FOOD CHEMICAL CONTAMINANTS

Determination of Ochratoxin A in Green Coffee by Immunoaffinity Column Cleanup and Liquid Chomatography: Collaborative Study

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A collaborative study was conducted to evaluate a method using immunoaffinity column cleanup with liquid chromatography (LC) for the determination of ochratoxin A (OTA) in green coffee at levels that could be included in possible future regulations of the European Union. The test portion was extracted with methanol–3% aqueous sodium hydrogen carbonate solution (50 + 50, v/v). The extract was filtered, and the filtrate was diluted with phosphate-buffered saline and applied to an immunoaffinity column containing antibodies specific for OTA. After washing, the toxin was eluted from the column with methanol and quantified by LC with fluorescence detection. Pairs of 4 homogeneous noncontaminated and naturally contaminated materials (mean levels of <0.12, 2.44, 5.15, and 13.46 ng/g) and blank samples (<0.12 ng/g) for spiking were sent to 20 participant laboratories from 8 countries. The materials were analyzed according to the method description and all difficulties encountered in the analysis were reported. Statistical analysis was carried out according to the Harmonized Protocol of the International Union of Pure and Applied Chemistry. The relative standard deviation for repeatability (RSD_r) ranged from 7.42 to 20.94%, and the relative standard deviation for reproducibility (RSD_R) ranged from 16.34 to 29.17%. The method showed acceptable within-laboratory and between-laboratories precision for green coffee materials, as evidenced by HorRat values of ≤0.85, at the studied range, for spiked and naturally contaminated materials. The mean recovery was 92.8% for green coffee material spiked with OTA at a level of 4.82 ng/g.

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Regulations and guidelines for ochratoxin A (OTA) in food have been established in many countries at levels ranging from 2 to 50 ng/g (1). Because the presence of OTA in foods represents a risk to human health, it is expected that the Commission of the European Union will establish a regulation for OTA in green and roasted coffee, along with sampling plans and method performance criteria, as a modification of European Commission (EC) Regulation No. 472/2002 (2) and EC Directive 2002/26/EC (3), respectively.

The existing AOAC method for the determination of OTA in green coffee (4) was published in 1975 (5). The method is based on thin-layer chromatography (TLC) and is not sufficiently sensitive (detection limit of 20 ng/g) for checking the compliance of green coffee consignments at possible future European regulatory levels. Moreover, the method does not have associated performance data. Therefore, there was a strong need for a validated method for green coffee that could be used by coffee-producing and -exporting countries to check the compliance of consignments with criteria set by importing countries.

OTA has been determined extensively in coffee since the first report of Levi et al. (6) in 1974. The toxin is usually extracted with organic solvent or water or a mixture of both, containing small amount of acid (7–11). The combination of aqueous methanol and bicarbonate has been the preferred extraction solvent (8, 12). Chromatography including liquid chromatography (LC) and TLC, has been the most widely used separation technique for the determination of OTA in various types of foods (13).

LC methods were introduced in the 1980s (14, 15) and produced limits of detection (LOD) 10–20 times lower than that of the original TLC-based AOAC method (5).

Nakajima et al. (16) introduced another significant improvement in 1990 by using for the first time an immunoaffinity column (IAC) cleanup for the determination of OTA in coffee. Since the introduction of the IAC as a cleanup step for the determination of OTA in coffee in combination with reversed-phase LC with fluorescence detection, it has been an attractive procedure to assess OTA contamination in coffee (17), giving clean extracts and clear chromatograms with no interference at the OTA retention time, and allowing LODs as low as 0.2 μg/kg (8). Nevertheless, interferences were still reported in the determination of OTA in roasted coffee by IAC with LC. Consequently, the use of phenyl silane and aminopropyl cartridges as a preliminary cleanup in combination with the IAC was proposed to overcome this problem (12, 18).

Collaborative Study

Test Materials

Bulk homogeneity of test materials.—Four samples of arabica green coffee beans, ca 20 kg each and previously analyzed (target values of <0.12, 2–4, 5–8, and 10–15 ng OTA/g), were prepared at LACQSA/LAV-MG. The beans were ground to pass through a 0.5–1.0 mm screen (80% of the material should be <0.5 mm particle size) and thoroughly homogenized in a blender for ≥6 h in 30 min cycles.

After each 30 min cycle, the ground material was mixed by hand with a spade. Ten samples of 100 g were randomly taken from each batch (bulk material) and analyzed in duplicate, by using the procedure described in this study, to check the homogeneity by 1-way analysis of variance (ANOVA) according to the International Harmonized Protocol for the Proficiency Testing of (Chemical) Analytical Laboratories (19).

Packaging of green coffee test materials.—The 4 homogeneous batches of materials were packed under vacuum in aluminium foil sachets and labeled and coded with 3 digit numbers. A total of 150 sachets filled with 50 ± 3 g ground arabica green coffee beans were packed for each batch of material.

Homogeneity testing of packaged materials.—During the packing of the 4 batches, 1 of every 10 sachets of packed material was taken from the filling sequence for final homogeneity testing. The contents of the sachets ($n \geq 10$) were analyzed in duplicate, by using the procedure described in this study.

The final homogeneity of the packaged materials was investigated by ANOVA according to the International Harmonized Protocol for the Proficiency Testing of (Chemical) Analytical Laboratories (19) as established by ISO 43-1-Annex A (20), at the 95% confidence level, by calculating the F-statistic and $S_s/\sigma(S_s/\sigma = 15\%)$. In order to decrease the influence of the variability of the sample homogenization in the variability of the analytical results (relative standard deviation for repeatability [RSD_r] and relative standard deviation for reproducibility [RSD_R]), a stricter σ of 15% instead of 22% (21) was used to evaluate the homogeneity of the test materials.

No significant difference was observed for the within-sachet and between-sachets standard deviations, either by the F-test (ANOVA; F-calculated < F-critical at 5%) or by the S_s/σ for any contaminated material.

The tests were also considered satisfactory because the variance between samples and the S_s/σ were significantly lower than those determined for 8 duplicate analyses of Food Analysis Performance Assessment Scheme test material (17), and also lower than the variance of the method itself.

As a result of this procedure, 4 homogeneous materials were coded as numbers 1, 3, 4, and 5 with OTA mean levels of <0.12, 2.44, 5.15, and 13.46 ng/g, respectively, which were considered as assigned values for the batches (material). In addition, 150 sachets of the material with a target contamination level of <0.12 ng/g were packed and labeled as "blank" (Material No. 2). These sachets were used as spiking materials.

Storage of collaborative trial samples.—All batches of test materials, before and after packaging, were stored at −18°C and protected from light.

Analysis control.—Blanks (<0.12 ng/g), spiked materials (5 ng/g), and naturally contaminated materials (5.23 ± 0.55 ng/g; $n = 42$) analyzed in duplicate were used as controls during the homogeneity testing, for both bulk and packaged batches of naturally contaminated materials.

Organization of the Collaborative Study

Twenty laboratories representing government institutions and the food industry from 8 countries, including coffee-producing countries, participated in the study. Nineteen laboratories submitted data and data from one laboratory were not used for statistical analysis because of sample misidentification.

Before the collaborative study, participants were provided with practice samples, IACs, OTA standard solution (10 μg/mL), and videotapes. The practice samples consisted of a pair of blind, naturally contaminated, green arabica coffee test samples containing OTA at 4.28 and 3.87 ng/g. The laboratories were asked to analyze the samples according to the method description, and to optimize their chromatography conditions.

A report containing the homogeneity test data for the 2 test materials, the statistical evaluation of the results, as well as all observations and comments returned by the participants was distributed before the final study. Horrat values were <1.0 for the 2 naturally contaminated green coffee test samples. A workshop was held to discuss the rationale behind the method being tested, the results obtained in the precollaborative study for the 2 practice samples, and the difficulties encountered during the analyses. The Study Coordinator outlined further details about the organization of the final study.

For the collaborative study, each laboratory received 4 pairs of frozen naturally contaminated blind arabica green coffee samples with assigned values of <0.12, 2.44, 5.15, and 13.46 ng OTA/g; 1 pair of frozen blank samples for spiking (<0.12 ng/g);

an ampoule of OTA working standard solution (calibrant) at 10 μg/mL; 2 ampoules of undisclosed OTA standard solutions A and B (0.804 μg/mL) for spiking (exactly 150 μL of each solution); 10 IACs containing OTA antibodies from the same lot (OchraTest–Vicam, 313 Pleasant St, Watertown, MA 02472, USA; www.vicam.com; Lot 221); instructions for the participants; reporting sheets for analytical results as well as criticisms and suggestions; a collaborative study material receipt form; an analytical work questionnaire; and a copy of the method.

The participants were required to analyze all 8 blind naturally contaminated and spiked materials, strictly adhering to the described method and the instructions. The participants were instructed to analyze all materials on the same day. Blank materials were to be spiked with undisclosed spiking solutions A and B. All results and difficulties as well as criticisms, comments, and suggestions were reported in the results sheets and in the analytical work questionnaire, respectively.

AOAC Official Method 2004.10
Ochratoxin A in Green Coffee
Immunoaffinity Column Cleanup and Liquid Chromatography
First Action 2004

(Applicable to the determination of ochratoxin A [OTA] in green coffee at ≥2.60 ng/g.)

Caution: OTA is a potent nephrotoxin with immunotoxic, teratogenic, and potential genotoxic properties. The International Agency for Research on Cancer (IARC) has classified OTA as a possible human carcinogen (group 2B). Protective clothing, gloves, and safety glasses should be worn at all times, and all standard and sample preparation steps should be carried out in a fume hood. Swab accidental spills of OTA with 1% NaOCl bleach solution, and wait 10 min before adding 5% solution of acetone in water. Rinse all glassware exposed to OTA with methanol, add 1% NaOCl solution, and after 2 h add acetone to bring the acetone concentration to 5% of the total volume. Let reaction proceed for 30 min, and then wash glassware thoroughly. Toluene is toxic. Operations involving this solvent must be performed in a fume hood. Methanol is hazardous. The blending step must be carried out by using an explosion-proof blender in a fume hood. All analyses should be carried out in a fume hood. Follow applicable environmental rules and regulations for disposal of waste solvents.

See Table **2004.10A** for the results of the interlaboratory study supporting acceptance of the method.

A. Principle

A test portion is blended with methanol–3% aqueous sodium bicarbonate solution (50 + 50). The extract is filtered, diluted with phosphate-buffered saline (PBS), and applied to an immunoaffinity column containing antibodies specific for OTA. After washing, the toxin is eluted from the column with methanol and quantified by liquid chromatography (LC) with fluorescence detection.

B. Apparatus

(a) *Immunoaffinity columns.*—Containing OTA antibodies. The columns should have a capacity of ≥100 ng OTA and should recover ≥85% OTA when 100 mL standard solution containing 5 ng toxin in methanol–3% sodium bicarbonate solution (50 + 50)/PBS solution (4 + 96) is passed through the column. Immunoaffinity columns should be brought to room temperature before use.

Table 2004.10A. Interlaboratory study results for determination of OTA in green coffee

Test material	Mean value found for OTA, ng/g	Rec., %	No. of sets of acceptable results	r, ng/g	s_r, ng/g	RSD_r, %	R, ng/g	s_R, ng/g	RSD_R, %	No. of outlier labs	HorRat
1[a]	NC[b]	—	18	NC	NC	NC	NC	NC	NC	NC	NC
2[c]	4.48	92.8	15[d]	0.93	0.33	7.42	2.05	0.73	16.34	2[d,e]	0.45
3[a]	2.60	—	15	1.22	0.44	16.78	1.50	0.53	20.51	3[f]	0.52
4[a]	6.32	—	17	3.70	1.32	20.94	5.16	1.84	29.17	1[g]	0.85
5[a]	12.89	—	15	3.33	1.19	9.24	7.30	2.60	21.15	3[h]	0.65

[a] Naturally contaminated green coffee samples.
[b] NC = Not calculated (because the OTA level was below the limit of detection).
[c] Blank green coffee (OTA at <0.12 ng/g) spiked with OTA at 4.82 ng/g.
[d] Data from Laboratory A were not included in the statistical analysis (noncompliance, results reported as <0.05 ng/g).
[e] Outliers = Laboratories C and K.
[f] Outliers = Laboratories A, K, and N.
[g] Outlier = Laboratory K.
[h] Outliers = Laboratories A, D, and K.

(b) *Homogenizer/blender.*—Motor speed: 0–16 000 rpm; equipped with glass vials with capacity of 946 mL (mason type) and stainless steel helix; Omnimixer Sorvall Model 17105, or equivalent.

(c) *Ultrasonic bath.*

(d) *Fast qualitative filter paper.*—24 cm diameter, folded; Whatman No. 4, or equivalent.

(e) *Fiber glass membrane.*—5.5 cm diameter, 1 μm retention, Whatman GF/B, or equivalent.

(f) *Membrane.*—0.45 μm Cellulose or cellulose nitrate for aqueous organic solvent.

(g) *Calibrated volumetric flasks.*—50, 100, 1000, and 2000 mL.

(h) *Analytical balance.*—Readability 0.1 mg.

(i) *Laboratory balance.*—Readability 0.01 g.

(j) *Disposable syringe barrels.*—For use as reservoirs (60 and 10 mL capacity); Luer locks; with attachments and adapters to fit to immunoaffinity columns.

(k) *Water bath or block heater.*—Maintaining temperature at 40°–45°C; equipped with gas flow adapter.

(l) *Vacuum system.*—With funnel top, funnel membrane support, aluminium clamp, stopper, and side-arm flask; Millipore type, or equivalent.

(m) *Calibrated volumetric pipets.*—4 and 25 mL.

(n) *Vortex mixer.*

(o) *Spectrophotometer.*—Measuring from 200 to 400 nm, with quartz cells.

(p) *LC system.*—Valve injection system of 20 μL loop size, or equivalent; mobile phase pump capable of pumping 1 mL/min with negligible pulsation; fluorescence detector, λ = 332 nm excitation and λ = 476 nm emission wavelengths; computer-based data processing system; reversed-phase (C18) column, 250 × 4.6 mm with 5 μm particles, Shimpack (Shimadzu), or equivalent; and degasser.

(q) *Vacuum manifold.*—Vac-Elut system from Varian, or equivalent.

(r) *Conical flasks.*—5 or 10 mL.

(s) *Calibrated displacement pipets.*—100–1000, 20–200, and 500–5000 μL, with appropriate tips.

(t) *Amber glass vials.*—2, 5, and 10 mL, with crimp caps, or equivalent.

C. Reagents

Unless otherwise specified, use only reagents of a recognized analytical grade and/or LC grade. Use ISO 3696 Grade 3 water.

(a) *OTA standard solutions.*—(*1*) Prepare a stock standard solution at ca 40 μg/mL in toluene–acetic acid (99 + 1). Using a spectrophotometer calibrated according to **970.44**, determine the exact concentration of this stock standard solution by using the following equation:

$$\text{OTAm } \mu\text{g/mL} = (A \times MW \times 1000)/\varepsilon$$

where MW = molecular weight of OTA (403.8); ε = molar absorptivity of OTA in toluene–acetic acid (99 + 1; 5440); and A = absorbance of the OTA stock standard solution at the wavelength of maximum absorption (λ_{max}, ca 333 nm).

(*2*) Prepare a working standard solution at 10 μg/mL by transferring an appropriate aliquot of stock standard solution to a 5 mL amber volumetric flask. Dilute to volume with toluene–acetic acid (99 + 1) and mix well.

(*3*) Pipet 500 μL OTA working standard solution (10 μg/mL) into a 5 mL amber volumetric flask, and dilute to volume with toluene–acetic acid (99 + 1) to obtain a concentration of 1 μg/mL. Pipet 200 μL diluted working standard solution (1 μg/mL) into a 5 mL amber volumetric flask. Evaporate the solution just to dryness under a stream of nitrogen at room temperature. Redissolve with 5000 μL LC mobile phase to obtain a concentration of 40 ng/mL (standard 1). Then use standard 1 to prepare additional calibrant solutions (standards 2–6) as indicated in Table **2004.10B**.

(b) *Water–glacial acetic acid.*—29 +1. Add 30 mL glacial acetic acid to 870 mL deionized water, and filter through a 0.45 μm membrane.

(c) *Aqueous sodium bicarbonate solution.*—3%. Add 30 g sodium bicarbonate to 1000 mL deionized water.

(d) *Anhydrous disodium hydrogen phosphate.*—Analytical grade.

(e) *Potassium dihydrogen phosphate.*—Analytical grade.

(f) *PBS solution.*—pH 7.0. Add 0.20 g potassium dihydrogen phosphate, 1.10 g anhydrous disodium hydrogen phosphate, 8.00 g NaCl, and 0.20 g KCl to 1000 mL deionized water and homogenize. Alternatively, use commercial PBS tablets, e.g., Oxoid Code BR14 (pH 7.3), or equivalent.

(g) *Methanol.*—LC grade.

(h) *Acetonitrile.*—LC grade.

(i) *Glacial acetic acid.*—LC grade.

(j) *Methanol–3% aqueous sodium bicarbonate solution 1 + 1 (v/v).*—Add 500 mL 3% aqueous sodium bicarbonate solution, **C(c)**, to 500 mL methanol.

(k) *Toluene–acetic acid.*—99 + 1. Add 1 mL glacial acetic acid to 99 mL toluene.

(l) *Nitrogen.*—Purity >99.9%.

(m) *Helium.*—Purified compressed gas or other degassing system.

Table 2004.10B. Preparation of calibrant standard solutions

Calibrant solution	Volume of standard 1, μL	Volume of LC mobile phase, μL	Final OTA concn, ng/mL
Standard 1	—	—	40
Standard 2	1500	500	30
Standard 3	1000	1000	20
Standard 4	500	1500	10
Standard 5	250	1750	5
Standard 6	100	1900	2

(**n**) *LC mobile phase.*—Combine acetonitrile, methanol, and water–glacial acetic acid (29 + 1) to obtain acetontrile–methanol–water–glacial acetic acid (35 + 35 + 29 + 1). The mobile phase should be degassed by ultrasonication or another degassing system, and by bubbling helium into the mobile phase reservoir during pumping.

(**o**) *Toluene.*—UV grade.

D. Extraction

Weigh, to the nearest 0.1 g, a 25 g test portion into a 500 mL flask. Add 200 mL methanol–3% aqueous sodium bicarbonate solution (1 + 1). Blend for 5 min with a blender at a minimum speed of 8000 rpm. Filter the mixture through a folded qualitative paper filter. Immediately after filtration, collect the filtrate and refilter through a fiber glass membrane, using the vacuum system. Immediately after the second filtration, transfer a 4 mL aliquot of filtered extract to a graduated cylinder. Dilute to 100 mL with PBS and homogenize.

E. Immunoaffinity Column Cleanup

Let the immunoaffinity column come to room temperature, connect it to the vacuum manifold, and attach a 60 mL reservoir. Using 2 portions of ca 50 mL each, transfer two 50 mL aliquots, one at a time, of diluted test extract from **D** to the 60 mL reservoir, and let it pass through the immunoaffinity column by gravity at a flow rate 2–3 mL/min. If necessary, push down with a rod or apply a slight vacuum. Do not exceed a flow rate of 3 mL/min and do not allow the column to run dry. Wash the column with 10 mL deionized water at a flow rate of 3 mL/min. Dry the column by applying a slight vacuum for 30 s, or apply pressure by pushing down the rod. Disconnect the immunoaffinity column, and replace the 60 mL reservoir with a 10 mL glass syringe.

F. Elution of OTA

Apply 4 mL methanol to the 10 mL glass syringe, and wait 3 min to allow the solvent to permeate the gel before elution. Elute the toxin from the column into a 5 or 10 mL conical flask at a flow rate of 2–3 mL/min, using positive pressure. Evaporate the eluate to dryness, using a gentle stream of nitrogen in a water bath or a block heater at ca 40°–45°C. Redissolve the residue with 200 µL LC mobile phase, and homogenize in a Vortex mixer and/or ultrasonic bath.

G. Determination of OTA

(**a**) *LC operating conditions.*—Standard and test solution injection volume, 20 µL. Follow instructions of the manufacturer for temperature of the injector and LC column, i.e., room temperature; elution flow rate, 0.8 mL/min; mobile phase, refer to **C**(**n**).

(**b**) *Column evaluation.*—Baseline resolution of the OTA peak from any other chromatographic peaks, with a retention time of approximately 10 min for OTA, can be achieved when a reversed-phase (C18) column, 250 × 4.6 mm, with 5 µm particles and a mobile phase of acetonitrile–methanol–water–glacial acetic acid (35 + 35 + 29 + 1), **C**(**n**).

(**c**) *Calibration curve.*—Prepare a calibration curve at the beginning of the analysis by injecting 20 µL of each of the LC calibrant standard solutions containing OTA at 2, 5, 10, 20, 30, and 40 ng/mL (Table **2004.10B**). Plot the peak area versus the weight of OTA injected, and check the curve for linearity. The calibration curve should have an r^2 value of ≥0.99.

(**d**) *Determination of OTA in test solutions.*—Inject 20 µL aliquot of each test solution into the LC system, using the same conditions as were used for the preparation of the calibration curve. Identify the OTA peak in the chromatogram of the test solution by comparing the retention time of the peak with that obtained for the OTA peak in the chromatograms of the standard solutions. If the area of the OTA peak in the chromatogram of the sample is greater than that of the highest standard, dilute the sample extract and re-inject.

H. Calculations and Expression of Results

From the calibration curve, determine the mass (ng) of OTA in the aliquot of test solution injected into the LC system.

Calculate the concentration (ng/g) of OTA, using the following equation:

$$\text{OTA, ng/g} = \frac{M}{W} = \frac{M}{M_s} \times \frac{V_1}{V_2} \times \frac{V_3}{V_4}$$

where M = mass of OTA (ng) in aliquot of extract (20 µL) injected into the LC system; W = equivalent weight of test portion injected into the LC system (0.05 g); M_s = mass of test portion (25 g); V_1 = volume of extraction solvent (200 mL); V_2 = volume of filtrate loaded onto the immunoaffinity column (4 mL); V_3 = volume of LC mobile phase used for taking up the dry residue (200 µL); and V_4 = volume of extract injected into the LC system (20 µL).

Reference: *J. AOAC Int.* **88**, 775–777 (2005).

Results and Discussion

Collaborative Trial Results

Data were received from 19 laboratories. Raw data for all materials are shown in Table 1. Laboratory S did not send any data. Data from Laboratory T were not considered because it was not possible to clearly correlate the results and samples.

For the pair of samples of uncontaminated material (Material 1, OTA <0.12 ng/g), all participants reported the concentration as ND (not detected) or <0.12 ng/g, except Laboratory D, which reported a higher value for OTA (<0.22 ng/g).

Pairs of results identified as outliers are shown in Table 1. Three pairs of results (from Laboratories A, K, and N) were identified as outliers for Material 3 by Grubbs tests. Six pairs of outlier results were identified by the Cochran test, 1 for Material 4 (from Laboratory K), 3 for Material 5 (from Laboratories A, D, and K), and 2 for Material 2 (spiked; from Laboratories C and K). The outlier results obtained by some laboratories may be related to the final extract volume and instrumental problems: Laboratories A and C commented that the final volume was too small, and Laboratory K had problems with the Aspec solid-phase extraction (SPE) station. There was

Table 1. Collaborative trial results (ng OTA/g) for LC determination of OTA in green coffee

	Material									
Laboratory	1[a]	1[a]	2[b]	2[b]	3[c]	3[c]	4[d]	4[d]	5[e]	5[e]
A	<0.05	<0.05	<0.05	<0.05	3.90[f]	5.74[f]	12.3	7.6	7.30[g]	22.80[g]
B	<0.08	<0.08	4.43	3.92	2.25	2.73	5.53	7.16	13.38	11.79
C	0	0.01	4.92[g]	7.50[g]	2.77	2.09	5.79	5.46	13.92	15.64
D	<0.22	<0.22	4.25	3.42	2.64	2.11	8.03	10.66	7.91[g]	15.82[g]
E	<0.12	<0.12	5.47	5.67	2.3	2.75	7.35	5.57	13.43	12.2
F	<0.1	<0.1	4.5	4.39	3.51	2.88	5.8	6.07	13.96	12.2
G	ND[h]	ND	4.45	4.35	3.16	1.84	5.4	5.64	13.53	11.21
H	—	—	4.49	3.81	2.58	2.65	6.2	4.77	10.31	8.63
I	ND	ND	4.87	4.87	2.1	2.56	6.91	6.25	14.22	17.04
J	0.04	0	5.1	4.97	3.12	3.02	6.4	8.04	12.56	12.95
K	0	0	20.73[g]	16.95[g]	9.57[f]	8.24[f]	42.07[g]	15.21[g]	66.12[g]	43.35[g]
L	<0.1	<0.1	4.89	4.60	3.05	2.59	6.28	7.84	15.75	18.69
M	ND	ND	3.86	4.00	3.42	3.42	5.6	7.7	11.69	12.56
N	ND	ND	2.82	2.88	0.90[f]	0.98[f]	2.34	4.7	9.4	9.14
O	0	0	5.03	5.04	1.55	2.65	5.42	6.86	14.03	14.63
P	ND	ND	6.04	4.94	2.71	3.26	6.02	5.45	14.14	12.75
Q	ND	ND	4.17	4.31	1.94	1.54	3.71	3.94	7.82	8.72
R	0	0	4.75	3.96	2.13	2.66	4.98	7.07	14.2	16.07

[a] Uncontaminated green coffee (OTA at <0.12 ng/g).
[b] Blank green coffee (OTA at <0.12 ng/g) spiked with OTA at 4.82 ng/g.
[c] Naturally contaminated sample (assigned value of OTA, 2.44 ng/g).
[d] Naturally contaminated sample (assigned value of OTA, 5.15 ng/g).
[e] Naturally contaminated sample (assigned value of OTA, 13.46 ng/g).
[f] Identified as an outlier (Grubbs test) and not included in the statistical analysis.
[g] Identified as an outlier (Cochran test) and not included in the statistical analysis.
[h] ND = Not detected.

no comment from Laboratory D that could explain the cause of failure.

The collaborative trial results were analyzed statistically according to the Harmonized Protocol of the International Union of Pure and Applied Chemistry; the results of the statistical analysis are given in Table **2004.10A**. The RSD_r values ranged from 7.42 to 20.94%, and the RSD_R values ranged from 16.34 to 29.17%. The method showed acceptable within-laboratory and between-laboratories precisions for green coffee materials, as evidenced by Horrat values of ≤0.85 obtained for spiked and naturally contaminated materials. The precision characteristics (RSD_r and RSD_R) obtained in this collaborative trial meet all criteria established by the European Committee for Standardization (22) for analytical methods for OTA, and they compare favorably with the values obtained by the AOAC method using SPE followed by immunoaffinity chromatography and LC for the determination of OTA in roasted coffee (12).

For determining the percent recovery of the method, participants were asked to spike the pair of blank coffee samples with 150 µL undisclosed OTA standard solutions A and B (0.804 µg/mL), respectively, which resulted in a contamination level of 4.82 ng/g. The results of the 2 spiking experiments are shown in Table 1. Mean recoveries ranged from 59.1 to 390.5% (n = 17) with an overall mean recovery of 112.4%. When the outliers are excluded (Laboratories C and K), mean recoveries ranged from 59.1 to 115.5% (n = 15) with an overall mean value of 92.8%.

Collaborators' Comments

The main comments and suggestions were related to the final extract volume and the filtration step. Five laboratories commented that the final volume was too small, and 4 laboratories reported that the filtration step was very slow. A few additional comments and suggestions concerned the injection volume and the composition of the mobile phase.

Recommendations

It is recommended that this method be adopted as an AOAC Official First Action Method for determination of OTA in green

coffee. The method is valid down to OTA concentrations of at least 2.6 ng/g and possibly lower.

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