

Determination of Fumonisin B₁ and B₂ in Corn by LC/MS with Immunoaffinity Column Cleanup: Interlaboratory Study

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An interlaboratory validation study was conducted to establish the method performance characteristics of an immunoaffinity column (IAC) cleanup procedure followed by LC/MS for the determination of fumonisins B₁ (FB₁) and B₂ (FB₂) and combined FB₁ + FB₂ in corn. The test portion is extracted with acetonitrile–methanol–water (25 + 25 + 50). The extract is filtered, diluted with phosphate-buffered saline solution, and applied to an IAC. FB₁ and FB₂ are removed with methanol, followed by water, then directly determined by RPLC with MS detection using selected-ion monitoring of two characteristic ions in each case. Naturally contaminated corn samples were milled to a fine powder and mixed to produce three samples with target levels of combined FB₁ + FB₂ ranging from 350 to 4000 g/kg. Of 15 initially participating laboratories, two failed to report results and another did not follow the prescribed method. Thus, valid results were obtained from 12 participants located in 11 countries. Statistical analysis of the results produced RSD_r values of 4.6–11.9, 1.9–12.6, and 1.4–11.5% for FB₁, FB₂, and combined FB₁ + FB₂, respectively; the corresponding RSD_R values were 19.8–23.8, 18.2–25.5, and 18.8–23.2%. The three concentration levels of combined FB₁ + FB₂ were 534, 1194, and 1954 g/kg. HorRat values for r and R were all <2.0, indicating that the method is suitable as a regulatory method for the enforcement of European Union limits for fumonisins in corn.

Fumonisin mycotoxins are fungal metabolites produced by several *Fusarium* species; they occur naturally in corn (1, 2). As a consequence of their stability, they can be transferred into processed corn products, including baby food (3, 4). Concern over potential human exposure to fumonisins has recently led the European Commission to regulate levels of fumonisins in corn and corn-based foods (5), with limits applied to the total content of fumonisin B₁ + fumonisin B₂ (FB₁ + FB₂). These regulations—covering processed and unprocessed corn, breakfast cereals, snacks, and baby foods—have some complexity in that different limits are applied, depending on intended use and type of finished products, with limits ranging from 200 to 4000 g/kg. Different limits also apply to different milled fractions with different Combined Nomenclature (CN) codes (6). In the United States, there are recommended maximum levels (rather than regulatory limits) for fumonisins in human food and in animal feed (7). Limits recommended by the U.S. Food and Administration are expressed as total fumonisins, but the total is the sum of FB₁ + FB₂ + FB₃—in contrast with European Union (EU) limits, which are based only on the total of FB₁ + FB₂. The advisory limits in the United States are also significantly higher than the EU limits, ranging from 2000 to 4000 g/kg for fumonisins in human food, and from 5000 to 100 000 for fumonisins in animal feed, depending on the intended species (8).

For analyses for regulatory purposes, AOAC *Official Method*SM 995.15 for FB₁, FB₂, and FB₃ in corn uses a strong anion-exchange column cleanup, precolumn *o*-phthalaldehyde derivatization, and LC analysis with fluorescence detection. This fully validated method (7) gives recoveries of 75–87% and RSD_R values of 14–27% for the three fumonisins in the concentration range of 800–12 800 g/kg (typical levels found in corn). This method performs less well when applied to processed corn products at lower levels of contamination, giving poor recoveries and precision values. The introduction of immunoaffinity columns (IACs) for fumonisins (9, 10) substantially improved

Received June 2, 2009. Accepted by AP July 28, 2009.

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cleanup, which is particularly important at lower levels of contamination, and has generally provided more robust methods for determining fumonisins in processed corn products like cornflakes (11). AOAC *Official Method 2001.04* (10) uses acetonitrile–methanol–water (25 + 25 + 50, v/v/v) for extraction of FB₁ and FB₂ from cornflakes, giving recoveries of 97–110%, repeatability values of 8–22%, and reproducibility values of 26–35% for FB₁ and FB₂ at contamination levels ranging from 130 to 920 g/kg. The method was extended to extruded corn, muffins, and infant formula when in-house validation demonstrated that acetonitrile–methanol–water (25 + 25 + 50, v/v/v) gave satisfactory recoveries from these processed corn matrices (12).

Increasingly, the use of MS (LC/MS or LC/MS/MS) is being advocated in the mycotoxin field for multimycotoxin analysis, frequently without sample cleanup, by making the determination directly with a crude sample extract (13–17). Although a number of multimycotoxin procedures have been published for the simultaneous determination of as many as 38 mycotoxins, including FB₁, FB₂, and hydrolyzed FB₁ (14), few papers have reported in-house validation. LC/MS/MS has also been combined with affinity column cleanup using multitoxin columns for the simultaneous determination of 11 toxins including FB₁ and FB₂ (18). Other methods for the LC/MS determination of fumonisins have used on-line IAC cleanup (19) and have critically compared fluorescence detection with LC/MS and LC/MS/MS (20). To our knowledge, no published interlaboratory validation studies of either LC/MS or LC/MS/MS methods for mycotoxins in food or feed have been published, as required for use in an accredited laboratory or for regulatory purposes. In a recent paper (21), we partially addressed this shortcoming for the determination of fumonisins by conducting a single-laboratory validation of an IAC cleanup procedure followed by LC/MS using selected-ion monitoring (SIM) of two ions. This method avoids the need for any precolumn derivatization, and was validated for the EU regulatory limits of total fumonisin levels of 800 g/kg for snack foods and breakfast cereals and 200 g/kg for infant foods (21), with recoveries of 90–101%, and repeatability values of 2.8 to 7.1%. In this paper, we have extended our previous work by reporting the results of a full interlaboratory study of this LC/MS method for fumonisins.

Interlaboratory Study Design

The study was conducted by using blind duplicates of corn naturally contaminated at four different levels (eight randomly coded containers) and a corn sample designated as the blank (four containers labeled “blank”) to be used for recovery analyses. Random identification numbers were assigned to each of the blind duplicate test samples. Participants were supplied with 25 IACs (R-Biopharm Rhône Ltd, Glasgow, UK), surplus Food Analysis Performance Assessment Scheme (FAPAS[®]) test material PT Sample No. 2246 (55 g) with assigned values of FB₁ = 1650 g/kg and

FB₂ = 461 g/kg, 15 phosphate-buffered saline (PBS) tablets, 12 glass syringe barrels (reservoirs), 10 plastic adaptors, three hand pumps, one Zorbax Eclipse XDB-C18 HPLC column, 4.6 × 100 mm, 3.5 μm (Agilent Technologies, Waldbronn, Germany), and 12 PTFE membrane filters. Collaborators were separately supplied by R-Biopharm Rhône Ltd with standard solutions to be used for calibration purposes, marked “Fumonisins Calibrant,” i.e., FB₁ 8 g/mL and FB₂, 2.4 g/mL in acetonitrile–water (50 + 50). Four spiking ampoules containing FB₁ and FB₂ in acetonitrile–water and labeled A, B, C, and D were also supplied.

Collaborators

Fifteen laboratories agreed to participate in this interlaboratory study and received test materials, although only 13 laboratories submitted valid results; one participant did not follow the prescribed method, and these results were excluded. The participants who submitted results were as follows: 10 from Europe, one from Canada, one from Turkey, and one from the United States. These results were used in the statistical analysis.

(a) *Test sample preparation.*—Corn materials that were naturally contaminated with fumonisins were used to prepare test materials with different target concentrations. The corn materials were first milled with a Romer mill (Romer Labs, Vienna, Austria) and then with a Centrifugal Mill Z200 (Retsch, Haan, Germany) by using a 1 mm mesh-size sieve at 18 000 rpm. Three corn samples containing different nominal levels of fumonisins were blended in kilogram amounts in various proportions to obtain three test materials with different target concentrations. Blank material was prepared from a corn sample that was found to contain fumonisins at <20 g/kg (FB₁ + FB₂). All blended materials were then homogenized in a Labormischer Typ L20 (Lödige, Paderborn, Germany) for 10 min.

All materials were analyzed to confirm target levels of fumonisins. The method used for confirmation of target levels was based on an unpublished HPLC method involving postcolumn derivatization, fluorescence detection, and IAC cleanup that was previously validated (single-laboratory validation). The total levels of FB₁ + FB₂ in the three samples were found to be 390, 670, and 1620 g/kg. The test materials were divided into portions of approximately 20 g (naturally contaminated) and 50 g (blank), packaged in polyethylene bottles, and kept frozen at –18 °C until they were distributed to collaborators.

(b) *Test material homogeneity.*—To assess homogeneity of the bulk test materials, 10 random portions were selected from each sample and analyzed in duplicate. The homogeneity of the bulk material was evaluated by using a one-way analysis of variance (ANOVA) and an *F*-test, as well as a comparison of the sampling SD with the target SD. The homogeneity of the individual portions of the test materials was established by random selection of 10 units from each of the three levels, followed by analysis in duplicate. Homogeneity was assessed by using an ANOVA and an

F-test, as well as a comparison of the sampling SD with the target SD.

(c) *FAPAS test material (TM2246)*.—This item was supplied primarily to confirm that the chromatographic conditions used by the participants provided adequate separation and that there were no coextractives that might cause ion suppression. It was suggested that participants follow the extraction procedure by using the procedures that were provided. The results for analysis of TM2246 were effectively used to test that participants could obtain quantitative results within the satisfactory range stipulated with the test certificate, but this analysis was not a formal part of the interlaboratory study, nor was TM2246 a qualifying practice sample.

(d) *Spiking procedure*.—Ten grams of the blank test material were weighed to the nearest 0.1 g into four different extraction containers marked A, B, C, and D. (For this experiment, a smaller sample size was used because the homogeneity of the test sample was not a critical issue, and the amount of fumonisin solution for spiking could be decreased; it was important that the sample-to-solvent ratio should remain the same.) To each of these extraction containers was added 500 μ L of the corresponding spiking solution (A, B, C, or D). The solvent was allowed to evaporate for approximately 60 min, but not for several hours or overnight. Spiked materials were extracted following the procedure described in the method, with the one modification that only 50 mL extraction solvent (20 g/100 mL = 10 g/50 mL) and 1 g NaCl were used in order to obtain the same sample-to-solvent ratio.

METHOD

(Applicable to the determination of FB₁ and FB₂ in corn and at levels of >500 ng/g for total fumonisins B₁ + B₂.)

Principle

The test portion is extracted with acetonitrile–methanol–water (25 + 25 + 50, v/v/v) by using an orbital shaker. The extract is filtered, diluted with PBS solution, and applied to an IAC. The purified fumonisins (FB₁ + FB₂) are eluted from the IAC with methanol and directly determined by RPLC with MS detection using SIM of two characteristic ions in each case.

Apparatus

Common laboratory apparatus and, in particular, the following are used:

(a) *Filter paper*.—With 24 cm diameter, prefolded (Whatman No. 4).

(b) *Reservoir*.—Ten mL with Luer tip connector for the IAC.

(c) *Hand pump*.—Twenty mL syringe with rubber stopper for the IAC.

(d) *Volumetric glassware*.—Including 3, 5, 10, 20, and 1000 mL with an accuracy of \pm 0.5%.

(e) *LC/MS system*.—A single-quadrupole, triple-quadrupole, or ion-trap mass spectrometer equipped with

electrospray ionization in the positive ion mode coupled to an HPLC system consisting of an HPLC pump, an autosampler, and a temperature-controlled column oven with computerized instrument control/data collection. Ensure that the LC system is operating efficiently and that the mass spectrometer is properly tuned for monitoring the ions at *m/z* 706 and 707 for FB₂ and at *m/z* 722 and 723 for FB₁. Inject the 10.4 ng/mL mixed standard (Fumonisin Calibrant) under the optimized conditions. Check that the S/N of the two quantification ions (*m/z* 722 and 706) is 10 in both cases, and measure the ion ratios of FB₁ (722/723) and FB₂ (706/707), 2.5 ± 0.2 and 2.6 ± 0.2 , respectively; check the retention times of FB₁ and FB₂ (ca 3.4 and 7.8 min, respectively), and establish the repeatability of retention times (*n* = 10), which should be \pm 10% or better.

(f) *LC pump*.—Suitable for flow rate of 0.5 ± 0.005 mL/min and gradient program at a flow rate of 0.5 mL/min.

(g) *Injection system*.—Total loop injection valve with a 20 μ L loop. The RSD of a series of ten 20 μ L injections of a standard solution of total fumonisins at 650 ng/mL should be \leq 5%.

(h) *RPLC columns*.—Agilent Technologies Zorbax XDB-C18, 3.5 μ m (4.6 \times 100 mm) operated at 40 $^{\circ}$ C.

(i) *Disposable filter unit (0.45 μ m)*.—Cellulose or cellulose nitrate can be used. Before use, it must be verified that no fumonisin losses occur during filtration (recovery testing), because it is possible that various filter materials can retain fumonisins.

(j) *Analytical balance*.—Capable of weighing \geq 0.1 mg.

(k) *Laboratory balance*.—Capable of weighing \geq 0.01 g.

(l) *Calibrated microliter syringe(s) or pipet(s)*.—10–5000 μ L.

(m) *Homogenizer*.—Heidolph Silent crusher M Ultra Turrax (Donau, Germany).

Reagents

All reagents shall be of recognized analytical grade.

(a) *PBS tablets, pH 7.4*.—Oxoid (Hampshire, UK). Dissolve 1 tablet in 100 mL water in a volumetric flask.

(b) *Methanol*.—LC grade.

(c) *Acetonitrile (pure)*.—LC grade.

(d) *Methanol (pure)*.

(e) *Water*.—LC and LC/MS grade.

(f) *NaCl*.—Extra pure <99.5%.

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

(h) *FB₁ and FB₂ crystalline standard*.—R-Biopharm Rhône Ltd. One vial contains the equivalent of FB₁ at 100 μ g/mL and FB₂ at 30 μ g/mL, in a crystalline form, when dissolved in a suitable volume of solvent (product code: P62A).

(i) *Fumoniprep IACs*.—R-Biopharm Rhône Ltd. The Fumoniprep columns use a monoclonal antibody that exhibits highly selective specificity for FB₁ and FB₂. The column contains monoclonal antibodies that have equal affinity for FB₁ and FB₂. The column has a minimum capacity of 10 μ g for any combination of both fumonisins (FB₁ + FB₂) and gives

recoveries of >70%. A column load of 10 g combined FB₁ + FB₂ gives a recovery of >90%.

(j) *Mobile phase solvents*.—(A) 0.005 M ammonium acetate (pH 3.18, adjusted with acetic acid). This solvent can be prepared by dissolving 0.385 g ammonium acetate in 1.00 L water. The pH should be adjusted with 100% acetic acid (ca 16 mL). (B) Methanol. The mobile phase solvents should be degassed.

(k) *Mixed fumonisin standard stock solution*.—Label this solution FUMO-ST1. To prepare an LC calibration curve from the crystalline fumonisin standard FB₁ at 100 g/mL and FB₂ at 30 g/mL, reconstitute the crystalline standard with 2 mL acetonitrile–water (1 + 1, v/v), date, and store at 2–4 °C when not in use. Note the expiration date of the standards, and do not use after the stated date because the composition of the standards cannot be guaranteed after that date.

(l) *Working standard solutions*.—Prepare dilutions of the stock solution (FUMO-ST1) in order to prepare the working standard solutions. These solutions are stored at 0–4 °C when not in use. To prepare the working standard solution, dilute the standard solution containing FB₁ at 100 g/mL and FB₂ at 30 g/mL with methanol to obtain a final concentration of FB₁ at 8 g/mL and FB₂ at 2.4 g/mL (dilute 400 L FUMO-ST1 to 5 mL with methanol). Label this solution FUMO-DS1. This solution must be freshly prepared on the day of use.

(m) *Dilution solvent*.—For dilution of calibrant solutions; methanol–water (50 + 50, v/v).

(n) *Working calibrant solutions for LC*.—Pipet 1.500 mL working solution (FUMO-DS1) into a 10 mL volumetric flask. Dilute contents of flask to volume with acetonitrile–water (1 + 1, v/v), and shake contents well to obtain a solution containing FB₁ at 1.2 g/mL and FB₂ at 0.36 g/mL. This is the calibrant stock solution. Use the calibrant stock solution to pipet (with either zero displacement pipets or calibrated syringes) the volumes listed in Table 1 into a set of 5 mL calibrated volumetric flasks, dilute to volume with dilution solvent, and shake contents well.

Extraction

Weigh, to the nearest 0.1 g, ca 20 g test portion into a beaker, and add 2 g NaCl and 100 mL extraction solvent. Homogenize, using an orbital shaker, for 60 ± 1 min to ensure the sample is mixed with the solvent. Filter the sample through Whatman No. 4 filter paper, and collect the filtrate in a 100 mL conical flask. Transfer 10 mL clear filtrate to a flask, add 40 mL PBS solution, and shake contents of flask.

IAC Cleanup

Pass 10.0 mL aliquot of PBS-diluted filtrate through the IAC at a flow rate of approximately 1 drop/s (approximately 3 mL/min) or by gravity. Wash the IAC with 10 mL PBS solution at a maximum flow rate of 5 mL/min. Wait for 30 s, and then pass 5 mL water through the IAC. Dry the column by applying a light vacuum for 5–10 s, or pass air through the IAC for 10 s by using a syringe.

Elution of Fumonisin by a Two-Step Procedure

Apply 2.5 ± 0.03 mL methanol at a rate of 1 drop/s to the IAC, wait for 30 s, then pass 2.5 ± 0.03 mL water through the IAC. Collect entire eluate in a 5 mL volumetric flask. Dilute contents of flask to volume with water, and shake contents of flask well. If the solution is clear, it can be used directly for LC/MS analysis. If the solution is not clear, pass it through a disposable filter unit (0.45 μm) before LC/MS injection. The injection by total loop mode guarantees maximum accuracy. It is recommended (depending on the injection system, i.e., manual injection) that a sample volume of three times the size of the injection loop be used, and that 2/3 of this volume be injected into the valve to ensure that the middle portion remains in the injection loop. Thus, the loop is rinsed with the injection solvent while enough solvent remains in the valve.

RPLC Parameters

Column temperature: 40 °C; flow rate: 0.5 mL/min; injection volume: 20 L; post time: 5 min; Solvent A: 0.005 M ammonium acetate, pH 3.18; Solvent B: methanol. The recommended gradient program is as follows: from 0 to 2 min, 35% Solvent A–65% Solvent B; from 2 to 15 min, changing to 5% Solvent A–95% Solvent B; from 15 to 16 min, changing to 35% Solvent A–65% Solvent B; from 16 to 20 min, maintained at 35% Solvent A–65% Solvent B.

LC/MS Analysis

Suitable LC/MS operating conditions—with the use of an Agilent Technologies 1100 HPLC system (Waldbronn, Germany) consisting of a binary pump, an autosampler, and a temperature-controlled column oven, coupled to an Agilent Technologies 1100 MS detector equipped with an electrospray interface—were as follows: drying gas flow, 10 L/h; nebulizer pressure, 50 psig; drying gas temperature, 350 °C; capillary voltage, 3.5(+) 3(–) kV; and fragmentor voltage, 150 eV at positive polarity. However, other conditions as appropriate for different LC/MS and

Table 1. Preparation of working calibrant solutions^a

Working standard	Volume of working solution, L	Volume of dilution solvent, L	Concn, ng/mL		Final concn of standard, ng/mL
			FB ₁	FB ₂	
1	15	4985	3.60	1.08	4.68
2	65	4935	15.50	4.68	20.18
3	115	4885	27.60	8.28	35.88
4	165	4835	39.60	11.88	51.48
5	265	4735	63.60	19.08	82.68
6	315	4685	75.60	22.68	98.28
7	365	4635	87.60	26.28	113.88

^a The diluted standard solution of total fumonisins should be freshly prepared on the day of use and used within 24 h.

LC/MS/MS systems were selected by participants and are shown in Table 2. For single-quadrupole operation, SIM was recommended for m/z 722 and 723 (FB₁) and m/z 706 and 707 (FB₂) with a dwell time of 218 ms. When the HPLC column provided and the recommended HPLC conditions were used, the identification of FB₁ was based on a retention time of 3.8 + 0.05 min and an ion ratio of $723/722 = 2.5 \pm 0.1$. The identification of FB₂ was based on a retention time of 8.4 + 0.05 min and an ion ratio of $706/707 = 2.6 \pm 0.05$. Some participants with triple-quadrupole instruments indicated that they had difficulties in recalibrating and tuning the tandem instrument as a single-quadrupole instrument because this takes time and can reduce sensitivity. Thus, the Study Director agreed to allow operation in the MS/MS mode, provided that the sensitivity and specificity criteria were not compromised. Nevertheless, all results that were included in the statistical analysis were based on quantification of parent ions (molecular ion plus one fragment ion), rather than on any multiple-reaction monitoring data. Table 2 gives details of the instrumentation used by the participants and indicates the selected operating conditions.

Quality Assurance

It was expected that participants would follow the general principles of analytical quality assurance as might be required in adherence to ISO 17025. For the study itself, once the instrument settings were shown to be acceptable, participants were asked to inject the extracts in the following suggested sequence: Injection 1: reagent blank; injections 2–8: calibrants; injections 9–16: eight test samples (individually coded); injection 17: fifth level of calibration standard (total 82.68 ng/mL, FB₁ + FB₂); injections 18–21: spiking levels A–D; injection 21: reagent blank. Injections were to be run in duplicate. Participants were advised to store the extracts at 20 °C if the analyses could not be performed immediately after sample preparation.

Results and Discussion

Analysis of Blank Samples

The sample that was supplied to be used as a nominal blank was known before the study was undertaken to contain low levels of FB₁ and FB₂. In fact, it is very difficult to procure corn in which the levels of fumonisins are below the LODs of today's sensitive detection techniques. The results from the analyses of the blank samples are given in Tables 3–5 for FB₁, FB₂, and total fumonisins, respectively. All participants reported that fumonisins were below their LODs, or they found low measurable amounts, 10–76 ng/g for FB₁, 5–26 ng/g for FB₂, and corresponding levels of 10–100 ng/g for total fumonisins. All pairs of results from analyses of blind duplicate blank samples showed good agreement, including those results reported by the participants that were below their LODs, which were similarly consistent. The mean levels for the blank samples were found to be 34 ng/g for FB₁, 15 ng/g for FB₂, and 49 ng/g for total fumonisins. Unfortunately, because there were only seven data sets for the blank samples

(excluding reported values below the LODs), it was not possible to calculate reliable precision data for these samples naturally contaminated with low levels of FB₁ and FB₂.

Recovery Experiments

The study design involved recovery experiments carried out by participants who analyzed four blank samples of corn spiked with calibrants of unspecified concentrations. The four spiked samples represented two pairs of blind duplicates spiked at levels of 230 and 800 ng/g for FB₁, and 70 and 240 ng/g for FB₂, respectively. The results of these experiments from 11 of the 13 participants showed recoveries ranging from 81 to 138% for FB₁, 71 to 138% for FB₂, and 77 to 138% for total fumonisins. These recoveries were background-corrected for average levels of fumonisins found in the blank samples, which made 14 and 21% contributions of FB₁ and FB₂, respectively, to the lower spiking level and 4 and 6% contributions of FB₁ and FB₂, respectively, to the higher spiking level. For nine of the participants who achieved recoveries in the range of 71–130%, the blind pairs showed good agreement for duplicate injections at each spiking level. In the case of the remaining four participants, the recovery results were excessively and inexplicably high, ranging from 138 to 306% for FB₁, 138 to 427% for FB₂, and 138 to 330% for total fumonisins. Participant 3, who reported recoveries of 88–100% and 71–94% for FB₁ and FB₂, respectively, in this study, also completed a separate spiking experiment with separate standards at the same time and reported recoveries of 94% for FB₁ and 88% for FB₂.

Despite these apparent anomalously high recoveries, the results for the blind duplicates were nevertheless consistent between pairs. No satisfactory explanation could be found for these high results, although it should be noted that the recoveries were based on spiking 10 g blank rather than the 20 g normally used, although participants were advised to adjust all other conditions accordingly. Further use of the recovery data set was therefore abandoned. However, it was clear from the analysis of the FAPAS test materials for which good agreement with the assigned values was achieved in all cases, and from the consistent results obtained in the study for the analysis of the samples of naturally contaminated corn, that good recoveries were achieved by all participants. The failure to achieve consistent recovery data from analysis of the spiked samples was thus attributed to some problems associated either with the spiking itself or with the spiking solutions. Further evidence that the method gives good recoveries can be derived from the single-laboratory validation in which recoveries from 90 to 101% were reported (21).

Statistical Analysis of Results

Despite the problems discussed above with respect to the recovery experiments, good precision data were obtained for the three blind duplicate sets of naturally contaminated corn. The individual results for the 12 valid data sets are given in Tables 3–5 for FB₁, FB₂, and total fumonisins (FB₁ + FB₂), respectively. Tables 3–5 include the results for Participant 2,

Table 2. Analytical instruments and columns used in the interlaboratory study

Lab	Liquid chromatograph	Type of pump	Column	Mass spectrometer	Type of mass spectrometer	Nebulizer gas pressure/flow	Temp. probe, C	Temp. cone/capillary, C	Spray voltage, kV	Source lens potentials, V
1	Agilent 1100	Quarternary gradient low pressure	Agilent, Zorbax XDB-C18 RR, 3.5 m, 4.6 100 mm	MDS SCIEX, API 2000	Triple-quadrupole	NA ^a	400	0	5500	70
2	Agilent	Quarternary gradient high pressure	Waters Atlantis T3, 5 m, 150 3 mm	Waters Quatro Premier XE	Triple-quadrupole	900 L/h	350	130	1	19000
3	Agilent 1100	Binary gradient high pressure	Atlantis dC18, 3 m, 2.1 50 mm	Applied Biosystems	Triple-quadrupole	NA	350	—	5	—
4	Waters Acquity UPLC	Binary gradient high pressure	Agilent, Zorbax XDB-C18 RR, 3.5 m, 4.6 100 mm	Waters/Micromass Quatro Micro	Triple-quadrupole	500 L/h	350	120	3	0.2
5	Waters Alliance 2695	Quarternary gradient high pressure	Agilent, Zorbax XDB-C18 RR, 3.5 m, 4.6 100 mm	Waters/Micromass Quattro Ultima Tandem MS	Single-quadrupole	550 L/h	350	120	3.4	110
7	Agilent 1100	Binary gradient high pressure	Phenomenex, LUNA 2 C-18 3 m, 2 150 mm	Applied Biosystems, API 5000	Triple-quadrupole	50 L/h	700	150	5	110
8	Agilent 1200	Binary gradient high pressure	Agilent, Zorbax XDB-C18 RR, 3.5 m, 4.6 100 mm	Agilent Technologies 6410	Triple-quadrupole	50 L/h	350	0	3.5	3.7
9	Gilson 307	Binary gradient high pressure	Agilent, Zorbax XDB-C18 RR, 3.5 m, 4.6 100 mm	Agilent 1100 MSD	Single-quadrupole	50 psi	350	—	3.5	—
10	Waters, HPLC Alliance 2695	Quarternary gradient low pressure	Agilent, Zorbax XDB-C18 RR, 3.5 m, 4.6 100 mm	Waters, Quatro Premiere XE	Triple-quadrupole	100 L/h	320	150	4.5	0.1
11	Agilent 1100 Series, micro	Binary gradient high pressure	Agilent, Zorbax XDB-C18 RR, 3.5 m, 4.6 100 mm	Applied Biosystems, QTrap	Hybrid triple-quadrupole/linear ion-trap	10 psi	350	—	4.5	55
12	Shimadzu SIL-HTc	Quarternary gradient low pressure	Agilent, Zorbax XDB-C18 RR, 3.5 m, 4.6 100 mm	Micromass quatro ultima	Single-quadrupole	—	350	120	2.99	—
13	Perkin-Elmer Series 200 micro pump	Binary gradient low pressure	Agilent, Zorbax XDB-C18 RR, 3.5 m, 4.6 100 mm	Applied Biosystems, API 3000	Triple-quadrupole	3.00	—	400	4.5	—
15	Agilent 1100	Binary gradient low pressure	Agilent, Zorbax XDB-C18 RR, 3.5 m, 4.6 100 mm	Agilent G1946 D SL	Single-quadrupole	—	350	120	2.99	—

^a NA = Not applicable.

who chose not to follow the prescribed procedure; the results from this participant were therefore excluded from the statistical analysis. The results for Participant 2 are nevertheless of interest and are discussed further below. Precision estimates were obtained by using the one-way ANOVA approach according to the Harmonized Protocol of the International Union of Pure and Applied Chemistry (22). Details of the average analyte concentrations, the SDs for repeatability (S_r) and reproducibility (S_R), the RSD_r and RSD_R values, the number of statistical outlier laboratories, and the HorRat values are given in Table 6. The results for Participant 8 were excluded by using the Cochran test for FB_1 only for sample level 1, which had a mean level of 443 ng/g; thus, the precision data were determined on the basis of 11 sets of results. The results for Participant 13 were excluded using Cochran's test for FB_2 only for level 2, which had a mean level of 197 ng/g, and the precision data were again determined based on 11 sets of results. For the statistical analysis of the results for total fumonisins ($FB_1 + FB_2$), results for Participant 8 were excluded for sample levels 1 and 2, and the results for Participant 13 were excluded from level 2 calculations. Thus, for total fumonisins the statistical analysis was based on 11 data sets for sample level 1 and 10 data sets for sample level 2. For levels 1, 2, and 3 for FB_1 , FB_2 , and total fumonisins, the statistical analysis was based in all cases on 12 data sets. None of the blind duplicate data sets were identified as outliers on the basis of the Grubbs test.

Table 6 shows that the method has RSD_r values of 5–12, 2–13, and 1.4–11% for FB_1 , FB_2 , and total fumonisins at levels of 450–1600, 90–230, and 530–1900 ng/g, respectively. These results are comparable with the

within-batch precision for each of three consecutive analyses which ranged from 2.8 to 5.7% for FB_1 and from 3.3 to 7.1% for FB_2 . The between-day (batch-to-batch) variabilities were 4.2 and 5.0% for FB_1 and 5.1 and 5.2% for FB_2 at the lower and higher levels of contamination, respectively, which were obtained in the single-laboratory method validation (20). The RSD_R data ranged from 20 to 24, from 18 to 25, and from 18 to 23% for FB_1 , FB_2 , and total fumonisins at levels of 450–1600, 90–230, and 530–1900 ng/g, respectively. HorRat values, which were all <2.0, clearly indicate the acceptability of this method as an AOAC *Official Method* in terms of its performance characteristics. These precision data compare favorably with previously published HPLC collaborative study data, where for example RSD_r ranged from 18 to 27%, and the RSD_R values ranged from 22 to 29% for FB_1 and FB_2 in corn (11).

Difficulties Associated with the Collaborative Study

The original single-laboratory validation was carried out with a single-quadrupole LC/MS instrument, and it was felt that with IAC cleanup there was little need to carry out MS/MS to improve specificity. However, in practice for this study it was difficult to find a sufficient number of laboratories equipped only with single-quadrupole instruments. In Table 2, we have listed the HPLC and MS instruments used by the 13 participants in this study. In fact, only Participants 5, 9, and 15 used a single-quadrupole mass spectrometer, with nine participants using a triple-quadrupole mass spectrometer, and one participant using a hybrid ion-trap instrument. Instruments were supplied by Waters (five participants), Applied Biosystems (four participants), Agilent

Table 3. Interlaboratory study results for determination of fumonisin B₁ (ng/g) in corn

Lab	Blank		Level 1 (low)		Level 2 (medium)		Level 3 (high)	
	Duplicate							
	1	2	1	2	1	2	1	2
1	47	40	462	443	943	971	1615	1684
2 ^a	51 ^a	43 ^a	639 ^a	683 ^a	1130 ^a	1080 ^a	2130 ^a	2570 ^a
3	20	21	370	375	843	862	1576	1038
4	<LOD	<LOD	322	360	837	834	1103	1111
5	36	38	446	471	1000	983	1725	1723
7	76	76	500	490	959	954	1598	1626
8	<LOD	<LOD	387 ^b	530 ^b	1030	1193	2112	1870
9	<35	<35	481	438	1060	1054	1931	1712
10	<45	<45	304	357	710	754	1397	1186
11	<LOD	<LOD	461	427	1101	1098	1759	1693
12	41	36	515	541	1079	1115	1906	1234
13	13	10	334	340	647	822	1265	1287
15	43	43	656	645	1430	1417	2407	2452

^a Excluded because the participant failed to follow the method.

^b Identified as an outlier by the Cochran test.

Table 4. Interlaboratory study results for determination of fumonisin B₂ (ng/g) in corn

Lab	Blank		Level 1 (low)		Level 2 (medium)		Level 3 (high)	
	Duplicate							
	1	2	1	2	1	2	1	2
1	26	21	104	100	195	198	333	340.5
2 ^a	<10 ^a	<10 ^a	83 ^a	78 ^a	163 ^a	178 ^a	270 ^a	304 ^a
3	5	8	56	71	158	156	324	216
4	<LOD	<LOD	58	68	162	173	296	282
5	8	8	102	105	213	200	348	343
7	24	24	125	109	203	199	349	361
8	<LOD	<LOD	74	113	228	227	477	509
9	<20	<20	104	93	215	216	388	337
10	<58	<58	64	74	141	140	287	246
11	<LOD	<LOD	98	74	193	193	292	246
12	14	13	97	101	190	190	326	223
13	<LOD	<LOD	72	63	111 ^b	165 ^b	261	235
15	<LOD	<LOD	134	128	272	273	431	428

^a Excluded because the participant failed to follow the method.

^b Identified as an outlier by the Cochran test.

Table 5. Interlaboratory study results for determination of total fumonisins (fumonisins B₁ + B₂; ng/g) in corn

Lab	Blank		Level 1 (low)		Level 2 (medium)		Level 3 (high)	
	Duplicate							
	1	2	1	2	1	2	1	2
1	73	62	566	543	1138	1169	1948	2025
2 ^a	51 ^a	43 ^a	722 ^a	761 ^a	1293 ^a	1258 ^a	2400 ^a	2874 ^a
3	25	29	425	446	1001	1018	1900	1255
4	<LOD	<LOD	380	429	999	1006	1399	1394
5	44	46	548	576	1213	1184	2073	2065
7	100	100	626	598	1162	1153	1948	1987
8	<LOD	<LOD	461 ^b	644 ^b	1258 ^b	1421 ^b	2588	2379
9	<55	<55	585	532	1275	1270	2319	2049
10	<100	<100	368	431	851	894	1684	1431
11	<LOD	<LOD	559	501	1294	1291	2051	1939
12	55	49	612	641	1270	1305	2232	1457
13	13	10	407	404	758 ^b	988 ^b	1526	1522
15	43	43	790	772	1702	1690	2838	2880

^a Excluded because the participant failed to follow the method.

^b Identified as an outlier by the Cochran test.

Table 6. International study results for determination of FB₁, FB₂, and total fumonisins by 13 laboratories

Analyte	Average, ng/g	S _r	RSD _r , %	S _R	RSD _R , %	No. outliers	HorRat	
							Ho _r	Ho _R
FB ₁								
Level 1	443	20.5	4.6	97.4	22.0	1	0.39	1.22
Level 2	987	50.9	5.1	195.8	19.8	0	0.49	1.24
Level 3	1626	194.0	11.9	387.7	23.8	0	1.22	1.60
FB ₂								
Level 1	91	11.4	12.6	23.3	25.5	0	0.83	1.11
Level 2	197	3.7	1.9	36.0	18.2	1	0.14	0.89
Level 3	328	35.8	10.9	78.8	24.0	0	0.87	1.27
FB ₁ + FB ₂								
Level 1	534	27.2	5.1	119.4	22.4	1	0.44	1.27
Level 2	1194	16.5	1.4	224.8	18.8	2	0.13	1.21
Level 3	1954	225.4	11.5	452.8	23.2	0	1.20	1.60

Technologies (three participants), and SCIEX (one participant), representing a good cross-section of instrument types and demonstrating the robustness of the method in terms of the LC/MS determination step.

To minimize method variations and to encourage participants to use columns with high resolution but short retention times, an Agilent Zorbax XDB-C18 3.5 μ m (4.6 \times 100 mm) column was supplied to all participants in the study. Initially, several participants indicated they were experiencing problems with the HPLC in terms of poor chromatographic peak shape and poor recovery. When these problems were reported, we did not insist that participants use the supplied column, and we advised the participants that they could use any column of their choice provided the selectivity and sensitivity could be achieved as prescribed in the instructions. In fact, 10 of the 13 participants used the column provided and achieved satisfactory results; of the three participants who used their own columns, one of these was Participant 2, who was excluded from the statistical analysis.

Comparison of LC/MS and LC/MS/MS Results

Participant 2 experienced difficulties with the prescribed LC/MS conditions. However, rather than using his own conditions with which he was familiar but nevertheless following the IAC method, he chose inexplicably to analyze the samples by using his own methodology. The results for Participant 2, therefore, were obtained by using methanol–water extraction of the sample, dilution with water, then direct analysis of the crude extract by LC/MS/MS. The results obtained by Participant 2 for FB₁ were higher by 12–50% than the mean levels obtained by the other participants, but the results obtained by Participant 2 for FB₂ were reasonably close to the mean levels for FB₂. It was not clear whether this participant used matrix-matched standards

to compensate for any ion suppression or other matrix effects that might have contributed to deviations in quantification from the mean values obtained by the other participants for samples cleaned up by IAC. Although a number of mycotoxin laboratories are advocating the use of LC/MS/MS to screen for a wide range of toxins in different classes (13–17), in fact no interlaboratory validation of any of these procedures has been conducted by analyzing crude extracts, and the method performance of these MS/MS procedures still remains to be established.

Feedback from Participants

Constructive comments were received from nine of the 13 participants. None of the participants indicated they had experienced any difficulties in following the method, and most comments suggested only minor improvements. Participant 4 questioned why the method did not use spiked samples for calibration, rather than solutions of the standards. However, with IAC cleanup the final extracts are essentially as clean as standards in solution and there is no advantage in undertaking the additional work of using extracts in addition to the difficulties of obtaining nominally blank samples for calibration purposes.

Participant 5 could not achieve the desired response (S/N ratio) with the recommended ammonium acetate solvent system. This participant, in fact, switched to 0.1% formic acid in both methanol and water and claimed this change increased analyte response; thus, this participant recommended the addition of formic acid to the mobile phase in place of ammonium acetate–acetic acid. It was suggested this change makes it easier to prepare the mobile phase components and greatly increases the response. Although this was not generally found to be the case, Participant 10 commented that the presence of ammonium acetate in the mobile phase was

not necessary because the originating ions are not ammoniacal adducts.

A number of participants commented that they routinely determined fumonisins by LC/MS/MS and found it difficult to adhere to the request to use the single-quadrupole mode. In fact, in retrospect we probably would have conducted this study with an LC/MS/MS method had we appreciated that most laboratories are now using MS/MS.

Conclusions

This paper reports for the first time the results of an LC/MS interlaboratory study for determination of mycotoxins in food—no previous mycotoxin interlaboratory studies have used LC/MS. The study results confirm that good precision data can be obtained by LC/MS, and they were consistent with previously reported data from a single-laboratory validation of the same method for FB₁ and FB₂ in corn. The method meets performance criteria set by the EU (23) and by the European Committee for Standardization (24), and is thus suitable for use as an official method for control purposes for determination of FB₁ and FB₂ in corn.

Acknowledgments

We acknowledge the TUBITAK-ATAL Directorate for financial support, Sincer Int. Trade and R-Biopharm for providing Fumoniprep columns and other consumables for this validation study, Agilent Technologies for providing participants with Zorbax XDB-C18 Rapid Resolution HPLC columns, and FAPAS [Food and Environment Research Agency (FERA), UK] for supplying surplus PT test materials. We also gratefully acknowledge the help of K. Mathieson (FERA, UK) for the statistical analysis and Carsten Mischke (Joint Research Centre, Institute for Reference Materials and Measurements) for the preparation and homogenization of the test samples.

This study could not have been conducted without the expert participation of the following collaborators and the support of their organizations:

- S. Biselli, Eurofins Analytik GmbH, Hamburg, Germany
- A. De Girolamo, Institute of Sciences of Food Production, National Research Council (CNR) Bari, Italy
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- S. MacDonald, FERA, York, UK
- G. Neumann, Health Canada, Winnipeg, Canada
- W.A. van Osenbruggen, TNO Quality of Life, Zeist, The Netherlands
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