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FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS

WORLD HEALTH ORGANIZATION

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Agenda Item 9 **CX/MAS 01/10-Add.1**

JOINT FAO/WHO FOOD STANDARDS PROGRAMME

CODEX COMMITTEE ON METHODS OF ANALYSIS AND SAMPLING
Twenty-Third Session
Budapest, Hungary, 26 February - 2 March 2001

ENDORSEMENT OF METHODS OF ANALYSIS PROVISIONS IN CODEX STANDARDS METHOD OF ANALYSIS FOR OCHRATOXIN A IN CEREALS

The 32nd Session of the Committee on Additives and Contaminants agreed to propose the following method of analysis for Ochratoxin A in cereals and cereal products for consideration by the CCMAS. The CCFAC is currently considering a Proposed Draft Maximum Level for Ochratoxin A in Cereals and Cereal Products of 5µg/kg (at Step 3) (ALINORM 01/12, paras. 94 - 96 and 138, Appendix X).

UDC 615.917:54.06	**NORDISK METODIKKOMMITTÉ FÖR LIVSMEDEL** **NORDIC COMMITTEE ON FOOD ANALYSIS**	**No 143** **2a uppl.** **2nd ed. 1997**

1. SCOPE AND FIELD OF APPLICATION

The method is applicable to the analysis of ochratoxin A (OTA) in cereals and cereal products in concentrations above 2 ng/g.

2. PRINCIPLE

OTA is extracted with chloroform-aqueous phosphoric acid and isolated by liquid-liquid partitioning into aqueous bicarbonate solution. The solution is applied to a C18 column, and OTA is eluted with ethyl acetate-methanol-acetic acid. The OTA is identified by reversed phase LC and quantified by fluorescence. Chromatography of OTA methyl ester derivative confirms the identification.

3. APPARATUS

3.1 High-speed blender. 1250 ml jar with cover.

3.2 Liquid chromatograph. Pump with variable flow from 0.5 to 5 ml/min., at up to 3000 psi. Flow reproducibility ±0.1%. Injection valve with 25 µl loop, fluorescence detector, compatible recorder or integrator.

3.3 LC analytical column. 250x4.6 mm i.d., packed with spherical 5 µm C18 material (Supelco Inc., Bellafonte, PA 16823, or equivalent).

3.4 Adsorption column. 500 mg 40 µm C18 material in 3 ml polypropylene tube (Supelco, Inc., or equivalent.

3.5 Vacuum manifold with stopcocks at each port for holding C18 columns. Can be replaced by a syringe (5-10 ml) with a suitable adapter (Luer).

3.6 Glass fiber filters. 0.33 mm thickness, 1.5 µm pore retention, 9.0 cm diameter (Whatman No. 934 AH, or equivalent).

3.7 Microfilter. 0.45 µm pore retention syringe filter (Gelman acrodisc 3CR PTFE, or equivalent).

4. REAGENTS

4.1 Solvents. Chloroform, dichloromethane, ethyl acetate, toluene and methanol, pro analysi, in glass. Acetonitrile, LC grade.

4.2 Chemicals. Phosphoric acid, sodium bicarbonate, acetic acid, pro analysi. All solutions are prepared using distilled water.

4.3 Diatomaceous earth. Soak about 900 g acid-washed Celite 545 overnight in methanol. Filter through a double layer of Whatman No. 1 paper in a 24 cm Buchner funnel, wash with 8 l water and dry for 12 hours at 150°C.

4.4 LC mobile phase. Water-acetonitrile-acetic acid (99+99+2). Use LC grade water. Degas.

4.5 Phosphoric acid solution, 0.1 mol/l. Dilute 5.75 g 85% phosphoric acid to 500 ml with water.

4.6 OTA standard solutions. About 24 µg/ml in toluene-acetic acid (99+1). Measure UV absorbance (333 nm, ε = 5440, MW = 403) to determine the exact concentration. Dilute the stock solution with toluene-acetic acid (99+1) solution to obtain a working standard solution (4 µg/ml).

4.7 BF_3-methanol solution. 14% BF_3 in methanol. *Caution:* Use in hood. Avoid contact with skin, eyes, and respiratory tract.

5. PROCEDURE

5.1 Sample preparation: Grind the sample to pass a 1.0 mm sieve. Blend.

5.2. Extraction: Weigh 50 g test portion into a blender jar (3.1) and add first 250 ml chloroform and then 25 ml 0.1 mol/l phosphoric acid (4.5). Blend for 3 min. at medium speed. Near the end of blending add 10 g (45 ml) diatomaceous earth (4.3). Filter the extract through glass fiber filter (3.6) covered with about 10 g diatomaceous earth on a 9 cm Buchner funnel (or by gravity through a 32 cm fluted paper). Collect >50 ml filtrate.

5.3 Partition: Transfer 50 ml of filtrate to a separation funnel. Add 10 ml 3% sodium bicarbonate solution and shake gently. Allow the phases to separate. If an emulsion forms, centrifuge 2 min. at 2000 rpm. Collect the upper (bicarbonate) phase for column extraction.

5.4 Column preparation: Attach the C18 columns (3.4) to vacuum manifold ports with 25 ml Erlen meyer flasks or beakers inside the manifold for collecting conditioning and washing solvents. Wash each column twice with about 2 ml methanol, 2 ml water, and 2 ml 3% sodium bicarbonate. DO NOT ALLOW THE COLUMN TO RUN DRY. To speed elutions, apply gentle suction. This procedure may also be performed manually by applying pressure with a 5-10 ml syringe adapted to the top of the column. Leave about 2 mm solvent on top of the frit.

5.5 Column extraction: Pipette 5 ml of the bicarbonate extract from 5.3 to the C18 column. Do not allow the column to run dry. Wash with 2 ml 0.1 mol/l phosphoric acid followed by 2 ml water. Discard the washing liquids.

Elute OTA with 8 ml ethyl acetate-methanol-acetic acid (95+5+0.5). Collect the eluate in a 10 ml test tube containing 2 ml water. Shake or stir the eluate with a glass rod to mix the two phases. Pipette OTA extract (upper phase) into another 10 ml test tube with a screw-cap. Wash the remaining upper phase from the tube with 2x1 ml ethyl acetate and add to the OTA phase. Continue as described under 5.6.3.

Used C18 columns may be regenerated by following the procedure under 5.4.

5.6 LC determination

5.6.1 Operating conditions: Adjust the flow rate to 1.0 ml/min. Select the sensitivity to give 4-8% of full scale response for 1.0 ng OTA and <2% noise. The retention time is 10-13 min. Use an injection volume of 20-25 µl and use 50 µl to fill the 25 µl loop. For fluorescence detectors with gratings, set the excitation at 333 nm and the emission at 460 nm; for fluorescence detectors with filters, use a 420 nm cut-off filter.

5.6.2 Standard curve: Prepare a standard curve at the start of the analysis and whenever the chromatographic conditions change. Dispense 2, 3, 5, 10, and 25 µl aliquots of working standard solution (4 ng/µl) into separate 4-5 ml Teflon-lined screw-topped vials using fixed-volume syringes. Evaporate just to dryness under nitrogen. Add 1.00 ml LC mobile phase (4.4) to each vial for final OTA concentrations of 0.2, 0.3, 0.5, 1.0 and 2.5 ng/25 µl.

Chromatograph each standard solution. Calculate (normalize) the response (R_{1-5}) of 1 ng OTA for each of the five standard concentrations (C_{1-5}). Calculate the mean response (R_a) for the five standard concentrations and determine the percentage deviation (D) of individual values from the mean as follows:

$$R_a = (R_1/C_1 + R_2/C_2 + R_3/C_3 + R_4/C_4 + R_5/C_5)/5$$

$$D = 100\ ((R/C) - R_a)/R_a$$

The deviation should be ≤5%. If D>5, omit one value and recalculate R_a using four standards. If four values do not agree, rechromatograph the standard solutions or prepare new standard solutions. When the deviation is acceptable plot peak heights or areas vs. 0.2, 0.3, 0.5, 1.0 and 2.5 ng OTA for linearity using the origin (0;0) as a fixed point.

5.6.3 Sample: Evaporate the extract from 5.3 just to dryness on a steam bath under nitrogen. Then immediately dissolve in 500 µl of the mobile phase (4.4) and filter through a 0.45 µm microfilter into a 5 ml screw-cap vial. Immediately chromatograph the sample. Identify OTA from the retention time (which must be the same as that of standard OTA). If the sample OTA response is outside the range of the standard curve, adjust the sample volume by concentrating or diluting the sample solution. Reserve the remaining sample solution for identity confirmation by formation of methyl ester.

Calculate the OTA concentration in the sample as follows:

$$\text{OTA (ng/g)} = (R_s x V_T)/(R_a x V_1 x W) = (R_s x F)/R_a$$

W = (50 g x 50 ml x 5 ml)/250 ml x 10 ml) = 5 g (weight of test sample represented by final extract)
R_s = response of test sample injected
R_a = calculated mean normalized respons (response for 1 ng OTA) of the five (*four*) working standard solution concentrations (5.1.1)
V_T = final test sample volume (500 µl)
V_I = volume of test sample injected (25 µl)
F = 4

6. CONFIRMATION OF OTA

Quantitatively transfer the remaining reserved sample (5.6.3) to a 25 ml separation funnel, using 3x1 ml dichloromethane to rinse the vial. Shake and allow the layers to separate. Collect the lower layer into a 5 ml vial and evaporate to dryness. Transfer 50 µl working standard solution to another 5 ml vial and evaporate to dryness. Add 0.5 ml 14% BF_3-methanol to each vial, cap and heat for 15 min. in a 50-60°C water bath. Evaporate to dryness on a steam bath under nitrogen. If water is present, add 1 ml acetonitrile and continue evaporation to dryness. Cool and dilute with mobile phase to the same volume as used for LC analysis. Chromatograph the derivatized sample and standard. Positive confirmation is provided by disappearance of the peak at the R_t for OTA (10-12 min.) and appearance of a new peak at the same R_t as that of the standard methyl ester of OTA (about 15 min. later).

7. RELIABILITY OF THE METHOD

7.1 First interlaboratory study

This method was first collaboratively studied in an interlaboratory study conducted by AOAC INTERNATIONAL in cooperation with the International Union of Pure and Applied Chemistry (IUPAC) and the Nordic Committee on Food Analysis (NMKL). A total of 16 laboratories in Europe, Canada and the United States participated in the study. OTA was added to barley and maize at 10, 20 and 50 ng/g. Mean recoveries of OTA ranged from 72 to 82%. Within-laboratory relative standard deviations were 7.9% and 20.1% for barley and maize, respectively. Between-laboratory relative standard deviations were 20.7-31.7% for all concentrations of OTA. Reference to the study report: *Journal of AOAC INTERNATIONAL* **75** (1992):3, 481-487.

7.2 Second interlaboratory study

In a second study 12 European laboratories analysed samples of naturally contaminated barley and samples of wheat-bran and rye to which OTA had been added. The concentrations of OTA ranged from 3 to 9 ng/g. Mean recoveries of OTA ranged from 64 to 72%. Within-laboratory relative standard deviations were 12-23% and between-laboratory relative standard deviations were 18-29%. Reference to this second study report: *Journal of AOAC INTERNATIONAL* **79** (1996):5, 1102-1105.

8. REFEREES OF THE METHOD

This method is based on method no. 991.44, Official Methods of Analysis of AOAC INTERNATIONAL. AOAC tested the method collaboratively on test materials containing OTA at levels higher than 10 ng/g (see 7.1). Later, in a second collaborative study (see 7.2) the method was tested on materials containing OTA at lower levels than those given in the AOAC method, 3-9 ng/g. The method was revised in accordance with the results of the study. The revision and study work was done by Kjell Larsson, National Laboratory for Agricultural Chemistry, Uppsala, Sweden (now working for Lantmännen Foderutveckling AB in Stockholm) in cooperation with Tord Möller, National Food Administration, Uppsala, Sweden.