FOOD CHEMICAL CONTAMINANTS

Immunoaffinity Column Cleanup with Liquid Chromatography Using Post-Column Bromination for Determination of Aflatoxins in Peanut Butter, Pistachio Paste, Fig Paste, and Paprika Powder: Collaborative Study

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A collaborative study was conducted to evaluate the effectiveness of an immunoaffinity column cleanup liquid chromatography (LC) method for the determination of aflatoxin B_1 and total aflatoxins at European regulatory limits. The test portion is extracted with methanol–water (8 + 2) for dried figs and paprika, and with methanol–water (8 + 2) plus hexane (or cyclohexane) for peanut butter and pistachios. The sample extract is filtered, diluted with phosphate buffer saline, and applied to an immunoaffinity column. The column is washed with water and the aflatoxins are eluted with methanol. Aflatoxins are quantitated by reversed-phase LC with post-column derivatization (PCD) involving bromination. PCD is achieved with either an electrochemical cell (Kobra cell) and addition of bromide to the mobile phase or pyridinium hydrobromide perbromide. Determination is by fluorescence. Peanut butter, pistachio paste, dried fig paste, and paprika powder samples, both naturally contaminated with aflatoxins and containing added aflatoxins, were sent to 16 collaborators in 16 European countries. Test portions of samples were spiked at levels of 2.4 and 9.6 ng/g for total aflatoxins which included 1.0 and 4.0 ng/g aflatoxin B_1, respectively. Recoveries for total aflatoxins ranged from 71 to 92% with corresponding recoveries for aflatoxin B_1 of 82 to 109%. Based on results for spiked samples (blind duplicates at 2 levels) as well as naturally contaminated samples (blind duplicates at 4 levels, including blank), the relative standard deviation for repeatability ranged from 4.6 to 23.3% for total aflatoxins and from 3.1 to 20.0% for aflatoxin B_1. The relative standard deviation for reproducibility ranged from 14.1 to 34.2% for total aflatoxins, and from 9.1 to 32.2% for aflatoxin B_1. The method showed acceptable within-laboratory and between-laboratory precision for all 4 matrixes, as evidenced by HORRAT values <1, at the low levels of determination for both total aflatoxins and aflatoxin B_1.

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The recommendation was approved by the Methods Committee on Natural Toxins and was adopted by the Official Methods Board of AOAC INTERNATIONAL. *See* "Official Methods Board Actions," (1999) *Inside Laboratory Management*, July, 1999 issue.

Methodology for determining aflatoxins in foods has greatly improved in recent years with the commercial availability of immunoaffinity columns having a particularly important impact (1). A collaborative trial with peanut butter was performed in the United Kingdom when these columns first became available (1989) and initially indicated promising results (2) although the trial was not performed strictly according to AOAC guidelines. In a subsequent international collaborative trial in 1990 (3) using post-column derivatization with iodine, some participants experienced problems with recoveries, and despite acceptable precision data, the method was not pursued for AOAC recognition. Another collaborative trial for peanut butter (4) organized in the United Kingdom for food control analysts

(21 public analyst laboratories) reported relative standard deviation for repeatability (RSD_r) values of 17 to 44% and relative standard deviation for reproducibility (RSD_R) values of 36 to 54% for concentrations of total aflatoxins from 7 to 47 ng/g. The precision parameters were regarded as rather high for this trial and the samples distributed were atypical, having aflatoxin G_1 as the principal aflatoxin in the peanut butter. The most successful collaborative trial, performed by Trucksess et al. in 1990 (5), used immunoaffinity column cleanup of samples and either solution fluorimetry or post-column derivatization with iodine for determining total aflatoxins in corn, peanuts, and peanut butter at levels >10 ng/g. This method was adopted by AOAC INTERNATIONAL as First Action (6).

European Commission Regulations (7) for aflatoxins which were to be implemented from January 1, 1999, set limits for aflatoxin B_1 and total aflatoxins of 2 and 4 ng/g, respectively in groundnuts, nuts, dried fruit, cereals (including buckwheat), and processed products for human consumption. The associated sampling requirements are to be implemented by December 31, 2000, although member states are encouraged to put the stipulated procedures into practice as soon as possible. Amendment of these regulations to set maximum limits for aflatoxins in spices is under consideration subject to the findings of a European Union (EU)-wide coordinated sampling program. Existing validated methods for aflatoxins, such as the AOAC First Action Method (6) for raw peanuts, corn, and peanut butter at >10 ng/g for total aflatoxins, do not fulfill European Regulatory requirements, either in terms of limits of detection or the wider range of materials such as dried fruit and spices covered by European regulations. It was therefore decided as part of a European Commission Standards, Measurement and Testing (SMT) Programme funded project on method validation to develop methodology and undertake a collaborative study for aflatoxins in peanut butter, pistachio paste, fig paste, and paprika at the lower limits required by the new regulatory controls. The validated method may ultimately be considered for adoption as a European Committee for Standardization (CEN) standard, and fulfill AOAC INTERNATIONAL requirements for a collaborative study.

Previous work at a European level for aflatoxin B_1 in animal feedings (8) has shown that with care and attention to detail during organization of a collaborative trial it is possible to achieve impressive performance characteristics for a method even at low limits of detection. Due to the low contamination levels involved in the present study and the attendant recognized difficulties in producing homogeneous test materials, particular care was taken in preparation (grinding to small particle size and mixing) and in demonstrating inter-unit homogeneity before undertaking the study. Although the methods used were not radically different from existing immunoaffinity column LC procedures widely used by many laboratories for determining aflatoxins (1), the extraction conditions and the use of post-column bromination with pyridinium hydrobromide perbromide (PBPB) are generally less accepted. Preliminary work showed that the proposed methanol–water extraction for dried figs and paprika and methanol–water plus hexane for peanut butter and pistachios gave recoveries at least as good as, if not better than, other solvent systems for naturally contaminated materials (9). To ensure that all collaborative trial participants rigorously followed the protocol, a precollaborative trial workshop was held in January 1998. The workshop did not involve hands-on analytical work, but provided opportunities to discuss and raise potential difficulties that could be encountered in the collaborative trial.

Test Materials for Collaborative Study

Preparation of Peanut Butter

Because of the heterogeneity of commercially available peanut butters, blank material (100 kg) was prepared from raw material containing shelled and blanched peanuts and selected from a number of different materials after preliminary analysis. The selected raw material was roasted in a band dryer (140°–170°C, 5 min) and processed to a paste by a roll mill. The processing was consistent with typical commercial practice. The blank material was blended with contaminated commercially available peanut butter (35 kg, 11.5 ng/g total aflatoxins) in the appropriate ng/g mass ratio to obtain samples with the contamination levels required. This material was blended in a high-shear blender in a warmed container. An amount of 0.4% lecithin was added using the roll mill to avoid separation of oil in the peanut butters. Lecithin is also used in commercially available peanut butter and does not cause any difficulty to the developed method.

Preparation of Pistachio Paste

Because no blank material was commercially available for a reasonable price, the blank sample was prepared from green, unripe pistachios (30 kg), which were dried (103°C, 2 × 6 h) and made into a paste using a cutter. Two different naturally contaminated batches of pistachios (20 kg of 1 ng/g or 17 ng/g total aflatoxins, respectively) containing ripe and shelled pistachios were dried and made into a paste in the same manner as described for peanuts. Finally, the pistachio pastes were stabilized with 0.4% lecithin using a microcutter.

Preparation of Fig Paste

Effort was made to procure non-contaminated figs from those available commercially. Unfortunately, from the analysis of 10 different batches of figs, the best blank sample obtainable contained 0.4–0.5 ng/g total aflatoxins. This material was therefore taken as the blank and blended (35 kg) with naturally contaminated materials (20 kg of 2 ng/g or 6 ng/g total aflatoxins, respectively). The material was first ground using a meat mincer equipped with a 3 mm sieve, dissolved in 4% glycerine containing 0.3% potassium sorbate, then treated with a cutter, and finally ground in a meat mincer again until the target aflatoxin content was reached.

Preparation of Paprika Powder

Paprika powder was obtained from various European countries. Naturally contaminated material was procured at 3 levels: 20 kg of 1 ng/g, 2.5 ng/g, or 4.5 ng/g total aflatoxin, respectively, as well as blank material (50 kg). The various lots were blended in a ribbon blender to the contamination levels required.

Bulk Homogeneity

The bulk homogeneity of pastes of peanuts, pistachios, and figs was investigated by analysis of variance (ANOVA), according to the International Harmonized Protocol for Proficiency Testing of Analytical Laboratories (10). Ten samples were randomly taken and analyzed in duplicate. The corresponding homogeneity testing of paprika powders was performed by analyzing 10 randomly selected samples. In addition, the variance of 10 blank samples spiked with a calibration solution was determined. A systematic difference of the respective variances was investigated by performing the F-test.

Packaging

For peanut butter, pistachio paste, and paprika, the homogenized material was filled manually into transparent glass containers. Two hundred containers of each matrix and of each level were filled with 51–53 g. The open containers were then heated in an oven to a core temperature of 80°C. Subsequently, the containers were closed manually with metal screw tops. For fig paste, the homogenized material was filled manually into transparent glass containers. Two hundred containers of each level were filled with 51–53 g. The containers were closed manually with metal screw tops.

Homogeneity Testing of Packaged Material

Every 10th sample was taken from the sequence and analyzed. The number of the first container from which the sampling started was randomly determined for each material, respectively. In the first part of the homogeneity test, the data were analyzed by the ANOVA that allows partitioning of the whole variance into individual components of variability. In this study, 10 samples were analyzed in duplicate; from each sample, 2 subsamples were analyzed. Using the ANOVA technique the following variances can be calculated:

Within-sample standard deviation =

$$\sqrt{\text{Within - Container - Variance}} = \sqrt{\sigma^2_{within}}$$

Assuming that the homogeneity within the containers can be considered as negligible, this component reflects the analytical error.

Between-Group-Variance ($\sigma^2_{between}$)

$$\text{Overall variance} = \sigma^2_{total} = \sigma^2_{between} + \sigma^2_{within}$$

Between-Sample-Standard-Deviation=

$$\sqrt{\frac{\sigma^2_{between} - \sigma^2_{within}}{2}} = \sigma_s$$

This component reflects the heterogeneity of the material and can only be calculated if the *F*-test of the ANOVA indicates a significant difference between $\sigma^2_{between}$ and σ^2_{within}.

The test material was deemed as satisfactory if the variances mentioned above did not differ significantly from the variance of the method itself. The equality of variances was checked by a second step using the *F*-test, which was applied to check for a drift in results, as follows:

- The samples of each material were arranged in the chronological order in which they had been analyzed, making up a row of 20 values.
- The mean ($\bar{x}_1$) of the first, second, and third value and the mean ($\bar{x}_2$) of the 18th, 19th, and 20th value were calculated, respectively. If there is a trend in the results the difference of these 2 values would be significant.
- To check for a significant difference of the means, the *t*-value was calculated according to the following equation, where SD is the standard deviation of the method determined in the in-house performance study:

$$t_{\text{calc}} = \frac{(\bar{x}_1 - \bar{x}_2)}{\text{SD}/\sqrt{3}}$$

The calculated *t*-value was compared with the critical *t*-value. If t_{calc} is within the range of $\pm\, t_{\text{calc}}$, the difference of the means is not significant, indicating that no trend of the results can be observed.

Storage of Collaborative Trial Samples

About 6 months elapsed between preparation/homogeneity testing and distribution of samples to collaborative trial participants. Although there is no evidence of aflatoxin instability in food matrixes, as a precautionary measure all samples were stored at –18°C before distribution.

Organization of Collaborative Study

The 16 collaborators from 16 different European countries represented a cross-section of government, food control, university, and food industry affiliations. Before the trial, each collaborator received a practice sample made up of a blank material and a calibrant solution for spiking. Collaborators met at a pre-collaborative trial workshop where any problems experienced with analysis of the practice sample were discussed, and the details and organization of the trial were outlined by the coordinators.

For the collaborative trial each participant received:

(**a**) Eight coded samples of peanut butter (blind duplicates at 4 content levels) plus 4 labeled blank units for spiking.

(**b**) Eight coded samples of pistachio paste (blind duplicates at 4 content levels) plus 4 labeled blank units for spiking.

(**c**) Eight coded samples of fig paste (blind duplicates at 4 content levels) plus 4 labeled blank units for spiking.

(**d**) Eight coded samples of paprika powder (blind duplicates at 4 content levels) plus 4 labeled blank units for spiking.

(**e**) One amber vial marked "Aflatoxin calibrants," containing a mixture of aflatoxins B_1, B_2, G_1, and G_2, which was to be used as the mixed calibrant aflatoxin solution described in the method.

(**f**) Two amber vials marked 'Spike solution A' and 'Spike solution B' to be used for spike recovery determinations.

(**g**) Fifty immunoaffinity columns (2 spares) for the cleanup of material extracts.

(**h**) A copy of the method.

(**i**) A report form for analytical data and reporting any criticisms and suggestions.

(**j**) A "Collaborative Study Materials Receipt" form.

Each participant was required to prepare one extract from each material, perform the cleanup using one immunoaffinity column, and analyze the extracts by LC. Additionally, each participant was required to spike 4 indicated blank materials of each matrix by adding 0.25 and 1.00 mL 'Solution A' and 0.25 and 1.00 mL 'Solution B,' which contained mixtures of aflatoxins at concentrations unknown to participants. After adding the spike solution, participants were instructed to mix by shaking and allow to stand for at least 30 min before extraction. The theoretical spiking levels were 1.0 and 4.0 ng/g aflatoxins B_1 and G_1 and 0.2 and 0.8 ng/g aflatoxins B_2 and G_2 (giving corresponding levels for total aflatoxins of 2.4 and 9.6 ng/g, respectively). Spiking was not done centrally by the collaborative trial organizers as this would have necessitated additional homogeneity testing and demonstration of aflatoxin stability in the spiked test material.

Participants were advised to analyze the 4 different matrixes on separate days. This would mean analyzing a batch of 12 samples (8 coded plus 4 spike samples per matrix) on separate days, thus completing the experimental work for the trial in 4 days, assuming LC analysis was performed overnight between days. Participants were instructed that samples should be analyzed in the numerical sequence of the sample codes (i.e., nested design).

999.07 Aflatoxin B_1 and Total Aflatoxins in Peanut Butter, Pistachio Paste, Fig Paste, and Paprika Powder—Immunoaffinity Column LC with Post-Column Derivatization

First Action 1999

(Applicable to determination of aflatoxin B_1 in peanut butter, pistachio paste, fig paste, and paprika powder, and of aflatoxins B_1, B_2, G_1, and G_2 in peanut butter, pistachio paste, fig paste, and paprika powder)

Caution: This method requires the use of solutions of aflatoxin B_1. Aflatoxins are carcinogenic to humans. Attention is drawn to the statement made by the WHO, International Agency for Research on Cancer (12).

Method Performance

See Table **999.07A** for method performance data.

Aflatoxins are subject to light degradation. Protect analytical work adequately from the daylight, and keep aflatoxin standard solutions protected from light by using amber vials or aluminium foil.

The use of non acid-washed glassware (e.g., vials, tubes, flasks) for aflatoxin aqueous solutions may cause a loss of aflatoxin. Special attention should be taken with new glassware. Thus, before use, soak the glassware in dilute acid (e.g., sulfuric acid, 2 mol/L) for several hours; then rinse extensively with distilled or deionized water to remove all traces of acid (this can be checked with pH paper).

A. Principle

Test portion is either extracted with MeOH–H_2O (8 + 2) or MeOH–H_2O (8 + 2) plus hexane (or cyclohexane). Sample extract is filtered, diluted with water to a specified solvent concentration, and applied to an affinity column containing antibodies specific to aflatoxins B_1, B_2, G_1, and G_2. Aflatoxins are removed from the affinity column with MeOH. Aflatoxins are quantitated by reversed-phase liquid chromatography (LC) with post-column derivatization (PCD) involving bromination. PCD is achieved with either electrochemically generated bromine (Kobra cell) or with pyridinium hydrobromide perbromide (PBPB) and determination is by fluorescence detection.

B. Performance Standards for Affinity Column

The affinity column should contain antibodies raised against aflatoxins B_1, B_2, G_1, and G_2. The column should have a maximum capacity of not less than 100 ng aflatoxin B_1 and should give a recovery of not less than 80% for aflatoxins B_1, B_2, and G_1 and not less than 60% for aflatoxin G_2 when applied as an aqueous standard solution (10% CH_3OH) containing 5 ng of each toxin.

C. Apparatus

(**a**) *Blender.*—Explosion proof (minimum 8000 rpm).

(**b**) *Vertical shaker.*—Adjustable (for maximum sample–extractant agitation); capable for 500 mL Erlenmeyer flasks.

(**c**) *Filter paper.*—24 cm diameter, prefolded, retention: 30 μm (or better).

(**d**) *Erlenmeyer flask.*—500 mL, screw top or glass stopper.

(**e**) *Glass microfiber filter paper.*—5 cm diameter, retention: 1.6 μm (or better).

(**f**) *Reservoir.*—75 mL with Luer tip connector for affinity column.

(**g**) *Hand pump.*—20 mL syringe with Luer lock or rubber stopper.

(**h**) *Volumetric glassware.*—2, 3, 10, and 20 mL (accuracy of at least 0.5%).

Table 999.07A. Method performance for aflatoxin B_1 and total aflatoxins in peanut butter, pistachio paste, fig paste, and paprika powder

Food	Contamin.	Av., ng/g	No. of labs[a]	S_r	RSD_r, %	S_R	RSD_R, %
			Aflatoxin B_1				
Peanut butter	Fortified	0.9	15	0.09	10	0.16	19
	Fortified	3.6	13	0.11	3	0.66	18
	Naturally	0.8	15	0.05	6	0.26	32
	Naturally	1.5	14	0.10	6	0.22	14
	Naturally	3.4	14	0.13	4	0.65	19
Pistachio paste	Fortified	0.9	15	0.13	14	0.15	16
	Fortified	3.3	12	0.13	4	1.02	31
	Naturally	0.7	13	0.08	11	0.12	17
	Naturally	1.5	15	0.27	18	0.36	23
	Naturally	2.9	14	0.59	20	0.61	21
Fig paste	Fortified	1.1	15	0.18	17	0.21	19
	Fortified	3.6	15	0.39	11	0.46	13
	Naturally	1.3	16	0.12	10	0.30	23
	Naturally	2.1	14	0.12	6	0.31	15
	Naturally	2.6	16	0.41	16	0.73	29
Paprika powder	Fortified	0.9	14	0.05	6	0.09	10
	Fortified	3.4	15	0.18	5	0.35	10
	Naturally	0.8	15	0.12	14	0.16	19
	Naturally	1.4	15	0.14	10	0.24	17
	Naturally	3.0	14	0.13	4	0.28	9
			Total aflatoxin[b]				
Peanut butter	Fortified	1.9	15	0.26	13	0.35	18
	Fortified	7.9	15	0.67	9	1.76	22
	Naturally	1.3	15	0.08	6	0.46	34
	Naturally	2.2	13	0.16	7	0.32	14
	Naturally	5.0	14	0.23	5	0.96	19
Pistachio paste	Fortified	2.0	14	0.24	12	0.36	18
	Fortified	7.8	14	1.82	23	1.82	23
	Naturally	0.8	13	0.10	12	0.17	21
	Naturally	1.7	15	0.31	18	0.42	24
	Naturally	3.3	14	0.66	20	0.72	22
Fig paste	Fortified	2.2	15	0.40	18	0.73	32
	Fortified	7.8	15	1.01	13	1.28	17
	Naturally	2.8	16	0.25	9	0.80	28
	Naturally	3.8	16	0.44	12	1.03	29
	Naturally	5.2	16	0.90	17	1.56	30
Paprika Powder	Fortified	1.7	13	0.11	6	0.34	20
	Fortified	7.1	15	0.72	10	1.01	14
	Naturally	0.9	16	0.16	17	0.31	34
	Naturally	2.0	16	0.23	12	0.55	28
	Naturally	4.5	14	0.22	5	0.66	15

[a] Number of laboratories that submitted acceptable results (the total number of participating laboratories was 16).

[b] The total aflatoxin parameter was subject to statistical evaluation after summarization of single aflatoxin results. The acceptance of each single result was not determined prior to summarization (pre-limitation of single results for further evaluation), thus allowing difference in number of accepted results for 'aflatoxin B_1' and 'total aflatoxin'.

Table 999.07B. Preparation of working calibration solutions

Working standard	Aliquot taken from working calibrant solution, µL	Final mass concentration of working calibrant, ng/mL			
		B_1	B_2	G_1	G_2
1	40	0.400	0.080	0.400	0.080
2	120	1.200	0.240	1.200	0.240
3	200	2.000	0.400	2.000	0.400
4	280	2.800	0.560	2.800	0.560
5	360	3.600	0.720	3.600	0.720

(**i**) *LC pump.*—Suitable for flow rate at 1.000 ± 0.005 mL/min.

(**j**) *Injection system.*—Valve with 200 µL loop or equivalent.

(**k**) *RP-LC column.*—(4.6 mm × 25 cm, 5 µm), e.g., LC-18 or ODS-2.

(**l**) *Post column derivatization system.*—(*1*) *With pyridinium hydrobromide perbromide (PBPB).*—Second LC pulseless pump, zero-dead volume T-piece, reaction tubing minimum dimensions 45 cm × 0.5 mm id polytetrafluoroethylene (PTFE). (*2*) *With electrochemically generated bromine.*—e.g., Kobra cell; Rhône Diagnostics Technologies Ltd., Lyon, France.

(**m**) *Fluorescence detector.*—Wavelength of λ = 360 nm excitation filter and a wavelength of λ > 420 nm cut-off emission filter, or equivalent.

(**n**) *Disposable filter unit.*—Cellulose or cellulose nitrate, 0.45 µm.

(**o**) *Pipets.*—Marked 10 mL capacity.

(**p**) *Analytical balance.*—Capable of weighing to 0.1 mg.

(**q**) *Laboratory balance.*—Capable of weighing to 0.1 g.

(**r**) *Calibrated microliter syringe(s) or microliter pipet(s).*—25 and 500 µL capacity.

D. Reagents

All reagents shall be of recognized analytical grade.

Unless otherwise stated, use water complying with grade 3 of ISO 3696:

ISO 3696 Grade 3.—Suitable for most laboratory wet chemistry work and preparation of reagent solutions; should be produced, for example, by single distillation, deionization, or reverse osmosis. Unless otherwise specified, it should be used for ordinary analytical work.

Note: It is assumed that the initial feed stock of water is potable and reasonably pure. If it is heavily contaminated in any respect, some pretreatment may be necessary.

(**a**) *Phosphate buffer saline (PBS), pH 7.4.*

(**b**) *Sodium chloride.*

(**c**) *PBPB.*—CAS: 39416-48-3.

(**d**) *Potassium bromide.*

(**e**) *LC grade acetonitrile.*

(**f**) *LC grade methanol.*

(**g**) *Methanol.*—Technical grade, pure or distilled.

(**h**) *LC grade water, complying with grade 1 of ISO 3696.*

(**i**) *Extraction solvent.*—Methanol–water solution (8 + 2, v/v).

(**j**) *Hexane or cyclohexane.*

(**k**) *Nitric acid, c(HNO_3) = 4 mol/L.*—The "c(HNO_3)" reflects the SI (Systeme International d'Unites) unit for concentration of a substance. In this case, nitric acid concentration is in mol/L. A solution of 4 mol/L HNO_3 can be made by diluting 28.1 mL concentrated HNO_3 (65%) in water to final volume of 100 mL.

(**l**) *LC mobile phase solvent (A).*—Water–acetonitrile–methanol (**f**) solution (6 + 2 + 3, v/v/v).

(**m**) *LC mobile phase solvent (B).*—For use with electrochemically generated bromine: water–acetonitrile–methanol solution (6 + 2 + 3, v/v/v). To each liter of mobile phase, a volume of 350 µL nitric acid [4 mol/L; *D*(**k**)] and 120 mg potassium bromide, *D*(**d**), must be added and dissolved.

(**n**) *Post column reagent (B).*—Dissolve 25 mg PBPB in 500 mL H_2O. Solution can be used for up to 4 days if stored in a dark place at room temperature.

(**o**) *Toluene–acetonitrile.*—(98 + 2, v/v).

(**p**) *Aflatoxin standard solutions for LC.*—(*1*) *Mixed aflatoxins calibrant solution X for LC.*—Prepare as in **971.22B-E** to contain 1000 ng B_1, 200 ng B_2, 1000 ng G_1, and 200 ng G_2/mL toluene–acetonitrile (98 + 2). (*2*) *Working calibrant solutions for LC.*—Prepare solution by pipetting exactly 2.0 mL calibrant solution X into 20.0 mL calibrated volumetric flask. Fill to mark with toluene–acetonitrile solution and shake well. Use this solution for pipetting volumes listed in Table **999.07B** into a set of 10.0 mL calibrated volumetric flasks. Evaporate toluene–acetonitrile solution just to dryness under a stream of nitrogen at room temperature. To each flask, add 4 mL methanol; then mix, dilute to 10.0 mL with water, and mix again. Prepare these working solutions daily.

E. Extraction

(**a**) *Peanut butter and pistachio paste.*—Weigh, to nearest 0.1 g, 50 g test portion into 500 mL Erlenmeyer flask, add 5 g NaCl, 200 mL methanol–water (ISO 3696, grade 3) extraction solvent, and 100 mL hexane or cyclohexane. Blend 3 min with high speed blender. Filter and pipette 10.0 mL clear filtrate into reservoir containing 60 mL PBS placed on conditioned immunoaffinity column. Mix with plastic spatula and rinse residues with 1–2 mL PBS from spatula into reservoir. Transfer solution on column as described in section *F*.

(**b**) *Paprika powder*.—Weigh, to nearest 0.1 g, 50 g test portion into 500 mL Erlenmeyer flask with screw top or glass stopper. Add 5 g NaCl and 300 mL methanol–water solvent. Shake intensively by hand for 15–30 s, and then for 30 min on a shaker. Filter extract using prefolded paper. Pipette 10.0 mL clear filtrate into reservoir containing 60 mL PBS placed on conditioned immunoaffinity column. Mix with plastic spatula and rinse residues with 1–2 mL PBS into reservoir. Apply solution on column as described in section *F*.

(**c**) *Dried figs*.—Weigh, to nearest 0.1 g, 50 g test portion into 500 mL Erlenmeyer flask, add 5 g NaCl and 300 mL methanol–water (ISO 3696, grade 3) extraction solvent. Blend 3 min with high speed blender. Filter and pipette 10.0 mL clear filtrate into reservoir containing 60 mL PBS placed on conditioned immunoaffinity column. Mix with plastic spatula and rinse residues with 1–2 mL PBS from spatula into reservoir. Transfer solution on column as described in section *F*.

F. Affinity Column Chromatography

Adjust columns to room temperature before conditioning. For conditioning, apply 10 mL PBS on top of column and let it pass at a speed of 2–3 mL/min through the column (gravity). Make sure that a small portion (0.5 mL) of PBS remains on the column until sample solution is applied.

[*Note*: Methods for loading onto affinity columns, washing the column, and elution vary slightly between column manufacturers, and specific instructions supplied with columns should be followed precisely. In general, procedures involve sample extraction with methanol–water, filtration, or centrifugation, possible sample dilution with PBS or water, loading under pressure onto (possibly pre-washed) column, washing of column with distilled water, and elution of aflatoxins with methanol or acetonitrile.]

Pass filtrate through column at flow rate of ca 1 drop/s (ca 3 mL/min; gravity). Do not exceed 5 mL/min. Wash column with 15 mL water and dry by applying little vacuum for 5–10 s or passing air through with a syringe for 10 s.

Elute aflatoxins by the following procedure: Apply 0.5 mL methanol on the column and let it pass through by gravity. Collect eluate in 3.0 mL calibrated volumetric flask. Wait 1 min and apply second portion of 0.75 mL methanol. Collect applied elution solvent by pressing air through. Fill to mark with water, and mix. If solution is clear, it can be used directly for LC analysis. If it is not clear, pass it through a disposal filter unit (0.45 μm) before injection on the LC column.

G. LC Determination with Fluorescence Detection and Post-Column Derivatization

When using PBPB, mount mixing T-piece and reaction tubing mentioned above; then operate using the following parameters: flow rates, 1.00 mL/min (mobile phase A) and 0.30 mL/min (reagent).

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

Inject 200 μL working standard mixture (1–4 ng/g for aflatoxin B_1) into injector, following manufacturer's instructions to ensure complete filling of the injection loop. Aflatoxins elute in the order G_2, G_1, B_2, and B_1, with retention times of ca 6, 8, 9, and 11 min respectively, and should be baseline resolved. Prepare a calibration curve using calibration solutions described and check curve for linearity (11). Inject 200 μL extract into injector and identify each aflatoxin peak in the chromatogram by comparing retention times with those of corresponding reference standards. Determine quantity of aflatoxin in eluate injected from the standard curve.

H. Calculations

Calculate concentration of aflatoxin in test sample as follows: Plot data [concentration of aflatoxin (ng/mL; y-axis) against the peak area (units; x-axis)] from the calibrant solution experiments into a table and calculate the calibration curve using linear regression. Use the resulting function ($y = ax + b$) to calculate the concentration of aflatoxin in the measured solution.

For a linear calibration, the formula describes the correlation between the detector signal (x) and the corresponding concentration of the analyte (y).

This means that (y) is a function of (x) [$y = (f)\ x$]. The constant (a) is the corresponding value of the slope of the linear function, while (b) is the value where the calibration function intercepts the y-axis of the coordinate system.

Wt (g) = sample material taken for analysis; solvent (mL) = solvent taken for extraction; aliquot (mL) = aliquot taken for immunoaffinity cleanup; elution (mL) = final volume collected after elution from IAC; C_{smp} (ng/mL) = concentration of aflatoxin calculated from linear regression; Contam. (ng/g) = contamination of sample material with aflatoxin; $Signal_{smp}$ (units) = area of aflatoxin peak obtained from the measured solution.

Calculation of the calibration curve (function) obtained by linear regression:

$$C_{smp},\ \text{ng/mL} = a \times \text{Signal}_{smp}\ (\text{units}) + b$$

$$\text{Contam.} = \frac{C_{smp} \times \text{Solvent} \times \text{Elution}}{\text{Wt} \times \text{Aliquot}} \left[\frac{\text{ng} \times \text{mL} \times \text{mL}}{\text{mL} \times \text{g} \times \text{mL}}\right]$$

Note that for sample preparation procedures involving the use of hexane or cyclohexane, the volume of these solvents added for extraction *must not* be taken into account for the calculation.

Add mass fractions of the 4 aflatoxins to obtain a total aflatoxin mass fraction.

Note: Soak all laboratory glassware in 10% solution of household bleach, which generally contains 5.25% NaOCl, before reusing or discarding. *See* **990.32J** for further details on decontamination. (*See* also reference 12).

Ref.: *J. AOAC Int*. **83**, 323–326(2000)

Table 1. Collaborative trial results of liquid chromatographic determination of aflatoxins in peanut butter

Aflatoxin B_1 concentration, ng/g (Blind duplicate pairs of naturally contaminated samples)												
Lab ID	1.0	1.0	4.0	4.0	A	A	B	B	C	C	D	D
A	1.0	0.8	3.7	3.5	<0.2	<0.2	0.9	0.9	1.4	1.5	3.2	3.6
B	0.9[a]	0.1	3.6	0.4	0.0	0.0	0.2	0.2	0.3	0.2	1.2	0.1
C	1.4	1.1	5.1	5.4	<0.01	<0.01	1.0	1.0	1.6	1.7	3.9	4.0
D	0.9	1.1	4.5	4.5	<0.05	<0.05	1.0	1.1	1.8	1.5	3.8	3.9
E	0.7	0.6	2.8	2.4	<0.10	<0.10	0.7	0.6	1.2	1.2	2.7	2.7
F	0.8	0.7	3.1	1.6	<0.12	<0.12	0.7	0.8	1.1	1.1	2.8	2.8
G	0.9	0.7	3.1	3.1	<0.3	<0.3	0.7	0.6	1.2	1.3	2.8	3.1
H	0.8	0.8	3.3	3.2	<0.1	<0.1	0.9	0.8	1.5	1.6	3.4	3.6
I	0.9	0.9	3.5	3.6	<0.1	<0.1	1.0	1.0	1.6	1.8	4.0	4.0
J	0.9	0.9	3.6	3.7	<0.1	<0.1	1.0	0.5	1.7	1.7	4.0	3.8
K	0.8	0.7	3.1	2.4	<0.1	<0.1	0.4	0.3	1.6	0.5	0.5	0.6
L	0.9	0.9	3.6	3.6	<0.01	<0.01	1.0	0.9	1.8	1.8	4.0	4.3
M	0.8	0.8	3.0	3.2	<0.10	<0.1	0.9	0.8	1.5	1.5	3.3	3.2
N	0.9	0.9	3.7	3.7	<0.1	<0.1	0.9	0.9	1.7	1.4	1.7	2.0
O	0.8	0.8	3.5	3.6	<0.02	<0.02	0.9	0.9	1.5	1.5	3.5	3.4
P	1.1	1.1	4.0	4.1	0.0	0.1	1.1	1.1	1.8	1.7	3.9	4.0
Aflatoxin B_2 concentration, ng/g												
A	0.2	0.2	0.7	0.7	<0.1	<0.1	0.1	0.2	0.3	0.3	0.7	0.8
B	0.2	0.1	0.8	0.3	0.0	0.0	0.1	0.1	0.1	0.1	0.4	0.2
C	0.3	0.2	1.0	1.1	<0.01	<0.01	0.2	0.2	0.3	0.3	0.8	0.8
D	0.2	0.3	0.9	0.9	<0.05	<0.05	0.2	0.2	0.4	0.3	0.8	0.8
E	0.2	0.2	0.6	0.7	<0.04	<0.04	0.2	0.1	0.3	1.3	0.7	0.7
F	0.2	0.2	0.8	0.4	<0.04	<0.04	0.1	0.2	0.3	0.3	0.6	0.7
G	0.2	<0.2	0.7	0.7	<0.16	<0.16	<0.2	<0.2	0.3	0.3	0.6	0.6
H	0.2	0.1	0.5	0.6	<0.04	<0.04	0.1	0.1	0.3	0.3	0.6	0.6
I	0.2	0.2	0.7	0.7	<0.05	<0.05	0.2	0.2	0.3	0.4	0.8	0.8
J	0.2	0.2	0.8	0.8	<0.04	<0.04	0.2	0.1	0.4	0.4	0.9	0.8
K	0.2	0.2	0.7	0.6	<0.05	<0.05	0.1	0.1	0.4	0.1	0.1	0.1
L	0.2	0.2	0.8	0.7	<0.01	<0.01	0.2	0.2	0.3	0.3	0.8	0.8
M	0.2	0.2	0.6	0.6	<0.07	<0.07	0.2	0.2	0.3	0.3	0.7	0.7
N	0.2	0.2	0.7	0.8	<0.1	<0.1	0.1	0.2	0.3	0.2	0.4	0.4
O	0.2	0.2	0.7	0.8	<0.02	<0.02	0.2	0.2	0.4	0.3	0.8	0.7
P	0.2	0.2	0.8	0.9	<0.02	<0.02	0.2	0.2	0.4	0.3	0.8	0.9
Aflatoxin G_1 concentration, ng/g												
A	0.8	0.8	3.5	3.4	<0.2	<0.2	0.3	0.4	0.3	0.3	0.6	0.8
B	2.0	0.2	7.9	1.2	0.0	0.0	0.2	0.1	0.1	0.1	0.6	0.0
C	1.1	0.8	4.4	4.2	<0.01	<0.01	0.4	0.5	0.3	0.3	0.8	0.7
D	0.7	0.9	3.6	4.1	<0.05	<0.05	0.3	0.3	0.3	0.2	0.7	0.7
E	0.7	0.7	2.9	3.4	<0.20	<0.20	0.4	0.3	0.4	0.4	0.7	0.7
F	0.7	0.7	2.8	2.2	<0.3	<0.3	0.4	0.5	0.4	0.3	0.6	0.6
G	0.7	0.6	2.9	2.8	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
H	0.8	0.8	3.2	3.3	<0.1	<0.1	0.4	0.4	0.3	0.4	0.8	0.8

Table 1. (*continued*)

Lab ID	1.0	1.0	4.0	4.0	A	A	B	B	C	C	D	D
I	0.9	0.8	3.2	3.2	<0.1	<0.1	0.4	0.4	0.3	0.4	0.8	0.8
J	0.8	0.7	3.1	3.2	<0.15	<0.15	0.3	0.2	0.4	0.3	0.8	0.7
K	0.8	0.4	1.5	0.9	<0.13	<0.13	<0.13	<0.13	0.4	<0.13	<0.13	<0.13
L	0.9	0.9	3.8	3.9	<0.01	<0.01	0.5	0.5	0.4	0.4	1.0	1.0
M	0.6	0.3	0.7	2.1	<0.2	<0.2	<0.2	<0.2	0.3	0.4	0.5	0.3
N	0.8	0.7	3.2	3.4	<0.1	<0.1	0.4	0.4	0.4	0.3	0.4	0.5
O	0.7	0.9	3.2	3.8	<0.02	<0.02	0.4	0.4	0.3	0.3	0.7	0.7
P	0.8	0.8	2.8	3.1	<0.04	<0.04	0.6	0.5	0.3	0.4	0.9	0.9
						Aflatoxin G_2 concentration, ng/g						
A	0.1	0.2	0.7	0.7	<0.1	<0.1	0.1	0.1	<0.1	0.1	0.2	0.2
B	0.4	0.2	1.4	0.5	0.0	0.0	0.2	0.1	0.1	0.2	0.3	0.2
C	0.2	0.1	0.9	0.8	<0.02	<0.02	0.1	0.1	0.1	0.1	0.2	0.2
D	0.2	0.2	0.8	0.8	<0.05	<0.05	0.1	0.1	0.1	0.1	0.2	0.2
E	0.2	0.1	0.7	0.7	<0.08	<0.08	0.1	0.1	0.1	0.1	0.2	0.2
F	0.1	0.2	0.4	0.5	<0.09	<0.09	0.1	0.1	0.1	0.1	0.2	0.5
G	0.2	0.1	0.7	0.7	<0.07	<0.07	0.1	0.1	<0.07	<0.07	0.2	0.2
H	0.1	0.0	0.2	0.5	<0.1	<0.1	0.1	0.1	0.1	0.1	0.2	0.2
I	0.2	0.2	0.7	0.7	<0.1	<0.1	0.1	0.1	0.1	0.1	0.2	0.2
J	0.2	0.2	0.7	0.7	<0.08	<0.08	0.1	<0.08	0.1	0.1	0.2	0.2
K	0.2	<0.1	0.3	0.2	<0.1	<0.1	<0.1	<0.1	0.1	<0.1	<0.1	<0.1
L	0.2	0.2	0.6	0.6	<0.01	<0.01	0.1	0.1	0.1	0.1	0.3	0.3
M	<0.05	<0.05	0.2	0.4	<0.05	<0.05	<0.05	<0.05	0.1	<0.05	0.2	<0.05
N	0.2	0.2	0.7	0.8	<0.1	<0.1	0.1	0.1	0.1	0.1	0.1	0.1
O	0.2	0.2	0.7	0.8	<0.02	<0.02	0.1	0.1	0.1	0.1	0.2	0.2
P	0.2	0.2	0.6	0.7	<0.03	<0.03	0.1	0.1	0.1	0.1	0.3	0.3
						Total aflatoxin concentration, ng/g						
A	2.1	2.0	8.6	8.4	0.0	0.0	1.4	1.5	2.0	2.3	4.7	5.4
B	3.5	0.6	13.7	2.4	0.0	0.0	0.7	0.5	0.5	0.6	2.5	0.5
C	3.0	2.2	11.4	11.4	0.0	0.0	1.7	1.8	2.3	2.4	5.7	5.7
D	2.0	2.5	9.8	10.4	0.0	0.0	1.6	1.7	2.5	2.1	5.5	5.6
E	1.7	1.6	7.0	7.3	0.0	0.0	1.3	1.1	2.0	3.1	4.3	4.3
F	1.8	1.7	7.0	4.7	0.0	0.0	1.4	1.5	2.0	1.7	4.2	4.6
G	1.9	1.4	7.3	7.3	0.0	0.0	0.8	0.7	1.5	1.6	3.6	3.9
H	1.9	1.7	7.1	7.6	0.0	0.0	1.5	1.4	2.2	2.3	5.0	5.2
I	2.2	2.1	8.1	8.2	0.0	0.0	1.6	1.6	2.3	2.6	5.9	5.9
J	2.1	1.9	8.1	8.4	0.0	0.0	1.5	0.8	2.5	2.5	5.8	5.5
K	2.0	1.3	5.7	4.0	0.0	0.0	0.5	0.4	2.5	0.6	0.6	0.7
L	2.1	2.1	8.8	8.8	0.0	0.0	1.8	1.6	2.7	2.7	6.0	6.4
M	1.6	1.3	4.5	6.4	0.0	0.0	1.0	1.0	2.2	2.3	4.7	4.2
N	2.0	1.9	8.3	8.6	0.0	0.0	1.5	1.5	2.4	2.0	2.6	3.1
O	1.9	2.0	8.1	8.9	0.0	0.0	1.6	1.6	2.2	2.3	5.1	5.1
P	2.3	2.3	8.3	8.7	0.0	0.1	2.0	1.9	2.5	2.5	5.9	6.1

[a] Shading indicates results identified as outliers and not included in statistical analysis.

Table 2. Collaborative trial results of liquid chromatographic determination of aflatoxins in pistachio paste

Aflatoxin B_1 concentration, ng/g (Blind duplicate pairs of naturally contaminated samples)												
Lab ID	1.0	1.0	4.0	4.0	A	A	B	B	C	C	D	D
A	0.9	1.0	3.6	3.7	<0.2[a]	<0.2	0.8	0.7	1.7	1.7	3.3	3.3
B	0.1	0.1	0.1	0.1	0.0	0.0	0.1	0.0	0.1	0.2	0.2	0.2
C	0.9	1.0	3.6	3.4	0.1	0.1	0.7	0.8	1.6	1.9	3.2	3.3
D	1.1	1.3	1.7	4.9	<0.05	0.1	0.9	0.8	2.4	1.6	3.4	2.5
E	0.7	0.9	3.5	3.5	0.1	0.1	0.6	0.6	1.3	1.4	2.8	2.0
F	0.9	0.9	3.7	3.7	0.2	0.1	0.6	0.6	1.4	1.4	2.8	2.7
G	0.6	1.0	1.9	2.7	<0.4	<0.4	0.6	0.6	1.4	1.4	3.0	1.3
H	1.0	1.0	3.4	3.7	0.2	0.2	0.7	0.8	1.6	1.6	4.5	3.1
I	1.1	1.1	3.8	3.8	0.2	0.2	0.8	0.8	1.8	1.9	3.3	3.3
J	1.0	1.1	3.8	3.6	0.2	0.4	0.7	0.4	0.5	1.4	3.3	1.6
K	0.7	1.0	1.6	?	<0.1	<0.1	0.3	0.2	1.4	0.6	0.8	0.8
L	1.0	1.0	3.7	3.7	0.1	0.1	0.8	0.8	1.8	1.7	3.5	3.3
M	1.0	0.9	3.3	3.0	<0.1	<0.1	0.8	0.8	1.4	1.2	2.8	2.5
N	1.0	1.1	3.8	3.4	0.2	0.6	0.2	0.7	1.6	1.7	3.0	2.7
O	0.8	0.9	3.7	3.7	0.1	0.1	0.8	0.8	1.8	1.9	3.2	2.9
P	1.0	0.7	4.6	1.6	0.2	0.3	0.9	1.0	1.5	1.9	3.1	2.6
Aflatoxin B_2 concentration, ng/g												
A	0.2	0.2	0.7	0.8	<0.1	<0.1	0.1	<0.1	0.2	0.2	0.3	0.3
B	0.1	0.1	0.1	0.1	0.0	0.0	0.0	0.0	0.1	0.1	0.1	0.1
C	0.2	0.2	0.8	0.8	0.0	<0.01	0.1	0.1	0.2	0.2	0.3	0.4
D	0.3	0.2	0.4	1.0	<0.05	<0.05	0.1	0.1	0.2	0.2	0.4	0.3
E	0.2	0.2	0.7	0.7	0.0	0.0	0.1	0.1	0.1	0.2	0.3	0.2
F	0.2	0.2	0.8	0.8	<0.03	<0.03	0.1	0.1	0.2	0.1	0.2	0.3
G	<0.2	0.2	0.4	0.6	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	0.3	<0.2
H	0.2	0.2	0.5	0.7	<0.04	<0.04	0.1	0.1	0.1	0.2	0.4	0.3
I	0.2	0.2	0.8	0.8	<0.05	<0.05	0.1	0.1	0.2	0.2	0.3	0.3
J	0.2	0.2	0.7	0.7	<0.04	0.1	0.1	0.1	0.1	0.1	0.3	0.2
K	0.2	0.2	0.3	?[b]	<0.04	<0.04	0.0	0.0	0.2	0.1	0.1	0.1
L	0.2	0.2	0.8	0.8	0.0	0.0	0.1	0.1	0.1	0.2	0.3	0.3
M	0.2	0.2	0.7	0.6	<0.08	<0.08	0.1	0.1	0.2	0.1	0.3	0.3
N	0.2	0.2	0.7	0.7	<0.1	<0.1	<0.1	0.1	0.2	0.2	0.3	0.2
O	0.2	0.2	0.8	0.7	<0.02	<0.02	0.1	0.1	0.2	0.2	0.3	0.3
P	0.2	0.1	0.9	0.4	0.0	0.0	0.1	0.1	0.2	0.2	0.3	0.3
Aflatoxin G_1 concentration, ng/g												
A	0.6	0.9	3.1	3.5	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2
B	0.2	0.0	0.3	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C	0.7	0.8	2.9	2.6	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	0.1
D	1.1	0.9	1.7	4.1	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	0.1	<0.05
E	0.8	0.7	3.5	3.3	<0.20	<0.20	<0.20	<0.20	<0.20	<0.20	<0.20	<0.20
F	0.8	0.8	3.7	3.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2
G	<0.5	0.8	1.9	2.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
H	0.9	0.9	3.2	3.7	<0.1	<0.1	<0.1	<0.1	<0.1	0.1	0.2	0.1

Table 2. (*continued*)

Lab ID	1.0	1.0	4.0	4.0	A	A	B	B	C	C	D	D
I	0.9	0.9	3.6	3.5	<0.1	<0.1	<0.1	<0.1	0.1	0.1	0.1	0.1
J	0.8	0.9	3.4	3.3	<0.2	<0.2	<0.15	<0.2	<0.2	<0.2	<0.2	<0.2
K	0.3	0.7	1.2	?[b]	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
L	1.0	0.9	3.9	3.8	<0.01	<0.01	<0.01	<0.01	0.1	0.1	0.2	0.2
M	0.4	0.3	2.7	1.8	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2
N	0.8	0.8	2.9	2.5	<0.1	<0.1	<0.1	<0.1	0.1	<0.1	<0.1	0.1
O	0.6	0.8	2.9	3.9	<0.02	<0.02	<0.02	<0.02	0.1	0.1	0.1	0.1
P	0.7	0.5	3.3	1.2	<0.04	<0.04	0.1	0.2	0.1	0.2	0.2	0.2
Aflatoxin G_2 concentration, ng/g												
A	0.1	0.1	0.6	0.7	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
B	0.2	0.1	0.3	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C	0.2	0.2	0.7	0.6	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02
D	0.2	0.2	0.4	0.9	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
E	0.2	0.2	0.7	0.7	<0.08	<0.08	<0.08	<0.08	<0.08	<0.08	<0.08	<0.08
F	0.2	0.2	0.8	0.6	<0.09	<0.09	<0.09	<0.09	<0.09	<0.09	<0.09	<0.09
G	0.1	0.2	0.4	0.6	<0.08	<0.08	<0.08	<0.08	<0.08	<0.08	<0.08	<0.08
H	0.2	0.1	0.3	0.7	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
I	0.2	0.2	0.7	0.7	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
J	0.2	0.2	0.7	0.7	<0.08	<0.08	<0.08	<0.08	<0.08	<0.08	<0.08	<0.08
K	0.1	0.1	0.3	?[b]	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
L	0.2	0.2	0.8	0.8	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	0.0
M	0.2	<0.10	0.6	0.4	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
N	0.2	0.2	0.7	0.6	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
O	0.1	0.2	0.6	0.8	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02
P	0.1	0.1	0.7	0.3	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03
Total aflatoxin concentration, ng/g												
A	1.8	2.2	7.9	8.6	0.0	0.0	0.9	0.7	1.8	1.8	3.6	3.7
B	0.6	0.3	0.8	0.5	0.0	0.0	0.1	0.0	0.2	0.3	0.3	0.3
C	1.8	2.1	8.0	7.4	0.2	0.1	0.8	0.9	1.8	2.1	3.5	3.8
D	2.7	2.7	4.1	10.8	0.0	0.1	1.1	0.9	2.6	1.8	3.9	2.8
E	1.8	1.9	8.4	8.1	0.1	0.1	0.7	0.7	1.4	1.6	3.1	2.2
F	2.0	2.0	8.9	8.3	0.2	0.1	0.7	0.7	1.6	1.5	3.0	3.0
G	0.7	2.1	4.6	6.3	0.0	0.0	0.6	0.6	1.4	1.4	3.2	1.3
H	2.3	2.2	7.3	8.8	0.2	0.2	0.8	0.8	1.7	1.8	5.1	3.5
I	2.4	2.5	8.9	8.8	0.2	0.2	1.0	0.9	2.1	2.2	3.7	3.8
J	2.2	2.3	8.6	8.3	0.2	0.4	0.8	0.5	0.6	1.5	3.6	1.8
K	1.3	2.1	3.5	0.0	0.0	0.0	0.3	0.3	1.6	0.7	0.9	0.9
L	2.4	2.3	9.2	9.0	0.1	0.1	0.9	0.9	1.9	2.0	4.0	3.8
M	1.8	1.4	7.2	5.9	0.0	0.0	0.9	0.9	1.6	1.3	3.1	2.8
N	2.1	2.3	8.1	7.2	0.2	0.6	0.2	0.8	1.9	1.8	3.3	3.0
O	1.6	2.1	8.0	9.1	0.1	0.1	0.9	0.9	2.1	2.2	3.7	3.3
P	2.1	1.4	9.4	3.4	0.2	0.3	1.1	1.3	1.8	2.3	3.6	3.1

[a] Shading indicates results identified as outliers and not included in statistical analysis.

[b] Results not reported (immunaffinity column blockage).

Table 3. Collaborative trial results of liquid chromatographic determination of aflatoxins in fig paste

Aflatoxin B_1 concentration, ng/g (Blind duplicate pairs of naturally contaminated samples)												
Lab ID	1.0	1.0	4.0	4.0	A	A	B	B	C	C	D	D
A	1.3	1.3	4.2	3.8	0.4[a]	>0.2	1.4	1.3	2.3	2.1	3.3	2.9
B	1.3	1.3	3.5	3.2	0.6	0.4	1.7	1.7	2.2	2.3	3.1	2.7
C	2.4	3.1	10.6	9.9	0.2	0.2	0.7	0.8	0.9	1.0	1.4	1.2
D	1.3	0.6	4.5	4.3	0.3	0.3	1.6	1.3	2.3	2.4	3.4	3.3
E	0.6	1.0	3.1	3.0	0.3	0.3	1.1	1.1	1.4	1.4	1.9	0.6
F	0.9	1.1	3.5	3.3	0.3	0.4	1.0	1.2	1.9	1.6	1.9	2.4
G	1.0	0.9	3.1	3.5	<0.5	<0.5	0.9	0.7	1.8	1.7	1.2	2.6
H	1.1	1.0	3.4	3.7	0.4	0.3	1.7	1.5	2.1	1.3	1.7	2.3
I	1.2	1.3	4.0	3.9	0.4	0.4	1.6	1.6	2.4	2.3	3.1	3.3
J	1.2	1.2	3.7	3.5	0.4	0.2	1.4	1.5	2.3	2.2	3.0	3.0
K	1.4	1.1	4.4	3.5	0.3	0.3	1.4	1.1	1.6	2.0	2.1	2.5
L	1.2	1.2	3.5	3.4	0.2	0.2	1.5	1.4	2.2	2.2	3.3	3.0
M	0.7	0.8	3.6	3.4	0.4	0.4	1.4	1.3	2.1	2.0	2.8	2.4
N	1.1	1.1	4.0	3.4	0.4	0.3	1.5	1.5	2.0	2.1	2.8	3.4
O	0.9	1.2	3.4	4.1	0.4	0.4	1.3	0.9	2.1	2.1	2.7	2.5
P	1.3	1.0	3.8	2.3	0.5	0.2	1.7	1.6	2.5	2.4	2.8	2.9
Aflatoxin B_2 concentration, ng/g												
A	0.3	0.3	0.8	0.8	>0.1	>0.1	0.5	0.5	0.9	0.8	1.5	1.5
B	0.3	0.3	0.7	0.6	0.1	0.1	0.6	0.6	0.8	0.8	1.4	1.4
C	0.5	0.6	2.1	2.0	0.1	0.0	0.2	0.3	0.3	0.3	0.6	0.5
D	0.3	0.1	1.0	0.9	0.1	0.1	0.6	0.5	0.9	0.9	1.5	1.6
E	0.2	0.3	0.8	0.7	0.1	0.1	0.4	0.4	0.6	0.6	1.1	0.3
F	0.2	0.2	0.8	0.7	0.1	0.1	0.4	0.4	0.7	0.7	1.3	1.4
G	<0.2	<0.2	0.7	0.7	<0.2	<0.2	0.3	0.3	0.6	0.6	0.6	1.1
H	0.2	0.2	0.5	0.7	0.1	0.1	0.5	0.4	0.6	0.4	0.7	0.8
I	0.3	0.3	0.8	0.8	0.1	0.1	0.5	0.5	0.8	0.8	1.5	1.5
J	0.2	0.2	0.8	0.8	0.1	0.1	0.5	0.5	0.7	0.8	1.4	1.4
K	0.3	0.3	1.0	0.8	0.0	0.1	0.5	0.4	0.6	0.7	1.1	1.2
L	0.3	0.3	0.8	0.7	0.1	0.1	0.5	0.5	0.8	0.7	1.4	1.3
M	0.3	0.2	0.8	0.7	0.1	0.1	0.5	0.5	0.7	0.7	1.4	1.3
N	0.2	0.3	0.8	0.8	0.1	0.1	0.4	0.5	0.6	0.6	1.2	1.4
O	0.2	0.3	0.8	0.9	0.1	0.1	0.5	0.3	0.8	0.8	1.2	1.3
P	0.3	0.2	0.8	0.5	0.1	0.1	0.6	0.5	0.9	0.8	1.3	1.5
Aflatoxin G_1 concentration, ng/g												
A	0.8	0.8	3.3	3.0	>0.2	>0.2	0.7	0.6	0.9	0.9	1.4	0.9
B	1.7	2.0	2.6	3.7	1.1	1.1	1.8	1.6	1.6	2.5	2.0	1.7
C	1.9	2.6	9.7	7.7	0.1	0.1	0.4	0.5	0.4	0.4	0.7	0.5
D	0.9	0.4	3.6	3.6	<0.05	0.1	0.7	0.7	1.1	1.0	1.3	1.4
E	0.4	0.7	3.1	2.9	0.3	0.9	1.0	1.2	0.3	1.6	1.2	0.3
F	1.0	0.7	2.1	3.2	0.3	<0.3	0.6	0.8	0.7	0.8	0.9	0.9
G	<0.8	<0.8	2.7	2.9	<0.8	<0.8	<0.8	<0.8	<0.8	<0.8	<0.8	0.9
H	1.1	0.9	3.3	3.8	0.2	0.2	1.3	1.0	1.1	0.7	0.9	1.1

Table 3. (*continued*)

Lab ID	1.0	1.0	4.0	4.0	A	A	B	B	C	C	D	D
I	1.0	1.0	3.5	3.2	0.2	0.2	0.9	0.9	1.0	1.1	1.3	1.4
J	0.6	0.6	2.4	2.2	<0.2	<0.2	0.8	1.0	0.9	0.8	0.9	0.9
K	0.5	0.8	1.1	1.1	0.1	<0.2	0.4	0.5	<0.2	0.2	0.2	0.4
L	1.0	1.0	3.5	3.7	0.0	0.1	1.0	0.9	1.1	1.0	1.6	1.4
M	0.1	0.2	3.7	1.7	<0.05	<0.05	0.4	0.4	0.3	0.4	0.6	0.4
N	1.1	1.2	3.7	3.3	0.5	0.4	1.2	1.1	1.1	1.1	1.3	1.2
O	0.4	0.9	1.3	3.5	0.2	0.2	0.8	0.6	0.9	1.0	1.1	1.1
P	0.7	0.6	1.7	1.4	0.7	0.2	0.8	0.5	1.0	1.0	1.2	1.2
					Aflatoxin G_2 concentration, ng/g							
A	0.2	0.2	0.6	0.7	>0.1	>0.1	0.2	0.2	0.4	0.4	0.7	0.6
B	0.3	0.4	0.5	0.7	0.2	0.3	0.5	0.4	0.5	0.6	0.8	0.7
C	0.5	0.5	2.1	1.7	<0.02	<0.02	0.1	0.1	0.1	0.2	0.3	0.2
D	0.2	<0.05	0.8	0.7	<0.05	<0.05	0.3	0.3	0.4	0.4	0.7	0.6
E	0.1	0.0	0.6	0.6	<0.08	<0.08	0.2	0.2	0.3	0.3	0.5	0.2
F	0.2	0.2	0.5	0.6	<0.09	<0.09	0.2	0.2	0.3	0.3	0.5	0.6
G	0.2	0.2	0.6	0.6	<0.10	<0.10	0.2	0.1	0.3	0.2	0.3	0.4
H	0.2	0.1	0.3	0.6	<0.15	<0.15	0.3	0.2	0.3	0.2	0.3	0.3
I	0.2	0.2	0.7	0.7	<0.1	<0.1	0.3	0.3	0.4	0.4	0.6	0.6
J	0.1	0.1	0.6	0.5	<0.1	<0.1	0.3	0.3	0.4	0.4	0.5	0.6
K	<	0.2	0.3	0.3	<0.15	<0.15	0.1	0.2	<0.15	<0.15	<0.15	0.2
L	0.2	0.2	0.7	0.7	0.0	<0.01	0.4	0.5	0.4	0.4	0.7	0.6
M	<0.1	<0.1	0.7	0.3	<0.1	<0.1	0.2	0.2	0.2	0.3	0.4	0.4
N	0.2	0.5	0.7	1.0	0.4	<0.1	0.3	0.5	0.6	0.3	0.5	0.5
O	0.1	0.2	0.4	0.8	<0.02	<0.02	0.3	0.2	0.4	0.5	0.6	0.6
P	0.1	0.1	0.4	0.3	<0.05	<0.05	0.2	0.2	0.4	0.4	0.6	0.6
					Total aflatoxin concentration, ng/g							
A	2.6	2.7	8.9	8.3	0.4	0.0	2.9	2.6	4.5	4.2	6.9	5.9
B	3.6	4.0	7.3	8.2	2.0	1.5	4.6	4.3	5.1	6.2	7.3	6.5
C	5.3	6.8	24.6	21.2	0.3	0.3	1.4	1.6	1.7	1.9	3.0	2.4
D	2.7	1.2	9.9	9.5	0.3	0.4	3.1	2.8	4.6	4.7	6.9	6.8
E	1.3	2.0	7.5	7.2	0.7	1.4	2.7	2.9	2.6	3.8	4.7	1.4
F	2.3	2.3	6.9	7.9	0.8	0.4	2.3	2.7	3.6	3.3	4.6	5.3
G	1.2	1.1	7.0	7.7	0.0	0.0	1.4	1.1	2.7	2.5	2.0	5.1
H	2.6	2.2	7.5	8.8	0.7	0.6	3.7	3.1	4.1	2.5	3.5	4.6
I	2.6	2.8	9.0	8.7	0.7	0.7	3.3	3.2	4.6	4.6	6.5	6.8
J	2.2	2.1	7.4	6.9	0.5	0.3	3.0	3.2	4.3	4.1	5.9	5.9
K	2.2	2.4	6.7	5.6	0.5	0.4	2.4	2.2	2.1	2.9	3.4	4.3
L	2.6	2.7	8.4	8.5	0.3	0.3	3.4	3.3	4.5	4.3	7.1	6.3
M	1.0	1.2	8.7	6.1	0.5	0.5	2.6	2.4	3.3	3.4	5.2	4.5
N	2.7	3.0	9.2	8.4	1.5	0.8	3.4	3.5	4.2	4.1	5.7	6.4
O	1.6	2.5	5.8	9.3	0.7	0.6	2.8	2.0	4.2	4.4	5.6	5.5
P	2.5	1.8	6.6	4.4	1.2	0.4	3.2	2.8	4.7	4.6	5.8	6.2

[a] Shading indicates results identified as outliers and not included in statistical analysis.

Table 4. Collaborative trial results of liquid chromatographic determination of aflatoxins in paprika

Aflatoxin B_1 concentration, ng/g (Blind duplicate pairs of naturally contaminated samples)												
Lab ID	1.0	1.0	4.0	4.0	A	A	B	B	C	C	D	D
A	1.0	0.8	3.5	3.4	<0.2	>0.2	1.1	0.7	1.3	1.2	2.9	3.2
B	0.0[a]	0.1	0.5	0.4	0.0	0.0	0.2	0.1	0.2	0.3	0.3	0.4
C	0.9	0.9	3.1	3.5	<0.01	0.1	0.8	0.8	1.5	1.5	3.1	3.2
D	0.4	0.8	3.8	4.0	<0.05	<0.05	1.1	1.0	1.4	1.6	3.8	1.6
E	0.8	0.8	4.0	3.9	<0.10	<0.10	0.7	0.6	1.4	1.3	2.9	3.2
F	0.7	0.7	2.9	2.9	<0.18	0.2	0.7	0.7	0.8	1.2	2.9	2.7
G	0.8	0.8	3.0	2.7	<0.5	<0.5	0.6	0.6	1.1	0.9	2.6	2.7
H	0.9	0.9	3.3	3.6	0.0	0.1	0.8	0.9	1.3	1.7	3.1	3.0
I	0.8	0.8	2.9	3.2	<0.1	<0.1	0.8	0.8	1.3	1.4	3.1	3.1
J	0.8	0.8	3.4	3.3	<0.2	<0.2	0.8	0.9	1.3	1.4	2.7	2.9
K	0.9	0.8	3.7	3.7	<0.2	<0.2	0.7	1.0	1.6	1.2	2.7	2.5
L	0.9	0.9	3.5	3.5	<0.01	<0.01	1.3	0.9	1.5	1.6	3.3	3.4
M	0.9	0.9	3.5	3.3	<0.1	<0.1	0.7	0.8	1.4	1.4	2.8	2.9
N	0.9	0.8	3.1	3.5	<0.1	<0.1	1.1	1.0	1.8	1.7	3.4	3.4
O	0.8	0.9	3.1	3.7	0.1	0.1	0.8	0.8	1.5	1.5	2.8	3.1
P	1.1	1.0	3.6	3.8	0.2	0.1	1.1	0.9	1.5	1.7	3.4	3.4
Aflatoxin B_2 concentration, ng/g												
A	0.2	0.1	0.7	0.7	<0.1	<0.1	<0.1	<0.1	0.1	<0.1	0.2	0.2
B	0.1	0.1	0.3	0.2	0.0	0.0	0.0	0.0	0.0	0.1	0.1	0.1
C	0.2	0.2	0.6	0.7	<0.01	<0.01	0.1	0.0	0.1	0.1	0.2	0.2
D	0.1	0.2	0.8	0.8	<0.05	<0.05	0.1	0.1	0.1	0.2	0.3	0.1
E	0.1	0.1	0.7	0.6	<0.04	<0.04	<0.04	<0.04	<0.04	<0.04	0.1	0.1
F	0.2	0.2	0.6	0.7	<0.04	<0.04	0.1	0.1	0.1	0.1	0.2	0.2
G	<0.2	<0.2	0.6	0.5	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2
H	0.1	0.1	0.3	0.6	<0.2	<0.2	0.0	0.1	0.1	0.1	0.2	0.2
I	0.2	0.2	0.6	0.7	<0.05	<0.05	0.1	0.1	0.1	0.1	0.2	0.2
J	0.2	0.2	0.7	0.7	<0.07	<0.07	0.0	0.0	0.1	0.1	0.2	0.2
K	0.2	0.2	0.8	0.8	<0.06	<0.06	0.1	0.1	0.1	0.1	0.2	0.2
L	0.2	0.2	0.7	0.7	<0.01	<0.01	0.1	0.1	0.1	0.1	0.2	0.2
M	0.2	0.2	0.7	0.7	<0.08	<0.08	<0.08	<0.08	0.1	0.2	0.2	0.2
N	0.2	0.1	0.6	0.6	<0.1	<0.1	0.7	<0.1	0.1	0.1	0.2	0.2
O	0.2	0.2	0.7	0.8	<0.02	<0.02	0.1	0.1	0.1	0.1	0.2	0.2
P	0.2	0.2	0.7	0.8	<0.03	<0.03	0.1	0.1	0.1	0.1	0.3	0.3
Aflatoxin G_1 concentration, ng/g												
A	0.7	0.6	2.5	2.2	<0.2	<0.2	<0.2	<0.2	0.4	0.4	1.0	1.2
B	0.1	0.0	0.8	2.0	0.0	0.0	0.2	0.1	0.8	0.7	0.2	0.4
C	0.8	0.9	2.3	2.9	<0.01	<0.01	0.0	0.1	0.7	0.8	1.2	1.6
D	0.3	0.6	2.9	3.0	<0.05	<0.05	0.1	0.1	0.5	0.5	1.3	0.6
E	0.8	0.7	3.5	3.3	<0.2	<0.2	<0.2	<0.2	0.6	0.7	1.3	1.5
F	0.7	0.8	2.2	2.3	<0.3	<0.3	<0.3	<0.3	0.5	0.6	1.0	1.1
G	<0.8	<0.8	2.4	2.0	<0.8	<0.8	<0.8	<0.8	<0.8	<0.8	1.0	1.1
H	0.9	0.9	3.4	3.9	<0.2	<0.2	0.1	0.2	0.8	0.9	1.7	1.7

Table 4. (*continued*)

Lab ID	1.0	1.0	4.0	4.0	A	A	B	B	C	C	D	D
I	0.4	0.5	1.5	1.6	<0.1	<0.1	<0.1	<0.1	0.2	0.3	0.8	0.8
J	0.6	0.6	2.4	2.2	<0.2	<0.2	<0.2	<0.2	0.4	0.5	0.9	1.1
K	0.3	0.2	2.7	1.5	<0.2	<0.2	<0.2	<0.2	0.5	0.2	0.5	0.4
L	0.9	0.8	3.7	3.7	<0.01	<0.01	0.1	0.0	0.8	0.9	1.8	1.9
M	0.5	0.5	3.6	2.4	<0.05	<0.05	<0.05	<0.05	0.5	0.3	1.2	1.1
N	0.5	0.6	2.0	2.2	<0.1	<0.1	<0.1	0.2	0.6	0.4	1.0	1.0
O	0.4	0.9	1.1	3.1	<0.02	<0.02	0.1	0.1	0.6	0.5	1.2	1.3
P	0.7	0.6	2.1	2.4	<0.06	<0.06	0.2	0.1	0.6	0.6	1.5	1.5
						Aflatoxin G_2 concentration, ng/g						
A	0.2	0.2	0.5	0.7	<0.1	<0.1	<0.1	<0.1	<0.1	0.1	0.1	0.1
B	0.1	0.1	0.4	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1
C	0.2	0.0	0.4	0.5	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	0.2	0.0
D	0.1	0.2	0.6	0.7	<0.05	<0.05	0.1	0.1	0.1	0.2	0.2	<0.05
E	0.2	0.1	0.6	0.7	<0.08	<0.08	<0.08	<0.08	<0.08	<0.08	<0.08	<0.08
F	0.1	0.2	0.5	0.6	<0.09	<0.09	<0.09	<0.09	<0.09	<0.09	0.1	0.1
G	0.2	0.2	0.6	0.4	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	0.1	0.1
H	0.1	0.1	0.1	0.4	<0.2	<0.2	<0.2	<0.2	<0.2	0.1	0.1	0.0
I	0.1	0.1	0.4	0.4	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
J	0.1	0.1	0.6	0.6	<0.1	<0.1	<0.1	<0.1	<0.1	0.0	0.1	0.1
K	<0.2	<0.2	0.4	0.6	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2
L	0.1	0.1	0.4	0.5	<0.01	<0.01	<0.01	0.2	1.0	0.3	0.8	0.2
M	<0.1	<0.1	0.5	0.5	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
N	0.1	0.1	0.5	0.5	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
O	interf[b]	interf	interf	interf	interf	interf	interf	interf	interf	interf	interf	interf
P	0.2	0.2	0.5	0.6	<0.05	<0.05	<0.05	<0.05	<0.05	0.1	0.1	0.1
						Total aflatoxin concentration, ng/g						
A	2.0	1.7	7.3	7.0	0.0	0.0	1.1	0.7	1.8	1.7	4.2	4.7
B	0.3	0.3	2.0	3.0	0.0	0.0	0.4	0.2	1.0	1.1	0.6	1.0
C	2.2	2.0	6.5	7.6	0.0	0.1	0.9	0.9	2.4	2.4	4.7	5.0
D	0.8	1.7	8.1	8.5	0.0	0.0	1.3	1.3	2.1	2.5	5.6	2.3
E	1.9	1.7	8.8	8.4	0.0	0.0	0.7	0.6	2.0	1.9	4.4	4.9
F	1.7	1.9	6.2	6.4	0.0	0.2	0.8	0.8	1.4	1.9	4.3	4.1
G	0.9	0.9	6.5	5.7	0.0	0.0	0.6	0.6	1.1	0.9	3.7	3.9
H	2.0	2.0	7.0	8.5	0.0	0.1	0.9	1.1	2.2	2.8	5.1	4.9
I	1.5	1.6	5.4	5.9	0.0	0.0	0.9	0.8	1.6	1.8	4.0	4.1
J	1.6	1.6	7.1	6.8	0.0	0.0	0.8	0.9	1.8	2.0	3.9	4.2
K	1.5	1.2	7.6	6.7	0.0	0.0	0.8	1.0	2.2	1.6	3.5	3.1
L	2.1	2.0	8.3	8.4	0.0	0.0	1.5	1.1	3.3	2.9	6.1	5.7
M	1.5	1.6	8.3	6.9	0.0	0.0	0.7	0.8	2.0	1.8	4.3	4.2
N	1.7	1.6	6.2	6.8	0.0	0.0	1.7	1.2	2.5	2.2	4.6	4.6
O	1.3	2.0	4.9	7.5	0.1	0.1	1.0	1.0	2.1	2.1	4.2	4.7
P	2.2	1.9	7.0	7.5	0.2	0.1	1.3	1.1	2.2	2.5	5.2	5.4

[a] Shading indicates results identified as outliers and not included in statistical analysis.

[b] interf = interference experienced in chromatogram.

Table 5. Statistical analysis of collaborative trial results for aflatoxin B_1 from 16 laboratories

Food	Added, ng/g	Avg., ng/g	S_r	RSD_r, %	S_R	RSD_R, %	No. outlier labs	HORRAT ratio	Rec., %
Peanut butter	0	<0.2	—	—[a]	—	—	1 (G)	—	—
	1.0	0.87	0.087	9.9	0.16	18.6	1 (B)	0.40	87
	4.0	3.65	0.111	3.1	0.66	18.2	3 (B, F, K)	0.49	91
	NC[b]	0.80	0.05	6.3	0.26	32.2	1 (J)	0.69	—
	NC	1.52	0.10	6.4	0.22	14.3	2 (B, K)	0.34	—
	NC	3.4	0.13	3.9	0.65	19.1	2 (B, K)	0.51	—
Pistachio paste	0	0.13	0.02	19.2	0.07	49.6	7 (A, D, G, J, K, M, N)	0.81	—
	1.0	0.94	0.13	13.9	0.15	16.1	1 (B)	0.35	94
	4.0	3.29	0.13	3.9	1.02	31.1	4 (D, G, K, P)	0.82	82
	NC	0.74	0.08	10.7	0.12	16.8	3 (B, K, N)	0.36	—
	NC	1.54	0.27	17.8	0.36	23.2	1 (B)	0.55	—
	NC	2.93	0.59	20.0	0.61	21.0	2 (B, K)	0.54	—
Fig paste	0	0.32	0.08	24.5	0.10	30.6	2 (A, G)	0.57	—
	1.0	1.10	0.18	16.8	0.21	19.3	1 (C)	0.43	109
	4.0	3.60	0.39	10.9	0.46	12.8	1 (C)	0.34	90
	NC	1.32	0.12	9.5	0.30	22.8	0	0.53	—
	NC	2.07	0.12	5.9	0.31	14.9	2 (C, H)	0.37	—
	NC	2.55	0.41	16.1	0.73	28.5	0	0.72	—
Paprika powder	0	<0.2	—	—	—	—	1 (G)	—	—
	1.0	0.86	0.05	6.0	0.09	10.3	2 (B, D)	0.22	86
	4.0	3.41	0.18	5.3	0.35	10.2	1 (B)	0.27	85
	NC	0.84	0.12	14.0	0.16	18.8	1 (B)	0.40	—
	NC	1.39	0.14	9.9	0.24	17.0	1 (B)	0.39	—
	NC	3.02	0.13	4.2	0.28	9.1	2 (B, D)	0.24	—

[a] —, statistical parameters not calculated; levels were below limits of detection.
[b] NC, naturally contaminated.

Results and Discussion

Homogeneity of Test Materials

The ANOVA showed that the difference between the between-container-variance and the within-container-variance regarding all 4 test materials at 3 concentration levels was not significant. Therefore the between-sample-standard-deviation was demonstrated to be negligible. The check for equality of the variances indicated that the within-container-variance and the overall-variance (*F*-test) of all 4 test materials did not differ significantly from the variance of the method's repeatability determined in the in-house performance study. All 4 test materials at 3 different levels of natural contamination together with blank material therefore met required homogeneity criteria for collaborative trial purposes.

Precollaborative Trial Workshop

At the precollaborative trial workshop, a number of points that required clarification of the method description were identified. The volume of hexane (or cyclohexane) must not be included in the calculation for aflatoxin content. A critical step was identified as the transfer of slurry from blender to filter paper, which needed to be done immediately without allowing any phase separation.

Collaborative Trial Results

All 16 collaborators who received test samples completed the study. All data submitted for the study for the 4 commodities are presented in Tables 1–4. Each table is subdivided, presenting individual results for aflatoxins B_1, B_2, G_1, G_2, and total aflatoxins. The data are given as individual pairs of results for each laboratory (A–P). Blank samples (sample 'A' in each case) were spiked with 1.0 and 4.0 ng/g of aflatoxins B_1 and G_1 and 0.2 and 0.8 ng/g of aflatoxins B_2 and G_2 (giving corresponding levels for total aflatoxins of 2.4 and 9.6 ng/g, respectively). Samples 'B', 'C', and 'D' are blind duplicates of naturally contaminated materials in each case.

Precision estimates were obtained using the one-way analysis of variance approach according to the IUPAC Harmonized Protocol (13). Details of food matrixes, average analyte concentration, standard deviations for repeatability (S_r) and reproducibility (S_R), relative standard deviations for repeat-

Table 6. Statistical analysis of collaborative trial results for aflatoxin B_2 from 16 laboratories

Food	Added, ng/g	Avg., ng/g	S_r	RSD_r, %	S_R	RSD_R, %	No. outlier labs	HORRAT ratio	Rec., %
Peanut butter	0	<0.05	—[a]	—	—	—	0	—	—
	0.2	0.19	0.03	14.5	0.04	19.3	1 (G)	0.33	93
	0.8	0.74	0.05	6.3	0.14	18.8	2 (B, F)	0.40	93
	NC[b]	0.15	0.01	6.8	0.03	21.0	2 (G, J)	0.35	—
	NC	0.32	0.02	7.6	0.04	12.6	3 (B, E, K)	0.23	—
	NC	0.72	0.03	3.8	0.12	17.0	2 (B, K)	0.36	—
Pistachio paste	0	<0.05	—	—	—	—	0	—	—
	0.2	0.18	0.02	13.1	0.02	13.1	3 (B, D, G)	0.22	91
	0.8	0.70	0.17	24.4	0.17	24.4	2 (B, K)	0.51	88
	NC	0.09	0.01	10.0	0.02	23.5	4 (A, B, G, N)	0.36	—
	NC	0.15	0.03	17.8	0.04	23.7	1 (G)	0.39	—
	NC	0.30	0.05	18.2	0.06	18.9	3 (B, G, K)	0.35	—
Fig paste	0	0.09	0.02	23.1	0.02	25.4	2 (A, G)	0.39	—
	0.2	0.24	0.04	18.0	0.05	19.1	2 (C, G)	0.34	123
	0.8	0.76	0.08	10.5	0.10	13.9	1 (C)	0.29	95
	NC	0.45	0.04	9.0	0.10	21.6	0	0.42	—
	NC	0.73	0.03	4.7	0.10	13.5	2 (C, H)	0.28	—
	NC	1.34	0.07	5.4	0.13	9.4	4 (C, D, G, H)	0.21	—
Paprika powder	0	<0.05	—	—	—	—	0	—	—
	0.2	0.15	0.03	21.2	0.04	27.3	1 (G)	0.46	77
	0.8	0.69	0.05	6.9	0.08	11.2	2 (B, H)	0.23	87
	NC	0.05	0.01	19.0	0.02	43.6	5 (A, E, G, M, N)	0.62	—
	NC	0.10	0.03	28.7	0.03	31.0	3 (A, E, G)	0.48	—
	NC	0.20	0.01	6.2	0.02	10.4	4 (B, D, E, G)	0.18	—

[a] —, statistical parameters not calculated; levels were below limits of detection.
[b] NC, naturally contaminated.

ability (RSD_r) and reproducibility (RSD_R), number of statistical outlier laboratories, HORRAT value, and percentage recovery are presented in Tables 5–9 for aflatoxins B_1, B_2, G_1, G_2, and total aflatoxins, respectively. The collaborative trial results had previously been examined for evidence of individual systematic error ($p < 0.025$) using Cochran's and Grubbs tests progressively (12). Pairs of results identified as outliers are indicated against the shaded background in Tables 1–4 and are individually identified by the laboratory code in Tables 5–9. For aflatoxin B_1, results given in Table 5 and for total aflatoxins given in Table 9 (excluding data for blank materials), the maximum numbers of outliers identified were 4 laboratories, giving acceptable data from 12 to 16 laboratories. Because of differences in reporting limits for not detectable, the results for blank materials in the cases of peanut butter and paprika powder were not analyzed statistically. The results, however, indicated clearly that all participants could identify the blank pairs of samples as not containing detectable aflatoxins or containing levels that were detectable but close to measurable limits. The results for the blank samples of pistachio and fig pastes showed that both samples contained low but measurable amounts of aflatoxins. The average levels of aflatoxin B_1 in the pistachio and fig pastes were 0.13 and 0.32 ng/g, respectively (corresponding to 0.2 and 0.6 ng/g for total aflatoxins, respectively). In the case of aflatoxins G_1 and G_2 in pistachio paste, the results for the 3 naturally contaminated samples (b, c, and d) contained levels below the limits of detection (3 × baseline noise); therefore, it was not possible to undertake statistical analysis in this instance (Tables 7 and 8). McClure (13) has shown that the usual statistical analysis, based on one-way ANOVA of total concentration data, to obtain estimates of repeatability and reproducibility is flawed and thus the variance components are not in keeping with AOAC INTERNATIONAL requirements. McClure recommends that precision estimates for total data continue to be obtained in the usual manner but that precision estimates so obtained be flagged as having been interpreted differently from the usual definitions of repeatability and reproducibility. This has accordingly been indicated in Table 9.

Table 7. Statistical analysis of collaborative trial results for aflatoxin G_1 from 16 laboratories

Food	Added, ng/g	Avg., ng/g	S_r	RSD_r, %	S_R	RSD_R, %	No. outlier labs	HORRAT ratio	Rec., %
Peanut butter	0	<0.2	—[a]	—	—	—	0	—	—
	1.0	0.73	0.12	16.5	0.15	19.3	1 (B)	0.41	76
	4.0	3.32	0.23	6.9	0.49	14.8	3 (B, K, M)	0.39	83
	NC[b]	0.37	0.04	11.1	0.11	28.3	3 (G, K, M)	0.54	—
	NC	0.34	0.04	11.5	0.05	16.3	3 (B, G, K)	0.31	—
	NC	0.68	0.13	18.9	0.21	31.0	2 (G, K)	0.65	—
Pistachio paste	0	0.07	—	—	—	—	0	—	—
	1.0	0.71	0.11	15.6	0.25	35.9	1 (G)	0.75	71
	4.0	3.05	0.68	22.2	0.72	23.8	2 (B, K)	0.62	76
	NC	0.07	—	—	—	—	—	—	—
	NC	0.06	—	—	—	—	—	—	—
	NC	0.13	—	—	—	—	—	—	—
Fig paste	0	—	—	—	—	—	—	—	—
	1.0	0.74	0.18	24.0	0.29	39.6	3 (B, C, G)	0.84	93
	4.0	2.82	0.63	22.2	0.88	31.2	1 (C)	0.80	70
	NC	0.83	0.11	12.8	0.35	41.9	1 (G)	0.90	—
	NC	0.85	0.05	6.3	0.25	29.0	5 (B, E, G, H, K)	0.63	—
	NC	1.06	0.14	13.2	0.42	39.9	2 (E, G)	0.89	—
Paprika powder	0	—	—	—	—	—	0	—	—
	1.0	0.58	0.08	13.1	0.24	42.1	2 (G, O)	0.86	58
	4.0	2.49	0.53	21.4	0.77	30.8	0	0.78	62
	NC	0.10	0.05	53.9	0.05	55.2	9	0.85	—
	NC	0.55	0.09	16.6	0.18	33.5	2 (B, G)	0.67	—
	NC	1.13	0.11	9.9	0.43	37.5	1 (D)	0.84	—

[a] —, statistical parameters not calculated; levels were below limits of detection.
[b] NC, naturally contaminated.

Comments from Collaborative Trial Participants

Comments were made on the reporting sheets from 13 of the 16 collaborative trial participants. In all cases, participants regarded the method description as being adequate. For laboratory B, where 13 pairs of results were rejected as outliers, the participant indicated a problem with the immunoaffinity columns he received, as he had not previously experienced such recovery problems, and achieved good results on the practice sample. Laboratory C did not use a blender as instructed in the method for sample homogenization, but performed extraction using a shaker. From this laboratory, 3 sets of results for figs were rejected as outliers, which could be explained by the difficulty of getting a good homogenization of dried fig paste using a shaker. Laboratory D failed to include NaCl in the extraction step for paprika for which they had 2 sets of outlier results. Laboratory K had 6 sets of outlier results with peanut butter and pistachio paste for which no obvious reasons were identified, except a blockage of the immunoaffinity column for one of the pistachio paste extracts. This laboratory also indicated degradation of aflatoxin G_1 and G_2 standards during autosampler injection into the LC after 10 h despite protection of samples from exposure to light. Laboratory O indicated an interference problem with the analysis of aflatoxin G_2 in paprika, resulting in exclusion of their results for aflatoxin G_2. This problem was not reported by other laboratories.

Performance Characteristics of Method

The precision data based only on spiked samples are shown in Table 999.07A (Method Performance) and for all samples are shown in Tables 5–9. Based on results for spiked samples (blind pairs at 2 levels), as well as naturally contaminated samples (blind pairs at 3 levels), the RSD_r values ranged from 5 to 23% for total aflatoxins and from 3 to 20% for aflatoxin B_1. The RSD_R values ranged from 14 to 34% for total aflatoxins, and from 9 to 32% for aflatoxin B_1. Where it is possible to make direct comparisons, the values for performance characteristics obtained in this trial are better than those obtained for the existing AOAC First Action Method (5). Thus, for the LC–post-column derivatization first action, RSD_R values of 30, 47, and 51% were obtained for peanut butter, raw

Table 8. Statistical analysis of collaborative trial results for aflatoxin G_2 from 16 laboratories

Food	Added, ng/g	Avg., ng/g	S_r	RSD_r, %	S_R	RSD_R, %	No. outlier labs	HORRAT ratio	Rec., %
Peanut butter	0	—[a]	—	—	—	—	0	—	—
	0.2	0.16	0.02	12.4	0.02	12.4	5 (B, C, H, K, M)	0.21	80
	0.8	0.61	0.09	14.8	0.19	32.0	1 (B)	0.66	76
	NC[b]	0.10	0.01	10.7	0.02	18.0	4 (B, J, K, M)	0.28	—
	NC	0.09	0.02	17.6	0.02	21.8	5 (A, B, G, K, M)	0.34	—
	NC	0.21	0.03	13.6	0.04	21.0	3 (F, K, M)	0.37	—
Pistachio paste	0	—	—	—	—	—	0	—	—
	0.2	0.16	0.03	20.7	0.04	24.1	1 (M)	0.40	80
	0.8	0.60	0.16	26.7	0.18	29.6	1 (K)	0.61	76
	NC	—	—	—	—	—	0	—	—
	NC	—	—	—	—	—	0	—	—
	NC	—	—	—	—	—	0	—	—
Fig paste	0	—	—	—	—	—	0	—	—
	0.2	0.21	0.06	31.4	0.13	63.2	3 (D, K, M)	1.10	104
	0.8	0.58	0.14	25.0	0.18	31.5	1 (C)	0.64	72
	NC	0.25	0.04	18.2	0.10	40.5	0	0.73	—
	NC	0.34	0.03	10.2	0.11	31.0	2 (K, N)	0.58	—
	NC	0.52	0.08	14.9	0.16	31.8	1 (K)	0.64	—
Paprika powder	0	—	—	—	—	—	0	—	—
	0.2	0.13	0.03	21.3	0.03	25.7	4 (C, K, M, O)	0.42	66
	0.8	0.50	0.09	17.6	0.12	25.1	1 (O)	0.50	62
	NC	0.05	—	—	—	—	0	—	—
	NC	0.24	—	—	—	—	0	—	—
	NC	0.09	—	—	—	—	0	—	—

[a] —, statistical parameters not calculated; levels were below limits of detection.
[b] NC, naturally contaminated.

peanuts, and corn at 10 ng/g total aflatoxins (5) compared to RSD_R values of 22, 23, 16, and 14% for peanut butter, pistachio paste, fig paste, and paprika powder, respectively, at a comparable level for total aflatoxins.

Although the method is only recommended for application at levels of aflatoxin B_1 at >1 ng/g and for total aflatoxins at >2.4 ng/g, there is evidence from the blank matrixes containing low levels of aflatoxins that the method in fact gives satisfactory performance at lower levels. Thus, for pistachio and fig pastes containing levels of 0.1 and 0.3 ng/g aflatoxin B_1 respectively (and corresponding total aflatoxin levels of 0.2 and 0.6 ng/g, respectively), RSD_r values of 19 and 24% and RSD_R values of 49 and 31% were obtained for aflatoxin B_1 (Table 5) and RSD_r values of 53 and 43% and RSD_R values of 58 and 53% were obtained for total aflatoxins. Although these precision values are higher than those for detection levels of the proposed method, the HORRAT values are nevertheless still below 2.0 and only in one instance slightly exceed 1.0. Thus, in principle the method could be claimed as operable with acceptable performance characteristics at levels as low as 0.1 ng/g for aflatoxin B_1 and 0.2 ng/g for total aflatoxins in pistachio paste.

The values for recoveries of aflatoxin B_1 derived from spiked samples ranged from 82 to 109% and for total aflatoxins ranged from 71 to 92% (Tables 5 and 9). The lowest recoveries were obtained for paprika powder, and the highest values for fig paste where the blank material used for spiking contained low levels of aflatoxins. When the average level of aflatoxin B_1 of 0.3 ng/g in the blank fig paste is deducted from measured values after spiking, recovery values decrease from 109 to 78% and from 90 to 82%. Similarly, when the measured level of total aflatoxins of 0.6 ng/g in the blank fig paste is deducted, recoveries decrease from 92 to 67% and from 81 to 75%. Recoveries for aflatoxins B_2, G_1, and G_2 ranged from 58 to 123%, with the lowest recoveries again for paprika powder and the high recoveries for fig paste, where if blank background levels are subtracted, recovery values decrease from 123 to 75%. Because the method may be used to determine either or both aflatoxin B_1 and total aflatoxins, the recoveries were considered acceptable.

Table 9. Statistical analysis of collaborative trial results for total aflatoxins from 16 laboratories[a]

Food	Added, ng/g	Avg., ng/g	S_r	RSD_r, %	S_R	RSD_R, %	No. outlier labs	HORRAT ratio	Rec., %
Peanut butter	0	—[b]	—	—	—	—	0	—	—
	2.4	1.9	0.26	13.4	0.35	18.1	1 (B)	0.44	81
	9.6	7.9	0.67	8.5	1.76	22.3	1 (B)	0.67	82
	NC[c]	1.3	0.08	6.2	0.46	34.2	1 (J)	0.79	—
	NC	2.2	0.16	7.0	0.32	14.1	3 (B, E, K)	0.35	—
	NC	5.0	0.23	4.6	0.96	19.3	2 (B, K)	0.54	—
Pistachio paste	0	0.2	0.10	53.4	0.11	58.2	6 (A, B, D, G, K, M)	1.00	—
	2.4	2.0	0.24	11.9	0.36	17.8	2 (B, G)	0.44	83
	9.6	7.8	1.82	23.3	1.82	23.3	2 (B, K)	0.70	81
	NC	0.8	0.10	12.2	0.17	20.6	3 (B, K, N)	0.44	—
	NC	1.7	0.31	17.8	0.42	24.3	1 (B)	0.58	—
	NC	3.3	0.66	20.2	0.72	22.1	2 (B, K)	0.58	—
Fig paste	0	0.6	0.26	43.3	0.32	52.9	3 (A, B, G)	1.10	—
	2.4	2.2	0.40	17.6	0.73	32.4	1 (C)	0.81	92
	9.6	7.8	1.01	13.0	1.28	16.5	1 (C)	0.50	81
	NC	2.8	0.25	8.8	0.80	28.4	0	0.73	—
	NC	3.8	0.44	11.5	1.03	28.9	0	0.73	—
	NC	5.2	0.90	17.2	1.56	29.7	0	0.84	—
Paprika powder	0	—	—	—	—	—	0	—	—
	2.4	1.7	0.11	6.4	0.34	19.6	3 (B, D, O)	0.47	71
	9.6	7.1	0.72	10.1	1.01	14.2	1 (B)	0.42	74
	NC	0.9	0.16	17.0	0.31	33.5	—	0.73	—
	NC	2.0	0.23	11.5	0.55	27.8	—	0.68	—
	NC	4.5	0.22	4.8	0.66	14.8	2 (B, D)	0.41	—

[a] Precision estimates for total aflatoxins data were interpreted differently from the usual definitions of repeatability and reproducibility (14).
[b] —, statistical parameters not calculated; levels were below limits of detection.
[c] NC, naturally contaminated.

Acceptability of the precision characteristics of the method were assessed on the basis of the HORRAT values (14), which compare the RSD_R at various levels and in various matrixes with statistically predicted values derived from previous collaborative trial studies taken from the published literature. When outliers were excluded, the HORRAT values for aflatoxin B_1 ranged from 0.3 to 0.7 for peanut butter, 0.4 to 0.8 for pistachio paste, 0.3 to 0.7 for fig paste, and 0.2 to 0.4 for paprika powder. The HORRAT values for aflatoxin B_2, G_1, and G_2 were generally of the same order as those for aflatoxin B_1 and only in one case (for aflatoxin G_2 in a sample of fig paste) were the HORRAT values = 1.1. For total aflatoxins, the HORRAT values ranged from 0.3 to 0.8 for peanut butter, 0.4 to 0.7 for pistachio paste, 0.5 to 0.8 for fig paste, and 0.4 to 0.7 for paprika powder. All HORRAT values were <1.1, which indicates acceptable precision, and were better than or comparable to values reported in the AOAC–IUPAC Official First Action Method (6), notwithstanding the low levels of aflatoxin contamination in this instance.

Recommendation

On the basis of the reseults of this study, it is recommended that the immunoaffinity column cleanup method by reversed-phase LC with post-column bromination for aflatoxin B_1 at > 1 ng/g and total aflatoxins at > 2.4 ng/g be adopted Official First Action for determinations in peanut butter, pistachio paste, fig paste, and paprika powder. The method is new in terms of validation for pistachio paste, fig paste, and paprika powder, and superior to the existing Official First Action method for peanut butter in terms of the lower limits, at which validation was performed for aflatoxin B_1 and total aflatoxins.

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