FOOD CHEMICAL CONTAMINANTS

Determination of Fumonisins B_1 and B_2 in Corn and Corn Flakes by Liquid Chromatography with Immunoaffinity Column Cleanup: Collaborative Study

ANGELO VISCONTI, MICHELE SOLFRIZZO, and ANNALISA DE GIROLAMO
Istituto Tossine e Micotossine da Parassiti Vegetali, Consiglio Nazionale delle Ricerche (CNR), V. le Einaudi, 51 – 70125 Bari, Italy

Collaborators: H. Bresch; P. Burdaspal; M. Castegnaro; I. Felgueiras; J. Gardikis; K. Jørgensen; E. Kakouri; H. Kretschmer; H. Lew; K. Meyer; J. Miller; T. Møller; K. Nuotio; S. Patel; A. Pietri; A. Pittet; E. Sizoo; M.C. Spanjer; W. Steiner; R. Tiebach; E. Usleber; C. von Holst; P. Wilson

Downloaded from https://academic.oup.com/jaoac/article/84/6/1828/5656541 by guest on 16 August 2022

A liquid chromatographic (LC) method for the determination of fumonisins B_1 (FB_1) and B_2 (FB_2) in corn and corn flakes was collaboratively studied by 23 laboratories, which analyzed 5 blind duplicate pairs of each matrix to establish the accuracy, repeatability, and reproducibility characteristics of the method. Fumonisin levels in the corn ranged from <0.05 (blank) to 1.41 μg/g for FB_1 and from <0.05 to 0.56 μg/g for FB_2, whereas in the corn flakes they ranged from <0.05 to 1.05 μg/g for FB_1 and from <0.05 to 0.46 μg/g for FB_2. The method involved double extraction with acetonitrile–methanol–water (25 + 25 + 50), cleanup through an immunoaffinity column, and LC determination of the fumonisins after derivatization with *o*-phthaldialdehyde. Relative standard deviations for the within-laboratory repeatability (RSD_r) of the corn analyses ranged from 19 to 24% for FB_1 and from 19 to 27% for FB_2; for the corn flakes analyses, RSD_r ranged from 9 to 21% for FB_1 and from 8 to 22% for FB_2. Relative standard deviations for the between-laboratories reproducibility (RSD_R) of the corn analyses ranged from 22 to 28% for FB_1 and from 22 to 30% for the FB_2; for corn flakes analyses, RSD_R ranged from 27 to 32% for FB_1 and from 26 to 35% for FB_2. Mean recoveries of FB_1 and FB_2 from corn spiked with FB_1 at 0.80 μg/g and with FB_2 at 0.40 μg/g were 76 and 72%, respectively; for corn flakes spiked at the same levels recoveries were 110 and 97% for FB_1 and FB_2, respectively. HORRAT ratios for the analyses of corn ranged from 1.44 to 1.53 for FB_1 and from 0.96 to 1.48 for FB_2, whereas for corn flakes they ranged from 1.60 to 1.82 for FB_1 and from 1.39 to 1.68 for FB_2.

Fumonisins B_1 (FB_1) and B_2 (FB_2) are mycotoxins produced mainly by *Fusarium verticillioides* (= *F. moniliforme* Sheld) and *F. proliferatum* that are frequently found as natural contaminants in corn and corn-based products worldwide (1, 2). FB_1 can cause acute mycotoxicoses in farm animals such as equine leukoencephalomalacia (3, 4) and pig pulmonary edema (5), and hypercholesterolemia and a number of immunological alterations in several animal species (6). It has been demonstrated to cause renal toxicity at low exposure rates in several animal species and is hepatotoxic in all animal species tested to date (6). Recently, FB_1 has been shown to induce renal tubule neoplasms and hepatocellular neoplasms in male rats and female mice, respectively (7). A possible association between human esophageal cancer and the consumption of corn with high levels of fumonisins in South Africa has been suggested (8), although at present no definitive conclusion can be made about cancer causation in humans.

The determination of fumonisins in corn-based food products is a challenge because most of the available methods for the analysis of corn do not perform well when applied to processed foods. Analytical problems in terms of low recoveries and insufficient cleanup were experienced by several laboratories when the official AOAC INTERNATIONAL method for the determination of fumonisins in corn (AOAC Method **995.15**; 9) was applied to the analysis of corn-based food products such as corn flakes, infant formula, muffins, mixed cereals, tortillas, and extruded products (10–13). In particular, the very low recoveries of fumonisins from corn flakes (5–40%) and the high variability of results experienced by our laboratory and other investigators with the AOAC method could explain the fact that significant levels of fumonisins are rarely found in corn flakes. As part of a project supported by the European Commission—Standards Measurements and Testing Programme (SMT), several factors (type and volume

Submitted for publication April 2001.
The recommendation was approved by the Methods Committee on Natural Toxins and Food Allergens as First Action. *See* "Official Methods Program Actions," (2001) *Inside Laboratory Management*, February issue.
Corresponding author's e-mail: visconti@area.ba.cnr.it.

of extraction solvent, test portion size, extraction mode, and cleanup) that may affect analytical performance were tested (13), and a widely applicable method based on immunoaffinity column cleanup and liquid chromatographic (LC) analysis was developed in our laboratory for the determination of FB_1 and FB_2 in corn, corn flakes, extruded corn, muffins, and infant formula (14). As part of the same SMT project a collaborative study was performed, with the participation of 23 laboratories, aimed at the validation of this method for the analysis of corn and corn flakes. The results of this collaborative study are reported herein together with the necessary information relevant to preparation of the materials, protocol of analysis, and statistical analysis of the results.

Collaborative Study

The objective of this study was to determine the accuracy, repeatability, and reproducibility characteristics of a new LC method with an immunoaffinity column (14) for the determination of fumonisins FB_1 and FB_2 at μg/g levels in corn and corn flakes by analyses of naturally contaminated and spiked samples of corn and corn flakes.

The method is quantitative and applicable to the determination of FB_1 and FB_2, at levels that can occur in commercially available corn and corn-based food products. Although the validation was performed only on corn and corn flakes, in-house experiments indicated that the method is also applicable to infant formula, muffins, and extruded corn (14). The proposed method was submitted to 24 participating laboratories, accompanied by 10 blind duplicate pairs of corn and corn flakes test samples (5 pairs per matrix), 2 practice samples of corn, 1 ampule of fumonisins standard solution, 22 immunoaffinity columns, and report sheets. Participants were instructed on how to store the test samples until analysis and how to proceed with the exercise. Participants were also asked to send the coordinator copies of chromatograms together with the report sheet.

The collaborative study of each matrix included blind duplicates of 1 blank material (fumonisins [B_1 + B_2] at <0.05 μg/g), 1 spiked material (FB_1 at 0.80 μg/g and FB_2 at 0.40 μg/g), and 3 naturally contaminated materials, each with a different fumonisins content. In particular, on the basis of the results (not corrected for recoveries) of the homogeneity study for the testing materials (*see* below), the 3 naturally contaminated corn materials had the following levels of fumonisins (not corrected for recoveries): (A) FB_1 at 0.39 μg/g and FB_2 at 0.07 μg/g; (B) FB_1 at 0.88 μg/g FB_1 and FB_2 at 0.23 μg/g; and (C) FB_1 at 1.41 μg/g and FB_2 at 0.53 μg/g; the 3 contaminated materials of corn flakes (commercial corn flakes mixed with naturally contaminated corn) had the following concentrations of fumonisins (not corrected for recoveries): (A) FB_1 at 0.38 μg/g and FB_2 at 0.15 μg/g; (B) FB_1 at 0.66 μg/g and FB_2 at 0.28 μg/g; and (C) FB_1 at 1.13 μg/g and FB_2 at 0.47 μg/g.

Test Materials

Blank corn (12 kg) was prepared by grinding (<1 mm particle size) and mixing corn containing fumonisins (B_1 + B_2) at <0.05 μg/g; blank corn flakes (12 kg) were prepared by grinding commercial product containing FB_1 + FB_2 at <0.05 μg/g. Blanks were analyzed by using the method under study. For both corn and corn flakes this level of contamination (fumonisins [B_1 + B_2] at <0.05 μg/g) was assumed to be acceptable for blank materials because of the low detection limit (0.03 μg/g, defined as a signal-to-noise ratio of 3) and the difficulty in finding fumonisin-free blank materials.

Contaminated corn containing the 3 levels of fumonisins (6 kg each) were prepared by appropriately mixing and grinding naturally contaminated corn. Contaminated corn flakes containing the 3 levels of fumonisins (6 kg each) were prepared by grinding and mixing small amounts of naturally contaminated corn (FB_1 + FB_2 at 65 μg/g) with commercial corn flakes.

Spiked test samples of corn and corn flakes were prepared as follows: 500 g ground blank material was spiked with 12 mL solution containing FB_1 at 400 μg/mL and FB_2 at 200 μg/mL in acetonitrile–water (50 + 50, v/v). After the solvent was allowed to evaporate overnight, the spiked materials were ground in a mortar and pestle to break up the lumps. Each material was blended consecutively with 500, 1000, 2000, and 2000 g blank material to obtain 6000 g spiked corn and 6000 g spiked corn flakes. Each laboratory sample was packed in aluminium foil sachets and stored at –20°C until the analyses were performed for the homogeneity study.

Ten corn test samples, individually marked from "Corn 1" to "Corn 10," and 10 corn flakes test samples, individually marked from "Corn flakes 1" to "Corn flakes 10," representing 5 blind duplicate pairs for each matrix, were sent to participating laboratories. Two practice samples of corn, each marked "practice sample," were also sent to participants for them to become familiar with the method. Participants were asked to verify that the results from analysis of the practice sample were within the range 0.5–1.0 μg/g for FB_1 and 0.2–0.4 μg/g for FB_2. In the case of out-of-range results, participants were asked to inform the coordinator so that the source of error could be found before they proceeded with the exercise.

For the homogeneity study, 10 sachets of each material were taken at regular intervals from the filling sequence, and duplicate determinations of FB_1 and FB_2 per sachet were performed at the Study Director's laboratory, by using the method to be tested in the collaborative study. When the in-house repeatability of the analytical procedure used (<20%) was taken into account, it was concluded that each of the tested materials was acceptably homogeneous. No significant drift of the FB_1 and FB_2 levels as a result of the filling sequence was detected for any of the contaminated materials.

AOAC Official Method 2001.04
Determination of Fumonisins B_1 and B_2 in Corn and Corn Flakes

Liquid Chromatography with Immunoaffinity Column Cleanup
First Action 2001

[Applicable to determination of fumonisins B_1 (FB_1) and B_2 (FB_2) in corn at total levels from 0.5 to 2 μg/g and in corn flakes at total levels from 0.5 to 1.5 μg/g.]

Downloaded from https://academic.oup.com/jaoac/article/84/6/1828/5656541 by guest on 16 August 2022

Caution: Fumonisins are nephrotoxic, hepatotoxic, and carcinogenic to rats and mice; effects on humans are not fully known. Wear protective gloves to reduce skin contact with extracts. Laboratory spills should be cleaned up by washing with a 5% dilution of commercial bleach (sodium hypochlorite) followed by water.

See Tables **2001.04A** and **B** for the results of the interlaboratory study supporting acceptance of the method.

A. Principle

Fumonisins are extracted from corn and corn flakes with methanol–acetonitrile–water (25 + 25 + 50, v/v/v), the filtered extract is cleaned up by an immunoaffinity column, and the fumonisins are eluted with methanol. The eluate is evaporated just to dryness, and the residue is dissolved in acetonitrile–water (50 + 50, v/v). *o*-Phthaldialdehyde and 2-mercaptoethanol is added to form fluorescent fumonisin derivatives, which are analyzed by reversed-phase liquid chromatography (LC) with fluorescence detection.

B. Reagents

(**a**) *Methanol*.—LC grade, or equivalent.

(**b**) *Acetonitrile*.—LC grade, or equivalent.

(**c**) *o-Phthaldialdehyde (OPA)*.—CAS 643-79-8.

(**d**) *2-Mercaptoethanol (MCE)*.—CAS 60-24-2.

(**e**) *Sodium dihydrogen phosphate solution.*—0.1M. Dissolve 15.6 g $NaH_2PO_4{\cdot}2H_2O$ in water and dilute to 1 L.

(**f**) *Sodium tetraborate solution*.—0.1M. Dissolve 3.8 g $Na_2B_4O_7{\cdot}10H_2O$ in water and dilute to 100 mL.

(**g**) *Hydrochloric acid*.—2M. Dilute HCl (12M) 1 + 5 with water.

(**h**) *Extraction solvent*.—Acetonitrile–methanol–water (25 + 25 + 50, v/v/v).

(**i**) *Acetonitrile–water.*—50 + 50, v/v.

(**j**) *Phosphate-buffered saline (PBS)*.—Dissolve 8.0 g NaCl, 1.2 g anhydrous Na_2HPO_4, 0.2 g KH_2PO_4, and 0.2 g KCl in approximately 990 mL water. Adjust pH to 7.0 with 2M HCl, and dilute to 1 L. Phosphate-buffered saline tablets can also be used.

(**k**) *Immunoaffinity columns.*—Specific for fumonisin cleanup with 100% cross reactivity for both FB_1 and FB_2. The column must have a total capacity of ≥10 µg fumonisins B_1 and B_2 and should give a recovery of ≥90% when a calibrant solution of fumonisins B_1 and B_2 in methanol–PBS containing 5 µg fumonisins is applied. Follow the manufacturer's instructions for the type of column used. Fumonitest™ from VICAM (Watertown, MA) was found to be suitable.

(**l**) *LC mobile phase.*—Methanol–0.1M NaH_2PO_4 (77 + 23, v/v), adjusted to pH 3.35 with H_3PO_4. Filter mobile phase through 0.45 µm membrane, and pump at 1 mL/min flow rate. Adjust composition to conform with individual LC column characteristics.

(**m**) *OPA reagent.*—Dissolve 40 mg OPA in 1 mL methanol, and dilute with 5 mL 0.1M $Na_2B_4O_7$ solution. Add 50 µL MCE and mix. Store in the dark for up to 1 week at room temperature in a capped amber vial.

(**n**) *Fumonisins B_1 and B_2.*—Crystalline form, purity of >95% (PROMEC, Medical Research Council, PO Box 19070, Tygerberg, 7505, South Africa, +27-21-938-0290).

(**o**) *Fumonisin stock solution for LC.*—Prepare calibrant solution containing fumonisins B_1 and B_2 in acetonitrile–water (50 + 50, v/v) at concentration of 100 µg/mL for FB_1 and 50 µg/mL for FB_2. Fumonisin calibrant solution is stable up to 6 months when stored at 4°C. Pipet 500 µL fumonisin calibrant solution into 5 mL calibrated volumetric flask. Dilute to volume with acetonitrile–water (50 + 50, v/v), and shake well to obtain stock solution containing FB_1 at 10 ng/µL and FB_2 at 5 ng/µL.

Table 2001.04A. Interlaboratory study results for the determination of fumonisins B_1 and B_2 in corn by liquid chromatography with immunoaffinity column cleanup

Toxin	Matrix, µg/g	$\bar{x}$, µg/g	No. of labs[a]	s_r	RSD_r, %	s_R	RSD_R, %	HORRAT	Recovery, %
FB_1	Blank (<0.05)	0.04	21(0)	—	—	—	—	—	—
	Spiked (0.80)	0.65	21(0)	0.14	21.1	0.16	25.7	1.48	75.6
	ncA (0.39 ± 0.03)[b]	0.37	21(0)	0.09	24.2	0.10	28.2	1.51	—
	ncB (0.88 ± 0.04)[b]	0.78	20(1)	0.15	18.8	0.20	25.5	1.53	—
	ncC (1.41 ± 0.06)[b]	1.41	21(0)	0.28	20.0	0.31	21.9	1.44	—
FB_2	Blank (<0.05)	0.01	21(0)	—	—	—	—	—	—
	Spiked (0.40)	0.30	21(0)	0.06	18.5	0.07	22.6	1.17	72.0
	ncA (0.07 ± 0.01)[b]	0.09	17(4)	0.02	22.0	0.02	22.1	0.96	—
	ncB (0.23 ± 0.01)[b]	0.20	21(0)	0.05	26.8	0.06	29.7	1.46	—
	ncC (0.53 ± 0.02)[b]	0.56	21(0)	0.13	22.5	0.14	25.9	1.48	—

[a] Number of laboratories retained after elimination of outliers; value in parentheses is number of laboratories removed as statistical outliers.

[b] nc = Naturally contaminated corn samples. Value in parentheses represents mean ± 2 SEM (standard error of the mean) of 20 measurements (10 sachets in duplicate) performed for the homogeneity study.

Downloaded from https://academic.oup.com/jaoac/article/84/6/1828/5656541 by guest on 16 August 2022

Table 2001.04B. Interlaboratory study results for the determination of fumonisins B_1 and B_2 in corn flakes by liquid chromatography with immunoaffinity column cleanup

Toxin	Matrix, µg/g	$\bar{x}$, µg/g	No. of labs[a]	s_r	RSD_r, %	s_R	RSD_R, %	HORRAT	Recovery, %
FB_1	Blank (<0.05)	0.04	21(0)	—	—	—	—	—	—
	Spiked (0.80)	0.92	20(1)	0.09	9.2	0.27	29.5	1.82	110.0
	A (0.38 ± 0.01)[b]	0.32	20(1)	0.07	20.6	0.10	31.8	1.68	—
	B (0.66 ± 0.03)[b]	0.57	21(0)	0.09	15.0	0.16	27.8	1.60	—
	C (1.13 ± 0.04)[b]	1.05	21(0)	0.12	11.1	0.29	27.4	1.73	—
FB_2	Blank (<0.05)	0.01	21(0)	—	—	—	—	—	—
	Spiked (0.40)	0.39	20(1)	0.03	7.7	0.12	30.9	1.68	97.0
	A (0.15 ± 0.01)[b]	0.13	21(0)	0.03	21.7	0.04	34.8	1.60	—
	B (0.28 ± 0.01)[b]	0.24	21(0)	0.04	14.6	0.07	27.5	1.39	—
	C (0.47 ± 0.02)[b]	0.46	21(0)	0.05	10.4	0.12	26.1	1.45	—

[a] Number of laboratories retained after elimination of outliers; value in parentheses is number of laboratories removed as statistical outliers.

[b] Contaminated corn flakes samples obtained by spiking ground corn flakes with minimal amounts (0.8–3.0%) of ground contaminated corn powder. Value in parentheses represents mean ± 2 SEM (standard error of the mean) of 20 measurements (10 sachets in duplicate) performed for the homogeneity study.

(**p**) *Fumonisin working calibrant solutions for LC*.—Prepare 4 LC calibrant solutions in separate 5 mL volumetric flasks according to Table **2001.04C**. Dilute contents of each flask to volume (5 mL) with acetonitrile–water (50 + 50, v/v).

C. Apparatus

(**a**) *Centrifuge bottle.*—Plastic, 250 mL, with screw cap.

(**b**) *Centrifuge.*—Capable of up to 2500 × *g*.

(**c**) *Filter papers.*—Whatman No. 4, 12 cm.

(**d**) *Glass microfiber filters.*—Whatman GF/A, 9 cm.

(**e**) *Reservoir.*—25 mL with Luer-tip connector for immunoaffinity column.

(**f**) *Calibrated microliter syringe* or *microliter pipet.*—25–1000 µL.

(**g**) *Vacuum manifold*.—To accommodate immunoaffinity columns.

(**h**) *Liquid chromatograph.*—LC pump delivering 1 mL/min constant flow rate and with injection system calibrated to deliver 20 µL; and data system.

(**i**) *LC column.*—Stainless steel (150 × 4.6 mm id), packed with 5 µm C18 deactivated reversed-phase material, preceded by corresponding reversed-phase guard column or guard filter (0.5 µm porosity; Rheodyne, L.P., PO Box 1909, Rohnert Park, CA 94927-1909, USA, +1-707-588-2000).

(**j**) *Fluorescence detector.*—Fitted with flow cell and set at 335 nm (excitation) and 440 nm (emission).

D. Extraction

Permit materials to reach room temperature before removing test portion. Weigh, to nearest 0.1 g, 20 g test portion of corn or corn flakes into 250 mL centrifuge bottle, and add 50 mL extraction solvent, **B**(**h**). Cover centrifuge bottle, and shake bottle for 20 min with orbital shaker. Centrifuge for 10 min at 2500 × *g*, and filter supernatant through filter paper, **C**(**c**), avoiding transfer of solid material on filter. Again extract remaining solid material by adding 50 mL extraction solvent, **B**(**h**), to centrifuge bottle and shaking bottle for 20 min. Centrifuge for 10 min at 2500 × *g*, and filter extract through the

Table 2001.04C. Preparation of working calibrant solutions

		Final fumonisin concentration of working calibrant solution and OPA-derivatized solution, ng/µL			
		FB_1		FB_2	
Working calibrant solution	Volume of stock solution taken, µL	Working solution	Derivatized solution	Working solution	Derivatized solution
1	25	0.05	0.025	0.025	0.0125
2	125	0.25	0.125	0.125	0.0625
3	500	1.00	0.500	0.50	0.250
4	2000	4.00	2.00	2.00	1.000

Downloaded from https://academic.oup.com/jaoac/article/84/6/1828/5656541 by guest on 16 August 2022

same filter paper. Collect and combine the 2 filtrates, and pipet 10 mL filtrate into 100 mL flask. Add 40 mL PBS, **B**(**j**), and mix well. Filter diluted extract through microfiber filter, **C**(**d**), and collect 10 mL filtrate (equivalent to 0.4 g test portion) for cleanup through immunoaffinity column.

E. Immunoaffinity Column Cleanup

Follow manufacturer's instructions for the type of column used. Remove top cap from column, and connect column with reservoir. Remove end cap from column and attach column to vacuum manifold. Pipet 10 mL filtrate into reservoir. Let filtrate flow through column at ca 1–2 drops/s and discard eluate. Wash column with 10 mL PBS, **B**(**j**), at rate of 1–2 drops/s until air comes through column. Place 4 mL vial under column. Elute fumonisins with 1.5 mL LC grade methanol at 1 drop/s, and collect fumonisins in vial. Evaporate eluate just to dryness under stream of N at ca 60°C. Retain dried residue at ca 4°C for derivatization and LC analysis.

F. Calibration Curve

Prepare calibration curves, using working calibrant solutions, **B**(**p**). These solutions cover the range of 0.025–2.000 μg/g for FB_1 and the range of 0.0125–1.000 μg/g for FB_2. Prepare calibration curves, before LC analysis, according to Table **2001.04C**, and check plots for linearity. If curve is not linear, repeat derivatization following instructions carefully and/or reduce the range of the calibrants.

G. Derivatization and LC Analysis

Redissolve purified residue in 200 μL acetonitrile–water (50 + 50, v/v), **B**(**i**). Transfer 50 μL aliquots of extract or standards to bottom of 1 mL test tube, and add 50 μL OPA reagent, **B**(**m**). Mix solution for 30 s with vortex mixer, and inject 20 μL derivatized solution (equivalent to 20 mg matrix) into LC system exactly 3 min after adding OPA reagent. With the described LC mobile phase, **B**(**l**), and column, **C**(**i**), satisfactory (baseline) resolution of FB_1–OPA and FB_2–OPA must be obtained, with expected retention times at ca 6 and 15 min, respectively. If fumonisin content of derivatized extract is higher than calibration range, dilute purified extract with acetonitrile–water (50 + 50, v/v), **B**(**i**), derivatize with OPA reagent, and repeat LC analysis.

H. Quantification of Fumonisins B_1 and B_2

Quantify FB_1 and FB_2 by measuring peak area (or peak height) at retention time of each fumonisin and comparing measured value with corresponding calibration curve.

From calibration curves determine amounts of FB_1 and FB_2 (in ng) in aliquot of test solution injected into LC column.

Separately calculate concentrations (C_{FB}) of FB_1 and FB_2 in micrograms per gram (μg/g), as follows:

$$C_{FB} = 10^{-3}\ M_A \times \frac{1}{10^{-3}\ M_B} = \frac{M_A}{20}$$

where M_A is mass of FB_1 or FB_2 (in ng) in aliquot of test solution injected on column, as determined from calibration curve, M_B is mass of matrix (in mg) injected on column (20 mg), obtained as follows:

$$\frac{20\text{ g (Extraction step)}}{100\text{ mL}} \times \frac{10\text{ mL (dilution)}}{50\text{ mL}} \times 10\text{ mL (purified aliquot)}$$

$$\times \frac{1\text{ (reconstitution volume)}}{0.2\text{ mL}} \times \frac{0.05\text{ mL (derivatization)}}{0.1\text{ mL}}$$

$$\times\, 0.02\text{ mL (injected aliquot)}$$

and 10^{-3} is factor to convert M_A and M_B from ng to μg and from mg to g, respectively.

Ref.: *J. AOAC Int.* **84**, 1829–1832(2001)

Results and Discussion

Results were received from 23 of 24 participating laboratories (Laboratory 17 did not perform the exercise). All participants except Laboratory 12 analyzed the practice sample. Only Laboratory 11 reported an FB_1 content of 1.0 μg/g in the practice sample; nevertheless, it provided good results in the collaborative study. Laboratory 16 did not report results for the practice sample because the chromatograms did not allow the identification and quantification of FB_1; however, Laboratory 16 was authorized by the coordinator to proceed with the collaborative study and was able to complete the study successfully.

All results are reported in Tables 1–4 for FB_1 and FB_2 in corn and corn flakes, respectively. Data reported by 2 participants were not included in the statistical evaluation of the results of the collaborative study because they were judged invalid. In particular, the data of Laboratory 3 were rejected because of a suspected possible mislabeling or exchange of code numbers between the blank and 1 of the contaminated samples for both corn flakes and corn (this conclusion was reached by the coordinator after thorough examination of the chromatograms, which showed no fumonisins in some contaminated samples and high levels of fumonisins in blank samples). The data of Laboratory 4 were rejected because of an apparent systematic error, giving rise to consistently higher values, ca 50% higher than the average results of participants, for all measurements except the blank. Because the chromatograms were well defined without apparent signs of possible interfering peaks, a possible error in the preparation of the fumonisin stock solution for LC, **B**(**o**), with consequent error in the preparation of the standard calibration curve, was envisaged by the coordinator.

Results of the remaining 21 laboratories were subjected to statistical analyses for identification of outliers by the Cochran and Grubbs tests to remove laboratories showing significantly greater variability among replicates and extreme averages, respectively. For corn, 5 pairs of data (1 pair for FB_1 in naturally contaminated sample B and 4 pairs for FB_2 in naturally contaminated sample A) were eliminated either by the Cochran test and/or the Grubbs test. For corn flakes, 3 pairs of data (2 pairs for FB_1 in the spiked sample and contaminated sample A, and 1 pair for FB_2 in the spiked sample) were eliminated by the Cochran test. Statistical analyses were performed

Downloaded from https://academic.oup.com/jaoac/article/84/6/1828/5656541 by guest on 16 August 2022

with all remaining data, based on the AOAC harmonized guidelines (15). Statistical data from the analyses for FB_1 and FB_2 in corn and corn flakes are reported in Tables **2001.04A** and **B**, respectively.

Mean recoveries of FB_1 from spiked corn and corn flakes were 76 and 110%, and the corresponding recoveries of FB_2 were 72 and 97%, respectively. The repeatability and reproducibility relative standard deviations (RSD_r and RSD_R) for the determination of FB_1 in corn materials (spiked or naturally contaminated in the range 0.37–1.41 μg/g) ranged from 19 to 24% and from 22 to 28%, respectively. RSD_r and RSD_R values for the determination of FB_2 (in the range 0.09–0.56 μg/g) in corn materials ranged from 19 to 27% and from 22 to 30%, respectively. RSD_r and RSD_R for the determination of FB_1 in corn flakes materials (spiked with standard or contaminated corn in the range 0.32–1.05 μg/g) ranged from 9 to 21% and from 27 to 32%, respectively. RSD_r and RSD_R values for the determination of FB_2 (in the range 0.13–0.46 μg/g) in corn flakes materials ranged from 8 to 22% and from 26 to 35%, respectively.

The recovery, repeatability (RSD_r), and reproducibility (RSD_R) data for corn flakes were all within the criteria established by the European Committee for Standardization (CEN) for the acceptability of mycotoxin analyses in the absence of official methods (16). These criteria with respect to fumonisins are: (*1*) recoveries between 70 and 110%, $RSD_r \leq 20\%$ and $RSD_R \leq 30\%$ for FB_1 or FB_2 levels in the range 0.5–5.0 μg/g; and (*2*) recoveries between 60 and 120%, $RSD_r \leq 30\%$ and $RSD_R \leq 60\%$ for FB_1 or FB_2 levels <0.5 μg/g. On the basis of these CEN criteria, the recoveries and reproducibility data for corn were also all acceptable, whereas only 2 of 8 repeatability values for this matrix were slightly higher than the 20% value established by CEN (i.e., 21% for the mean FB_1 value of 0.65 μg/g and 22% for the mean FB_2 value of 0.56 μg/g).

The acceptability of the performance characteristics of the method was also assessed on the basis of the HORRAT values

Table 1. Results of the interlaboratory study for the determination of fumonisin B_1 (μg/g) in blind duplicates of spiked and naturally contaminated corn samples

Lab	Practice sample	Blank (<0.05 μg/g)		Spiked (0.80 μg/g)		Naturally contaminated A		Naturally contaminated B		Naturally contaminated C	
1	0.86	0.03	0.05	0.77	0.71	0.40	0.41	0.96	0.87	1.69	1.27
2	0.97	0.08	0.07	0.64	0.74	0.40	0.37	0.91	0.92	1.36	1.32
3[a]	0.78	0.26	0.09	0.43	0.53	0.07	0.34	2.00	0.64	0.88	1.84
4[a]	0.86	0	0.03	1.21	0.83	0.55	0.46	1.25	1.19	2.35	0.94
5	0.84	0	0	0.60	0.61	0.42	0.29	0.89	0.82	1.58	1.35
6	1.09	0.01	0	0.54	0.80	0.35	0.43	0.69	0.97	1.37	1.49
7	0.94	0.22	0.18	0.73	0.75	0.37	0.50	0.76[b]	1.79[b]	1.60	1.54
8	0.86	0.01	0.04	0.70	0.75	0.37	0.38	0.95	0.83	1.31	1.69
9	0.95	0	0.05	0.73	0.76	0.42	0.44	0.91	0.95	1.56	1.69
10	0.61	0	0.03	0.41	0.70	0.11	0.46	0.47	0.87	0.96	1.52
11	1.18	0.01	0.06	0.68	1.03	0.29	0.39	0.81	1.02	1.11	1.40
12	—[c]	0.02	0.03	0.45	0.62	0.36	0.29	0.44	0.59	1.78	0.75
13	0.99	0.02	0.02	0.82	0.67	0.41	0.37	0.53	1.05	1.85	1.82
14	0.67	0.02	0.05	0.53	0.34	0.32	0.25	0.48	0.57	0.89	0.82
15	0.87	0.06	0.05	0.67	0.76	0.41	0.47	1.00	1.06	1.47	1.64
16	—[d]	0.01	0	0.38	0.36	0.15	0.17	0.49	0.51	0.82	1.15
18	0.75	0	0	0.69	0.70	0.50	0.46	0.55	0.84	2.10	1.07
19	0.97	0.02	0.02	0.50	0.42	0.23	0.17	0.73	0.62	1.37	1.63
20	0.98	0.04	0.06	0.66	1.04	0.38	0.48	1.00	1.01	1.48	1.85
21	1.03	0.07	0.06	0.83	0.65	0.39	0.49	0.97	0.97	1.59	1.37
22	0.90	0.12	0.03	0.51	0.52	0.32	0.44	0.68	0.45	1.01	1.19
23	0.83	0.03	0.03	0.38	0.75	0.31	0.40	0.53	0.83	1.27	1.53
24	0.61	0.01	0.07	0.48	0.75	0.31	0.62	0.73	0.74	1.44	1.80

[a] Data from Laboratories 3 and 4 were invalid because the laboratories were considered technical outliers.
[b] Data rejected on the basis of the Cochran test.
[c] Sample was not analyzed.
[d] Problems during analysis.

Downloaded from https://academic.oup.com/jaoac/article/84/6/1828/5656541 by guest on 16 August 2022

(17), which compare the RSD_R obtained for a particular matrix with the RSD_R statistically predicted on the basis of collaborative trials taken from the published literature. When the outliers were excluded in the present collaborative study, the HORRAT values ranged from 1.44 to 1.53 for FB_1 and from 0.96 to 1.48 for FB_2 in the corn materials, whereas for corn flakes they ranged from 1.60 to 1.82 for FB_1 and from 1.39 to 1.68 for FB_2. These HORRAT values indicated that results obtained for all spiked and naturally contaminated corn and corn flakes materials were acceptable for method validation; a HORRAT of <2 normally indicates acceptable precision.

Collaborators' Comments

All participants found the method description clear and easy to follow. Participants reported limits of detection (signal-to-noise ratio of 3) ranging from 0.002 to 0.028 μg/g for FB_1 and from 0.001 to 0.089 μg/g for FB_2. The limits of quantification (signal-to-noise ratio of 6) ranged from 0.004 to 0.1 μg/g for FB_1 and from 0.003 to 0.178 μg/g for FB_2. Seven laboratories (1, 2, 5, 8, 11, 15, and 18) diluted some corn and corn flakes extracts before OPA derivatization in order to bring the FB_1 and FB_2 responses within the calibration range. Laboratories 13 and 10 dissolved their final extracts in 1 and 2 mL, respectively, instead of 0.2 mL because their autosamplers needed larger volume for automatic injection. Laboratory 12 used an additional point in the calibration curve to analyze samples with high fumonisin concentrations. Some laboratories (1, 2, 5, 8, 16, and 18) found late-eluting components or components that interfered with FB_1; some laboratories solved the problem of late-eluting components by washing the LC column with methanol after the elution of FB_2. Laboratory 16 also analyzed all crude sample extracts (before immunoaffinity cleanup) by enzyme-linked immunosorbent assay (ELISA), obtaining results higher (2–5 times higher)

Downloaded from https://academic.oup.com/jaoac/article/84/6/1828/5656541 by guest on 16 August 2022

Table 2. Results of the interlaboratory study for the determination of fumonisin B_2 (μg/g) in blind duplicates of spiked and naturally contaminated corn samples

Lab	Practice sample	Blank (<0.05 μg/g)		Spiked (0.80 μg/g)		Naturally contaminated A		Naturally contaminated B		Naturally contaminated C	
1	0.22	0	0	0.34	0.32	0.13	0.10	0.24	0.21	0.63	0.50
2	0.28	0	0	0.28	0.34	0.12	0.10	0.21	0.22	0.61	0.53
3[a]	0.20	0.08	0	0.32	0.27	0	0.10	0.83	0.19	0.18	0.74
4[a]	0.32	0	0	0.51	0.35	0.10	0.13	0.26	0.25	0.94	0.39
5	0.21	0.02	0.02	0.26	0.27	0.10	0.07	0.23	0.23	0.65	0.51
6	0.28	0	0	0.26	0.40	0.08	0.09	0.17	0.22	0.55	0.56
7	0.19	0	0	0.29	0.30	0.15	0.08	0.15	0.08	0.31	0.30
8	0.21	0	0.01	0.31	0.34	0.07	0.09	0.23	0.21	0.50	0.64
9	0.22	0	0.01	0.32	0.34	0.09	0.09	0.22	0.25	0.62	0.62
10	0.17	0	0	0.23	0.32	0.02[b]	0.10[b]	0.12	0.23	0.39	0.64
11	0.40	0	0.01	0.30	0.40	0.06	0.08	0.22	0.24	0.45	0.50
12	—[c]	0	0.02	0.25	0.30	0.08	0.08	0.16	0.19	0.69	0.39
13	0.25	0	0	0.37	0.36	0.09	0.07	0.17	0.36	0.73	0.74
14	0.18	0	0	0.25	0.19	0.08	0.07	0.13	0.15	0.36	0.29
15	0.23	0.03	0.02	0.31	0.35	0.24[d]	0.11[d]	0.26	0.28	0.61	0.77
16	—[e]	0	0	0.17	0.17	0[b]	0.38[b]	0.14	0.14	0.36	0.45
18	0.25	0	0	0.29	0.28	0.11	0.09	0.07	0.25	0.91	0.46
19	0.29	0	0	0.23	0.17	0.08[f]	0.05[f]	0.19	0.19	0.58	0.64
20	0.32	0.03	0.02	0.31	0.48	0.10	0.11	0.26	0.28	0.60	0.81
21	0.26	0.03	0.02	0.34	0.30	0.08	0.10	0.26	0.24	0.60	0.53
22	0.22	0.05	0.01	0.27	0.26	0.07	0.10	0.17	0.12	0.42	0.50
23	0.22	0	0	0.17	0.34	0.06	0.09	0.22	0.15	0.50	0.62
24	0.21	0.01	0.01	0.26	0.37	0.07	0.12	0.16	0.32	0.45	0.80

[a] Data from Laboratories 3 and 4 were invalid because the laboratories were considered technical outliers.
[b] Data rejected on the basis of the Cochran test and the single Grubbs test.
[c] Sample was not analyzed.
[d] Data rejected on the basis of the Cochran test.
[e] Problems during analysis.
[f] Data rejected on the basis of the single Grubbs test.

Table 3. Results of the interlaboratory study for the determination of fumonisin B_1 (μg/g) in blind duplicates of spiked and contaminated corn flakes samples

Lab	Blank (< 0.05 μg/g)		Spiked (0.80 μg/g)		Naturally contaminated A		Naturally contaminated B		Naturally contaminated C	
1	0.05	0.06	1.00	0.97	0.40	0.37	0.60	0.53	0.9	1.09
2	0.05	0.07	0.89	0.96	0.37	0.31	0.51	0.56	0.96	0.93
3[a]	0.03	0.72	0.26	0.61	0.28	0.02	0.67	1.09	1.00	0.30
4[a]	0.36	1.29	0.68	1.63	0.15	0	1.81	1.43	0.9	0.33
5	0.03	0.07	0.96	0.92	0.28	0.27	0.53	0.71	0.93	1.03
6	0	0	0.75[b]	1.48[b]	0.29	0.42	0.37	0.56	1.52	1.49
7	0.09	0.09	0.57	0.48	0[b]	0.34[b]	0.54	0.61	1.00	0.67
8	0.03	0.03	0.92	1.16	0.25	0.31	0.49	0.48	0.87	0.93
9	0.04	0.04	1.29	1.20	0.41	0.41	0.72	0.70	1.35	1.41
10	0.04	0	0.85	1.02	0.26	0.47	0.68	0.74	1.55	1.51
11	0.04	0.05	0.96	0.86	0.28	0.21	0.38	0.56	1.00	0.93
12	0.02	0.04	0.68	0.69	0.14	0.37	0.33	0.47	0.62	0.79
13	0.04	0.04	1.21	1.36	0.52	0.38	0.73	0.77	1.53	1.26
14	0.02	0.02	0.59	0.51	0.13	0.15	0.22	0.24	0.51	0.44
15	0.08	0.08	1.26	1.22	0.44	0.42	0.65	0.68	1.35	1.33
16	0.05	0	0.32	0.57	0.14	0.13	0.39	0.23	0.79	0.73
18	0	0	0.84	1.01	0.46	0.40	0.58	0.87	1.03	0.79
19	0.03	0.05	1.07	0.89	0.30	0.31	0.64	0.64	1.10	0.91
20	0.07	0.06	0.93	0.95	0.32	0.46	0.81	0.69	0.98	1.25
21	0.06	0.06	1.09	1.07	0.42	0.34	0.70	0.64	1.18	1.17
22	0.04	0.04	1.32	1.39	0.40	0.35	0.56	0.65	1.15	1.37
23	0.02	0.02	0.94	0.98	0.24	0.23	0.64	0.50	1.04	0.82
24	0.09	0.08	0.44	0.54	0.28	0.29	0.37	0.44	0.92	0.79

[a] Data from Laboratories 3 and 4 were invalid because the laboratories were considered technical outliers.

[b] Data rejected on the basis of the Cochran test.

than those obtained by LC. Moreover, it was suggested by this laboratory that the second extraction is time consuming and could be avoided by using a higher solvent–matrix ratio and longer extraction time to obtain acceptable fumonisin recovery. In this regard, a preliminary study for method development performed with a ruggedness test according to a factorial design within the same SMT-sponsored project (13) and additional experiments performed in the Study Director's laboratory indicated that these factors (different solvent–matrix ratio and extraction times) had a minor or no effect on fumonisin recovery.

Recommendation

It is recommended that the immunoaffinity column cleanup method with reversed-phase LC determination and fluorescence detection be adopted First Action by AOAC INTERNATIONAL for determination of fumonisin B_1 and fumonisin B_2 at levels higher than 0.4 and 0.1 μg/g, respectively, in corn, and at levels higher than 0.3 and 0.1 μg/g, respectively, in corn flakes.

Acknowledgments

This project was conducted under the auspices and with the financial support of the European Commission—Standards, Measurements and Testing Programme, contract No. SMT4 CT97 2193.

The authors are grateful to the following collaborators for their participation in the study:

E. Sizoo, National Institute of Public Health and the Environmental Protection (RIVM), Bilthoven, The Netherlands

P. Wilson, Central Science Laboratory, York, UK

C. von Holst, Joint Research Centre – ISPRA Institute for Health and Consumer Protection, Ispra, Italy

K. Meyer, Lehrstuhl fur Tierhygiene, Freising, Germany

I. Felgueiras, Instituto Nacional de Engenharia e Tecnologia Industrial, Lisboa, Portugal

T. Møller, National Food Administration, Uppsala, Sweden

P. Burdaspal, Centro Nacional de Alimentacion, Instituto de Salud Carlos III Ministerio de Sanidad y Consumo, Majadahonda (Madrid), Spain

Downloaded from https://academic.oup.com/jaoac/article/84/6/1828/5656541 by guest on 16 August 2022

Table 4. Results of the interlaboratory study for the determination of fumonisin B_2 (µg/g) in blind duplicates of spiked and contaminated corn flakes samples

Lab	Blank (< 0.05 µg/g)		Spiked (0.80 µg/g)		Naturally contaminated A		Naturally contaminated B		Naturally contaminated C	
1	0	0	0.45	0.40	0.14	0.13	0.24	0.21	0.39	0.45
2	0	0	0.38	0.41	0.14	0.12	0.22	0.23	0.41	0.38
3[a]	0	0.34	0.12	0.27	0.12	0	0.32	0.56	0.48	0.11
4[a]	0.15	0.54	0.32	0.71	0	0	0.84	0.64	0.32	0.11
5	0.01	0.02	0.41	0.38	0.13	0.12	0.22	0.28	0.43	0.47
6	0	0	0.32[b]	0.62[b]	0.13	0.18	0.17	0.26	0.64	0.62
7	0	0.03	0.10	0.07	0.07	0	0.15	0.18	0.39	0.26
8	0.01	0	0.39	0.49	0.10	0.13	0.21	0.21	0.37	0.40
9	0	0	0.53	0.48	0.17	0.16	0.28	0.31	0.59	0.60
10	0	0	0.40	0.47	0.10	0.18	0.28	0.29	0.69	0.66
11	0	0	0.40	0.34	0.12	0.08	0.21	0.20	0.43	0.37
12	0.01	0.01	0.33	0.33	0.08	0.16	0.18	0.22	0.35	0.41
13	0	0	0.51	0.55	0.19	0.15	0.30	0.30	0.53	0.64
14	0	0	0.25	0.23	0.05	0.06	0.10	0.11	0.23	0.21
15	0.01	0.02	0.52	0.49	0.19	0.17	0.28	0.30	0.59	0.57
16	0	0	0.25	0.25	0.07	0.08	0.16	0.13	0.40	0.35
18	0	0	0.38	0.45	0.20	0.18	0.27	0.41	0.45	0.37
19	0	0	0.46	0.43	0.13	0.15	0.27	0.28	0.47	0.43
20	0.02	0.02	0.44	0.44	0.13	0.19	0.36	0.29	0.46	0.60
21	0	0	0.43	0.44	0.16	0.13	0.29	0.25	0.47	0.47
22	0	0	0.57	0.60	0.16	0.15	0.22	0.28	0.51	0.62
23	0	0	0.39	0.41	0.10	0.09	0.27	0.22	0.45	0.36
24	0.01	0.01	0.20	0.22	0.10	0.12	0.17	0.17	0.37	0.35

[a] Data from Laboratories 3 and 4 were invalid because the laboratories were considered technical outliers.

[b] Data rejected on the basis of the Cochran test.

M. Castegnaro, Unit of Gene-Environment Interactions, IARC, Lyon, France

K. Nuotio, Finnish Customs Laboratory, Espoo, Finland

E. Kakouri, State General Laboratory, Nicosia, Cyprus

H. Kretschmer, Institut für Getreideverarbeitung GmbH, Bergholz-Rehbrucke, Germany

H. Bresch, Bundesforschungsanstalt für Ernahrung, Karlsruhe, Germany

J. Miller, UNILEVER Research Colworth SEAC Contaminants, Bedford, UK

S. Patel, RHM - Technology Limited, The Lord Rank Centre, High Wycombe, UK

M.C. Spanjer, Food Inspection Service, Alkmaar, The Netherlands

W. Steiner, Migros Scientific Services, Zurich, Switzerland

E. Usleber, Institute for Hygiene and Technology of Food of Animal Origin, University of Munich, Munich, Germany

R. Tiebach, Bundesinstitut für Gesundheitlichen Verbraucherschutz und Veterinärmedizin, Berlin, Germany

J. Gardikis, General Chemical State Laboratory Division of Environment, Athens, Greece

K. Jørgensen, Danish Veterinary and Food Administration, Søborg, Denmark

H. Lew, Bundesamt für Agrarbiologie, Linz, Austria

A. Pittet, NESTLE Research Centre Quality & Safety Assurance Department, Lausanne, Switzerland

A. Pietri, Università Cattolica del Sacro Cuore, Piacenza, Italy

References

(1) Shephard, G.S., Thiel, P.G., Stockenstrom, S., & Sydenham, E.W. (1996) *J. AOAC Int.* **79**, 671–687

(2) Visconti, A. (1996) *Adv. Exp. Med. Biol.* **392**, 193–204

Downloaded from https://academic.oup.com/jaoac/article/84/6/1828/5656541 by guest on 16 August 2022

(3) Caramelli, M., Dondo, A., Cantini Cortellazzi, G., Visconti, A., Minervini, F., Doko, M.B., & Guarda, F. (1993) *Ippologia* **4**, 49–53

(4) Marasas, W.F.O. (1995) *Nat. Toxins* **3,** 193–198

(5) Harrison, L.R., Colvin, B.M., Greene, J.T., Newman, L.E., & Cole, J.R. (1990) *J. Vet. Diagn. Invest.* **2,** 217–221

(6) Visconti, A., Marasas, W.F.O., Miller, J.D., & Riley, R. (1999) *Proceedings of Third Joint FAO/WHO/UNEP International Conference on Mycotoxins*, March 3–6, Tunis, Tunisia, http://www.fao.org/WAICENT/FAOINFO/ECONOMICS/ESN/mycoto/papers/myco5a.pdf

(7) U.S. NTP (1999) *NTP TR 496, NIH Publication No. 99-3955* U.S. Department of Health and Human Services, Washington, DC, 146 pp

(8) Rheeder, J.P., Marasas, W.F.O., Thiel, P.G., Sydenham, E.W., Shephard, G.S., & Van Schalkwyk, D.J. (1992) *Phytopathology* **82**, 353–357

(9) Sydenham, E.W., Shephard, G.S., Thiel, P.G., Stockenstrom, S., Snijman, P.W., & VanSchalkwyk, D.J. (1996) *J. AOAC Int.* **79**, 688–696

(10) Schneider, E., Usleber, E., & Maertlbauer, E. (1995) *J. Agric. Food Chem.* **43**, 2548–2552

(11) Scott, P.M., & Lawrence, G.A. (1996) *Food Addit. Contam.* **13**, 823–832

(12) Meister, U. (1999) *Mycotoxin Res.* **15**, 13–23

(13) Visconti, A., Solfrizzo, M., De Girolamo, A., & Boenke, A. (1999) Progress Report, Contract No. SMT4-CT97-2193, EUR 19096 EN, edited by the European Commission, Directorate General XII—Science, Research and Development, Luxembourg, 156 pp

(14) Solfrizzo, M., De Girolamo, A., & Visconti, A., Sizoo, E., & van Egmond, H.P. (2000) in *Proceedings of the X International IUPAC Congress on Mycotoxins and Phycotoxins,* May 21–25, 2001, Guarujà, Brazil, pp 87–94

(15) *AOAC Official Methods Program* (1995), *Associate Referee's Manual on Development, Study, Review, and Approval Process*, AOAC INTERNATIONAL, Gaithersburg, MD

(16) CEN (European Committee for Standardization) (1999) CEN/TC 275/WG5 Food Analysis—Biotoxins—Criteria of Analytical Methods of Mycotoxins, *CEN Report CR 13505,* Berlin, Germany, 10 pp

(17) Horwitz, W., & Albert, R. (1991) *J. Assoc. Off. Anal. Chem.* **74**, 718–744

Downloaded from https://academic.oup.com/jaoac/article/84/6/1828/5656541 by guest on 16 August 2022

Downloaded from https://academic.oup.com/jaoac/article/84/6/1828/5656541 by guest on 16 August 2022