FOOD CHEMICAL CONTAMINANTS

Determination of Aflatoxin B_1 in Baby Food (Infant Formula) by Immunoaffinity Column Cleanup Liquid Chromatography with Postcolumn Bromination: Collaborative Study

JOERG STROKA and ELKE ANKLAM
Institute for Health and Consumer Protection, Joint Research Centre of the European Commission, Food Products and Consumer Goods Unit, 21020, Ispra (VA), Italy
URBAN JOERISSEN
Dr. Wierz–Dipl.-Chem. Eggert–Dr. Joerissen GmbH, Stenzelring 14b, 21107 Hamburg, Germany
JOHN GILBERT
Ministry of Agriculture, Fisheries and Food, Central Science Laboratory, Sand Hutton, York YO41 1LZ, UK

Collaborators: A. Barmark; C. Brera; B. Dias; I. Felgueiras; J. Gardikis; L. Macho; J.Y. Michelet; K. Noutio; A. Pittet; M. Reutter; M.C. Spanjer; E. Strassmeier; L. Szymanski; R. Worswick

A collaborative study was conducted to evaluate the effectiveness of an immunoaffinity column cleanup liquid chromatography (LC) method for determination of aflatoxin B_1 in a milk powder based infant formula at a possible future European regulatory limit (0.1 ng/g). The test portion was extracted with methanol–water (8 + 2 [v + v]), filtered, diluted with water, and applied to an immunoaffinity column. The column was washed with water to remove interfering compounds, and the purified aflatoxin B_1 was eluted with methanol. The separation and determination of the aflatoxin B_1 was performed by reversed-phase LC and detected by fluorescence after postcolumn derivatization (PCD) involving bromination. PCD was achieved with either pyridinum hydrobromide perbromide (PBPB) or an electrochemical (Kobra) cell by addition of bromide to the mobile phase. The baby food (infant formula) test samples, both spiked and naturally contaminated with aflatoxin B_1, were sent to 14 laboratories in 13 different European countries. Test portions were spiked at levels of 0.1 and 0.2 ng/g for aflatoxin B_1. Recoveries ranged from 101 to 92%. Based on results for spiked test samples (blind pairs at 2 levels) and naturally contaminated test samples (blind pairs at 3 levels), the relative standard deviation for repeatability (RSD_r) ranged from 3.5 to 14%. The relative standard deviation for reproducibility (RSD_R) ranged from 9 to 23%. Nine participants used PBPB derivatization, and 5 particpants used the Kobra cell. There was no evidence of method performance depending on the derivatization method used. The method showed acceptable within- and between-laboratory precision for baby food matrix, as evidenced by HORRAT values, at the target levels of determination for aflatoxin B_1.

Submitted for publication November 2000.
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," (2000) *Inside Laboratory Management,* April issue.

Methods for determining aflatoxins in food products have greatly improved in recent years with the commercial availability of immunoaffinity columns (1). A recently completed European collaborative trial (2) with 4 different matrixes (peanut butter, pistachio paste, fig paste, and paprika powder) demonstrated performance characteristics at ng/g levels that were better than anticipated from predictions based on the Horwitz curve. For spiked test samples (blind pairs at 2 levels) and naturally contaminated test samples (blind pairs at 3 levels) the relative standard deviation for repeatability (RSD_r) ranged from 5 to 23% for total aflatoxins and 3 to 20% for aflatoxin B_1. The relative standard deviation for reproducibility (RSD_R) ranged from 14 to 34% for total aflatoxins and from 9 to 32% for aflatoxin B_1. This method was adopted as First Action by AOAC INTERNATIONAL (3) and as a European Standard by the European Standardization Committee (CEN; 4) on the basis of acceptable within- and between-laboratory precision for all 4 matrixes, as evidenced by HORRAT values, at the regulatory limits of determination for both total aflatoxins and aflatoxin B_1.

European Commission Regulations (5) exist for aflatoxin B_1 and total aflatoxins in cereals, nuts, nut products, and spices, setting limits of 2 and 4 ng/g, respectively. The Commission has also focused attention on the need to protect the health of infants and young children, and regulations have been introduced (6) setting limits of 1 ng/g for pesticides in processed cereal-based foods and baby foods. These controls

will be extended to cover infant formulas and follow-on formulas (7). Both regulations state that the analytical method used shall be validated and have a limit of quantification that allows detection of the substance at the established maximum residue limit. It is anticipated that mycotoxin regulations will be extended to set limits for aflatoxin B_1 in baby food, for which a level of 0.1 ng/g has been proposed as appropriate, and for which a validated method will be required.

Previous experience with 4 different matrixes (2) has shown that with care and attention to detail during organization of a collaborative trial, impressive performance characteristics for a method are possible, even when it is necessary to operate close to the limit of detection. The present study has required validation at exceptionally low levels of aflatoxin B_1. It was recognized that difficulties in producing homogenous powdered test materials would be likely. Therefore, particular care was taken in the preparation step (grinding to small particle size and extensive mixing) and in demonstrating interunit homogeneity before undertaking the study.

Collaborative Study

Test Materials

(**a**) *Blank baby food*.—Rice flour free of aflatoxin B_1 contamination (16 kg) was mixed in a bag with 24 kg milk powder based proprietary infant formula-type baby food, and then ground in a special mill (Bauermeistermühle; Bauermeister Verfahrenstechnik GmbH, Norderstedt, Germany) using a 0.3 mm sieve. The flour was subsequently mixed again for 2 h in a special mixer (Lödige Chargenmischer Typ FKM 130017; Lödige Maschinenbau GmbH, Paderborn, Germany). The aflatoxin B_1 content was determined to be <0.02 ng/g by the method described.

(**b**) *Baby food with target level of 0.05 ng/g*.—Naturally contaminated rice flour (8 kg) was mixed in a bag with milk powder based proprietary baby food (12 kg) and then ground in the special mill (Bauermeistermühle) using a 0.3 mm sieve. The flour was mixed again for 2 h in the special mill. The aflatoxin B_1 was determined to be 0.07 ng/g by the method described below.

(**c**) *Premixes for target levels 0.10 and 0.20 ng/g baby foods*.—Crushed naturally contaminated corn kernels were dried for 8 h at 103°C, ground to 0.3 mm, and then mixed for 5 h in a rotating (barrel) drum equipped with 50 stainless steel balls. Analysis showed that this material contained 74 ng/g aflatoxin B_1. To 150 g of this corn flour, various amounts of noncontaminated rice flour were added as follows: 0.15, 0.30, 0.60, 1.20, and 2.40 kg.

After each addition, the material was mixed for 2 h in a rotating (barrel) drum. The resulting 4.8 kg contaminated rice flour was divided into 2 portions each of 2.4 kg (rice flour A, rice flour B). A and B were blended with noncontaminated rice flour, resulting in contents of aflatoxin B_1 of 1.3 and 1.8 ng/g, respectively.

(**d**) *Baby food with a target level of 0.10 ng/g*.—A mixture of noncontaminated rice flour (2.5 kg) and 0.5 kg rice flour A was blended for 2 h in a rotating drum. The same mixing procedure was carried out with 0.5 kg rice flour B added to 2.5 kg noncontaminated rice flour. The resulting 6 kg of rice flour was then premixed with milk powder based proprietary baby food (9 kg) in a bag and ground to 0.3 mm in the special mill (Bauermeistermühle). The baby food was then mixed for 2 h in the special mill. The aflatoxin B_1 content was determined to be 0.10 ng/g by the method described below.

(**e**) *Baby food with a target level of 0.20 ng/g*.—Noncontaminated rice flour (2 kg) and rice flour A (1 kg) were mixed for 2 h in a rotating drum. Another portion of aflatoxin-free rice flour (2 kg) was mixed with rice flour B (1 kg) by the same procedure. The resulting 6 kg rice flour was premixed with 9 kg of milk powder based proprietary baby food in a bag, ground to pass a 0.3 mm sieve, and mixed for 2 h. The aflatoxin B_1 was determined to be 0.20 ng/g by the method described below.

Particle size measurement of test materials.—All test materials were analyzed for particle size and particle size distribution. All test materials (except the 0.10 ng/g material), on the basis of microscopic photographs and particle size measurements, revealed a well-distributed and discreetly distributed particle size pattern. Because of poor particle size distribution, the 0.1 ng/g test material was reground and remixed.

Homogeneity.—The baby food test materials prepared above were analyzed for aflatoxin B_1 using this method and the results demonstrated that in all cases the bulk homogeneity was satisfactory. The bulk materials were subdivided into appropriate glass containers. Every 10th sample was taken from the sequence and analyzed. The number of the first container from which the sampling started was randomly selected for each material, respectively. For example, baby food with the target content of 0.10 μg/kg aflatoxin B_1 was checked by taking the 4th, the 14th, the 24th … sample.

Statistical tests indicated that all test materials were homogeneous and met the requirements for the collaborative trial. The mean contents of aflatoxin B_1 of the 4 test materials analyzed for homogeneity was <0.02, 0.09, 0.13, and 0.21 ng/g.

Organization of Collaborative Study

Fourteen participants from 13 different European countries representing a cross-section of government, food control, university, and food industry affiliations took part in this collaborative trial. In advance of the trial, each participant received a practice sample which included a blank material for spiking. Participants met at a precollaborative trial workshop where any problems experienced with analysis of the practice sample were discussed, and details and organization of the trial were outlined by the coordinators.

For the collaborative trial study, each participant received (*1*) 8 coded test samples of baby food (blind duplicates at 4 content levels) plus 4 labeled 'blank' units for spiking; (*2*) 1 amber vial marked 'Aflatoxin calibrant' containing aflatoxin B_1 to be used as the calibrant aflatoxin B_1 solution described in the method; (*3*) 2 amber vials marked 'Spike solution A' and 'Spike solution B' to be used for spiking procedures; (*4*) 14 immunoaffinity columns (including

2 spares) for the cleanup of material extracts; (*5*) a copy of the method; (*6*) a report form for analytical data and reporting any criticisms and suggestions (in electronic form and paper copy); (*7*) 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 purified aflatoxin B_1 by liquid chromatography (LC). Each participant was also required to spike the 4 indicated 'blank' test materials by adding the provided 'Spike solutions A and B' with a syringe. After adding the spike solution, the participants were to let the solvent evaporate for at least 2 h prior to extraction. They were instructed to analyze the samples in the numerical sequence of the sample codes (i.e., nested design).

AOAC Official Method 2000.16
Aflatoxin B_1 in Baby Food

Immunoaffinity Column HPLC Method
First Action 2000

(Applicable to determination of ≥0.1 ng/g aflatoxin B_1 in baby food.)

See Table **2000.16A** for the results of the interlaboratory study supporting acceptance of the method.

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 International Agency for Research on Cancer (8). Aflatoxins are subject to light degradation. Protect analytical work adequately from daylight and keep aflatoxin standard solutions protected from light by using amber vials or aluminum foil. The use of non-acid-washed glassware (e.g., vials, tubes, flasks) for aflatoxin aqueous solutions may cause a loss of aflatoxin. Soak new glassware before use in dilute acid (e.g., sulfuric acid, 2 mol/L) for several hours; then rinse extensively with distilled water to remove all traces of acid (check using pH paper).

A. Principle

Test portion is extracted with methanol–H_2O (8 + 2). Extract is filtered, diluted with water to specified solvent concentration, and applied to affinity column containing antibodies specific to aflatoxin B_1. Aflatoxin B_1 is removed from affinity column with methanol and quantified by reversed-phase liquid chromatography (LC) with postcolumn derivatization (PCD) involving bromination. PCD is achieved with either electrochemically generated bromine (Kobra cell) or with pyridinium hydrobromide perbromide (PBPB), and measurement is made by fluorescence.

B. Performance Standard for Affinity Column

The affinity column must contain antibodies raised against aflatoxin B_1 with a capacity of not less than 50 ng aflatoxin B_1 and should give recovery of not less than 80% when applied as a standard solution in methanol–H_2O containing 5 ng aflatoxin B_1.

C. Apparatus

(**a**) *Vertical or horizontal shaker.*

(**b**) *Filter paper.*—24 cm, prefolded, Whatman 2V, or equivalent.

(**c**) *Erlenmeyer flask.*—Screw top or glass stopper.

(**d**) *Glass microfiber filter paper.*—5 cm.

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

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

(**g**) *Volumetric glassware.*—5, 10, and 20 mL (accuracy of at least 0.5%).

(**h**) *LC pump.*—With flow rates between 0.20 and 1.00 mL/min.

(**i**) *Injection system.*—Total loop injection valve with loop between 100 and 1000 µL. For the volume (100–1000 µL) of the injection system, it must be guaranteed that the relative standard deviation (RSD) of the aflatoxin B_1 peak for a multiple injection (n = 10) of a standard solution of aflatoxin B_1 reflecting a contamination level of 0.1 ng/g results in a value of maxium 10%.

Table 2000.16A. Interlaboratory study results for aflatoxin B_1 in baby food

ID, ng/g	$\bar{x}$, ng/g	Rec., %	No. of labs[a(b)]	s_r, ng/g	RSD_r, %	r	s_R, ng/g	RSD_R, %	R	HORRAT
0.1	0.10	101	11 (3)	0.004	3.5	0.010	0.012	12	0.034	0.2
0.2	0.18	92	14 (0)	0.024	13	0.067	0.042	23	0.118	0.4
a (NC)[c]	<0.02	—	14	—	—	—	—	—	—	—
b (NC)	0.07	—	14 (0)	0.010	14	0.028	0.017	23	0.046	0.3
c (NC)	0.09	—	11 (3)	0.007	8	0.020	0.008	9	0.022	0.1
d (NC)	0.17	—	13 (1)	0.021	12	0.059	0.039	23	0.110	0.4

[a(b)] Where a = number of laboratories retained after eliminating outliers, (b) = number of laboratories removed as outliers.

[c] a–d = Identifications for samples; NC = naturally contaminated.

(**j**) *Reversed-phase LC column.*—4.6 mm × 25 cm, 5 μm, ODS-2 column of 5 μm pore size, 12% carbon loading; not end-capped is suitable.

(**k**) *Postcolumn derivatization system.*—(*1*) *With PBPB.*—Second LC pulseless pump, zero-dead volume T-piece, reaction tubing minimum. 45 cm × 0.5 mm id PTFE, or (*2*) *With electrochemically generated bromine.*—e.g., Kobra cell, Rhône Diagnostics Ltd. (Glasgow, UK).

(**l**) *Fluorescence detector*.—Wavelength 360 nm excitation filter and 420 nm cut-off emission filter.

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

(**n**) *Pipets.*—Marked, 1–10 mL capacity.

(**o**) *Analytical balance.*—Weighing to 0.1 mg.

(**p**) *Laboratory balance.*—Weighing to 0.01 g.

(**q**) *Calibrated microliter syringe(s) or microliter pipet(s).*—20 and 500 μL capacity.

(**r**) *Immunoaffinity columns specific for aflatoxin B_1 cleanup*.—*See* **B** above for performance standard for column. For example, columns from Vicam (Watertown, MA) and Rhône Diagnostics (Glasgow, UK) meet these criteria.

(**s**) *UV spectrophotometer*.—Calibrate as follows: Determine absorbance (A) of the 3 solutions of $K_2Cr_2O_7$ (0.25, 0.125, 0.0625mM) in H_2SO_4 (0.009mM), at maximum absorption near 350 nm, against 0.009mM H_2SO_4 as solvent blank. Calculate molar absorptivity (ε) at each concentration: ε = (A × 1000)/concentration in mM. If the 3 values vary by more than guaranteed accuracy of A scale, check either technique or instrument. Average the 3 ε values to obtain ε. Determine correction factor (CF) for particular instrument and cells by substituting in equation: CF = 3160/ε, where 3160 is value for ε of $K_2Cr_2O_7$ solutions. If CF is <0.95 or >1.05, check either technique or instrument to determine and eliminate cause. (Use same set of cells in calibration and determination of purity.)

D. Reagents

Unless otherwise stated, use water complying with grade 3 of ISO 3696 (standard mineral-free water).

(**a**) *Phosphate-buffered saline (PBS), pH 7.4.*—Dissolve 0.2 g KCl, 0.2 g KH_2PO_4, 1.16 g anhydrous Na_2HPO_4, and 8.0 g NaCl in 900 mL water. Adjust pH to 7.4 with 0.1M NaOH and dilute to 1.0 L.

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

(**c**) *Pyridinium hydrobromide perbromide (PBPB)*.—CAS 39416-48-3.

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

(**e**) *Acetonitrile*.—LC grade.

(**f**) *Methanol*.—LC grade.

(**g**) *Methanol*.

(**h**) *Water*.—LC grade.

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

(**j**) *Nitric acid*.—4 mol/L (1 + 3).

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

(**l**) *LC mobile phase solvent B.*—For use with electrochemically generated Br: water–acetonitrile–methanol solution, 6 + 2 + 3 (v + v + v), containing 120 mg/L KBr and 350 μL/L HNO_3 at 4 mol/L.

(**m**) *Postcolumn reagent*.—Dissolve 25 mg PBPB in 500 mL H_2O. Store in the dark at room temperature (22–25°C) for ≤ 4 days.

(**n**) *Toluene–acetonitrile*.—98 + 2 (v + v).

(**o**) *Aflatoxin B_1 standard solutions for LC.*—(*1*) *Option A.*—(For aflatoxin B_1 standard received as dry films or crystals.) To container of dry aflatoxin B_1 add volume of toluene–acetronitrile (9 + 1), calculated to give concentration of 8–10 μg/mL. Use label statement of aflatoxin weight as guide. Vigorously agitate solution 1 min on Vortex shaker and transfer without rinsing to convenient-sized glass-stoppered flask. (Dry films on glass are not completely recoverable because of adsorption. Continued contact with solvent may result in slow dissolution.) Do not transfer dry aflatoxin for weighing or other purposes unless facilities are available to prevent dispersion of aflatoxin to surroundings because of electrostatic charge on particles.

(*2*) *Option B.*—(For aflatoxin B_1 standard received as solution or crystals.) Transfer solution to convenient-sized glass-stoppered flask. Dilute, if necessary, to adjust concentration to 8–10 μg/mL. Record UV spectrum of aflatoxin B_1 solution from 200 to 400 nm against solvent used for solution in reference cell. Determine concentration of aflatoxin B_1 solution by measuring absorbance (A) at wavelength of maximum absorption close to 350 nm, using following equation:

$$1\ \mu g\ \text{Aflatoxin } B_1/mL = \frac{A \times MW \times 1000}{\varepsilon}$$

where MW (molecular weight) = 312 and ε (molar absorptivity) = 19300 [from *J. AOAC Int.* **82**, 252(1999)].

Return aflatoxin solution to original glass-stoppered flask and dilute with toluene–acetronitrile (9 + 1) to obtain a concentration of 5.00 ng/mL.

For option A, use this solution for pipeting the volumes listed in Table **2000.16B** into a set of 10 mL calibrated volumetric flasks. Evaporate toluene–acetonitrile solution just to dryness under stream of N_2 at room temperature (22–25°C). To each flask, add 3.5 mL methanol, and mix; dilute to volume with water and mix again. Prepare these working solutions daily.

(**p**) *Sulfuric acid*.—C = 0.009; dissolve 1 mL H_2SO_4 in 2 L H_2O.

(**q**) *Potassium dichromate standard solutions.*—(*1*) *C = 0.25mM.*—Accurately weigh 78 mg $K_2Cr_2O_7$ (primary standard) and dissolve in 1.0 L 0.009M H_2SO_4; calculate molarity to 3 significant figures (MW $K_2Cr_2O_7$ = 294.2). (*2*) *C = 0.125mM.*—Dilute 25 mL 0.25mM $K_2Cr_2O_7$ to 50 mL with 0.009M H_2SO_4 in volumetric flask. (*3*) *C = 0.0625mM.*—Dilute 25 mL 0.125mM $K_2Cr_2O_7$ to 50 mL with 0.009M H_2SO_4 in volumetric flask.

For option B, pipet from aflatoxin standard solution (5 ng/mL) volumes as listed in Table **2000.16C** into a set of 10 mL calibrated volumetric flasks; then evaporate the toluene–acetonitrile solution just to dryness under stream of N_2 at

Table 2000.16B. Preparation of working calibration solutions (option A)

Working standard	Aliquot taken from stock solution, μL	Final concentration of working calibrant, ng AfB_1/mL
1	20	0.01
2	40	0.02
3	60	0.03
4	80	0.04
5	100	0.05
6	120	0.06
7	140	0.07

room temperature (22–25°C). Add 3.5 mL methanol, let aflatoxins dissolve, fill to the mark with methanol, and shake well.

Transfer exactly 1 mL of this working calibrant into an acid-washed vial and evaporate to dryness according to option B. Redissolve in exactly the same amount of aqueous methanol that will be used for test solutions. Calculate concentration of aflatoxin B_1 in the redissolved working calibrant solution in ng/mL. Use these values for the calculation (the calibration range in ng/g will remain unchanged).

E. Extraction

Weigh to nearest 0.1 g ca 50 g test portion of baby food into 500 mL Erlenmeyer flask with screw top or glass stopper. Add 5 g NaCl and 250 mL methanol–water solvent. Shake intensively by hand for first 15–30 s and then for 30 min with a shaker. Filter extract using prefolded filter paper. Pipet 15 mL clear filtrate into calibrated 150 mL volumetric flask and fill with PBS or water. Refilter through glass fiber filter and apply volume of 50 or 100 mL clear filtrate in a reservoir placed on a conditioned immunoaffinity column. A volume of 50 mL will normally be adequate, although 100 mL can be used if fluorescence detection does not provide adequate sensitivity.

F. Affinity Column Chromatography

Adjust columns to room temperature (22–25°C) prior to conditioning. For conditioning, apply 10 mL PBS on top of column and let it pass at 2–3 mL/min through the column by gravity. Make sure that a small portion (ca 0.5 mL) of the PBS remains on the column until test solution is applied.

Note that methods for loading onto affinity columns, washing the column, and elution vary slightly between column manufacturers; follow precisely specific instructions supplied with the columns. In general, procedures involve extraction with methanol–water, filtration or centrifugation, possible dilution with PBS or water, loading under pressure onto (possibly prewashed) 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) under gravity. Do not exceed flow rate of 5 mL/min. Wash column with 15 mL water applied in 5 mL portions, and dry by applying small vacuum for 5–10 s or passing air through by means of syringe for 10 s.

Elute the aflatoxins in a 2-step procedure. First, apply 0.5 mL methanol on the column and let it pass through by gravity. Collect eluate in either 5 mL volumetric flask (option A below) or LC injection vial (option B below). Wait 1 min and apply a second portion of 0.75 mL methanol. Collect applied elution solvent by pressing air through.

(**a**) *Option A* (*recommended*).—This option requires appropriate fluorescence detector and injection system. Option B only applies if detector signal is insufficient for analysis by option A.

Collect elute in calibrated 5 mL volumetric flask. Fill to mark with water and shake well. If solution is clear, it can be used directly for LC analysis. If solution is not clear, pass it through disposable 0.45 μm filter unit prior to LC injection. Injection by total loop mode provides maximum accuracy. Depending on injection system, e.g., syringe or autosampler, take volume of 3 times the injection loop size and inject at least 2/3 this volume into the valve to ensure that the middle fraction remains in the injection loop. Thus, the loop is rinsed with the filtered eluate while enough liquid remains in the valve.

(**b**) *Option B* (*only if applicable*).—If detector signal is not sufficient to provide the required RSD (10%), include an additional evaporation step to meet the required RSD, **C**(*i*).

Collect methanol eluate from affinity column in an acid-washed LC injector vial. Evaporate methanol to dryness under gentle stream of N_2 at 40°C. Redissolve residue in aqueous methanol solution (3.5 mL methanol diluted to 10 mL with water). Use exactly the same volume for the evaporated analyte residues as that used for evaporated calibrants. The volume for redissolving will depend on the size of injection loop. Use total loop mode for injection as in option A.

G. LC Determination with Fluorescence Detection and Postcolumn Derivatization

When using PBPB, mount the mixing T-piece and reaction tubing, **C**(**k**)(*1*), operate with the following parameters: flow rates, mobile phase A: 1.00 mL/min; reagent: 0.30 mL/min.

Table 2000.16C. Preparation of working calibration solutions (option B)

Working standard	Aliquot taken from stock solution, μL	Final concentration of working calibrant, ng AfB_1/mL
1	100	0.05
2	200	0.10
3	300	0.15
4	400	0.20
5	500	0.25
6	600	0.30
7	700	0.35

When using electrochemically generated bromine (Kobra cell) follow the manufacturer's instructions for installing the cell and operate with the following parameters: flow rate, mobile phase B: 1.00 mL/min; current, 100 μA.

Inject working standard mixture (covering range of 0.05–0.35 ng/g for aflatoxin B_1) into injector, following manufacturer's instructions to ensure complete filling of injection loop. Aflatoxins elute in the order G_2, G_1, B_2, and B_1 with retention times of approximately 6, 8, 9, and 11 min, respectively, and should be base-line resolved to measure aflatoxin B_1 as a discrete peak. Prepare calibration curve using calibration solutions. Check curve for linearity. Inject same volume of working standards and 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 B_1 in injected eluate from the standard curve.

H. Calculations

Plot the data: concentration of aflatoxin (ng/mL) as the *y*-axis against peak area (units) as the *x*-axis, from the calibrant solutions. Calculate the resulting function, $y = ax + b$, from linear regression, where a is the slope and b is the *y*-value where the line intercepts the *y*-axis ($x = 0$).

Concentration of aflatoxin B_1 in the test sample =

$$\frac{C}{g} \times \frac{V_{extraction}}{V_{extract\ aliquot}} \times \frac{V_{diln} \times V_{elutn}}{V_{AFC}} = AfB_1 \text{ in sample}$$

$$\frac{ng / mL \times mL \times mL \times mL}{g \times mL \times mL} = AfB_1 \text{ in sample}$$

$$\frac{ng \times mL \times mL \times mL}{mL \times g \times mL \times mL} = AfB_1 \text{ in sample}$$

$$\frac{ng}{g} = AfB_1 \text{ in sample}$$

where C = concentration of aflatoxin B_1 (ng/g) from linear regression; g = test portion (g; 50); $V_{extraction}$ = volume extraction solvent (250); $V_{extract\ aliquot}$ = volume aliquot extraction solvent (15); V_{diln} = volume diluted with PBS or water (150); V_{AFC} = volume applied to column (50 or 100); V_{elutn} = volume after elution (5).

Ref. *J. AOAC Int.* **84**, 1118–1121(2001)

Results and Discussion

Collaborative Trial Results

All laboratories that received the test samples completed the study. The full set of results for the trial are given in Table 1 as individual pairs of results for each laboratory (identified as 1–14). Blank samples (a) were spiked with 0.1 and 0.2 ng/g aflatoxin B_1 as blind duplicates. Samples b–d were also blind duplicates but of naturally contaminated materials.

Statistical Analysis of Results

Precision estimates were obtained by using one-way analysis of variance according to the IUPAC Harmonized Protocol

Table 1. Collaborative trial results of LC determination of aflatoxin B_1 in baby food[a]

	Aflatoxin B_1 concentration ng/g											
Lab ID	0.1	0.1	0.2	0.2	a	a	b	b	c	c	d	d
1	0.09	0.09	0.18	0.17	<0.02	<0.02	0.05	0.05	0.08	0.07	0.19	0.16
2[b]	0.12	0.12	0.22	0.23	0.02	0.02	0.09	0.10	0.11[c]	0.12[c]	0.25	0.21
3[b]	0.11[c]	0.19[c]	0.10	0.19	0.02	0.01	0.08	0.08	0.09	0.09	0.21	0.17
4	0.11	0.11	0.19	0.20	<0.01	<0.01	0.11	0.08	0.13[c]	0.09[c]	0.17	0.21
5[b]	0.11	0.10	0.20	0.22	<0.01	<0.01	0.07	0.08	0.10	0.09	0.18	0.23
6	0.11	0.11	0.20	0.20	<0.02	<0.02	0.07	0.08	0.10	0.09	0.18	0.19
7[b]	0.09	0.08	0.18	0.17	<0.02	<0.02	0.07	0.07	0.10	0.09	0.18	0.19
8	0.09	0.09	0.17	0.16	<0.02	<0.02	0.07	0.06	0.08	0.07	0.15	0.15
9	0.03[c]	0.04[c]	0.11	0.06	0.02	0.02	0.05	0.02	0.06[c]	0.05[c]	0.08	0.10
10[b]	0.09	0.10	0.19	0.21	<0.04	<0.04	0.08	0.07	0.08	0.09	0.15	0.15
11	0.10	0.11	0.20	0.20	0.01	0.01	0.07	0.07	0.09	0.09	0.21	0.17
12	0.11	0.10	0.27	0.21	<0.02	<0.02	0.07	0.07	0.08	0.09	0.39[c]	0.18[c]
13	0.07[c]	0.10[c]	0.20	0.20	0.03	0.02	0.09	0.07	0.09	0.07	0.15	0.19
14	0.09	0.09	0.16	0.15	0.01	0.01	0.07	0.05	0.08	0.08	0.13	0.12

[a] a–d = Naturally contaminated samples.

[b] Laboratories using Kobra cell; all other participants used PBPB for derivatization.

[c] Results identified as outliers and not included in statistical analysis.

(9). Details of average aflatoxin B_1 contents of various baby food test samples, the RSD_r and RSD_R, the number of statistical outlier laboratories, HORRAT values, and mean percentage recovery are presented in Table **2000.16A**. The collaborative trial results were examined for evidence of individual systematic error ($p < 0.025$) using Cochran and Grubbs tests progressively (9). Pairs of results identified as outliers are indicated against the shaded background in Table 1 and are individually identified by code in Table **2000.16A**. For the results for aflatoxin B_1 given in Table **2000.16A** (excluding data for blank materials) the maximum number of outliers identified was 3 laboratories, giving acceptable data from 11 to 14 laboratories.

Collaborators' Comments

Comments on the trial were made by 7 of the 14 participants. In all cases participants regarded the method description as adequate. For laboratory 9 where 2 pairs of results were rejected as outliers, no comments were given. Laboratory 2 and laboratory 4 also made no comments. Laboratory 3 had prepared the standards in methanol as solvent. Laboratory 12 expressed doubt about its results for spiked samples, but did not give a technical reason. However, the value for aflatoxin B_1 was in compliance (i.e., this laboratory did not produce an outlier value for spiked samples). Laboratory 13 again used only an injection volume of 200 μL, although no further comments or problems were reported.

Precision Characteristics of the Method

Due to differences in reporting limits for not detectable, the results for blank materials were not analyzed statistically. The results, however, indicated clearly that all participants could identify the blank pairs of samples as not containing detectable aflatoxin B_1 or containing a level that was detectable but close to their limit of determination. The precision data for all samples are summarized in Table **2000.16A**. Based on results for spiked test samples (blind pairs at 2 levels) and naturally contaminated test samples (blind pairs at 3 levels) the RSD_r ranged from 3.5 to 14%, and the RSD_R ranged from 9 to 23%. The values for recoveries of aflatoxin B_1 derived from spiked baby food samples ranged from 101 to 92%.

Interpretation of Results

Acceptability of the precision characteristics of the method was assessed on the basis of the HORRAT values (10), which compare the RSD_R at various levels with those predicted from published, collaborative trial studies. When outliers were excluded, the HORRAT value for aflatoxin B_1 ranged from 0.l to 0.4, being significantly below 2.0, indicating acceptable precision.

Recommendation

It is recommended that the immunoaffinity column cleanup method by reversed-phase LC with postcolumn bromination for determination of aflatoxin B_1 in baby food at $\geq$0.1 ng/g be adopted Official First Action. The method is the first study of an aflatoxin B_1 method for baby food and its level of applicability is significantly lower for aflatoxin B_1 than any previous validation studies for other matrixes.

Acknowledgments

This study was funded by the European Commission, Standards, Measurement, and Testing Programme (4th Framework Programme). Those who contributed to the success of this study include A. Boenke (EC SMT-Programme Scientific Officer for project); K. Mathieson (CSL Food Science Laboratory); and C. von Holst (Institute for Health and Consumer Protection, JRC Ispra) who supported the statistical evaluation; A. Williams (Leatherhead Food RA), who prepared calibrant standards; Ir. H.P. van Egmond (RIVM, Bilthoven, The Netherlands), who took responsibility for the pretrial workshop for participants; A.E. Buckle (CSL, York, UK) for assisting with the workshop; G. Niedwetzki and A. Peper (WEJ, Hamburg, Germany) for test material preparation; H. Schimmel (Institute for Reference Materials and Measurements, JRC Geel) for particle size measurements and M. Petz (University of Wuppertal, Germany) for scientific support. We also express appreciation to the following collaborators for their participation in the study:

A. Barmark, National Food Administration, Uppsala, Sweden

C. Brera, C., Istituto. Sup. di Sanita, Rome, Italy

B. Dias, D.GF.C.Q, Lisbon, Portugal

I. Felgueiras, INETI/D, Lisbon, Portugal

J. Gardikis, General Chemist State Laboratory, Athens, Greece

L. Macho, Gobierno Vasco, Bilbao, Spain

J.Y. Michelet, ISP Ministry of Health, Brussels, Belgium

K. Noutio, Finnish Customs Laboratory, Espoo, Finland

A. Pittet, Nestle Research, Lausanne, Switzerland

M. Reutter, LUFA/ITL, Kiel, Germany

M.C. Spanjer, IHP–Food Inspection Service, Amsterdam, The Netherlands

E. Strassmeier, Bundesa. Lebensm. U.F., Vienna, Austria

L. Szymanski, Laboratoire Interregional de la Repression des Fraudes, Massey, France

R. Worswick, LGC, Teddington, UK

References

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

(2) Stroka, J., Anklam, E., Joerissen, U., & Gilbert, J. (2000) *J. AOAC Int.* **83**, 320–340

(3) *Official Methods of Analysis* (2000) 17th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, Method **999.07**

(4) European Standardization Committee (1999) CEN TC 275/WG 5, Draft document

(5) Commission Regulation 466/2001/EC (May 8, 2001) *Off. J. Eur. Commun.* L077/1

(6) Commission Directive 1999/39/EC (May 6, 1999) *Off. J. Eur. Commun.* L124/8

(7) Draft Commission Directive (1998) Amending Directive 91/321/EEC on infant formulae and follow-up formulae, Document III/5572/98-EN
(8) IARC (1993) *Working Group on the Evaluation of Carcinogenic Risks to Humans*, Vol. 56, IARC, Lyon, France
(9) IUPAC (1995) *Pure Appl. Chem.* **67**, 331–343
(10) Horwitz, W., & Albert, R. (1991) *J. Assoc. Off. Anal. Chem.* **74**, 718–744