	119.2	101.7	239.5	250.0	3.2	3.2	12.8	12.8	51.0	47.5	85.5	99.0	110.1	99.7	227.6	217.1
D	(1.9) ^a	3.4	13.6	13.5	58.5	42.4	67.4	87.5	98.7	88.8	214.1	182.8	2.8	3.1	13.8	14.0	60.0 ^b	37.0	71.3	84.1	96.3	85.0	181.2	197.5
E	3.6	3.9	13.4	10.9	48.7	46.1	85.2	91.6	95.4	79.8	200.8	213.5	3.7	2.8	11.9	10.8	45.6	49.4	97.2	81.7	90.9	78.4	204.4	200.1
F	3.3	3.5	12.7	12.7	44.3	45.2	84.6	83.1	91.8	87.2	191.6	199.5	2.9	3.1	11.7	11.9	42.0	39.1	79.9	81.2	88.7	82.6	161.3	172.8
G	4.0	3.0	11.6	12.7	48.8	49.8	89.5	89.7	91.6	96.5	192.5	193.6	3.9	2.9	11.5	12.6	47.8	44.5	90.9	87.3	92.8	89.7	178.4	183.1
H	2.3	3.1	16.2	14.7	57.9	61.8	84.6	92.7	89.8	95.1	232.7	231.1	2.6	2.5	16.8	13.3	54.6	57.4	83.1	77.7	84.8	82.2	199.4	190.5
I	2.9	3.3	11.2	11.4	46.9	38.3	86.0	76.7	101.8	95.4	179.3	198.9	2.3	3.2	12.2	11.1	45.6	37.4	85.8	67.0	92.0	100.6	186.3	189.1
Mean (µg/kg)	3.2	12.5	47.5	86.1	86.1	94.0	201.0						3.0	12.3	46.2	83.1	89.7						186.2	
Mean recovery (%)	—	92.8	88.7	83.0	83.0	—	—						—	92.1	86.3	80.1	—						—	
Outlier (Cochran test)	—	0	0	0	0	0	0						—	0	1	0	0	0						0
Outlier (single Grubbs test)	—	0	0	0	0	0	0						—	0	0	0	0	0						0
Outlier (paired Grubbs test)	—	0	0	0	0	0	0						—	0	0	0	0	0						0
Repeatability relative SD [RSD _r , %]	—	8.1	9.7	7.1	7.1	7.4	5.2						—	8.3	6.5	9.3	6.1						4.3	
Reproducibility relative SD [RSD _R , %]	—	16.0	14.2	12.3	12.3	9.3	12.5						—	13.0	11.6	9.6	9.9						10.9	
HorRat	—	0.7	0.6	0.5	0.5	0.4	0.6						—	0.6	0.5	0.4	0.4						0.5	

^a : Parentheses indicate a value between LOD and LOQ

^b : Underline means an outlier

Table 5. Comparison of the performance of the two strong anion-exchange cartridges. (A) fumonisin B₁, (B) fumonisin B₂, (C) fumonisin B₃

(A)

		Spiked sample			Naturally contaminated corn	
		100 µg/kg	500 µg/kg	1,000 µg/kg	I	II
Mean (µg/kg)	Bond Elut LRC-SAX	109.8	440.1	852.6	610.3	1185.2
	InertSep LSC SAX	105.1	421.5	829.7	578.1	1103.7
Difference between the two cartridges (µg/kg)		4.7	18.6	22.9	32.3	81.4
Critical difference (µg/kg)		7.1	29.6	66.8	44.4	83.6

(B)

		Spiked sample			Naturally contaminated corn	
		10 µg/kg	50 µg/kg	100 µg/kg	I	II
Mean (µg/kg)	Bond Elut LRC-SAX	20.8	54.3	92.3	344.3	663.4
	InertSep LSC SAX	21.4	54.7	89.7	333.3	626.8
Difference between the two cartridges (µg/kg)		0.6	0.4	2.6	11.0	36.5
Critical difference (µg/kg)		3.0	5.2	7.6	22.4	65.4

(C)

		Spiked sample			Naturally contaminated corn	
		10 µg/kg	50 µg/kg	100 µg/kg	I	II
Mean (µg/kg)	Bond Elut LRC-SAX	12.5	47.5	86.1	94.0	201.0
	InertSep LSC SAX	12.3	46.2	83.1	89.7	186.2
Difference between the two cartridges (µg/kg)		0.2	1.3	3.0	4.3	14.8
Critical difference (µg/kg)		1.6	5.2	7.5	7.2	20.6

ter-laboratory study. InertSep LSC SAX was used for purification. Applicability of the method to analysis of fumonisins in corn grits was confirmed by a recovery test.

Results and Discussion

Inter-laboratory study

The measurement conditions used in each laboratory are shown in Table 1. In order to separate FB₂ and FB₃, two laboratories (A and I) changed the gradient HPLC method. Good separation of FB₂ and FB₃ was not achieved in the two laboratories by the method described in the protocol. This may be due to differences in the type or condition of the HPLC instruments. LOQs ranged from 0.03 to 5.0 µg/kg for all analytes.

The results of the inter-laboratory study are shown in Table 2 (FB₁), Table 3 (FB₂) and Table 4 (FB₃). The results of two laboratories were eliminated because of pro-

cedure violation. In the fortification test, all laboratories detected FB₁, FB₂ and FB₃ in the fumonisin-negative sample and the mean values were 22.7, 11.3 and 3.2 µg/kg (Bond Elut LRC SAX) and 22.9, 11.1 and 3.0 µg/kg (InertSep LSC SAX), respectively. To calculate recovery, the mean value of the fumonisin-negative sample was subtracted from each measured value for each laboratory. The recovery, RSD_r and RSD_R were in the ranges of 79.7–87.2%, 3.7–8.0% and 6.3–10.1% for FB₁, 78.6–103.2%, 2.6–15.3% and 5.9–18.7% for FB₂ and 80.1–92.8%, 4.3–9.7% and 9.3–16.0% for FB₃.

According to the criteria for analytical methods mentioned in Commission Regulation (EC) No. 401/2006²⁴⁾, the recovery, RSD_r and RSD_R for ≤500 µg/kg of FB₁ or FB₂ are required to be in the ranges of 60–120%, ≤30% and ≤60%, respectively and for >500 µg/kg of FB₁ or FB₂, the levels are required to be in the ranges of 70–110%, ≤20% and ≤30%, respectively. Although criteria for FB₃

Table 6. Natural fumonisins detected in corn grits in 2010–2012

Analyte	LOQ ($\mu\text{g/kg}$)	No. of samples	No. of samples below LOQ	Mean of positives ($\mu\text{g/kg}$)	Maximum ($\mu\text{g/kg}$)
2010					
FB ₁	2	20	0	193.3	582.5
FB ₂	2	20	0	44.4	149.0
FB ₃	2	20	0	25.2	78.7
FB ₁ +FB ₂ +FB ₃	—	20	—	262.9	810.2
2011					
FB ₁	2	19	0	88.1	321.0
FB ₂	2	19	0	19.3	78.5
FB ₃	2	19	0	14.5	57.1
FB ₁ +FB ₂ +FB ₃	—	19	—	122.0	456.6
2012					
FB ₁	2	31	0	87.8	498.8
FB ₂	2	31	0	37.3	232.9
FB ₃	2	31	0	17.9	103.0
FB ₁ +FB ₂ +FB ₃	—	31	—	143.0	834.7
Totals					
FB ₁	2	70	—	118.1	—
FB ₂	2	70	—	37.3	—
FB ₃	2	70	—	17.9	—
FB ₁ +FB ₂ +FB ₃	—	70	—	171.6	—

were not provided, all the results obtained in this study met the above criteria. A range of 0.5–1.5 for the HorRat value is acceptable according to AOAC International²²⁾. In this study, the HorRat values were in the ranges of 0.2–0.6 for FB₁, 0.3–0.9 for FB₂ and 0.4–0.7 for FB₃. None of the values exceeded 1.5, but the values of some samples were less than 0.5, although the study was performed without any problems. One of the reasons why low HorRat values were obtained might be that most of the laboratories conformed to the protocol. The trueness of the two SAX cartridges was compared (Table 5). Although the mean values obtained by using Bond Elut LRC-SAX tended to be larger than the InertSep LSC SAX-derived values, all of the mean differences between the two SAX cartridges were less than the critical differences. This result shows that the difference in trueness between the two cartridges is not significant ($p>0.05$).

Sydenham *et al.*¹⁷⁾ reported an inter-laboratory study for FB₁, FB₂ and FB₃ in corn using an LC-MS method coupled with a SAX cartridge. In their study, the concentrations of the spiked samples ranged from 500 to 8000 $\mu\text{g/kg}$ for FB₁, from 200 to 3200 $\mu\text{g/kg}$ for FB₂ and from 100 to 1600 $\mu\text{g/kg}$ for FB₃. The recovery, RSDr and RSDr were in the ranges of 81.1–84.2%, 5.8–13.2% and 13.9–22.2% for FB₁, 75.9–81.9%, 7.2–17.5% and 15.8–26.7% for FB₂ and 75.8–86.8%, 8.0–17.2% and 19.5–24.9% for FB₃. The HorRat values ranged from 0.75 to 1.73. Visconti *et al.*²⁶⁾ reported an inter-laboratory study for FB₁ and FB₂ in corn and corn flakes using an HPLC method coupled with an immunoaffinity column. In their study, fumonisin levels in corn ranged from 0.39 to 1.41 mg/kg for FB₁ and from 0.07 to 0.53 mg/kg for FB₂. The recov-

eries of FB₁ and FB₂ were 75.6% and 72.0%, respectively, and the RSDr, RSDr and HorRat values were in the ranges of 18.8–24.2%, 21.9–28.2% and 1.44–1.53 for FB₁ and 18.5–26.8%, 22.1–29.7% and 0.96–1.48 for FB₂. LOQs ranged from 0.004 to 0.1 mg/kg for FB₁ and from 0.003 to 0.178 mg/kg for FB₂. Although the concentrations of spiked samples in our study were lower than those of previous studies, similar results were obtained with the LC-MS/MS method mentioned in this report.

Based on the results of this study, the developed LC-MS/MS method was successfully validated for the simultaneous determination of FB₁, FB₂ and FB₃ in corn. It is recommended that this method can be adopted for the determination of FB₁ in the range of 100 to 1,000 $\mu\text{g/kg}$, and both FB₂ and FB₃ in the range of 10 to 100 $\mu\text{g/kg}$. Relative to the methods validated previously, this method can detect lower concentrations of fumonisins.

Occurrence of FB₁, FB₂ and FB₃ in corn grits

Surveillance for fumonisins in corn grits was performed using the method validated in this study. Recovery tests were carried out by analyzing corn grits samples fortified with FB₁, FB₂ and FB₃ at two concentrations. At the fortified concentrations of 50 and 300 $\mu\text{g/kg}$, the recoveries were in the ranges of 94.2 to 116.2%, 88.4 to 106.9% and 87.8 to 98.2% for FB₁, FB₂ and FB₃, respectively. In total, 70 samples were analyzed over three years and the results are shown in Table 6. FB₁, FB₂ and FB₃ were detected in all samples and the mean concentrations of FB₁, FB₂, FB₃ and total fumonisins in the entire collection were 118.1, 37.3, 17.9 and 171.6 $\mu\text{g/kg}$, respectively. The mean concentrations

in this study were similar to those in a four-year (2004–2007) surveillance in Japan (104 µg/kg for FB₁, 36.5 µg/kg for FB₂ and 22.8 µg/kg for FB₃)⁴⁾. The fumonisin concentrations in the samples in 2010 were significantly higher than those in the other two years. No sample exceeded the maximum level set in the European Union (sum of FB₁ and FB₂, 1,000 µg/kg for maize-based foods for direct human consumption). The method validated in this study is concluded to be useful to obtain data concerning the state of fumonisin contamination in corn. Continual monitoring is needed to provide data for evaluating the risk of fumonisin exposure in Japan.

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