FOOD CHEMICAL CONTAMINANTS

Immunoaffinity Column Cleanup with Liquid Chromatography for Determination of Aflatoxin B_1 in Corn Samples: Interlaboratory Study

CARLO BRERA, FRANCESCA DEBEGNACH, VALENTINA MINARDI, ELENA PANNUNZI, BARBARA DE SANTIS, and MARINA MIRAGLIA
Italian National Institute for Health (ISS), National Center for Food Quality and Risk Assessment, GMO and Mycotoxins Unit, Viale Regina Elena, 299–00161 Rome, Italy

Collaborators: C. Bergamini; A. Biancardi; M. Bodda; L. Bonassisa; P. Burdaspal; L. Cantamessa; G. Chessa; I. Commissati; A. Corrao; J. Dömsödi; G. Esposito; C. Focardi; D. Garbini; M. Gatti; C. Gibellino; K. Kroeger; F.M. Lombardi; P. Mambelli; J. Mastrantoni; J.Y. Michelet; T. Møller; M. Pascale; C. Petrini; A. Pietri; M. Piombino; R. Piro; A. Pittet; N. Rizzi; J. Stroka; A.M. Thim; A. Ubaldi; A. Villani; F. Zanon

An interlaboratory study was conducted to evaluate the effectiveness of an immunoaffinity column cleanup liquid chromatography (LC) method for the determination of aflatoxin B_1 levels in corn samples, enforced by European Union legislation. A test portion was extracted with methanol–water (80 + 20); the extract was filtered, diluted with phosphate-buffered saline solution, filtered on a microfiber glass filter, and applied to an immunoaffinity column. The column was washed with deionized water to remove interfering compounds, and the purified aflatoxin B_1 was eluted with methanol. Aflatoxin B_1 was separated and determined by reversed-phase LC with fluorescence detection after either pre- or postcolumn derivatization. Precolumn derivatization was achieved by generating the trifluoroacetic acid derivative, used by 8 laboratories. The postcolumn derivatization was achieved either with pyridinium hydrobromide perbromide, used by 16 laboratories, or with an electrochemical cell by the addition of bromide to the mobile phase, used by 5 laboratories. The derivatization techniques used were not significantly different when compared by the Student's *t*-test; the method was statistically evaluated for all the laboratories. Five corn sample materials, both spiked and naturally contaminated, were sent to 29 laboratories (22 Italian and 7 European). Test portions were spiked with aflatoxin B_1 at levels of 2.00 and 5.00 ng/g. The mean values for recovery were 82% for the low level and 84% for the high contamination level. Based on results for spiked samples (blind pairs at 2 levels) as well as naturally contaminated samples (blind pairs at 3 levels), the values for relative standard deviation for repeatability (RSD_r) ranged from 9.9 to 28.7%. The values for relative standard deviation for reproducibility (RSD_R) ranged from 18.6 to 36.8%. The method demonstrated acceptable within- and between-laboratory precision for this matrix, as evidenced by the HorRat values.

Received November 29, 2006. Accepted by AP January 16, 2007.
Corresponding author's e-mail: carlo.brera@iss.it

Specific, selective, and accurate methods of analysis for determining mycotoxins, including aflatoxins in food and feed products, have been used extensively with commercially available immunoaffinity columns (ACS; 1). Currently, existing methods for determining aflatoxins in cereals are outdated, and a specific method for ground corn in the range of 2.00–20.00 ng/g is needed. The lack of a suitable method is even more relevant because corn undoubtedly is the major cereal contributor of aflatoxin contamination in food and feed.

Furthermore, it should be noted that the presence of aflatoxin B_1 in corn is strictly correlated with the presence of aflatoxin M_1 in milk and derived products. The formulation of management approaches, for the entire agrifood chain, is needed with the objective of controlling and reducing aflatoxin B_1 contamination in corn, starting at the field level.

Corn is also widely involved in import/export commercial transactions, necessitating the satisfaction of certain safety requirements that are in compliance with existing legislation worldwide.

The European Union (EU) has set a differentiated legal limit, compared with those for all the other cereals, setting a maximum tolerable limit of 5.0 ng/g for raw corn (2) as a consequence of scientific evidence reported in some studies on the effects of industrial milling processing (3, 4). As for feeds, aflatoxin B_1 is the only mycotoxin currently regulated

Table 1. Test results for aflatoxin B_1 levels of naturally contaminated test materials

Material	B_1, ng/g[a]	RSD, %[b]	SE, ng/g[c]	Range, ng/g[d]
Corn (blank)	0.04 (<0.10)[e]	24.79	0.01	0.02–0.06
Corn	2.20 (2.00)[e]	14.88	0.10	1.31–2.03
Corn	17.60 (20.00)[e]	3.90	0.14	17.26–17.87

[a] Mean value from the homogeneity testing.
[b] Relative standard deviation among containers obtained from the analysis of variance results (n = 10).
[c] SE = Standard error; $SE = \sqrt{\frac{MS_{container}}{n}}$, where $MS_{container}$ is the mean square among containers obtained from the analysis of variance results (n = 10).
[d] Range = mean ± 2.2 SE.
[e] Value in parentheses = desired target value for validation study.

by the EU, with the level set at 0.02 mg/kg for corn and a range of 0.005–0.020 mg/kg for different types of feeds (5).

Additionally, corn milled fractions obtained from industrial processing reach different markets, because bran, germ, and meal are used for animal feed, but grits and flour are commonly intended for human consumption. Therefore, starting from the same raw commodity, different sanitary requirements should characterize all these fractions.

The availability of a unique methodology for determining aflatoxin B_1 in all milled fractions, including the raw kernels, would evidently be a worthwhile and versatile diagnostic tool in solving legal debates in this respect.

Interlaboratory Study

Preparation of Test Materials

Test materials consisted of 2 batches of naturally contaminated corn at aflatoxin B_1 levels of 2.20 and 17.60 ng/g (Table 1) and a batch of blank material. Each batch was milled, initially mixed manually, and then mixed with an electrical device. The test materials were analyzed for homogeneity before and after the packaging of test samples.

Homogeneity

Bulk homogeneity.—The preliminary homogeneity was checked for 2 sets of results from analysis of samples randomly taken from the bulk, and on 2 different days. The results were subjected to 1-way analysis of variance (ANOVA), and no significant difference was observed as detected by the F-test at the 95% confidence level (Table 2).

Packed-material homogeneity.—Five packages were randomly selected from each batch (including the blank) and analyzed in duplicate. The between- and within-package variances were calculated. The within-package variance was calculated on the set of results from the analysis of 2 samples taken from each package. The results were subjected to 1-way ANOVA. When the F-test was applied at the 95% confidence level, both between- and within-package variances demonstrated proper homogeneity of the test materials (Table 2).

Organization of the Interlaboratory Study

Twenty-nine participants from 7 different European countries representing a cross section of government food regulators, universities, and food industry affiliations took part in this interlaboratory trial. According to AOAC guidelines (6), 5 materials were prepared. For the interlaboratory trial, each participant received 6 sample packages (blind duplicates at 3 content levels) and 4 blank samples intended for spiking analysis. Each participant was also given an additional sample for familiarization purposes. Furthermore, one amber vial marked "STD" containing aflatoxin B_1 standard solution (described in *Method*), 2 amber vials marked "SPKA" and "SPKB" to be used for spiking procedures, 12 IACs (including one spare), a report form for

Table 2. Results of ANOVA test for homogeneity

		ANOVA test for preliminary homogeneity		ANOVA test for final homogeneity	
Material	B_1, ng/g	F_{crit}	F_{calc}	F_{crit}	F_{calc}
Corn (coded and blank)	0.04	5.19	ND[a]	5.19	ND
Corn (coded)	2.20	3.86	0.81	5.19	0.48
Corn (coded)	17.60	5.19	0.12	5.19	1.90

[a] ND = Not determined.

analytical data, a "Confirmation of the Receipt" form, and the analytical protocol were also sent to participants.

Participants were advised to perform sample analyses by following a working instruction schedule. This schedule required each participant to prepare one extract from each material, perform a cleanup using the IAC, and analyze the purified aflatoxin B_1 by liquid chromatography (LC). Additionally, each participant was required to fortify the blank materials with the spiking solutions provided. Participants were instructed to add known volumes of spiking solutions to samples and to leave them in a fume hood overnight, to allow the solvent to evaporate before extraction.

METHOD

(Applicable to the determination of aflatoxin B_1 at levels of >0.10 ng/g.)

Caution: This method requires the use of aflatoxin B_1 solutions. Aflatoxins are carcinogenic for humans. Attention is drawn to the statement released by the International Agency for Research on Cancer. Aflatoxins are subject to light degradation. Adequately protect analytical work from daylight, and keep aflatoxin standard solutions protected from light by using amber vials or aluminum foil. Use acid-washed glassware (vials, tubes, and flasks) to prevent loss of aflatoxins. Take special care when using new glassware. Soak new glassware in dilute acid (2 N sulfuric acid) for several hours, and rinse extensively with distilled water to remove all traces of acid (check with pH litmus paper).

Principle

The test portion is extracted with methanol–water (8 + 2, v/v). The extract is filtered, diluted with phosphate-buffered saline solution (PBS), filtered a second time, and applied to an IAC containing antibodies specific to aflatoxin B_1. After elution with methanol, aflatoxin B_1 is quantified by LC with postcolumn derivatization followed by fluorescence detection.

Performance Standard and General Procedures for IACs

The aflatoxin IACs (R-Biopharm Rhône Ltd., Glasgow, UK) contain monoclonal antibodies raised against aflatoxin B_1. The IACs should have a minimum capacity of ≥40 ng aflatoxin B_1 and should give a recovery of ≥80%, when a standard solution in methanol–water containing toxin at 0.25 ng/mL toxin (corresponding to 160 mL) is applied.

Allow the columns to reach room temperature before conditioning. For conditioning, apply 5 mL PBS on top of the column (gravity). Make sure that a small portion (0.5 mL) of the PBS remains on the column until the extract is applied.

Apparatus

(**a**) *Mill*.—Romer Laboratories (Union, MO).

(**b**) *Blender*.—Explosion proof, with 1 L jar and cover, capable of operating at a high speed (ca 20 000 rpm).

(**c**) *Filter paper*.—24 cm diameter, prefolded.

(**d**) *Glass microfiber filter paper*.—Particle retention, 1.6 μm.

(**e**) *Vacuum system*.—For use with IACs.

(**f**) *Volumetric glassware*.—5, 10, and 50 mL (accuracy of ≥0.5%).

(**g**) *LC pump*.—With flow rates of 0.20–1.00 mL/min.

(**h**) *Injection system*.—Total loop-injection valve with loop between 100 and 1000 μL.

(**i**) *Reversed-phase LC column*.—4.6 × 250 mm, 5 μm, LC-18 or ODS-2.

(**j**) *Postcolumn derivatization system*.—Either with pyridinium hydrobromide perbromide [PBPB; second LC pulseless pump, zero-dead volume T-piece, reaction tubing minimum 450 × 0.5 mm id polytetrafluoroethylene (PTFE)] or with electrochemically generated bromine (Kobra cell, R-Biopharm Rhône Ltd.).

(**k**) *Fluorescence detector*.—Fitted with flow cell and set at 365 nm (excitation wavelength) and 435 nm (emission wavelength). For trifluoroacetic acid (TFA) precolumn derivatization, wavelengths are set at 365 nm for excitation and 450 nm for emission.

(**l**) *Pipet*.—Marked with 25 mL capacity.

(**m**) *Analytical balance*.—Accuracy of ±0.1 mg.

(**n**) *Laboratory balance*.—Accuracy of ±0.01 g.

(**o**) *Calibrated microliter syringe or microliter pipet*.—20–1000 μL.

(**p**) *IACs.—See Performance Standard and General Procedures for IACs.*

Reagents

All reagents should be of a recognized analytical grade. Unless otherwise stated, use water complying with Grade 3, ISO 3696.

(**a**) *PBS*.—Prepare from potassium chloride (0.20 g), potassium dihydrogen phosphate (0.20 g), anhydrous disodium hydrogen phosphate (1.16 g; or $Na_2HPO_4 \cdot 12H_2O$ [2.92 g]), and sodium chloride (8.00 g) added to 900 mL purified water. After dissolution, adjust pH to 7.4 (with 0.1 M HCl or 0.1 M NaOH as necessary). Dilute to 1.0 L, or alternatively use commercially available PBS tablets.

(**b**) *PBPB*.—CAS No. 39416-48-3.

(**c**) *Potassium bromide*.

(**d**) *TFA*.—Spectrophotometric grade.

(**e**) *Acetonitrile*.—LC grade.

(**f**) *Methanol*.—LC grade.

(**g**) *LC grade water*.—Obtained by distillation or deionization. Equivalent to Grade 1, ISO 3696.

(**h**) *Extraction solvent*.—Methanol–water (80 + 20, v/v).

(**i**) *Nitric acid–water (1 + 3, v/v)*.—This will result in a concentration of ca c(HNO_3) = 4 mol/L.

(**j**) *LC mobile phase solvent A*.—Water–acetonitrile–methanol (54 + 17 + 29, v/v/v).

(**k**) *LC mobile phase solvent B*.—For use with electrochemically generated bromine: water–acetonitrile–methanol (54 + 17 + 29, v/v/v), containing 120 mg potassium bromide, (**c**), and 350 μL nitric acid, (**i**), in 1 L final reagent.

Table 3. Preparation of aflatoxin B_1 working calibrant solutions

Working calibrant solution	Aliquot of working standard solution, μL	Calibrated volumetric flask, mL	Concn of aflatoxin B_1, ng/mL
1	20	50	0.04
2	40	10	0.4
3	80	10	0.8
4	200	10	2.0
5	240	10	2.4

(**l**) *Postcolumn reagent*.—Dissolve 25 mg PBPB in 500 mL water. Solution can be used for ≤4 days if stored in the dark at room temperature.

(**m**) *Toluene–acetonitrile*.—90 + 10, v/v.

(**n**) *Aflatoxin B_1 standard material*.—Crystals or dry film.

(**o**) *Aflatoxin B_1 stock solution*.—Containing aflatoxin B_1 at 10.0 μg/mL in toluene–acetonitrile (90 + 10, v/v).

(**p**) *Aflatoxin B_1 working standard solution*.—Pipet 100 μL stock solution, (**o**), into a 10 mL volumetric flask. Evaporate the toluene–acetonitrile solution under a gentle stream of nitrogen. Dilute contents of flask to volume with methanol–water (4 + 6, v/v) to obtain a calibrant stock solution containing aflatoxin B_1 at 100 ng/mL.

(**q**) *Aflatoxin B_1 working calibrant solutions*.—Pipet aliquots of working standard solution, (**p**), into a set of calibrated flasks as listed in Table 3. Dilute contents of flasks to volume with methanol–water (4 + 6, v/v), and shake well.

The concentration of the aflatoxin B_1 stock solution was spectrophotometrically checked by following the AOAC guidelines (7).

Extraction

Weigh a 50 g test portion (to the nearest 0.1 g) into a high-speed blender jar. Add 5 g sodium chloride and 250 mL methanol–water extraction solvent. Cap the jar, and blend contents at high speed for 3 min. Filter the extract through prefolded filter paper. Pipet 20 mL filtrate into a volumetric flask, and dilute with 20 mL PBS. Mix thoroughly, and filter the diluted sample through a microfiber glass filter. Apply 20 mL diluted sample to the IAC. Avoid overheating the jar during the extraction step.

IAC

Connect the IAC to the vacuum manifold or equivalent, and attach the reservoir to the IAC. Pass 20 mL (equivalent to 2.0 g sample) diluted extract through the IAC at a flow rate of ca 3 mL/min, or under gravity (flow rate should be ≤5 mL/min). The IAC must not run dry during this step. Wash the IAC with 10 mL deionized water, and dry either by applying a light vacuum for 5–10 s or by passing air through the IAC by means of a syringe for 10 s; the volume of air should be ≥3–4 times the IAC volume.

Elute aflatoxin B_1 in a 2-step procedure. First, apply 1.0 mL methanol to the IAC and let it flow through under gravity. Collect eluate in a calibrated 5 mL volumetric flask. Wait 1 min, and apply a second portion of 1.0 mL methanol. Use a 10 mL syringe to pass air through the column to collect the remaining few drops. Dilute contents of the 5 mL volumetric flask to volume with water, shake well, and store the sample at 4°C until analysis.

LC Determination with Fluorescence Detection and Postcolumn Derivatization

When using PBPB, mount the mixing T-piece and reaction tubing previously mentioned. The derivatizing solution is pumped at a flow rate of 0.4 mL/min, and mobile phase A, at a flow rate 1.00 mL/min.

When using electrochemically generated bromine (Kobra cell), follow the instructions for cell installation as supplied by the manufacturer, and use the following parameters: flow rate, 1.00 mL/min (mobile phase B); current, 100 μA.

Aflatoxins elute in the order G_2, G_1, B_2, and B_1 with retention times of ca 7, 9, 10, and 12 min, respectively, and should be baseline resolved in order to measure aflatoxin B_1 as a discrete peak.

LC Determination with Fluorescence Detection and Precolumn Derivatization

After the cleanup step elute the sample into a test tube, and evaporate the methanol under a gentle stream of nitrogen at 40°C. Add 50 μL TFA, and shake tube for 30 s. Add 600 μL methanol–water (4 + 6, v/v), shake tube for 1 min, and let tube stand for 15 min. Shake tube for another 30 s, and then inject solution into the LC system. In order to detect the different derivatives, the fluorescence detector is set to the wavelengths 365 nm for excitation and 450 nm for emission. It should be noted that only a few laboratories (8 out of 29) used a precolumn derivatization TFA.

Calibration Curve

Prepare a calibration curve by using the 5 working calibrant solutions covering the range 0.04–2.40 ng/mL for aflatoxin B_1, and obtain the calibration curve by injecting in triplicate each of the 5 concentrations. The volume of each injection should be the same as that of the test solution samples (150 μL). Check the curve for linear regression according to the harmonized guidelines of the International Union of Pure and Applied Chemistry (IUPAC; 8). Identify the aflatoxin B_1 peak in the chromatogram by comparing retention time with the corresponding reference standard.

Calculations

Calculate the toxin concentration in the test sample by plotting the concentration of aflatoxin B_1 (ng/mL) versus the peak area (units). Use the resulting function (linear regression) to calculate the concentration of aflatoxin B_1 in the measured solution.

Table 4. Single results (ng/g) from interlaboratory study for determination of aflatoxin B_1 in corn samples

		Fortified sample						Naturally contaminated sample			
Lab	Derivatization method	2.00 ng/g		5.00 ng/g		Blank		2.20 ng/g		17.60 ng/g	
1	Kobra	0.91	1.01	2.21	2.41	<0.16	<0.16	1.03	1.07	9.40	8.04
2	PBPB	1.80	1.60	4.30	4.30	<0.50	<0.50	1.50	1.50	16.10	16.10
3[a]	PBPB	12.20	NA[b]	NA	32.20	0.00	0.50	16.80	95.40	126.40	104.20
4	PBPB	1.80	1.80	4.10	4.10	<0.20	<0.20	1.00	1.70	15.40	15.10
5	TFA	0.80	1.91	2.19[c]	0.81[c]	0.02	0.01	1.18	1.43	7.61	7.64
6	Kobra	1.73	1.72	4.51	4.08	NA	NA	1.36	1.48	15.14	16.09
7	TFA	2.00	2.92	5.66	6.01	<0.6	<0.6	3.23	2.94	19.94	21.77
9	TFA	1.85	1.94	4.22	4.76	0.15	0.09	2.15	1.66	15.00	16.00
10[a]	PBPB	1.50	0.05	4.23	0.08	0.00	0.00	2.30	0.00	14.34	0.33
11	PBPB	1.95	1.85	4.49	4.67	0.09	0.10	2.82	1.97	19.62	19.85
12	TFA	1.51	1.42	5.14	4.04	0.13	0.13	1.47	1.73	20.26	18.87
14[a]	PBPB	7.01	6.70	7.00	6.70	0.60	0.60	2.90	NA	24.90	26.30
15	PBPB	2.10	2.00	5.10	4.80	<0.20	<0.20	2.80	2.30	17.90	16.80
16	PBPB	1.40	1.30	3.70	3.30	0.05	0.10	1.90	1.40	14.40	12.70
17	Kobra	2.14	1.91	4.78	4.73	0.04	0.04	1.76	2.10	18.57	17.31
18	TFA	1.85	1.85	4.20	4.70	<0.50	<0.50	2.55	2.00	11.20	17.50
19	PBPB	1.44	0.79	3.88	4.21	0.02	0.02	1.81	4.07	15.02	17.45
20	PBPB	17.23[c]	39.79[c]	33.31[c]	95.69[c]	<1.00	<1.00	31.27[c]	30.84[c]	170.93[c]	50.03[c]
21	PBPB	2.39	1.17	5.11	3.84	<0.10	<0.10	2.89	0.75	11.79	9.39
22	PBPB	1.29	1.34	3.25	4.34	0.03	0.03	1.69	1.70	11.20	16.20
23	TFA	1.75	1.50	4.65	4.74	0.03	0.00	1.21	1.00	13.31	11.72
24[a]	TFA	1.73	1.23	3.78	—	<0.01	<0.01	1.09	0.58	11.51	5.52
25	PBPB	2.10	2.00	5.80	4.80	<0.10	<0.10	2.70	1.90	18.80	20.00
26[a]	PBPB	1.29	1.40	2.94	2.92	0.09	0.34	2.62	2.48	28.49	16.87
27	PBPB	1.90	1.60	4.20	5.00	0.10	0.20	2.30	1.60	12.70	15.30
28	TFA	1.60	1.66	3.81	4.28	0.03	0.03	1.37	1.61	15.46	15.69
29	Kobra	2.00	2.01	4.75	4.69	0.07	0.03	2.63	2.68	18.84	17.44
30	Kobra	2.17	2.01	5.37	4.97	0.26	0.47	2.67	2.36	23.08	17.76
31	PBPB	1.48	0.97	3.51	2.60	<0.04	<0.04	1.48	1.13	11.89	12.92

[a] Laboratory did not follow the analytical protocol.
[b] NA = Not analyzed.
[c] Result identified as an outlier; not included in the statistical analysis.

Determine the content of aflatoxin B_1 in the test material, in ng/g, from the following equation:

$$AFB_1, \text{ng/g} = \frac{k \times solvent \times elution}{W \times aliquot \times V_{inj}}$$

where k = ng aflatoxin B_1 in V_{inj} (from the standard curve), ng; *solvent* = extraction solvent (250 mL), mL; *elution* = final volume achieved after elution from the IAC (5 mL), mL; W = sample material taken for analysis (50 g), g; *aliquot* = aliquot of the extract passed through the IAC (10 mL), mL; V_{inj} = volume injected into the LC system (0.150 mL), mL.

It is possible to simplify the above equation as follows:

$$AFB_1, \text{ng/g} = k/0.06$$

In cases where the aflatoxin B_1 content in the test sample is outside the calibration range, dilute the injection solution to an appropriate concentration within the range of the calibration curve.

Table 5. Results of the comparison between Kobra/PBPB and TFA derivatization procedures

	Fortified material		Naturally contaminated material	
Contamination level, ng/g	2.00	5.00	2.20[a]/1.90[b]	17.60[a]/15.44[b]
t-Value, obtained	0.47	1.23	0.437	0.26
t-Value, critical (α = 0.05)	2.08	2.08	2.08	2.09
RSD[c] for Kobra/PBPB, %	21.91	18.47	28.92	21.20
RSD for TFA, %	20.84	12.93	37.11	29.11
F-value, obtained	1.01	1.68	0.69	0.56
F-value, critical (α = 0.05)	3.94	4.62	3.94	3.94
RSD for all, %	21.19	17.24	30.73	23.15

[a] Desired target level.
[b] Mean level from study.
[c] RSD = Relative standard deviation.

Results and Discussion

Interlaboratory Trial

Single results for the interlaboratory trial are given in Table 4 as individual pairs of results for each participating laboratory. Both naturally contaminated and spiked corn samples were blind duplicates. Blank samples were spiked at 2 different levels with aflatoxin B_1 at 2.00 and 5.00 ng/g.

Results from Laboratories 3, 10, 14, 24, and 26 were rejected because the analyses were not performed according to the protocol supplied by the coordinator.

Laboratory 20 was identified as an outlier. The source of this outlier is due to a calculation error, since results reported by Laboratory 20 when divided by a value of 10, fell within the range of the mean results reported by the other participants.

Statistical Analysis

Before statistical analysis of the interlaboratory trial results were examined for systematic errors ($P < 0.05$) by using the Cochran and Grubbs tests progressively (9), and the identified outliers were removed.

A preliminary statistical comparison of single results for the Kobra cell and PBPB derivatization methods was conducted to verify if the 2 data sets were significantly different and therefore required a separate statistical evaluation of the results. As also reported by Stroka et al. (10), no differences were highlighted between the 2 derivatization methods; thus, from now on, these results will be treated and discussed as one data set. The Student's *t*-test and *F*-test were performed to compare the Kobra cell/PBPB method (16 laboratories) with the TFA derivatization method (7 laboratories). Outliers were removed before the tests. Results are given in Table 5. No significant differences between the averages and the relative standard deviation (RSD) values of the 2 data sets were found. Consequently, an advance evaluation was carried out for the total number of results. Precision estimates were obtained by using a 1-way ANOVA according to the IUPAC harmonized protocol (9). The average aflatoxin B_1 contents of the corn samples, the values for repeatability relative standard deviation (RSD_r) and reproducibility relative standard deviation (RSD_R), the number of laboratories that were found to be statistical outliers, the HorRat values, and the mean recovery values are shown in Table 6.

In Table 4, laboratories that did not follow the analytical protocol, and the pairs of results identified as outliers are indicated and identified individually by laboratory. As shown in Table 6, after the data for the blank material were excluded, the number of statistically acceptable laboratories was 22 or 23, depending on the number of outliers detected (1 or 2).

Collaborators' Comments

Fifteen of the 29 participating laboratories in the interlaboratory trial made comments. The results of Laboratories 14, 24, and 26 were rejected; their comments confirm that they did not correctly follow the analytical protocol.

Laboratories 2, 5, 6, 15, 17, and 23 reported a change in the sample volume injected into LC system; however, this does not represent an actual method modification, because differences in injected volumes are taken into account in the calculation step.

Laboratory 15 made a 4-point calibration curve because it was not able to measure the lowest level, whereas Laboratory 2 decided to prepare the calibration curve by injecting as the lowest concentration a standard solution containing aflatoxin B_1 at 0.06 ng/g instead of 0.04 ng/g as suggested by the coordinator.

For Laboratories 23 and 0 27–29, samples D and F were outside the range of the calibration curve; in all cases, the samples were re-injected after adequate dilution.

Precision Characteristics of the Method

Because of differences in reporting limits of "not detectable", the results for blank materials were not analyzed

Table 6. Interlaboratory study results for determination of aflatoxin B_1 in corn samples

Added, ng/g	No. of labs[a]	Avg., ng/g	S_r, ng/g[b]	S_R, ng/g[c]	RSD_r, %	RSD_R, %	r, ng/g[d]	R, ng/g[d]	HorRat	Mean recovery, %
2.00	24 (1)	1.70	0.32	0.42	18.59	24.93	0.88	1.19	0.60	82
5.00	24 (2)	4.37	0.43	0.81	9.86	18.59	1.21	2.27	0.51	84
NC[e] (0.04)	24 (0)	<0.10	—	—	—	—	—	—	—	—
NC (2.20)	24 (1)	1.90	0.55	0.70	28.71	36.83	1.53	1.96	0.90	—
NC (17.60)	24 (1)	15.44	1.70	3.77	11.03	24.42	4.77	10.56	0.82	—

[a] Number of laboratories retained after removal of outliers; each number of outliers is in parentheses.
[b] S_r = Repeatability standard deviation.
[c] S_R = Reproducibility standard deviation.
[d] R (reproducibility) and r (repeatability) are calculated according to the IUPAC harmonized protocol.
[e] NC = Naturally contaminated.

statistically. Most of the laboratories were able to identify the blank pairs of samples as not containing aflatoxin B_1, or containing a level not detectable but close to the limit of determination.

The precision data for all samples are summarized in Table 6. For spiked samples (blind pairs at 2 levels) and for naturally contaminated samples (blind pairs at 3 levels), the RSD_r values ranged from 9.9 to 28.7%, and the RSD_R values ranged from 18.6 to 36.8%. The estimated parameter values obtained in this study satisfy the requirements recently presented in Regulation 401/2006 of the EU (11). The different RSD_r and RSD_R values can be ascribed to the different order of magnitude of the concentration levels in the study. As for the 2 levels of spiked corn samples, the mean recoveries were 82 and 84% for the low (2.00 ng/g) and high (5.00 ng/g) contamination levels, respectively.

Interpretation of Results

The original basis for assessing performance characteristics of interlaboratory trial data was the HorRat values (12). An improved version of this function has been introduced (11) that better fits with recent data. After the exclusion of the outliers, the HorRat values for aflatoxin B_1 ranged from 0.51 to 0.90; these values are significantly <2.0, which indicates acceptable precision.

Conclusions

The results of the interlaboratory study demonstrated that the present method can accurately detect aflatoxin B_1 in corn samples over a wide range of concentrations, covering the European regulatory limits for aflatoxin B_1 in both foods intended for human consumption and in feeds.

In general, all 3 derivatization procedures used in the study produced good results, confirming the reliability of the method.

Finally, no statistical differences were observed in the study between the results for the spiked samples and those for the naturally contaminated samples from all laboratories; this finding is consistent with efficient extraction from the matrix.

Acknowledgments

We thank the following collaborators for their participation in the study:

Cecilia Bergamini, ARPA Emilia Romagna, Bologna, Italy

Marisa Bodda, ARPA Piemonte, Torino, Italy

Lucia Bonassisa and Paolo Mambelli, Bonassisa Laboratory, Foggia, Italy

Pedro Burdaspal, Instituto de Salud Carlo III, Madrid, Spain

Liliana Cantamessa, Chemical Control, Cuneo, Italy

Gianna Chessa, IZS Sardegna, Sassari, Italy

Italo Commissati, Chelab, Treviso, Italy

Antonino Corrao, IZS Sicilia, Palermo, Italy

Jozsef Dömsödi, National Institute for Agricultural Quality Control, Budapest, Hungary

Giovanna Esposito, ARPA Campania, Napoli, Italy

Claudia Focardi, IZS Lazio e Toscana, Firenze, Italy

Davide Garbini, COOP, Bologna, Italy

Marcello Gatti, Neotron, Modena, Italy

Cristina Gibellino, ARPA Valle d'Aosta, Aosta, Italy

Fabio Massimo Lombardi, VEGEZIO, Cesena, Italy

Joanna Mastrantoni, Ministry of Finance, Piraeus, Greece

Jean-Yves Michelet, ISP Ministry of Health, Brussels, Belgium

Tord Møller and Anna Maria Thim, National Food Adminstration, Uppsala, Sweden

Michelangelo Pascale, CNR, Bari, Italy

Cesare Petrini, GREIT, Bologna, Italy

Amedeo Pietri, Piacenza University, Piacenza, Italy

Matteo Piombino, Pioneer Hi-Bred, Cremona, Italy

Roberto Piro and Alberto Biancardi, IZS Lombardia, Brescia, Italy

Alain Pittet, Nestlé, Lausanne, Switzerland

Nicoletta Rizzi, ARAL, Cremona, Italy

Joerg Stroka and Katy Kroeger, IRMM, Geel, Belgium

Alessandro Ubaldi, IZS Lazio e Toscana, Rome, Italy
Andera Villani, AGER, Bologna, Italy
Francesca Zanon, ARPA Veneto, Venezia, Italy

We also wish to express our thanks to Viviana Renzi for her helpful technical assistance.

References

(1) Scott, P.M., & Trucksess, M.W. (1997) *J. AOAC Int*. **80**, 941–949

(2) Commission Regulation (EC) No. 1881/2006 (December 19, 2006) Setting maximum levels for certain contaminants in food, *Off. J. Eur. Union* **L364**, 5

(3) Brera, C., Debegnach, F., Grossi, S., & Miraglia, M. (2004) *J. Food Prot.* **67**, 1261–1266

(4) Brera, C., Catano, C., De Santis, B., Debegnach, F., De Giacomo, M., Pannunzi, E., & Miraglia, M. (2006) *J. Agric. Food Chem.* **54**, 5014–5019

(5) *Commission Directive 2003/100/EC* (October 31, 2003) Amending Annex I to Directive 2002/32/EC of the European Parliament and of the Council on undesirable substances in animal feed, *Off. J. Eur. Union* **L285**, 33

(6) AOAC Official Methods Program (1995) *J. AOAC Int.* **78**, 143A–160A

(7) *Official Methods of Analysis* (2000) 17th Ed., AOAC INTERNATIONAL, Gaithersburg, MD

(8) IUPAC (2002) *Pure Appl. Chem.* **74**, 835–855

(9) IUPAC (1995) *Pure Appl. Chem.* **67**, 331–343

(10) Stroka, J., von Holst, C., Anklam, E., & Reutter, M. (2003) *J. AOAC Int.* **86**, 1179–1186

(11) *Commission Regulation (EC) No. 401/2006* (February 23, 2006) Laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs, *Off. J. Eur. Union* **L70**, 12

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