FOOD CHEMICAL CONTAMINANTS

Determination of Ochratoxin A in Black and White Pepper, Nutmeg, Spice Mix, Cocoa, and Drinking Chocolate by High-Performance Liquid Chromatography Coupled with Fluorescence Detection: Collaborative Study

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A method validation study for the determination of ochratoxin A in black and white pepper (*Piper* spp.), nutmeg (*Myristica fragrans*), spice mix (blend of ginger, turmeric, pepper, nutmeg, and chili), cocoa powder, and drinking chocolate was conducted according to the International Harmonized Protocol of the International Union of Pure and Applied Chemistry. The method is based on the extraction of samples with aqueous methanol, followed by a cleanup of the extract with an immunoaffinity column. The determination is carried out by reversed-phase LC coupled with a fluorescence detector. The study involved 25 participants representing a cross-section of research, private, and official control laboratories from 12 European Union (EU) Member States, together with Turkey and Macedonia. Mean recoveries ranged from 71 to 85% for spices and from 85 to 88% for cocoa and drinking chocolate. The RSD_r values ranged from 5.6 to 16.7% for spices and from 4.5 to 18.7% for cocoa and drinking chocolate. The RSD_R values ranged from 9.5 to 22.6% for spices and from 13.7 to 30.7% for cocoa and drinking chocolate. The resulting Horwitz ratios ranged from 0.4 to 1 for spices and from 0.6 to 1.4 for cocoa and drinking chocolate according to the Horwitz function modified by Thompson. The method showed acceptable within-laboratory and between-laboratory precision for each matrix, and it conforms to requirements set by current EU legislation.

Ochratoxin A (OTA) is a secondary metabolite mainly produced by *Aspergillus ochraceus, A. carbonarius*, and *A. niger* in tropical regions and by *Penicillium verrucosum* in temperate climates. Of the various mycotoxins that can contaminate food and feed, OTA is detectable in a diverse range of matrixes. Its presence has been reported in all types of cereals, cereal-derived products, pulses, dried fruit, wine, grape juice, beer, coffee, cocoa, nuts, spices, licorice, botanicals, and meat products, the latter resulting from OTA-contaminated feed. OTA has been classified as group 2B by the International Agency for Research on Cancer (IARC), meaning that there is sufficient evidence of its renal carcinogenicity to animals and possibly to humans (1). In addition, with regard to chronic effects, OTA has been reported to have nephrotoxic, teratogenic, and immunotoxic properties and has been linked to nephropathy in humans (1, 2). The European Food Safety Authority (EFSA) undertook a risk assessment of OTA in 2006 (3), deriving a Tolerable Weekly Intake (TWI) of 120 ng/kg bodyweight and estimating that adult European consumers had dietary exposures ranging from 12 to 50% of the TWI. A subsequent evaluation in 2010 (4) of more recent toxicological data did not change the earlier evaluation. There are no regulatory controls of OTA in the United States, but since 2006, the European Union (EU) has applied limits (5) to cereals, cereal-derived products, vine fruit, coffee, wine, and grape juice ranging from 0.5 μg/kg for processed cereal-based foods and baby foods to 5.0 μg/kg for unprocessed cereals, and limits of 10.0 μg/kg are applied to both dried vine fruit and soluble coffee (5). Controls in the EU were extended to spices and licorice, with a limit of 20 μg/kg for licorice root (6); 15 μg/kg for white and black pepper, ginger, nutmeg, and turmeric (7); 20 μg/kg for dried chilies, chili powder, cayenne,

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and paprika; and 15 μg/kg for mixtures containing one or more of the aforementioned spices (7). At the present time, there are no EU limits for cocoa and cocoa products, although these are presently under consideration (5).

Regulations in the EU based on maximum limits are supported by the development and validation of reference methods that are made available through the European Committee for Standardization (CEN) as CEN Standards (8). CEN Standards are currently available as fully validated methods with complete performance data for the analysis of OTA (8) in cereals and cereal products (two standards); cereal-based foods for infants and young children (one standard); currants, raisins, sultanas, mixed dried fruit, and dried figs (one standard); wine and beer (one standard); and barley and roasted coffee (one standard). In 2013, as part of a program to continue to provide reference methods in support of EU Regulations, the CEN published a tender for the development and full validation of 11 new standardized methods covering a range of mycotoxins. This program included a requirement to validate a single method for OTA covering a range of spices, as well as cocoa and cocoa products.

There are a number of published methods for the analysis of OTA in spices, with various amounts of data from single-laboratory validations (9–21). These methods are very similar to one another, essentially being based on the extraction of ground spices with acetonitrile–water (9, 10, 12–14) or methanol–water (12, 15–22) in various proportions. The filtered or centrifuged extract is then diluted with phosphate-buffered saline (PBS), sometimes with the addition of a detergent (10, 15, 17) such as Triton X or Tween 20 before immunoaffinity column (IAC) cleanup, and OTA is determined by LC with fluorescence detection (FD). A typical IAC-based method for spices (9) has an LOQ of 0.2 μg/kg, a recovery on spiked samples of 85%, and an RSD_r of 1.4%. Naturally contaminated and spiked spice samples have been analyzed for OTA using these IAC-based methods and have performed well for sweet and hot dried red peppers (9, 10, 15, 17, 19), black and white pepper (21), paprika (20), chili (9, 12, 14, 15, 19), prickly ash, cinnamon, aniseed, fennel, and cumin (15), ginger (18, 19), and curry powders (15, 16) without any evident background interferences. The only full collaborative study for OTA in spices was conducted using an IAC LC–FD method for the analysis of spiked and naturally contaminated samples of paprika and chili (22). The study involved 21 participants from 14 EU Member States and Singapore. The method was shown to be applicable for the quantification of OTA in paprika and chili in the range of 2–80 μg/kg and had mean recoveries of 83.7–87.5%. The RSD_r values ranged from 1.7 to 14.3% and the RSD_R values from 9.1 to 27.5%, giving Horwitz ratio (HorRat) values from 0.4 to 1.3. This method met the performance requirements set by the EU for a reference method (23) and is under consideration by CEN for standardization.

There are only a few publications concerning the analysis of OTA in cocoa and in cocoa products such as chocolate (24–27). Again, the methods are essentially similar to the IAC-based methods used for spices. In one publication (25), chocolate was extracted with methanol–water containing sodium chloride. Analyses for both OTA and aflatoxins were conducted after cleanup on an IAC specific for both classes of mycotoxins. For cocoa beans and various samples of chocolate, the extraction involved sodium bicarbonate solution without methanol, and after filtration, the extract was diluted with PBS plus Tween 20 before LC–FD analysis (26). Others have used the same extraction of cocoa nibs, but carried out a defatting of the extracts before purification on the IAC by flocculation and filtration of fats (25). Many of these variations relate to the optimization of the extraction recovery, as well as the minimization of any potentially interfering coextractives that might influence the ability of the LC column to achieve sufficient separation. One collaborative study (27) was conducted with naturally contaminated and spiked cocoa samples by extraction with sodium bicarbonate solution and dilution with PBS before IAC cleanup and HPLC analysis. Recoveries averaged 80% for spiked samples, and repeatability ranged from 21 to 31% and reproducibility from 29 to 40% at levels of OTA from 0.2 to 1.4 μg/kg (27).

In this paper, we report the results of a full collaborative study of a method for the analysis of OTA in black and white pepper, nutmeg, spice mix, cocoa, and drinking chocolate. The method development was based on the previously successful IAC-based method that was validated for OTA in paprika and chili (22), and aimed to extend the method to the analysis of more difficult spice matrixes such as black pepper and white pepper, as well as the analysis of cocoa and cocoa products. It is intended that the derived method would be considered as an EU reference method and ultimately adopted as a CEN Standard (8).

Interlaboratory Study for Validation of the Method

Test Materials

Samples of blank ground black pepper, whole white pepper, and ground turmeric were purchased as the commercial products "Pfeffer schwarz gemahlen" (Fuchs Gewürze GmbH, Wald, Germany), "Weißer Pfeffer ganz" (Horeca Select, MCC Trading International GmbH, Düsseldorf, Germany), and Kurkuma gemahlen (Horeca Select, MCC Trading International GmbH), respectively. Samples of nutmeg, ginger, chili, and curry mix, which were both blank and naturally contaminated with OTA, were kindly supplied by various EU food control laboratories. Blank drinking chocolate and cocoa samples included "Suchard Caona," "Suchard Kakao Express," and "deZaan Cocoa," available in the market as commercial products. Naturally contaminated cocoa powder was kindly provided by another EU food control laboratory.

All test materials were analyzed for their OTA content by the method described below to establish blank and natural contamination levels in order to determine proportions of each matrix required to be mixed to achieve target levels. Black pepper, white pepper, turmeric, nutmeg, and curry powder all contained <0.5 μg/kg OTA and were used as test materials for spiking and/or as ingredients in the spice mix. Naturally contaminated samples of nutmeg contained around 1.0 and 10.0 μg/kg of OTA, and ginger and chili contained around 3.0 and 8.5 μg/kg of OTA, respectively. These samples were used either directly as naturally contaminated test samples, or various amounts were used to prepare two samples of naturally contaminated spice mix with a composition as shown in Table 1.

Two commercial samples of drinking chocolate and one commercial sample of cocoa powder contained <0.4 μg/kg

Table 1. Composition of spice mix test material

	Samples A and D		Samples B and C	
Ingredient	Weight, g	OTA level, µg/kg	Weight, g	OTA level, µg/kg,)
Black pepper	250	<0.5	1000	<0.5
Turmeric	250	<0.5	250	<0.5
Ginger	500	3.0	500	3.0
Chili	250	8.5	1000	8.5
Nutmeg	750	1.0	250	1.0
Curry mix	1500	<0.5	500	<0.5
Spice mix	3500	Target = 1.2	3500	Target = 3.1

OTA, whereas a further noncommercial sample of cocoa obtained from a food control laboratory contained around 10 µg/kg OTA. The composition of the two commercial drinking chocolate samples were indicated on the label to contain 27% or 18% cocoa powder, plus sugar (glucose), milk powder, soy lecithin, salt, and aroma. The commercial cocoa powder contained no other added ingredients. The blank drinking chocolate was prepared by mixing equal amounts (500 g) of the two commercial products together with cocoa (500 g). The naturally contaminated drinking chocolate was prepared by mixing a 300 g portion of the two commercial products together with 300 g blank cocoa and 700 g naturally contaminated cocoa. Naturally contaminated cocoa was also used directly to provide samples B and D, whereas cocoa samples A and C were prepared by mixing 500 g naturally contaminated material containing 10 µg/kg OTA with 1200 g commercial cocoa powder containing <0.4 µg/kg OTA.

All samples of individual spices, spice mix, cocoa, and drinking chocolate were ground using a centrifugal mill (Retsch ZM 200) with a sieve size of 500 µm. Test samples (about 15 g) of each material were taken from different parts of the milling tray and packaged in individual polyethylene sample containers of 100 mL capacity. Samples were individually labeled with a code to enable the matrix type to be distinguished (BP, WP, NM, SM, CO, and DC for black pepper, white pepper, nutmeg, spice mix, cocoa, and drinking chocolate, respectively). In addition to matrix codes, samples were labeled SP1, SP2, SP3, or SP4 to indicate to participants which samples to spike with the respective spike solutions. Other samples were labeled A, B, C, or D to indicate the matrixes that were naturally contaminated. It was not disclosed to participants that these samples were pairs of blind duplicates.

Homogeneity of Test Materials

All naturally contaminated test materials were checked for homogeneity according to the International Harmonized Protocol of the International Union of Pure and Applied Chemistry (IUPAC; 28) and ISO 13528:2015 (29). For this, 10 sample units were randomly selected and analyzed in duplicate after the units were split into two subfractions of 5 g. The analytical method used for homogeneity testing was the one described in this protocol, keeping the same ratio of test portion to extraction solvent. The RSD of the obtained data under repeatability conditions did not exceed 6.6%, which is the requirement for sufficient homogeneity (30).

OTA Solutions

An OTA stock solution was prepared by dissolving a crystalline OTA standard (Sigma Chemical Co.) in 100 mL 1% acetic acid in acetonitrile to give a concentration of approximately 10 µg/mL. This solution was then used to prepare the spike solutions containing 75, 250, and 750 ng/mL (to spike 500 µL onto a 12.5 g portion of blank test sample) and a standard solution of 1 µg/mL. The absolute values were checked following the OTA method sent to the participants using a six-point calibration curve.

Approximately 750 µL aliquots of the spiking solutions were distributed to each participant in 10 mL amber ampules. The spiking solutions were labeled SP-1, SP-2, SP-3, and SP-4 followed by the code of the sample into which they were to be spiked. An approximately 3 mL aliquot of the standard solution containing 1 µg/mL was distributed to participants in 10 mL amber ampules for preparation of the calibration solutions.

Organization of the Method Validation Study

Twenty-five laboratories participated in the study, representing a cross-section of academia, official control, and private laboratories. The participants were divided into three groups so that one group took part only in the analysis of spices, one group analyzed only cocoa and drinking chocolate, whereas the third group analyzed all the matrixes. In January 2016, participants received parcels containing their confidential laboratory code, a method description, a spiking protocol, and outlines of the study with specific instructions.

Participants who took part in only the spice analysis received nine coded test materials for direct analysis (SM-A, SM-B, SM-C, SM-D, SM-E, NM-B, NM-C, NM-D, and BP-C); 16 test materials identified for spiking with WP-1, WP-2, WP-3, WP-4, and so on, with the number indicating the spike solution to be used; OTA stock solution; 16 spiking solutions labeled SP-1, SP-2, SP-3, and SP-4 followed by the code of the sample they had to be spiked into; and 25 IACs.

Participants who took part in only the cocoa and drinking chocolate analysis received eight coded test materials for direct analysis (CO-A, CO-B, CO-C, CO-D, CO-E, DC-A, DC-B, and DC-C); six test materials identified for spiking (CO-1, CO-2, DC-1, DC-2, DC-3, and DC-4); OTA stock solution; six spiking solutions labeled SP-1, SP-2, SP-3, and SP-4 followed by the code of the sample they had to be spiked into; and 15 IACs (included one spare column).

Participants who took part in both the spice and the cocoa and drinking chocolate analysis received nine coded spice test materials for direct analysis (SM-A, SM-B, SM-C, SM-D, SM-E, NM-B, NM-C, NM-D, and BP-C); 16 blank spice test materials identified for spiking with WP-1, WP-2, WP-3, WP-4, and so on, with the number indicating the spike solution to be used; eight coded cocoa test materials for direct analysis (CO-A, CO-B, CO-C, CO-D, CO-E, DC-A, DC-B, and DC-C); six test materials identified for spiking (CO-1, CO-2, DC-1, DC-2, DC-3, and DC-4); OTA stock solution; 22 spiking solutions; and 40 IACs.

Participants were asked to confirm that the content of parcels was received undamaged. They were told to store the IACs in the refrigerator (at about 4°C) and to store samples and solutions in the freezer (at approximately –18°C). Polyethylene bottles

contained sufficient material (about 15 g) to perform a single determination. Each participant had to prepare one extract from each test material unit and report its content of OTA. For spiked samples, participants were asked to fortify the material with the respective spiking solution (with an evaporation time of at least 1 h) before the determination. Participants were also asked to complete questionnaires, providing details on their institutional profile and specified analytical details. Results had to be expressed in micrograms per kilogram and rounded to the nearest 0.01 μg/kg. As a result, method performance figures with one decimal could be obtained from reported data with at least one additional decimal to follow the recommendation in ISO 5725-2:1994 (32). In addition, chromatograms were requested for samples and at least one calibration standard. All laboratories reported results, although laboratory 12 reported results only for cocoa but not for spices. Twenty-two participants who reported results also supplied chromatograms.

Experimental

Safety Precautions

Use of this protocol involves hazardous materials, operations, and equipment. This protocol does not address all the safety problems associated with its use. It is the responsibility of the user of this protocol to establish appropriate safety and health practices and determine the applicability of regulatory limitations before use. OTA is a potent nephrotoxin with immunotoxic, teratogenic, and potential genotoxic properties; the 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 stages should be carried out in a fume cupboard. Disposal of waste solvents should be carried out according to applicable environmental rules and regulations of the IARC (31).

Principle

A test portion of black or white pepper, nutmeg, or spice mix is extracted with a mixture of methanol and aqueous sodium bicarbonate solution, whereas a test portion of cocoa or drinking chocolate is extracted with aqueous methanol. The extract is filtered, diluted with PBS and Tween 20, and applied to an IAC containing antibodies specific to OTA. The OTA is isolated, purified, and concentrated on the column and then released using methanol. The purified extract is quantified by reversed-phase (RP) LC–FD.

Apparatus

In addition to the usual laboratory glassware (such as graduated cylinders, glass funnels, beakers, pipets, screw-cap flasks, screw-cap amber vials, etc.) and equipment, the following is necessary for carrying out the analysis (listed in order of use):

(a) *Flasks*.—Volumetric flasks of class A (ISO 1042:2000) of various capacities (e.g., 10, 20, 25, and 50 mL) and conical flasks (100 and 500 mL with screw caps).

(b) *Laboratory balance*.—Capable of 0.01 g mass resolution.

(c) *Analytical balance*.—Capable of 0.0001 g mass resolution.

(d) *Shaker*.—Adjustable vertical or horizontal shaker.

(e) *Pipets*.—Calibrated volumetric pipets or calibrated microsyringes of various capacities (e.g., 100–2000 μL) and displacement pipets of 100 and 1000 μL capacity, with appropriate tips.

(f) *Filters*.—Glass microfiber filter paper with 1.6 μm retention size and 150 mm diameter, or equivalent.

(g) *Reservoirs*.—Disposable syringe barrels of adequate capacity to be used as reservoirs, and attachments to fit to IACs.

(h) *IACs*.—Equipped with antibodies specific to OTA. The columns applicable for the method should have a capacity of not less than 100 ng OTA and should give a recovery of not less than 85% when applied as a standard solution of OTA in a mixture of MeOH–PBS solution containing 3 ng OTA (15 + 85, v/v). OCHRAPREP® IACs from R-Biopharm Rhône Ltd (Glasgow, United Kingdom) were shown to meet these criteria and were supplied to participants for the study.

(i) *SPE system*.—Optional. Automated SPE vacuum system as an alternative to gravimetric, manual elution.

(j) *Vortex mixer.*

(k) *Glass vials*.—Glass vials of about 2 mL capacity with crimp caps (or equivalent) and glass insert vials of about 250 μL capacity with crimp caps (or equivalent).

(l) *HPLC system*.—HPLC apparatus comprising the following: an eluent reservoir; a mobile phase pumping device capable of generating a gradient and maintaining a volume flow rate of 0.8 and 1 mL/min pulse-free; an injector system capable of injecting in full loop mode (approximately 20 μL); a fluorescence detector suitable for measurements at an λ_{ex} = 332 nm and an λ_{em} = 476 nm; a recorder, integrator, or computer-based data processing system; and a column oven with temperature control. Optionally, a degasser could be used for degassing the mobile phases.

(m) *Analytical RP-HPLC column*.—A suitable column and appropriate HPLC conditions (isocratic or gradient program) should be selected so that the maximum overlap of OTA with any other peaks shall be less than 10%. A C18 column with a length of 25 cm, an inner diameter of 4.6 mm, and a particle size of 5 μm has been found to be suitable for some spices, cocoa, and drinking chocolate, but interference problems could be encountered with black and white pepper and spice mixes containing pepper. Sufficient resolution can be achieved with a phenyl-hexyl-HPLC column with a length of 10 cm, an inner diameter of 4.6 mm, and a particle size of 2.7 μm at 50°C (column oven). This column was supplied to all participants for the purpose of this collaborative study. A precolumn with the same or similar stationary phase material as the analytical column and of suitable dimensions can be used.

Reagents

Only reagents of recognized analytical grade and water complying with grade 1 of ISO 3696(EN), unless otherwise specified, may be used. Solvents should be of quality for HPLC analysis, unless otherwise specified. Use of commercially available solutions with properties equivalent to those listed is allowed. Necessary reagents and preparations are listed in order of use:

(a) *Nitrogen: purified compressed gas (N_2)*.—Purity equivalent to 99.95% or better.

(b) *Methanol (CH_3OH)*.—Technical and HPLC grade.

(c) *Acetonitrile (CH_3CN).*— HPLC grade.

(d) *Glacial acetic acid (CH_3COOH).*—Mass fraction w ≥96%.

(e) *Sodium hydrogen carbonate ($NaHCO_3$).*

(f) *Sodium chloride (NaCl).*—Minimum 99% purity.

(g) *Disodium hydrogen orthophosphate ($Na_2HPO_4 \cdot 12\ H_2O$).*—Minimum 99% purity.

(h) *Potassium dihydrogen phosphate (KH_2PO_4).*—Minimum 99% purity.

(i) *Potassium chloride (KCl).*—Minimum 99% purity.

(j) *Sodium hydroxide (NaOH).*—Minimum 99% purity.

(k) *Hydrochloric acid (HCl).*—Mass fraction w = 37% in water.

(l) *HCl solution.*—For substance concentration c = 0.1 M, dilute 8.28 mL HCl solution to 1 L with water.

(m) *Sodium hydroxide solution.*—For c(NaOH) = 0.2 M, dissolve 8 g NaOH in 1 L water.

(n) *Acetic acid solution.*—For ρ(acetic acid) = 1 g/L (1%), add 10 g glacial acetic acid to 1000 mL grade 3 water.

(o) *Tween 20.*

(p) *Tween 20 solution.*—For ρ(Tween 20) = 20 g/L, add 20 g Tween 20 to 1000 mL grade 3 water.

(q) *PBS solution.*—For pH = 7.4, dissolve 8 g NaCl, 2.9 g $Na_2HPO_4 \cdot 12H_2O$, 0.2 g KH_2PO_4, and 0.2 g KCl in 900 mL water. Adjust the pH of the solution to 7.4 with HCl or NaOH solution as appropriate, and then dilute to 1 L with water. Alternatively, a PBS solution with equivalent properties can be prepared from commercially available PBS material.

(r) *Sodium hydrogen carbonate solution.*—For $\rho(NaHCO_3)$ = 30 g/L (3%), add 30 g sodium hydrogen carbonate to 1000 mL grade 1 water.

(s) *Extraction solvent (black and white pepper, nutmeg, and spice mix).*—Mix 50 parts methanol with 50 parts sodium hydrogen carbonate solution (v/v).

(t) *Extraction solvent (cocoa and drinking chocolate).*—Mix 80 parts methanol with 20 parts grade 3 water (v/v).

(u) *HPLC mobile phase A.*—Mix 35 parts methanol and 35 parts acetonitrile with 29 parts grade 1 water and 1 part glacial acetic acid (v/v/v/v).

(v) *HPLC mobile phase B (proven to provide adequate separation of OTA from other interfering peaks in chromatograms for black and white pepper and spice mix).*—Mix 56 parts methanol– acetonitrile (1 + 1) with 39 parts grade 1 water and 1 part glacial acetic acid (v/v/v/v).

(w) *OTA standard solution, ρ(OTA) approximately 1 μg/mL.*—An OTA standard solution with a mass fraction 10 times lower than the stock solution was prepared in 1% acetic acid in acetonitrile. This gives a standard solution containing approximately 1000 ng/mL OTA (the exact concentration depends on the concentration of the stock solution). For the purpose of the collaborative study, this solution was distributed to the participants. Store this solution in a freezer at approximately −18°C.

(x) *OTA spiking solution.*—An appropriate volume of OTA stock solution was pipetted into a calibrated volumetric flask and diluted with a mixture of acetonitrile–glacial acetic acid (99 + 1, v/v) to obtain spiking solutions containing 75, 250, and 750 ng/mL OTA. These solutions were distributed to the participants.

Procedure

(a) *Spiking.*—For all matrixes, weigh a 12.5 g test portion of blank test sample into a conical flask or similar recipient of 500 mL. Pipet a 500 μL aliquot of the corresponding OTA spiking solution onto the blank matrix. After addition of the spike solution, let the solvent evaporate in a fume cupboard for at least 1 h before extraction.

(b) *Extraction.*—Mix or stir the samples thoroughly before removing an analytical test portion. For all matrixes, weigh a 12.5 g test portion (w in Equation 2) of the sample into a conical flask or similar recipient of 500 mL. Add 100 mL [v_1 in Equation 2)] extraction solvent, mix by hand for a few seconds to obtain a homogeneous suspension, and then shake for 40 min with a shaker. Transfer at least 10 mL extract onto the 150 mm glass fiber filter paper, conically folded. Collect the filtered extract in a screw-cap flask for further analysis. Proceed immediately with the IAC cleanup procedure.

(c) *Cleanup.*—Attach a syringe barrel or reservoir to the IAC. Place the IAC on a suitable support. Columns should be allowed to reach room temperature prior to use. Mix 50 mL PBS, 1 mL 2% Tween 20, and 4 mL (v_2 in Equation 2) filtered extract before transferring to the reservoir in two portions. Draw the mixture (extract Tween 20 + PBS) through the column by gravity at a steady flow rate (the flow rate should result in a dropping speed of 1 drop/s, which is about 3 mL/min) until all extract has passed through the column and the last solvent portion reaches the column frit. If necessary, the process can be accelerated by applying slight pressure to the IAC using a syringe or by applying a little vacuum (e.g., by using the vacuum system described). In both cases, attention should be paid not to exceed the flow rate of 3 mL/min (1 drop/s).

(d) *Preparation of injection solution for HPLC analysis.*—After the extract has passed through the column, wash it with 1.0 mL Tween 20, followed by 10 mL water at a rate not exceeding 3 mL/min. Dry the column by pushing 50 mL air through it with a syringe. Discard all the eluent from this stage of the cleanup procedure.

Lastly, place a 2 mL volumetric flask under the column and pass 1.5 mL methanol through the column, collecting the eluate. Carefully push 50 mL air through the column with a syringe in order to collect any final drops without spilling. Dilute to the mark by addition of 1% acetic acid solution. Close the vial and shake vigorously. This 2 mL eluate will be analyzed directly. Optionally, the eluate can be filtered before injection using a syringe filter PTFE membrane with a pore size of 0.45 μm.

Caution: Because all the IAC eluate is used for the quantitative analysis, it is very important to dry the IAC effectively by air after the washing step and after the elution by methanol. Shaking the vials before injection is also critical.

(e) *HPLC operating conditions.*—When the specified C18 column and mobile phase A [*see* **(u)** under *Reagents*] were used, the following settings were found to be appropriate: flow rate, 0.8 mL/min; column oven temperature (including the guard column), 22 ± 1°C; injection volume, 20 μL; autosampler (optional) temperature, 15–20°C; and detector wavelengths, λ_{ex} = 332 nm and λ_{em} = 476 nm. OTA elutes with retention of approximately 6.5 min. Other column dimensions may be used, provided that the required resolution is achieved and can be demonstrated by a maximum overlap of OTA with any other peaks (if present) less than 10%. The flow rate may be adjusted according to the column dimension.

When a phenyl-hexyl column and mobile phase B [*see* **(v)** under *Reagents*] were used, the following settings were found to be appropriate: flow rate, 1.0 mL/min; column oven temperature (including the precolumn), 50 ± 1°C; injection volume, 20 μL; autosampler (optional) temperature, 15–20°C; and detector

wavelengths, λ_{ex} = 332 nm and λ_{em} = 476 nm. OTA elutes with retention of approximately 5.7 min. Other column dimensions may be used, provided that the required resolution is achieved and can be demonstrated by a maximum overlap of OTA with any other peaks (if present) less than 10%. The flow rate may be adjusted according to the column dimension.

(f) *Calibration.*—Prepare a set of calibration solutions from the OTA standard solution provided [*see* **(w)** under *Reagents*] as described in Table 2. These solutions have to be injected before the analytical sequence starting with the mobile phase, to prove linearity and generate a calibration curve. Plot the peak signal against the concentration of OTA in the calibration solutions injected. These six solutions cover a range from approximately 1–100 µg/kg for OTA for all spices and cocoa matrixes.

(g) *Determination of OTA in test solutions*.—The following calculations were used to determine the concentrations of OTA.

Plot the peak signals as area or height (*y*-axis) against the concentration of the OTA calibration standard solutions (*x*-axis), and prepare the calibration curve using linear regression. Calculate the concentration of OTA in the injected purified sample extract (the test solution) by using the relationship $y = ax + b$ and Equation 1:

$$\rho_{OTA} = \frac{S_{OTA} - b}{a} \quad (1)$$

where ρ_{OTA} (ng/mL) = the concentration of OTA in the aliquot of test solution injected and corresponding to the area of the OTA peak; S_{OTA} = the signal of the OTA peak obtained from the chromatogram of the test solution; b = the value where the calibration function intercepts the *y*-axis; and a = the value of the slope of the linear function.

Calculate the concentration of OTA in the test sample using Equation 2:

$$c_{OTA} = \frac{\rho_{OTA} \times v_1 \times v_3}{w \times v_2} \quad (2)$$

where $c_{OTA} = \rho_{OTA} \times 4$, and where c_{OTA} = the concentration of OTA (µg/kg) in the test sample; ρ_{OTA} = the concentration of OTA (ng/mL) in the aliquot of test solution injected and corresponding to the area of the OTA peak; v_1 = the volume (mL) of the extraction solvent used for the extraction of the test sample; v_3 = the final volume (mL) of the test solution; w = the weight (g) of the test sample extracted; and v_2 = the volume (mL) of the test sample extract aliquot applied onto the IAC.

Table 2. Preparation of HPLC calibration solutions

Standard	OTA standard solution, µL	Final volume, mL	Nominal concn, ng/mL
1	15	50	0.3
2	15	25	0.6
3	25	25	1
4	50	10	5
5	150	10	15
6	250	10	25

Results and Discussion

Preliminary Evaluations

The 25 participants were divided into three groups for which group A (10 participants) received only spice samples, group B (10 participants) received only cocoa and drinking chocolate samples, and group C (5 participants) received both sets of samples. In group C, laboratory 12 failed to report results for spices.

From the data submitted by participants, calibration functions were found to be linear according to Mandel's test. Chromatograms were checked for consistency in the retention time of the OTA peak between standards and samples for correct identification, and peak shape and resolution were critically examined. Overall, as anticipated, the only issues associated with the chromatograms concerned the spice mix and black and white pepper samples, in which there was evidence of coextractives. Most participants using the column provided with the recommended mobile phase achieved a good baseline separation of two nonidentified peaks that eluted immediately before OTA. For cocoa and drinking chocolate samples, no interfering signals were observed that challenged the integration of OTA peaks. None of the laboratories reported deviations from the method that could justify their classification as noncompliant.

HPLC Analytical Column

A fully capped stable bond C18 RP column (250 × 4.6 mm id, 5 µm) using methanol–acetonitrile–water–acetic acid (35 + 35 + 29 + 1) as the mobile phase was suitable for the analysis of cocoa and drinking chocolate and some spice samples, but not those containing black or white pepper. A phenyl-hexyl column (100 × 4.6 mm id, 2.7 µm) was found to be appropriate for the analysis of black and white pepper, because this column provided good separation of OTA from interfering peaks in pepper matrixes. Methanol–acetonitrile–water–acetic acid (28 + 28 + 39 + 1) as the mobile phase gave optimum separation with this column. It might be expected that the IAC containing antibodies specific to OTA should provide extracts free of any interferences. However, because of either cross-reactivity with natural components from pepper samples or nonspecific binding, this problem appears to occur irrespective of the supplier of the IAC column. Indeed, laboratory 25 used a different brand of IAC from the one supplied for the study, and examination of the chromatograms for pepper samples showed an identical split peak eluting before OTA but with good resolution from OTA.

An example of a typical chromatogram for a white pepper sample is shown in Figure 1. It illustrates a well-resolved OTA peak from the coeluting coextractives using a phenyl-hexyl column. A chromatogram for a naturally contaminated sample of cocoa is shown in Figure 2, in which a single peak for OTA and the absence of any coextractive can be seen. To achieve the simplicity of having a single method for spice and cocoa matrixes, the use of a phenyl-hexyl column was recommended for this study. This column, which is not normally available in most laboratories for routine use, was supplied for this study. However, the use of this column is not essential for the analysis of some spices and for cocoa.

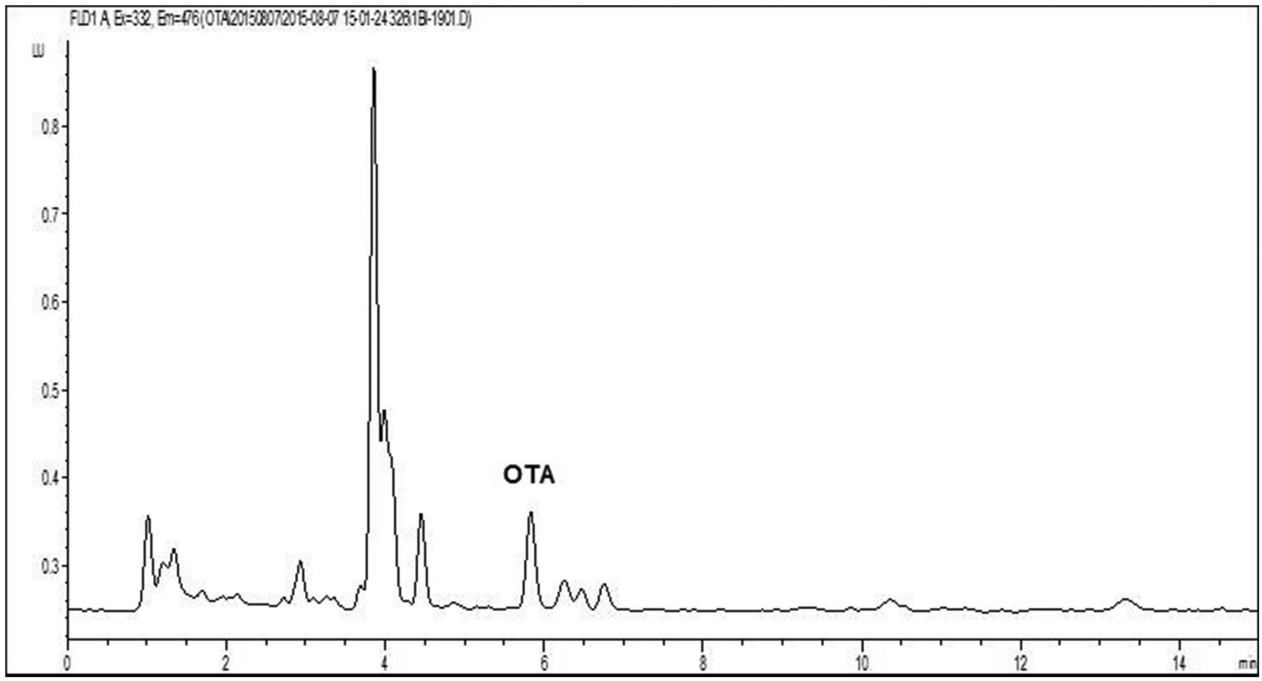

Figure 1. Chromatogram of white pepper (OTA concentration approximately 5 µg/kg).

Comments from Participants

Twenty-four participants reported that they had between 4 and 30 years of experience in the analysis of OTA for food and feed, with an average of 13.7 years of experience. Twenty-one laboratories (84%) had ISO 17025 accreditation for the analysis of OTA for a wide range of matrixes, including cereals, dried fruit, coffee, wine, and beer, with 12 participants indicating prior experience in the analysis of OTA in a range of spices, but only three participants indicating experience in the analysis of OTA in cocoa. Five participants reported that they routinely analyzed between 150 and 500 samples per annum for OTA, and four participants analyzed more than 500 samples per annum.

From the questionnaires distributed, none of the participants reported any difficulty in understanding the instructions that were supplied for the study, but several commented that the

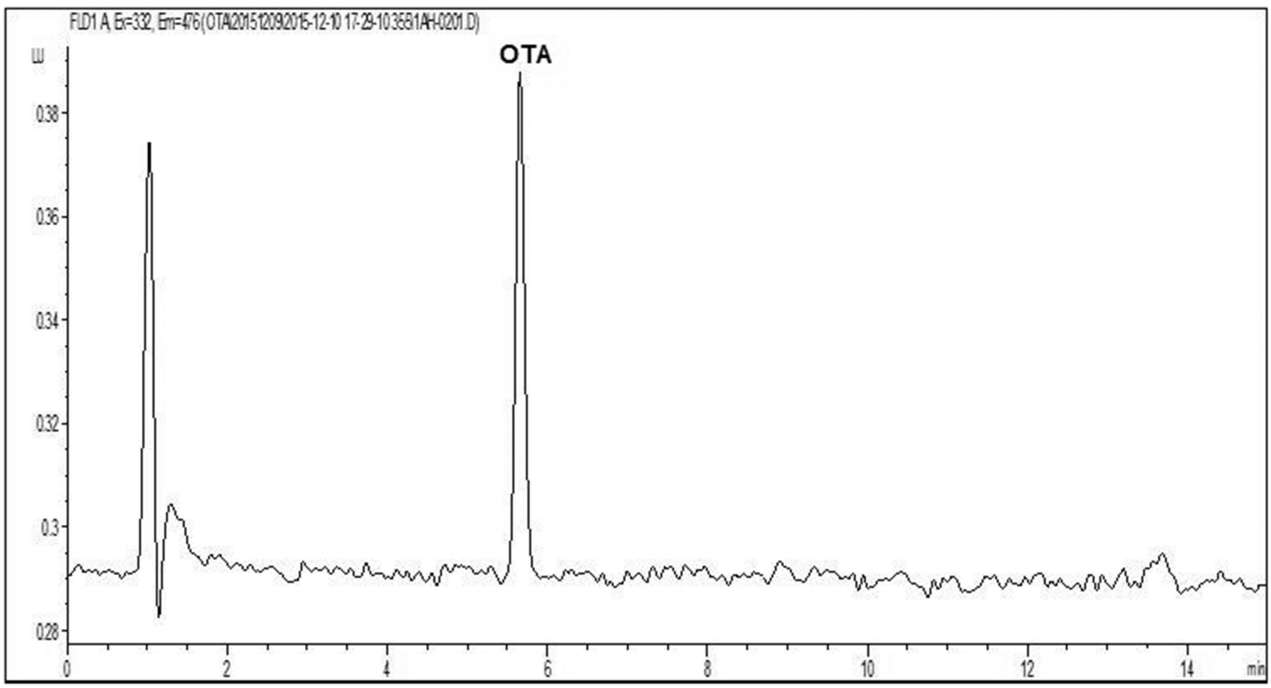

Figure 2. Chromatogram of cocoa powder (OTA concentration approximately 5 µg/kg).

reservoir for the IAC column, which was stated initially as being 50 mL, was inadequate, because the method required the addition of 55 mL of solution (50 mL PBS + 4 mL extract + 1 mL Tween 20). This instruction was subsequently changed in the protocol. Seven participants reported that they experienced some difficulties with the analysis, which they overcame with minor variations in the way they carried out the method, but these were steps that were indicated in the method protocol as being discretionary.

Several participants found that an additional filtration step after IAC cleanup was necessary for some samples because the extracts were cloudy after the addition of 1% acetic acid before HPLC analysis. Syringe filters with a PTFE membrane (pore size, 0.45 μm), as well as Millipore Ultrafree-MC SV or Durapore PVDF 5.0 μm centrifugal filters, were found to be effective. One of the participants also reported that they centrifuged these samples at 15 000 rpm before injection into the HPLC.

Some participants experienced slow running of nutmeg and cocoa samples through the IAC. This was solved by applying a vacuum, using the vacuum system described in the method protocol.

Inexplicably, one participant reported difficulties with the IACs supplied for the study and instead used a brand from another supplier, which was routinely used by that participant. This deviation was acceptable because the method allows for any IAC to be used, provided it meets the described performance characteristics in terms of capacity and recovery. The chromatograms from this participant were indistinguishable from those chromatograms in which sample cleanup used the supplied IAC. Three participants indicated that they made small changes to the mobile phase composition in order to achieve the desired separation of OTA, but most participants did not report any problems and returned example chromatograms that were judged as being acceptable.

Evaluation of Chromatograms

Chromatograms were provided by 22 of the 25 participants who sent back results for the study. Of the laboratories whose results were identified as outliers, only laboratory 12, for whom one set of results for drinking chocolate was an outlier, failed to supply chromatograms. Laboratory 4 had pairs of results for spice mix and black pepper identified as outliers. The chromatograms for these matrixes for this laboratory contained far more background coextractive peaks than other participants experienced. Laboratory 10 had to change the composition of the mobile phase to achieve adequate resolution for OTA in spice mix and pepper samples, but had one outlier result. Laboratory 13 had one outlier result for white pepper, but the chromatograms showed good resolution from coextractives, so chromatographic issues were not responsible. Pairs of outlier results for laboratories 19 and 25 could not be explained by chromatographic issues because in all cases, only single peaks for OTA were evident, with no evidence of background coextractives. Of the eight outliers, seven were from spiked samples, which could indicate that they were due to errors in pipetting the spiking solution.

Interlaboratory Study Results

Repeatability, reproducibility, and recovery data were obtained according to ISO 5725-2:1994 (32) and the Protocol for the design, conduct and interpretation of method -performance studies (33). All individual results submitted by participants are given in Table 3 for naturally contaminated and spiked spices and in Table 4 for

Table 3. Collaborative trial results for OTA in black and white pepper, nutmeg, and spice mix[a]

	Sample 1		Sample 2		Sample 3		Sample 4		Sample 5		Sample 6		Sample 7		Sample 8		Sample 9		Sample 10		Sample 11	
	Black pepper, 3 μg/kg spike		Nutmeg, 3 μg/kg spike		Spice mix, natural contamination		Spice mix, natural contamination		Spice mix, 10 μg/kg spike		Nutmeg, natural contamination		White pepper, 10 μg/kg spike		Black pepper, 30 μg/kg spike		Nutmeg, 30 μg/kg spike		Spice mix, 30 μg/kg spike		White pepper, 30 μg/kg spike	
Lab code	1	2	1	2	A	D	B	C	1	2	1	2	1	2	1	2	1	2	1	2	1	2
1	2.55	2.52	2.89	2.52	0.96	1.03	2.90	2.24	9.16	7.98	11.63	9.49	8.95	7.66	20.99	19.86	24.28	25.16	23.18	23.16	27.62	31.50
2	2.28	2.05	1.51	1.44	0.73	0.71	2.02	2.22	8.22	6.92	3.54	8.39	6.51	7.80	17.86	20.08	18.72	17.66	19.71	19.14	19.79	21.29
3	2.20	2.30	2.70	2.70	0.90	1.00	2.90	2.70	9.10	8.30	12.10	11.50	8.00	9.90	24.40	22.20	29.20	29.00	25.30	26.40	28.20	30.60
4	1.51[b]	1.50[b]	2.21	2.52	0.88	0.89	2.58	2.06	8.06	8.04	8.80	10.93	8.24	7.35	18.35	18.48	23.32	25.00	10.13[b]	10.35[b]	24.93	27.78
5	1.85	2.43	1.15	1.70	0.88	1.09	2.92	2.46	8.35	8.59	11.70	11.79	8.27	7.92	22.70	23.48	21.88	21.80	24.76	23.87	22.52	23.72
6	2.69	2.25	2.34	1.70	1.22	1.31	2.53	3.05	9.14	9.43	13.66	11.53	7.10	6.51	24.56	24.77	22.63	23.20	24.99	28.97	26.82	26.65
7	2.18	2.24	2.38	2.48	0.98	1.07	2.25	2.13	7.81	8.44	6.69	6.59	7.98	8.04	24.64	19.69	23.96	21.17	20.90	22.55	21.70	27.82
8	2.80	2.27	2.20	2.15	0.99	0.89	2.21	2.37	8.73	8.41	9.78	7.25	8.38	8.27	21.84	22.76	26.28	26.12	22.84	27.55	24.76	24.34
9	2.60	2.80	1.80	1.40	1.20	1.00	1.30	2.80	8.60	8.80	10.80	10.80	6.10	6.90	25.30	25.70	24.80	17.00	20.70	25.00	22.70	27.50
10	2.24[c]	5.25[c]	2.48	2.70	0.63	0.95	1.86	2.24	7.62	7.77	11.17	8.78	6.65	7.08	23.92	23.30	18.81	13.74	20.86	18.64	20.72	24.92
13	2.59	2.41	2.07	1.64	1.00	1.04	2.68	2.16	10.25	8.93	7.72	8.05	3.31[b]	4.36[b]	23.49	25.46	24.25	23.84	26.35	28.23	25.83	16.88
14	2.34	2.36	1.74	1.75	—	—	2.46	2.22	8.74	8.97	8.67	8.76	8.69	8.48	22.28	22.47	15.69	17.64	24.00	25.66	24.96	25.62
15	2.50	2.23	2.30	2.47	1.17	0.92	2.24	2.76	8.84	7.90	10.76	10.66	8.00	7.01	22.98	24.54	23.74	24.42	23.64	22.69	22.70	22.98
16	2.42	2.49	2.49	2.09	0.92	1.10	2.86	2.39	8.75	8.77	11.22	11.91	9.03	8.87	23.33	24.06	24.44	24.76	25.35	25.27	25.63	25.85

[a] Results reported as micrograms per kilogram OTA.

[b] Outlier determined by the Grubbs test (differing laboratory mean).

[c] Outlier determined by the Cochran test (excessive SD mean).

Table 4. Collaborative trial results for OTA in cocoa and drinking chocolate[a]

	Sample 1		Sample 2		Sample 3		Sample 4		Sample 5		Sample 6	
	Cocoa, natural contamination		Drinking chocolate, natural contamination		Cocoa, natural contamination		Drinking chocolate, 10 µg/kg spike		Cocoa, 30 µg/kg spike		Drinking chocolate, 30 µg/kg spike	
Lab code	A	C	1	2	B	D	1	2	1	2	1	2
12	1.05	1.69	1.87	1.84	6.19	5.69	5.25	5.87	12.76	12.04	15.45[b]	11.01[b]
13	2.69	1.46	2.38	3.04	8.24	7.80	8.15	9.47	25.96	22.68	25.36	25.68
14	2.43	—	3.42	3.53	2.53	4.99	9.41	9.41	27.43	26.59	28.31	26.54
15	2.08	2.16	2.88	3.50	7.43	7.53	9.08	9.11	24.76	25.23	27.18	22.32
16	2.43	2.34	4.06	3.95	8.07	7.84	9.49	9.51	28.67	27.32	26.97	27.47
18	2.59	2.74	3.48	2.97	8.21	7.79	9.20	10.38	28.25	28.18	24.47	27.98
19	2.45	1.93	1.04	0.63	2.98	6.86	6.90	6.37	15.98[c]	26.77[c]	27.16	21.50
20	2.26	2.43	3.19	3.44	8.16	8.66	7.67	9.26	25.97	28.88	22.96	19.32
21	1.90	1.53	2.54	2.30	6.50	6.07	8.23	8.10	23.98	22.77	23.32	26.08
22	3.10	2.91	3.15	3.28	9.82	8.77	8.99	9.57	31.22	30.41	28.78	27.57
23	2.28	2.44	3.16	3.20	6.51	6.67	2.16	7.96	25.16	23.04	23.66	22.66
24	1.98	2.45	3.42	2.89	7.60	8.01	10.07	9.50	26.32	24.89	26.99	27.81
25	0.87	1.27	2.26	1.96	17.20[c]	4.15[c]	5.08	5.17	14.66	16.16	14.15[b]	4.42[b]
26	2.55	1.88	3.23	2.89	8.36	8.15	9.55	9.32	27.52	27.89	27.11	26.66
27	2.60	1.52	1.32	2.16	7.28	8.96	15.80	14.00	37.84	36.04	35.72	34.96

[a] Results reported as micrograms per kilogram OTA.

[b] Outlier determined by the Grubbs test (differing laboratory mean).

[c] Outlier determined by the Cochran test (excessive SD mean).

naturally contaminated and spiked cocoa and drinking chocolate test materials. Results corresponding to noncompliant values and outliers, as well as the statistical tests, are indicated.

For outlier detection, Cochran's test and single and double Grubbs' tests were applied at a significance level of $P < 0.025$. The results of laboratory 4 included an outlier for one pair of spice mix and black pepper samples. The results of laboratory 10 included an outlier for one pair of black pepper samples and one pair of spice mix samples, whereas laboratory 12 provided outlier results for drinking chocolate. Furthermore laboratory 13 provided outlier results for a pair of white pepper samples. The results of laboratory 19 included an outlier for a cocoa sample, whereas laboratory 25 had outliers for a cocoa and drinking chocolate sample. The reasons for outliers were investigated by consulting the questionnaire and the respective participant, but no apparent pattern was evident.

Method performance characteristics were calculated after removal of noncompliant results. For all materials, the number of identified outliers was 2/14 for spices and 1/15 for cocoa, which is below the maximum of 2/9 recommended in the AOAC INTERNATIONAL guidelines for conducting interlaboratory studies (34).

Calculation of Precision and Recovery Estimates

All relevant method performance characteristics are reported in Table 5 and indicate that the method performed well and compared favorably with similar published studies (21). The RSD_R values ranged from 7.7 to 22.6% for spices and from 13.7 to 30.7% for cocoa and drinking chocolate. This resulted in HorRat values from 0.4 to 1 for spices and from 0.6 to 1.4 for cocoa and coca products, applying the Horwitz equation (35) modified by Thompson (30) for concentrations below 120 µg/kg.

Recoveries ranged from 71 to 85% for spices and from 85 to 88% for both cocoa and drinking chocolate samples (Table 5). The method was validated with a view to be applied for compliance testing by EU official food control laboratories. Therefore, it was aimed at achieving method performance characteristics to meet the provisions in Commission Regulation No. 401/2006/EC (23). For levels of OTA from 1.0 to 10.0 µg/kg, relative repeatability should be better than 20%, whereas reproducibility should be less than 30%. This was clearly achieved because the largest repeatability was 18.7% for all matrixes, and the reproducibility ranged from 7.7 to 25.9% (except one sample of naturally contaminated drinking chocolate for which a reproducibility of 30.7% was obtained). Recovery values across all matrixes varied from 71 to 88%, thus demonstrating that the method fulfils the requirement of recoveries (70–110%) for application as an official method in the EU (23). Legal limits of 15 and 20 µg/kg in different individual spices and 15 µg/kg for mixtures of spices have been set in EU legislation for OTA (6, 7). Although no limit is yet in place for cocoa and cocoa products (5), a similar limit was assumed for the purpose of evaluating the method performance. The required precision parameters were achieved at levels of 1.0–2.4 µg/kg for spice mix, nutmeg, and white pepper. For cocoa and drinking chocolate, levels of OTA of 2.1 and 2.8 µg/kg, respectively, could be reliably measured, which are well below any anticipated statutory limits. Therefore, Codex recommendations concerning the LOQ to be less than 1/5th of the anticipated decision limit (legal limit) as mentioned in the 30th Report of the Codex Committee are met (36).

Table 5. OTA content and method performance obtained from the study

Material description	No. of valid sets	Mean content, µg/kg	RSD_r, %	RSD_R, %	Mean recovery, %	HorRat[a]
Black pepper, 3.0 µg/kg spike	12	2.4	8.7	9.5	79.7	0.4
Black pepper, 30.0 µg/kg spike	14	22.6	5.6	10.0	75.4	0.5
White pepper, 10.0 µg/kg spike	13	7.8	7.9	11.8	78.3	0.5
White pepper, 30.0 µg/kg spike	14	24.9	10.5	13.0	82.9	0.6
Nutmeg, 3.0 µg/kg spike	14	2.1	11.1	22.2	71.0	1.0
Nutmeg, 30.0 µg/kg spike	14	22.6	8.5	17.1	75.3	0.8
Nutmeg, natural contamination	14	9.8	13.7	22.6	NA[b]	1.0
Spice mix, 10.0 µg/kg spike	14	8.5	5.9	7.7	85.2	0.4
Spice mix, 30.0 µg/kg spike	13	23.8	7.1	11.5	79.4	0.5
Spice mix, natural contamination	14	1.0	11.4	16.0	NA	0.7
Spice mix, natural contamination	14	2.4	16.7	16.7	NA	0.8
Cocoa, 30.0 µg/kg spike	14	25.4	4.5	23.7	84.8	1.1
Cocoa, natural contamination	15	2.1	18.7	26.1	NA	1.2
Cocoa, natural contamination	14	7.2	13.5	23.1	NA	1.1
Drinking chocolate, 10.0 µg/kg spike	15	8.8	6.7	25.9	88.0	1.2
Drinking chocolate, 30.0 µg/kg spike	13	26.3	7.3	13.7	87.7	0.6
Drinking chocolate, natural contamination	15	2.8	10.6	30.7	NA	1.4

[a] HorRat for reproducibility (35) modified by Thompson (30).

[b] NA = Not applicable.

Conclusions

The method was validated in an international collaborative study and meets the required method performance criteria, with HorRat ≤1.4 in all cases. The method fulfils the provisions of Commission Regulation No. 401/2006/EC (23), and Codex recommendations (36) regarding LOQ were also met. This is the first method validated for the determination of OTA in black pepper, white pepper, nutmeg, and spice mix following the provisions given in the AOAC/IUPAC International Harmonized Protocol (28) and is complementary to a similar method previously validated for chili and paprika (22). This is also the first method to be fully validated for the determination of OTA in drinking chocolate, but the second study for OTA in cocoa, although producing superior performance characteristics to those of the earlier study (27). The method covering a wide range of different spices and cocoa matrixes is, therefore, suitable to serve as a basis for a standard within the CEN and as a First Action method for AOAC.

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