FOOD CHEMICAL CONTAMINANTS

Determination of Ochratoxin A in Wine and Beer by Immunoaffinity Column Cleanup and Liquid Chromatographic Analysis with Fluorometric Detection: Collaborative Study

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The accuracy, repeatability, and reproducibility characteristics of a liquid chromatographic method for the determination of ochratoxin A (OTA) in white wine, red wine, and beer were established in a collaborative study involving 18 laboratories in 10 countries. Blind duplicates of blank, spiked, and naturally contaminated materials at levels ranging from ≤0.01 to 3.00 ng/mL were analyzed. Wine and beer samples were diluted with a solution containing polyethylene glycol and sodium hydrogen carbonate, and the diluted samples were filtered and cleaned up on an immunoaffinity column. OTA was eluted with methanol and quantified by reversed-phase liquid chromatography with fluorometric detection. Average recoveries from white wine, red wine, and beer ranged from 88.2 to 105.4% (at spiking levels ranging from 0.1 to 2.0 ng/mL), from 84.3 to 93.1% (at spiking levels ranging from 0.2 to 3.0 ng/mL), and from 87.0 to 95.0% (at spiking levels ranging from 0.2 to 1.5 ng/mL), respectively. Relative standard deviations for within-laboratory repeatability (RSD_r) ranged from 6.6 to 10.8% for white wine, from 6.5 to 10.8% for red wine, and from 4.7 to 16.5% for beer. Relative standard deviations for between-laboratories reproducibility (RSD_R) ranged from 13.1 to 15.9% for white wine, from 11.9 to 13.6% for red wine, and from 15.2 to 26.1% for beer. HORRAT values were ≤0.4 for the 3 matrixes.

Ochratoxin A (OTA) is a widely distributed mycotoxin produced mainly by *Aspergillus ochraceus* and *Penicillium verrucosum*. OTA commonly occurs in various foods and beverages, including a variety of cereals (mainly wheat, barley, maize, and oats), beans, groundnuts, spices, dried fruits, pig kidney and blood, coffee, milk, wine, and beer, and has been shown to be nephrotoxic, hepatotoxic, teratogenic, and immunotoxic to several animal species and to cause kidney and liver tumors in mice and rats (1–6). The International Agency for Research on Cancer (IARC) has classified OTA as a possible carcinogen to humans (Group 2B; 1). OTA is suspected to be involved in Balkan endemic nephropathy (BEN), a fatal kidney disease occurring in some areas of southeastern Europe, and with urinary tract tumors (7).

Currently, several countries have specific regulations for OTA in various commodities at levels ranging from 1 to 50 ng/g in foods and from 5 to 300 ng/g in animal feeds (8). Recently, the Italian Ministry of Health issued a directive setting guidelines for OTA in several products, including beer for which a maximum limit of 0.2 μg/L has been set (9). No tolerance levels for OTA in wine have been established, although the topic is under discussion by European authorities. The occurrence of OTA in wine and beer has been shown in several surveys, with higher toxin concentrations and incidence usually recorded for red wines (10–15). Provisional estimates of the Codex Alimentarius Commission, based on limited data, suggest that about 23% of the total intake of this toxin is due to wine and beer (16).

The availability of reliable methods for the determination of OTA in these matrixes is therefore highly desirable to fulfill the need to protect the health of consumers from the risk of exposure to the toxin. Few methods using a silica gel cartridge or an antibody-based immunoaffinity column for cleanup, combined with liquid chromatography (LC), have been specifically proposed for the determination of OTA in wine (10–15); most of them require the use of hazardous extraction solvents (aromatic or chlorinated solvents) and time-consuming sample preparation procedures. Analytical methods using immunoaffinity column cleanup have been specifically proposed for the determination of OTA in beer (13, 17, 18).

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The increased awareness of the potential risk to consumer health from exposure to OTA through the consumption of wine and beer prompted the European Committee for Standardization to request more accurate analytical methods that can be applied to the determination of OTA in these 2 beverages that are widely consumed in Europe (19). A rapid and accurate method for the determination of OTA in wine and beer by means of immunoaffinity column cleanup and LC was recently developed in our laboratory (20, 21). The purpose of this collaborative study was to establish the accuracy, repeatability, and reproducibility parameters of this method by analysis of spiked and naturally contaminated materials.

Collaborative Study

Test Materials

The following test materials were prepared: 5 white wine samples, 5 red wine samples, and 5 beer samples representing, for each matrix, 1 blank material (containing ≤0.01 ng OTA/mL), 3 spiked materials, and 1 naturally contaminated material.

A spiking solution of OTA (1.0 μg/mL) in methanol was prepared from a stock solution (20 μg/mL) in toluene–acetic acid (99 + 1, v/v). Aliquots of the spiking solution were added to 1.5 L of each blank material, and the solutions were stirred for 30 min. The spiked materials contained OTA at levels of 0.1, 1.1, and 2.0 ng/mL for white wine; 0.2, 0.9, and 3.0 ng/mL for red wine; and 0.2, 0.8, and 1.5 ng/mL for beer. In addition, 2 naturally contaminated samples for each matrix (practice samples) were prepared to allow participants to become familiar with the analytical method before proceeding with the collaborative study.

A 25 mL aliquot of each test material was dispensed into a labeled plastic container and stored at +4°C until shipped.

The study was designed and conducted in accordance with guidelines prescribed by AOAC INTERNATIONAL (22). Eighteen laboratories from 10 countries participated in the collaborative study. Each laboratory was assigned a laboratory code number. The laboratories were selected on the basis of their proven experience in the determination of OTA in wine and/or other matrixes and/or their responsibilities in food quality control (public or private).

Each participant was supplied with the following materials: 2 practice samples of white wine marked "white wine A" and "white wine B"; 2 practice samples of red wine marked "red wine A" and "red wine B"; 2 practice samples of beer marked "beer A" and "beer B"; 10 collaborative test samples marked "white wine No. 1–10"; 10 collaborative test samples marked "red wine No. 1–10"; 10 collaborative test samples marked "beer No. 1–10"; 1 amber vial containing OTA standard solution (2 μg/mL); 1 sachet containing 10 g polyethylene glycol (PEG 8000); 36 OchraTest immunoaffinity columns; a copy of the method of analysis; and a collaborative study report form. Participants were left free to use their own OTA standards, provided that this was clearly noted in the general comments of the report form. In that case the concentration of the standard should have been compared with that of the standard provided by the coordinator, and the relevant correction factor ($C_{std\ coordinator}/C_{std\ participant}$) reported to the coordinator.

Participants were requested to analyze 2 naturally contaminated practice test samples for each matrix before proceeding with the collaborative study. OTA concentrations for practice samples were based on levels commonly found in naturally contaminated samples and included low levels of contamination in sample A (OTA at ca 0.5 ng/mL in white wine, red wine, and beer) and high levels in sample B (OTA at ca 1.5 ng/mL in white wine and beer and ca 2.0 ng/mL in red wine). The OTA content of these samples was determined by the same method as proposed for collaborative study. Acceptable OTA concentrations for practice sample A were considered to be between 0.1 and 0.9 ng/mL for white wine, red wine, and beer; acceptable OTA concentrations for practice sample B were considered to be between 0.9 and 2.0 ng/mL for white wine and beer, and between 1.3 and 2.5 ng/mL for red wine. Participants were requested to ensure that their results fell within the specified range before proceeding with the study. Participants obtaining results outside the specified ranges were instructed to contact the coordinator before proceeding with the analyses.

After performing the practice study, participants analyzed, in nested code order, 10 white wine samples, 10 red wine samples, and 10 beer samples, representing, for each matrix, blind duplicates of 1 blank material, 3 spiked materials, and 1 naturally contaminated material. All samples of the same matrix were analyzed on the same day. For each test sample, participants prepared a single cleaned-up extract and performed a single LC run.

AOAC Official Method 2001.01
Determination of Ochratoxin A in Wine and Beer

Immunoaffinity Column Cleanup/
Liquid Chromatographic Analysis
First Action 2001

[Applicable to the determination of ochratoxin A (OTA) in white wine at 0.1–2.0 ng/mL, red wine at 0.2–3.0 ng/mL, and beer at 0.2–2.0 ng/mL.]

Caution: OTA is a potent nephrotoxin and liver toxin and has been reported to have immunosuppressant properties. It is classified by the International Agency for Research on Cancer (IARC) as possibly carcinogenic to humans (Group 2B). Wear gloves and safety glasses when handling OTA, and perform all preparation steps in a fumehood. Decontaminate glassware and laboratory wastes. Toluene is highly flammable and harmful. Perform standard preparation involving this solvent in a fumehood.

See Table **2001.01A** for the results of the interlaboratory study supporting acceptance of the method.

Table 2001.01A. Interlaboratory study results for determination of OTA in wine and beer by immunoaffinity column cleanup and liquid chromatographic analysis with fluorometric detection

Matrix	Spiking level, ng/mL	Mean, ng/mL	No. of labs[a]	S_r	RSD_r, %	S_R	RSD_R, %	HORRAT	Rec., %
White wine	<0.01[b]	—	—	—	—	—	—	—	—
	0.10	0.105	13(4,1)	0.01	7.9	0.02	15.9	0.3	105.4
	1.10	0.998	14(2,2)	0.07	6.6	0.13	13.3	0.3	90.7
	2.00	1.764	14(2,2)	0.15	8.4	0.23	13.1	0.3	88.2
	NC[c]	0.283	15(2,1)	0.03	10.8	0.04	14.6	0.3	—
Red wine	<0.01[b]	—	—	—	—	—	—	—	—
	0.20	0.186	12(4,2)	0.01	6.5	0.02	11.9	0.2	93.1
	0.90	0.813	14(3,1)	0.08	9.9	0.10	12.5	0.3	90.3
	3.00	2.530	15(3,0)	0.22	8.9	0.35	13.6	0.3	84.3
	NC	1.690	14(3,1)	0.18	10.8	0.23	13.6	0.3	—
Beer	<0.01[b]	—	—	—	—	—	—	—	—
	0.20	0.190	13(4,1)	0.02	10.6	0.04	20.9	0.4	95.0
	0.80	0.696	15(3,0)	0.05	7.2	0.13	18.3	0.4	87.0
	1.50	1.403	13(3,2)	0.07	4.7	0.21	15.2	0.4	93.6
	NC	0.070	14(4,0)	0.01	16.5	0.02	26.1	0.4	—

[a] Each value is the number of laboratories retained after elimination of outliers; the first value in parentheses is the number of laboratories removed as technical outliers; the second value in parentheses is the number of laboratories removed as statistical outliers.

[b] Detection limit = 0.01 ng/mL (based on a signal-to-noise ratio of 3:1).

[c] NC = naturally contaminated.

A. Principle

Wine and beer are diluted with a solution containing polyethylene glycol and $NaHCO_3$, and the diluted solutions are filtered and cleaned up on an immunoaffinity column. OTA is eluted with methanol and quantified by reversed-phase liquid chromatography (LC) with fluorometric detection.

B. Apparatus

(**a**) *Microbalance*.—Measuring to within ± 0.01 mg.

(**b**) *Glass vials*.—4 mL. (*Note:* Certain types of vials might lead to losses of OTA during evaporation. To avoid this, silanization can be used. Prepare vials by filling them with silanizing reagent [e.g., SurfaSil™, Pierce Chemical Co., 3747 N. Meridian Rd, Rockford, IL 61101-0747, USA; +1-815-968-0747; Fax: +1-815-968-7316; www.piercenet.com], and leave this reagent in vials for 1 min. Rinse vials twice with a solvent [toluene, acetone, or hexane] followed by water [twice], and dry vials.)

(**c**) *Volumetric flasks*.—5 mL, with accuracy of at least ± 0.5%.

(**d**) *Vacuum manifold*.—To accommodate immunoaffinity columns.

(**e**) *Reservoirs and attachments*.—To fit immunoaffinity columns.

(**f**) *Glass microfiber filters*.—Whatman GF/A, or equivalent.

(**g**) *Immunoaffinity columns*.—Containing antibodies against OTA with a total binding capacity of ≥100 ng OTA and a recovery of ≥85% when a diluted wine solution containing 100 ng OTA is applied. The OchraTest™ column from VICAM L.P. (313 Pleasant St, Watertown, MA 02472, USA) has been found suitable. Other types of immunoaffinity columns meeting these performance characteristics may be used, following the manufacturer's instructions.

(**h**) *Solvent evaporator*.

(**i**) *Syringe and microliter pipet(s)*.—250 µL.

(**j**) *LC pump*.—Isocratic; delivering constant flow rate of 1.0 mL/min.

(**k**) *Injection system*.—Syringe-loading injection valve with 100 µL injection loop, or equivalent.

(**l**) *LC analytical column*.—Stainless steel (150 × 4.6 mm id) packed with 5 µm C18 reversed-phase material preceded by a reversed-phase guard column (i.e., 20 × 4.6 mm id, 0.5 µm particle size) or guard filter (i.e., 0.5 µm, Rheodyne, L.P., PO Box 1919, Rohnert Park, CA 94927-1909, USA; +1-707-588-2000; Fax: +1-707-588-2020; rheodyne@rheodyne.com). Columns of different dimensions may be used, if they adequately resolve the OTA peak from all other peaks.

(**m**) *Fluorescence detector*.—Fitted with a flow cell and set at 333 nm (excitation) and 460 nm (emission) indicating a peak from ≥0.02 ng of OTA.

(**n**) *Data collection system*.

(**o**) *UV spectrophotometer*.

C. Reagents

(**a**) *Polyethylene glycol (PEG)*.—PEG 8000.

(**b**) *Methanol*.—LC grade.

(**c**) *Acetonitrile*.—LC grade.

(**d**) *Water*.—LC grade.

(**e**) *Glacial acetic acid*.—99% purity.

(**f**) *Diluting solution*.—1% PEG + 5% $NaHCO_3$, pH 8.3. Dissolve 10 g PEG, (**a**), and 50 g $NaHCO_3$ in ca 950 mL water, and dilute to 1 L with water.

(**g**) *Washing solution*.—2.5% NaCl + 0.5% $NaHCO_3$, pH 8.1. Dissolve 25 g NaCl, and 5 g $NaHCO_3$ in ca 950 mL water, and dilute to 1 L with water.

(**h**) *LC mobile phase*.—Water–acetonitrile–glacial acetic acid (99 + 99 + 2, v/v/v), pH 3.2. Mix 990 mL water, (**d**), with 990 mL acetonitrile, (**c**), and 20 mL acetic acid, (**e**), filter through 0.45 μm filter, and degas (e.g., with He).

(**i**) *Toluene*.—Analytical grade.

(**j**) *Ochratoxin A (OTA)*.—Crystalline form, film, or solution.

(**k**) *Solvent mixture*.—Toluene–acetic acid (99 + 1, v/v). Mix 99 parts, by volume, of toluene, (**i**), with 1 part, by volume, of acetic acid, (**e**).

(**l**) *OTA stock solution*.—Dissolve 1 mg OTA, (**j**), or the contents of 1 ampule (if OTA has been obtained as a film) in the solvent mixture, (**k**), to prepare a solution containing OTA at approximately 20–30 μg/mL. To determine the exact concentration, record the absorption curve between 300 and 370 nm in a 1 cm quartz cell with the solvent mixture, (**k**), as the reference. Identify the maximum absorption, and calculate the mass concentration of OTA, c_{OTA}, in μg/mL, using the following equation:

$$c_{OTA} = A_{max} \times M \times 100/\epsilon \times \delta$$

where A_{max} is the absorption determined at the maximum of the absorption curve (at 333 nm); M is the relative molecular mass of OTA (M = 403.8 g/mol); ϵ is the relative molar absorption coefficient of OTA in the solvent mixture, (**k**), (ϵ = 544 m^2/mol); and δ is the pathlength of the quartz cell in cm.

This solution is stable at –18°C for ≥4 years.

(**m**) *OTA standard solution*.—2 μg/mL, in toluene–acetic acid (99 + 1, v/v). Dilute stock solution, (**l**), with solvent mixture, (**k**), to obtain a standard solution with a mass concentration of OTA of 2 μg/mL. Store standard solution at +4°C.

(**n**) *Calibration solutions*.—Pipet 0.5 mL standard solution containing OTA at 2 μg/mL, (**m**), into a glass vial, and evaporate the solvent under a stream of N. Redissolve contents of vial in 10 mL LC mobile phase, (**h**), which has been filtered through a 0.45 μm filter. This gives a solution containing OTA at 100 ng/mL. Prepare 6 LC calibration solutions in separate 5 mL volumetric flasks according to Table **2000.01B**. Dilute each standard solution to volume (5 mL) with filtered LC mobile phase, (**j**). Inject 100 μL of each calibration solution into the LC system.

D. Sample Preparation and Immunoaffinity Column Cleanup

Cool beer at +4°C for 30 min to prevent fast foam formation. Degas by sonicating for 1 h.

Pour 10 mL wine or beer into a 100 mL conical flask. Add 10 mL diluting solution, **C**(**f**). Mix vigorously. Filter through glass microfiber filter, **B**(**f**), if solution is cloudy solutions or if solid residue is formed after dilution. Connect the immunoaffinity column, **B**(**g**), to the vacuum manifold, **B**(**d**), and attach the reservoir, **B**(**e**), to the immunoaffinity column. Add 10 mL (equivalent to 5 mL wine/beer) diluted solution to the reservoir, and pass solution through the immunoaffinity column at a flow rate of about 1 drop/s. Do not permit the immunoaffinity column to run dry. Wash the immunoaffinity column with 5 mL washing solution, **C**(**g**), and then with 5 mL water at a flow rate of 1–2 drops/s. Dry the column by passing air through it. Elute OTA into the vial by passing 2 mL methanol, **C**(**b**), at a flow rate of 1 drop/s. Evaporate the eluate to dryness at 50°C under N. Redissolve eluate immediately in 250 μL LC mobile phase, **C**(**h**), and store at +4°C until LC analysis.

E. LC Analysis

Set flow rate of the mobile phase, **C**(**h**), at 1.0 mL/min. Inject 100 μL reconstituted extract (equivalent to 2 mL wine or beer) into the LC system.

Quantify OTA by comparing OTA peak area (or peak height) with the relevant calibration curve. If the content of OTA in the test solutions fall outside the calibration range, dilute test extracts.

Prepare a calibration curve at the beginning of every day of analysis and whenever chromatographic conditions change.

Table 2000.01B. Preparation of working calibration solutions

	Standard					
Variable	1	2	3	4	5	6
Filtered LC mobile phase, **C**(**h**), μL	4970	4900	4700	4000	3000	2000
OTA solution (100 ng/mL) added, μL	30	100	300	1000	2000	3000
OTA concentration, ng/mL	0.6	2.0	6.0	20	40	60
OTA injected, ng	0.06	0.20	0.60	2.00	4.00	6.00

Table 1. Interlaboratory study results for determination of OTA (ng/mL) in blind duplicates of spiked and naturally contaminated white wine samples

		Laboratory																	
Sample	Code	2	3	4	5[a]	6	7	8	9	10[a]	11	12	13	14	15	16	17	18	19
A	Practice	0.47	0.40	0.50	0.22	0.36	0.39	—[b]	0.42	0.57	0.45	0.44	0.46	0.44	0.48	0.42	0.30	0.42	0.43
B	Practice	1.45	1.51	1.32	1.03	1.19	1.42	—[b]	1.30	1.83	1.33	0.91	1.38	1.25	1.25	1.45	1.73	1.29	1.46
1[c]	8	ND[d]	ND	ND	ND	ND	0.01	ND	ND	0.06	0.02	ND	0.04	ND	ND	0.02	ND	0.01	0.02
	10	ND	ND	ND	ND	<0.01	0.01	ND	ND	0.06	0.01	0.03	0.02	ND	ND	ND	ND	0.01	0.02
2[e]	2	0.10	0.11	ND	ND	**0.02**[f]	0.09	ND	0.10	0.16	0.11	0.11	0.14	0.10	0.12	0.09	0.08	0.10	0.12
	6	0.11	0.08	ND	0.01	**0.02**[f]	0.10	ND	0.10	0.15	0.12	0.12	0.13	0.10	0.13	0.09	0.07	0.11	0.11
3[g]	7	0.78	0.87	1.00	0.01	**0.36**[h]	0.94	**0.46**[i]	0.96	0.19	1.00	0.90	1.35	1.00	1.16	1.05	1.08	0.90	0.92
	9	1.00	0.97	1.10	ND	**0.90**[h]	0.97	**0.71**[i]	0.89	0.08	0.97	0.83	1.33	0.88	1.16	0.96	1.00	0.97	1.00
4[j]	3	1.74	1.66	2.24	0.06	**0.50**[f]	1.75	1.65	1.69	1.25	1.83	1.54	2.36	**1.58**[h]	1.99	1.89	1.58	1.58	1.88
	5	1.62	1.62	1.72	0.25	**0.99**[f]	1.52	1.37	1.74	1.16	1.67	1.51	2.16	**0.62**[h]	1.98	1.88	1.89	1.62	1.70
5[k]	1	0.22	0.19	0.36	0.22	**0.11**[f]	0.24	0.23	0.26	1.88	0.29	0.27	0.34	0.25	0.31	0.27	0.28	0.31	0.33
	4	0.30	0.30	0.32	0.37	**0.04**[f]	0.24	0.27	0.26	0.59	0.26	0.28	0.35	0.28	0.32	0.28	0.30	0.25	0.35

[a] Data from Laboratories 5 and 10 were considered invalid because of problems with glassware and the automatic injection system, respectively.
[b] Problems during analysis.
[c] Blank sample (containing <0.01 ng OTA/mL).
[d] ND = not detected (Laboratories 4 and 8 reported a detection limit of 0.2 ng/mL).
[e] Sample spiked with 0.1 ng OTA/mL.
[f] Data rejected on the basis of the Single Grubbs test.
[g] Sample spiked with 1.1 ng OTA/mL.
[h] Data rejected on the basis of the Cochran test.
[i] Data rejected on the basis of the Double Grubbs test.
[j] Sample spiked with 2.0 ng OTA/mL.
[k] Naturally contaminated sample at a mean concentration of 0.283 ng OTA/mL.

Table 2. Interlaboratory study results for determination of OTA (ng/mL) in blind duplicates of spiked and naturally contaminated red wine samples

		Laboratory																	
Sample	Code	2	3	4	5[a]	6	7	8[a]	9	10[a]	11	12	13	14	15	16	17	18	19
A	Practice	0.47	0.42	0.62	0.40	0.49	0.47	0.32	0.46	0.58	0.45	0.46	0.59	0.57	0.44	0.46	0.44	0.44	—
B	Practice	1.94	1.74	2.08	1.45	1.73	1.92	1.44	1.87	1.68	1.64	1.78	2.28	2.24	1.99	1.89	2.02	1.74	1.78
1[b]	7	ND[c]	ND	ND	ND	<0.01	<0.01	ND	ND	2.36	0.02	0.04	ND	ND	0.04	ND	ND	0.01	0.07
	10	ND	ND	ND	ND	ND	0.03	ND	ND	0.11	0.02	0.03	ND	ND	0.03	ND	ND	0.01	0.08
2[d]	1	0.19	0.17	ND	0.01	**0.06**[e,f]	0.19	ND	0.14	0.16	0.18	0.20	0.21	**0.21**[e]	0.21	0.17	0.15	0.19	0.21
	3	0.21	0.16	ND	0.01	**0.02**[e,f]	0.19	ND	0.17	0.15	0.19	0.18	0.21	**0.12**[e]	0.23	0.16	0.18	0.18	0.20
3[g]	2	0.89	0.81	0.88	0.13	**0.11**[e,f]	0.80	0.62	0.74	0.31	0.80	0.79	0.89	0.82	0.87	0.81	0.88	0.82	0.83
	5	0.91	0.69	0.92	0.07	**0.74**[e,f]	0.70	0.55	0.71	0.56	0.67	0.79	1.04	0.55	1.00	0.79	0.73	0.78	0.85
4[h]	6	2.13	2.44	2.62	0.48	2.62	2.29	1.47	2.70	2.31	2.49	2.52	3.10	1.78	3.00	2.49	1.92	2.61	2.63
	8	2.58	2.68	2.64	0.37	1.79	2.68	1.71	2.60	0.96	2.43	2.53	3.16	1.97	3.00	2.70	2.48	2.66	2.66
5[i]	4	1.74	1.76	1.68	0.30	**0.35**[f]	1.61	0.74	1.70	1.53	1.58	1.67	1.85	0.92	2.00	1.89	1.56	1.57	1.77
	9	1.95	1.60	1.82	0.82	**1.27**[f]	1.64	1.20	1.24	0.98	1.56	1.70	1.98	1.67	2.00	1.93	1.54	1.76	1.63

[a] Data from Laboratories 5, 8, and 10 were considered invalid because of problems with glassware, filtration, and the automatic injection system, respectively.
[b] Blank sample (containing <0.01 ng OTA/mL).
[c] ND = not detected (Laboratory 4 reported a detection limit of 0.2 ng/mL).
[d] Sample spiked with 0.2 ng OTA/mL.
[e] Data rejected on the basis of the Cochran test.
[f] Data rejected on the basis of the Single Grubbs test.
[g] Sample spiked with 0.9 ng OTA/mL.
[h] Sample spiked with 3.0 ng OTA/mL.
[i] Naturally contaminated sample at a mean concentration of 1.690 ng OTA/mL.

Table 3. Interlaboratory study results for determination of OTA (ng/mL) in blind duplicates of spiked and naturally contaminated beer samples

		Laboratory																	
Sample	Code	2	3	4	5[a]	6	7	8[a]	9	10[a]	11	12	13	14	15	16	17	18	19
A	Practice	0.43	0.53	0.44	0.24	0.49	0.46	0.3	0.46	0.54	0.47	0.37	0.58	0.12	0.52	0.46	0.46	0.46	—
B	Practice	1.51	1.50	1.16	0.33	1.20	1.38	1.19	1.60	1.76	1.28	1.51	1.77	1.17	1.51	1.44	1.42	1.33	1.62
1[b]	3	ND[c]	0.02	ND	ND	<0.01	0.01	ND	ND	0.07	0.01	ND	ND	ND	ND	ND	ND	0.02	0.01
	10	ND	0.01	ND	ND	<0.01	0.01	ND	ND	0.06	0.01	0.04	0.01	0.01	0.02	ND	ND	0.01	0.02
2[d]	2	0.27	0.20	ND	0.06	**0.20**[e]	0.17	ND	0.18	1.55	0.19	0.17	0.23	0.19	0.23	0.20	0.13	0.21	0.22
	7	0.21	0.21	ND	0.02	**0.08**[e]	0.14	0.13	0.16	0.29	0.16	0.14	0.24	0.18	0.24	0.19	0.10	0.16	0.22
3[f]	6	0.85	0.74	0.72	0.11	0.62	0.60	0.40	0.61	0.75	0.65	0.48	0.95	0.58	0.85	0.73	0.74	0.72	0.82
	9	0.86	0.78	0.62	0.19	0.51	0.57	0.43	0.69	0.62	0.66	0.49	0.87	0.52	0.76	0.80	0.61	0.65	0.83
4[g]	4	1.45	1.52	1.44	0.29	**1.28**[e]	1.28	0.63	1.29	1.59	1.19	0.78	1.62	**0.36**[e]	1.57	1.50	1.58	1.22	1.52
	8	1.56	1.60	1.44	0.20	**0.38**[e]	1.38	0.63	1.25	1.38	1.28	0.99	1.69	**0.88**[e]	1.65	1.43	1.46	1.26	1.54
5[h]	1	0.10	0.07	ND	<0.01	0.07	0.05	ND	0.07	0.14	0.07	0.07	0.07	0.07	0.10	0.09	0.03	0.08	0.09
	5	0.07	0.08	ND	<0.01	0.06	0.05	ND	0.05	0.16	0.07	0.05	0.07	0.04	0.10	0.07	0.05	0.07	0.09

[a] Data from Laboratories 5, 8, and 10 were considered invalid because of problems with glassware, filtration, and the automatic injection system, respectively.
[b] Blank sample (containing <0.01 ng OTA/mL).
[c] ND = not detected (Laboratory 4 reported a detection limit of 0.2 ng/mL).
[d] Sample spiked with 0.2 ng OTA/mL.
[e] Data rejected on the basis of the Cochran test.
[f] Sample spiked with 0.8 ng OTA/mL.
[g] Sample spiked with 1.5 ng OTA/mL.
[h] Naturally contaminated sample at a mean concentration of 0.070 ng OtA/mL.

F. Calculations

Determine from the calibration curve the amount of OTA (in ng) in the aliquot of test solution injected into the LC system. Calculate the concentration of OTA (C_{OTA}; in ng/mL) from the following equation:

$$C_{OTA} = M_A \times (2/V_1) \times (V_3/V_2)$$

where M_A is the mass of OTA (in ng) in the aliquot injected on column, determined from the calibration graph; *2* is the dilution factor; V_1 is the volume of solution taken for analysis (10 mL); V_2 is the volume of test solution injected on column (100 µL); and V_3 is the volume of solution used to dissolve the dried eluate (250 µL).

Refs.: *J. AOAC Int.* **84**, 1819–1825(2001); Castegnaro, M., Barek, J., Fremy, J.M., Lafontaine, M., Miraglia, M., Sansone, E., & Telling, G. (1991) IARC Scientific Publication No. 113, International Agency for Research on Cancer, Lyon, France

Results and Discussion

All 18 participants completed the collaborative study. Data submitted by 2 participants were considered invalid because of problems encountered during the analysis and were not included in the statistical analysis. In particular, 1 participant (Laboratory 5) submitted results that showed very low recoveries (from 2 to 32%) and very high variability of results for the blind duplicate samples (relative standard deviations from 12 to 100%). To identify the source of the error, 12 spiked samples (randomly taken from leftover test materials of Laboratory 5) of the 3 matrixes were returned to the coordinator and reanalyzed with the collaboratively tested method. Results obtained by the coordinator were similar to those obtained by the other participants (recoveries of >85%). The participant (Laboratory 5) did not find a plausible explanation and indicated *"the possibility of adsorption of ochratoxin A to glassware"* as the major source of error. Another participant (Laboratory 10) had problems with the autosampler, indicating that the *"automatic injection system did not work well."* In addition, a third participant (Laboratory 8) submitted valid data only for white wine; this participant used deviations (in the filtration step) from the described method for the analysis of red wine and beer. Consequently, data subjected to statistical analysis were from 16 laboratories for white wine and from 15 laboratories for red wine and beer. Moreover, Laboratories 4 and 8 reported a detection limit of 0.2 ng/mL; therefore their data relative to samples with OTA contamination levels of ≤0.2 ng/mL were excluded from the statistical evaluation. Data of all laboratories that returned results relevant to the collaborative study for white wine, red wine, and beer are presented in Tables 1–3, respectively. Table **2001.01A** summarizes the statistical data of the entire study, including OTA mean concentrations, mean recoveries, within-laboratory repeatability standard deviations (s_r) and relative standard deviations (RSD_r), between-laboratories reproducibility standard deviations (s_R) and relative standard deviations (RSD_R), number of sets of acceptable results, number of outlier laboratories, and the values for HORRAT, which is considered a measure of acceptability of method performance (23). Determination of outliers was assessed by the Cochran test and the Single Grubbs and Paired Grubbs tests. Pairs of results identified as outliers are marked by code and indicated in bold in Tables 1–3. Laboratory 6 reported data that were outliers in most cases (9 of 12), and its remaining data contributed to a considerable increase in the relative standard deviations of the entire study. The participant was contacted by the coordinator to determine the causes of these odd results, but no valid reason to eliminate the submitted results was found.

With respect to the naturally contaminated practice test samples (Tables 1–3), all but 1 participant (Laboratory 1) reported results within the acceptable concentration range for all matrixes. Two laboratories did not return results: Laboratory 8, because of in-house problems with the LC system during the analysis (white wine practice samples A and B), and Laboratory 19, which did not receive practice samples A for red wine and beer. All of these laboratories contacted the coordinator before proceeding with the collaborative study. Laboratory 1 did not participate in the collaborative study.

Statistical analysis was not performed for test sample 1 (blank material, ≤0.01 ng OTA/mL), which was also used as the blank for spiking the samples. Most participants did not detect OTA in this sample or detected OTA at levels close to the detection limit. Only 1 laboratory (Laboratory 13) found appreciable OTA contamination in the white wine sample (average value of blind duplicates was 0.03 ng/mL), and 3 laboratories (Laboratories 12, 15, and 19) found appreciable OTA contamination in the red wine sample (average values of blind duplicates were 0.03, 0.03, and 0.071 ng/mL, respectively).

Interlaboratory study results for the determination of OTA in white wine, red wine, and beer by LC after immunoaffinity column cleanup are reported in Table **2001.01A**. For the spiked samples (blind duplicates), average recoveries ranged from 88.2 to 105.4% for white wine, from 84.3 to 93.1% for red wine, and from 87.0 to 95.0% for beer. Within-laboratory relative standard deviations (RSD_r) ranged from 6.6 to 8.4% for white wine, from 6.5 to 9.9% for red wine, and from 4.7 to 10.6% for beer. Between-laboratories relative standard deviations (RSD_R) were higher than the corresponding within-laboratory values. RSD_R values ranged from 13.1 to 15.9% for white wine, from 11.9 to 13.6% for red wine, and from 15.2 to 20.9% for beer. Repeatability and reproducibility levels for naturally contaminated samples were comparable to those for spiked samples (RSD_r = 10.8%, and RSD_R = 14.6% for white wine; RSD_r = 10.8%, and RSD_R = 13.6% for red wine; and RSD_r = 16.5%, and RSD_R = 26.1% for beer).

The acceptability of the performance of a method is indicated by the HORRAT, which is a measure of the ratio between the RSD_R determined in this collaborative study and the RSD_R statistically predicted for the determined or known concentration, calculated from the following equation:

$$RSD_R = 2^{(1–0.5 \log C)} = 2C^{(–0.15)}$$

where C = concentration expressed as a decimal factor (23). Considering that HORRAT values of <2 are acceptable, the HORRAT values calculated for the present study (ranging from 0.2 to 0.4) are far below the acceptability threshold and much better than the values derived from the literature with respect to most mycotoxin collaborative studies (23, 24).

The performance characteristics of the method (accuracy, repeatability, and reproducibility) are better than those obtained in a collaborative study organized in 1998 by the OIV (Office International de la Vigne et du Vin) for determining OTA in a spiked white wine sample (spiked at 0.2 ng/mL) and a naturally contaminated red wine (mean value of 0.191 ng/mL) by a method based on silica cartridge cleanup (25). In addition, the proposed method presents a number of advantages with respect to the OIV method. In particular, the use of large volumes of hazardous solvents and tedious liquid–liquid extraction is avoided, and the rapid sample preparation and cleanup saves time.

Collaborators' Comments

All participants indicated that the method was well described and easy to perform. Two participants (Laboratories 4 and 16) used their own standards in preparing the calibration curve, submitting results that fitted well only after the correction factor ($C_{\text{std coordinator}}/C_{\text{std participant}}$) was applied. One participant (Laboratory 17) judged the calibration range too large and suggested the use of a different calibration curve for reliable determination at low levels of contamination.

One participant (Laboratory 15) suggested dissolving the dried extract in a larger solvent volume and allowing the loop to overfill by 3–5 times the injection volume. Some laboratories (Laboratories 3, 6, 9, 13, and 19) injected volumes that were smaller (from 20 to 50 μL) than the suggested volume (100 μL) into the LC system, indicating 250 μL was too small a dissolving volume. In processing the results, the coordinator judged this deviation from the prescribed method of analysis irrelevant.

One participant (Laboratory 5) suggested that glass vials should be silanized to prevent OTA adsorption and ensure the stability of OTA in aqueous solvents, having experienced this kind of problem in his laboratory. However, the good results of this collaborative study, obtained without using silanized glassware, demonstrated that the use of silanized vials is not essential for the determination of OTA in wine and beer (this was further confirmed by additional experiments in the coordinator's laboratory). Nevertheless, on the basis of the suggestion of Laboratory 5, silanization or acid-washing procedures may be advisable when new glassware of poor quality is used.

Conclusions

The HORRAT values obtained indicate that the method for the determination of OTA in wine and beer is reproducible with results superior to those obtained in a large number of other studies involving mycotoxins in various matrixes. In particular, method performance is much better than that of the only method tested so far in a collaborative study of a method for the determination of OTA in wine (25). No collaborative study of a method for the determination of OTA in beer has been previously performed.

Recommendation

On the basis of the results of this study, it is recommended that the method for the determination of OTA in wine and beer be adopted First Action.

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