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DRAFT TANZANIA STANDARD

Edible cottonseed oil – Specification

TANZANIA BUREAU OF STANDARDS

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0 Foreword

Cottonseed oil is extracted from the seeds of cotton plant of various species.Cottonseed have similar structures to other oil seeds such as sunflower seeds, having an oil bearing kernel surrounded by a hard outer hull; during processing, the oil is extracted from the kernel. Edible Cottonseed oil is used for salad oil, mayonnaise, salad dressing, and similar products because of stability of its flavour.It is one of traded edible vegetable oils in Tanzania.

This Tanzania Standard has been prepared to ensure the safety and quality of edible cottonseed oil produced in,exported or imported into the country.

In preparation of this Tanzania standard considerable help was derived from:

CODEX STAN 210 -1999 (Amended 2015) *Codex standard for named vegetable oils* published by the Codex Alimentarius Commission

ISO 15141-2018 *Cereals and cereal products — Determination of ochratoxin A — High performance liquid chromatographic method with immunoaffinity column cleanup and fluorescence detection* published by International Organization for Standardization.

In reporting the results of a test or analysis made in accordance with this Tanzania Standard, if the final value, observed or calculated is to be rounded off, it shall be done in accordance with TZS 4.

1 Scope

This Tanzania Standard prescribes the requirements and methods of sampling and tests for edible cottonseed oil.

2 Normative references

The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies;

CODEX STAN 192, *General standards for food additives*

TZS 4, *Rounding off numerical values*

TZS 54, *Animal and Vegetable fats and oils – Sampling*

TZS 76, *Methods for determination of arsenic*

TZS 109, *Food processing units – Code of hygiene — General*

TZS 268, *General atomic absorption – Spectro – Photometric method for determination of lead in food stuffs*

TZS 288-1*, Animal and vegetable fats and oils — Preparation of methyl esters of fatty acids*

TZS 288-2, *Animal and vegetable fats and oils — Analysis by gas chromatography of methyl ester of fatty acids*

TZS 538 , *Packaging and labeling of foods*

TZS 799*, Foodstuffs – Determination of aflatoxin B1, and the total content of aflatoxins B1, B2, G1 and G2 in cereals, nuts and derived products – High\-performance liquid chromatographic method*

TZS 1313*, Fortified edible oils and fats — Specification*

TZS 1322, *Oils and fats Sampling and test methods – Purity test*

TZS 1324, *Animal and vegetable fats and oils – Determination of peroxide value-Iodometric (visual) end point determination*

TZS 1325, *Animal and vegetable fats and oils - Determination of saponification value*

TZS 1326, *Animal and vegetable fats and oils – Determination of moisture and volatile matter*

TZS 1327, *Animal and vegetable fats and oils – Determination of iodine value*

TZS 1328, *Essential oils – Determination of relative density at 20 ℃ – Reference method*

TZS 1329, *Animal and vegetable fats and oils – Determination of refractive index*

TZS 1330, *Animal and vegetable fats and oils – Determination of lovibond colour*

TZS 1331, *Animal and vegetable fats and oils – Determination of acid value and acidity*

TZS 1332, *Animal and vegetable fats and oils – Determination of unsaponifiable matter-method using diethyl ether extraction*

TZS 1335, *Animal and vegetable fats and oils – Determination of copper, iron and nickel content-graphite furnace atomic absorption*

TZS 1336, *Animal and vegetable fats and oils – Determination of insoluble impurities content*

TZS 1369, *Animal and Vegetable fats and oils – Determination of Butylhydroxyanisole (BHA) and Butylhydroxytoluene (BHT) – Gas liquid chromatographic method*

TZS 1370, *Animal and Vegetable fats and oils – Determination of tocopherol and tocotrienol content by High Performance Liquid Chromatography*

TZS 1371 *Animal and vegetable fats and oils — Determination of individual and total sterol contents-Gas chromatographic method*

TZS 1775, *Animal and Vegetable fats and oils - Detection and identification of antioxidants - Thin-layer chromatographic method*

3 Terms and definitions

For the purpose of this Tanzania Standard the definitions below shall apply;

3.1 edible oils
food stuffs which are composed of glycerides of fatty acids of vegetable, animal or marine origin which is suitable for human consumption. They may contain small amounts of other lipids such as phosphatides, of unsaponifiable constituents and of free fatty acids naturally present in the oil.

3.2 vegetable oil
any of a large group of oils that are esters of fatty acids and glycerol, obtained from the leaves, fruit, or seeds of plants.

3.3 edible cottonseed oil
edible oil derived from the seeds of various cultivated species of *Gossypium spp.*

3.4 virgin cottonseed oil
cottonseed oil obtained, without altering the nature of the oil, by mechanical procedures, for example, expelling or pressing, and the application of heat only. It may have been purified by washing with water, settling, filtering and centrifuging only.

3.5 refined cottonseed oil
cottonseed oil obtained, by mechanical procedures and/or solvent extraction and subjected to refining processes.

4.Requirements

4.1 General requirements
Edible cottonseed oil shall

a) be fit for human consumption;

b) be free from foreign and rancid odour and taste;
c) have colour characteristic of designated product ;
d) be clear and free from adulterants, sediments, suspended or foreign matter and separated water and;
e) be free from admixture with mineral or other oils of vegetable or animal origin when tested in accordance with the methods prescribed in TZS 1322.
f) be free from gossypol when tested according to the method prescribed in **Annex B.**

4.2 The clarity of the oil shall be judged by the absence of turbidity after keeping the filtered sample at 30 ^{0}C for 24 hours.

4.3 Specific requirements

Edible cottonseed oil shall comply with specific requirements given in table 1 when tested in accordance with the methods specified therein**;**

Table 1-Specific requirements for edible cottonseed oil

S. No.	Parameter	Requirement	Test method
i.	Relative density(at 20 ^{o}C/water at 20 ^{o}C)	0.918-0.926	TZS 1328
ii.	Refractive index,(ND 40 ^{o} C)	1.458 - 1.466	TZS 1329
iii.	Saponification value, mg KOH/g oil	189 - 198	TZS 1325
iv.	Iodine value (Wijs), g/100	100– 123	TZS 1327
v.	Unsaponifiable matter, g/kg, max.	15	TZS 1332
vi.	Fatty acids composition,%of total fatty acids	As in Annex A	TZS 288(Part 1&2)
vii.	Moisture and matter volatile at 105 ^{o} C, % m/m, max.	0.2	TZS 1326
viii.	Insoluble impurities, % m/m, max.	0.05	TZS 1336
ix.	Free Fatty Acid(FFA) as oleic acid, %max	Refined oils: 0.3 Cold pressed and virgin oils: 2	TZS 1331
x.	Peroxide value, mEq peroxide- oxygen/kg oil, max.	Refined oils :10 Cold pressed and virgin oils: 15	TZS 1324
xi.	Colour, in a 0.635 cm cell in Lovibond unit, expressed as (Y+5R) not deeper than	10	TZS 1330
xii.	Total sterols(mg/kg)	2700-6400	TZS 1371
xiii.	Halphen test	Positive	TZS 1322

4.3 Food additives

4.3.1 General

4.3.1.1 Food additives may be used during processing of edible cottonseed oil and shall be in accordance to Codex Stan 192.

4.3.1.2 In addition food additives shall not be used in virgin or cold pressed oils. In Refined the additives may be used subject to tables 2, 3, and 4.

4.3.2 Flavours

Natural flavours and their identical synthetic equivalents, and other synthetic flavours may be used, except those which are known to be hazardous.

4.3.4 Antioxidants

4.3.4.1 Antioxidants specified in Table 2 may be used;

Table 2– Requirements for antioxidant in edible cottonseed oil

INS No.	Antioxidant	Requirements	Test method
304	Ascorbyl palmitate, mg/kg, max	500 (singly or in combination)	TZS 1775
305	Ascorbyl stearate , mg/kg, max		
307a	Tocopherol, d-*alpha- , mg/kg, max*	300 (singly or in combination)	TZS 1370
307b	Tocopherol concentrate, mixed , mg/kg, max		
307c	Tocopherol, dl-*alpha , mg/kg, max*		
310	Propyl gallate , mg/kg, max	100	TZS 1775
319	Tertiary butyl hydroquinone (TBHQ) , mg/kg, max	120	
320	Butylated hydroxyanisole (BHA) , mg/kg, max	175	TZS 1369
321	Butylated hydroxytoluene (BHT),mg/kg, max	75	
Any combination of gallates, BHA, BHT, and/or TBHQ , mg/kg, max		200 within individual limits	TZS 1775
389	Dilauryl thiodipropionate , mg/kg, max	200	

4.3.4.2 Antioxidant synergists specified in Table 3 may be used.

Table 3 — Antioxidant synergists

INS No.	Antioxidant synergist	Requirements	Test method
330	Citric acid , mg/kg, max	GMP	TZS 1775
331(i)	Sodium dihydrogen citrate, mg/kg, max		
331(iii)	Trisodium citrate , mg/kg, max		
384	Isopropyl citrates , mg/kg, max	100 (Singly or in combination)	
472c	Citric and fatty acid esters of glycerol , mg/kg, max		

4.3.5 Antifoaming agents (deep frying oil)

Antifoaming agents specified in Table 4 may be used;

Table 4 — Antifoaming agents

INS No.	Antifoaming agent	Requirements	Test method
900a	Polydimethylsiloxane, mg/kg, max	10	TZS 1775

4.4 Fortification
Edible cottonseed oil may be fortified in accordance with TZS 1313.

5 Hygiene

Edible cottonseed oil shall be produced, processed, handled and stored in accordance with TZS 109 .

6 Contaminants

6.1 Pesticide residues
Edible cottonseed oil shall comply with relevant maximum pesticide residue limits established by the Codex Alimentarius Commission.

6.2 Soap content
Edible cottonseed oil shall have the **soap content not exceed 0.005%** when tested according to **TZS 1322**

6.3. Metals contaminants

Edible cottonseed oil shall comply with maximum limits established by Codex Alimentarius Commission as specified in Table 5.

Table 5– Acceptable levels of metal contaminants in edible cottonseed oil

Characteristics	Maximum level	Method of test
Iron (Fe) mg/kg	Virgin 5.0 Refined 1.5	TZS 1335
Copper (Cu) mg/kg	Virgin 0.4 Refined 0.1	TZS 1335
Lead (Pb) mg/kg	0.1	TZS 268
Arsenic (As) mg/kg	0.1	TZS 76

6.4 Mycotoxins

6.4.1 Total aflatoxin shall not exceed 10 µg/kg while aflatoxin B1 shall not exceed 5 µg/kg when tested in accordance with TZS 799.

6.4.2 Ochratoxin A level shall not exceed 5µg/kg when tested in accordance with the method specified in Annex C.

6.4.3 Edible cottonseed oil shall be free from Poly-Hydroxy alkanoates (PHA).

7 Sampling and Tests

7.1 Sampling
Edible cottonseed oil shall be sampled as prescribed in TZS 54.

7.2 Tests
7.2.1 Testing shall be in accordance with TZS 1322 and as provided in the respective tables and Annexes of this Tanzania Standard.

7.2.2 Quality of reagents
Unless specified otherwise, analytical grade chemicals and distilled water shall be used in tests.

8 Packaging, Marking And Labeling

Edible cottonseed oil shall be packed, marked and labeled in accordance with TZS 538.

8.1 Packaging
Edible cottonseed oil shall be packaged in food grade containers and sealed in manner to ensure the safety and quality requirements specified in this standard are maintained throughout the shelf life of the product.

8.2 Marking and labeling
8.2.1 In addition each Container of product shall be legibly and indelibly marked with the following information:

a) Name of the product
b) The words virgin or refined shall be declared on the label to indicate the type of oil
c) Name, physical and postal address of the manufacturer and/or packer
d) Date of manufacture and expiry date
e) Language-Kiswahili/English or Kiswahili and English
f) A complete list of ingredients in descending order of proportion
g) Net content
h) Batch number
i) Manufacturers registered trade mark
j) Country of origin
k) The phrase “Place the products away from direct sunlight”.
l) Nutritional information(option)

8.2.2 The containers may also be marked with the TBS Mark of Quality.

NOTE - The TBS Mark of Quality may be used by the packers only under licence from TBS. Particulars of conditions under which the licenses are granted, may be obtained from TBS.

ANNEX A
(normative)
Free fatty acids composition

Carbon configuration	Fatty acid composition(%)	Test Methods
C6:0	ND	**TZS 288(Part 1&2)**
C8:0	ND	
C10:0	ND	
C12:0	ND-0.2	
C14:0	0.6-1.0	
C16:0	21.4-26.4	
C16:1	ND-1.2	
C17:0	ND-0.1	
C17:1	ND-0.1	
C18:0	2.1-3.3	
C18.1	14.7-21.7	
C18:2	46.7-58.2	
C18:3	ND-0.4	
C 20:0	0.2-0.5	
C 20:1	ND-0.1	
C20:2	ND-0.1	
C22:0	ND-0.6	
C22:1	ND-0.3	
C22:2	ND-0.1	
C24:0	ND-0.1	
C24:1	ND	
NOTE- Free fatty acid composition is expressed as % of total fatty acids ND-none detectable,defined as ≤0.05		

ANNEX B
(normative)
Determination of Free and Total Gossypol

1. Introduction

Free gossypol refers to gossypol and gossypol derivatives in cottonseed products which are soluble in aqueous acetone under the conditions of this method.

2. Apparatus

2.1 Mechanical shaker
2.2 Spectrophotometer
2.3 Glass beads about 6 mm diameter
2.4 Erlenmeyer flasks 250 ml
2.5 Pipettes,
2.6 Filter paper, (Whatman No 2 or equivalent)
2.7 Volumetric flasks 25, 200 ,250 ml
2.8 Water bath for operation at 100°C.

3. Reagents

Solvents

3.1 Aqueous acetone – Mix 700 ml acetone with 300 ml distilled water
3.2 Aqueous Isopropyl alcohol (2 – propanol) – Mix 800 isopropyl alcohol with 200 ml water.
3.3 Aniline – distilled over a small amount of zinc dust. Redistill when the reagent blank exceeds 0.022 absorbance (95 % transmittance)
3.4 Thiourea solution – Dissolve 10 gm thiourea in water and make up to 100 ml
3.5 HCl – 1.2 N Dilute 106 ml conc. HCl (35-37%) to 1 litre with water.
3.6 Gossypol - Primary standard or gossypol acetic acid (89.61 % gossypol by wt) to be used for calibration.
3.7 Standard Gossypol solution – Prepare by accurately weighing 25 mg primary standard gossypol or 27.9 mg gossypol acetic acid and transferring quantitatively to a 250 ml volumetric flask using 100 ml of acetone. Add 1 ml glacial acetic acid, 75 ml water, dilute to volume with acetone and mix well. Pipette 50 ml of this solution into a 200 ml volumetric flask, add 100 ml acetone, 60 ml water and dilute to volume with acetone. Mix well. This standard gossypol solution contains 0.025 mg of gossypol per ml if exactly 25 mg gossypol or 27.9 mg of gossypol acetate were weighed. It is stable for 24 hrs when protected from light.

4. Procedure

4.1 The weight of the sample and the aliquot of the acetone extract to be taken for test shall depend on the gossypol content but sample size should not exceed 2-5 gm if the free gossypol is expected to be between 0.2 – 0. 5 % and the aliquot of extract to be taken for test should be 10 ml.
4.2 Transfer the accurately weighed sample to a 250 ml Erlenmeyer flask Add a few glass beads and 50 ml aqueous acetone, stopper and shake vigorously on a mechanical shaker for 1 hour.
4.3 Filter through a dry filter paper discarding the first 5 ml and collect filterate in a small flask.
4.4 Pipette duplicate aliquots into 25ml volumetric flasks
4.5 To one sample solution designated as solution A, add 2 drops of 10 % aqueous thiourea, 1 drop of 1.2 N HCl and dilute to volume with aqueous isopropyl alcohol.
4.6 To the second sample designated as solution B, add 2 drops of 10 %,aqueous thiourea, 1 drop of 1.2 N HCl and 2 ml of redistilled aniline. A rapid delivery pipette may be used for dispensing aniline.

4.7 Prepare a reagent blank containing a volume of aqueous acetone solution equal to that of the sample aliquot and add 2 drops of 10% thiourea and 2 ml of aniline(do not add any 1.2 N HCl).

4.8 Heat the sample aliquot B and the reagent blank in a boiling water bath for 30 minutes.

4.9 Remove the solutions from the bath, add about 10 ml of aqueous isopropyl alcohol; to effect homogeneous solution and cool to room temperature.

4.10 Dilute to volume with aqueous isopropyl alcohol.

4.11 Determine the absorbance of sample aliquot A at 440 nm using aqueous isopropyl alcohol to set the instrument at zero absorbance (100% transmittance).

4.12 With the instrument at zero absorbance with aqueous isopropyl alcohol, determine the absorbance of reagent blank. If the reagent blank exceeds 0.022 absorbance units, the analysis must be repeated using freshly distilled aniline.

4.13 Determine the absorbance of sample aliquot B at 440 nm using the reagent blank to set instrument at 0 absorbance.

Calculate the corrected absorbance of the aliquot as follows;
Corrected absorbance = (absorbance of B – absorbance of A)

4.14 From the corrected absorbance of the sample, determine the mg of gossypol in the sample aliquot by reference to a calibration graph prepared by taking1 , 2 , 3, 4, 5, 7 , 8, 10 ml aliquot of standard gossypol solution (0.025 mg / ml) into 25 ml volumetric flask.

4.15 To one set of aliquots designated C add 2 drops of 10 % aqueous thiourea, 1 drop of 1.2 N HCl and dilute to volume with aqueous isopropyl alcohol and determine its absorbance.

4.16 To the other set of aliquots designated D add 2 drops of aqueous thiourea, 2 drops of 1.2 N HCl and 2 ml of redistilled aniline.

4.17 Prepare a reagent blank containing 10 ml of aqueous acetone, 2 drops of aqueous thiourea and 2 ml of aniline (do not add HCl). Heat the standards and the reagent blank in boiling water bath for 30 minutes, cool and dilute to volume with aqueous isopropyl alcohol and determine their absorbance.

4.18 Determine corrected absorbance. Plot the corrected absorbance for each gossypol standard against mg of gossypol in 25 ml volume to obtain the calibration graph.

Total Gossypol

1. Introduction

Total gossypol refers to gossypol and gossypol derivatives both free and bound in cottonseed products which are capable of reacting with 3 - amino -1 propanol in dimethylformamide solution to form diaminopropanel complex which then reacts with aniline to form dianilinogossypol under the conditions specified in this method

2. Apparatus

(As described in determination of free gossypol)

3 Reagents

3.1 Solvents

- **Isopropyl alcohol** (n – propanol),
- **n – hexane** (B.P 68-69 ^{0}C), dimethyl formamide,
- **3 – amino 1 propanol** (propanolamine), free of colour,
- **glacial acetic acid** and
- **aniline**, the aniline should be redistilled over zinc dust using water cooled condenser.

3.2 Isopropyl alcohol- hexane mixture (60 + 40)

3.3 Complexing reagent prepared by pipetting 2 ml of 3 amino-1 propanol and 10 ml glacial acetic acid into a 100 ml volumetric flask, cooling to room temperature and diluting to volume with dimethyl formamide. Prepare reagent weekly and store in a refrigerator when not in use.

3.4 Gossypol or **Gossypol acetic acid** as primary standard.

3.5 Standard Gossypol solution prepared by weighing 25 mg of primary standard gossypol or 27.9 mg of gossypol acetic acid into a 50 ml volumetric flask. Dissolve in and make up to volume with complexing reagent. Solution is stable for 1 week if stored in refrigerator. The solution contains 0.50 mg gossypol per ml .Multiply gossypol acetic acid with 0.8962 to obtain mg of gossypol

4 Procedure

4.1 Weigh 0.5 – 0.75 gm sample accurately and transfer to a 50 ml volumetric flask

4.2 Add 10 ml complexing reagent. Prepare reagent blank containing 10 ml of complexing reagent in a 50 ml volumetric flask.

4.3 Heat sample and blank in a water bath at 100°C for 30 minutes, cool, dilute to volume with isopropyl alcohol- hexane mixture.

4.4 Filter through 11 cm filter paper into a 50 ml glass stoppered Erlenmeyer flask discarding first 5 ml of the filterate.

4.5 Pipette 2 ml of duplicate sample extract into 25 ml volumetric flasks. Pipette duplicate blank aliquots of same volume as sample aliquot into 25 ml volumetric flasks.

4.6 Dilute one set of sample and blank aliquots with isopropyl – hexane mixture and reserve as reference solutions for absorption measurement.4.7

4.7 Add 2 ml of aniline by pipette to the other set of samples and reagent blank aliquots, heat in a water bath for 30 minutes, cool, dilute to volume with isopropyl – hexane mixture and mix well. Allow to stand for 1 hour.

4.8 Measure the absorbance at 440 nm of reagent blank treated with aniline using blank aliquot without aniline as reference solution.

4.9 Determine absorbance of sample aliquot reacted with aniline using diluted sample aliquot without aniline as reference solution.

4.10 Subtract absorbance of reagent blank from that of sample aliquot treated with aniline to obtain corrected absorbance.

4.11 From corrected absorbance of sample aliquot determine mg gossypol in sample aliquot reference to a calibration graph prepared

ANNEX C
(normative)
Determination of ochratoxin A — High performance liquid chromatographic method with immunoaffinity column cleanup and fluorescence detection

1.Principle

Ochratoxin A (OTA) is extracted by acetonitrile-water. The extract is purified using an immunoaffinity column and ochratoxin A is determined by high performance liquid chromatography (HPLC) on a reverse-phase column and fluorescence detection. The result is verified, if required, by derivatization with boron trifluoride in methanolic solution.

2.Reagents

During the analysis, unless otherwise stated, use only reagents of recognized analytical grade and only distilled water or water of grade 1. Solvents shall be of quality for HPLC analysis.

2.1 Acetonitrile.

2.2 Methanol.

2.3 Sodium chloride (NaCl).

2.4 Glacial acetic acid, $\varphi(CH_3COOH) \geq 98$ %.

2.5 Tween-20.

2.6 Sodium bicarbonate ($NaHCO_3$).

2.7 Disodium hydrogen phosphate (Na_2HPO_4).

2.8 Potassium dihydrogen phosphate (KH_2PO_4).

2.9 Potassium chloride (KCl).

2.10 Hydrochloric acid, c(HCl) = 12 mol/l.

2.11 Ochratoxin A, in crystal form or as a film in ampoules.

2.12 Extraction solvent, mix 60 volume parts of acetonitrile (2.1) and 40 volume parts of water.

2.13 Phosphate buffered saline (PBS), dissolve 8 g NaCl (2.3), 1.2 g Na_2HPO_4 (2.7), 0.2 g KH_2PO_4 (2.8) and 0.2 g KCl (2.9) in about 990 ml water. Adjust pH to 7 with HCl (2.10) and dilute to 1 l with water.

2.14 Washing solution, dissolve 25 g NaCl (2.3), 5 g $NaHCO_3$ (2.6) and 0.1 ml Tween-20 (2.5) in 1 l water.

2.15 Mobile phase, mix 48 volume parts of acetonitrile (2.1) with 51 volume parts of water and 1 volume parts of glacial acetic acid (2.4) and degas this solution before use.

2.16 Toluene.

2.17 Solvent mixture, mix 99 volume parts of toluene (2.16) with 1 volume parts of glacial acetic acid (2.4).

2.18 Ochratoxin A stock solution.

Dissolve 1 mg of the ochratoxin A (crystals) (2.11) or the contents of 1 ampoule (if ochratoxin A has been obtained as a film) (2.11) in solvent mixture (2.17) to give a solution containing approximately 20 µg/ml to 30 µg/ml of ochratoxin A.

To determine the exact concentration, record the absorption curve between a wavelength of 300 nm and 370 nm in 5 nm steps in a 1 cm quartz cell (3.12) with solvent mixture (2.17) as reference. Identify the wavelength for maximum absorption by recording in 1 nm steps around the maximum as reference.

Calculate the mass concentration of ochratoxin A, ρOTA, in micrograms per millilitre of solution using Formula (1):

$$\rho_{OTA} = A_{max} \times \frac{M \times 100}{\kappa \times \delta} \quad (1)$$

where

A_{max} is the absorption determined at the maximum of the absorption curve(here: at 333 nm);

M is the relative molecular mass of ochratoxin A (M = 403 g/mol);

κ is the molar absorption coefficient of ochratoxin A, in solvent mixture(here: 544 m^2/mol);

δ is the path length of the cell in centimetres.

Store this solution at approximately −18 °C. A solution stored in this way is usually stable for 12 months. Check the concentration of the solution if it is older than 6 months.

2.19 Ochratoxin A standard solution, ρOTA = 1 µg/ml.

Evaporate under a nitrogen flow 1 ml of the stock solution (2.18) or the aliquot portion which is equivalent to an absolute amount of 100 µg of ochratoxin A to dryness and dilute to 100 ml with the mobile phase (2.15).

This solution can be stored in a refrigerator at 4 °C. Stability shall be checked.

2.20 Ochratoxin A calibration solutions.

Pipette suitable volumes of ochratoxin A standard solution (2.19), e.g. 0.05 ml, 0.1 ml, 0.25 ml, 0.5 ml and 1 ml into, for example, a 100 ml volumetric flask (3.15) and dilute to the mark with the mobile phase (2.15). The amount of ochratoxin A in the calibration solutions should cover the range of 0.05 ng to 1.0 ng per 100 µl injection volume. The calibration solutions should be freshly prepared from ochratoxin A standard solution (2.19) before each HPLC analysis.

2.21 Sodium hypochlorite solution, ρ(NaOCl) = 4 g/100 ml.

2.22 Boron trifluoride.

2.23 Boron trifluoride in methanol solution, $\rho(BF_3)$ = 14 g/100 ml.

2.24 Dichloromethane.

2.25 Sodium sulfate, anhydrous.

2.26 Elution solvent, mix 98 volume parts of methanol (2.2) and 2 volume parts of glacial acetic acid (2.4).

WARNING — Use a well maintained fume hood. Avoid contact with skin, eyes, and respiratory tract.

3.Apparatus and equipment

Usual laboratory equipment and, in particular, the following.

3.1 Analytical balance, accurate to 10 mg.

3.2 Blender, 1 l jar and cover, explosion-proof.

3.3 Filter paper,

a) folded filter paper, or

b) glass microfibre filter.

3.4 Centrifuge tube, 50 ml.

3.5 Membrane filter for aqueous solutions, made of polytetrafluoroethylene (PTFE), with a diameter of 25 mm and a pore size of 0.2 µm.

3.6 Immunoaffinity column, which shall contain antibodies raised against ochratoxin A, ToxinFast® Ochratoxin A Immunoaffinity Column (Huaan Magnech)1) or equivalent.

3.7 Glass syringe, 10 ml.

3.8 Vacuum pump.

3.9 Rotary evaporator, with a water bath capable of being controlled between 20 °C and 50 °C.

3.10 Laboratory mill, suitable to grind to 1 mm.

3.11 UV-Spectrometer, suitable for measurement at wavelengths of 300 nm up to 370 nm, having a spectral band width of not more than ± 2 nm.

3.12 Quartz cells, with 1 cm optical path length and no significant absorption between wavelengths of 300 nm and 370 nm.

3.13 Conical flask, 150 ml.

3.14 Sieve, with an aperture size of not more than 1 mm.

3.15 Volumetric flask, 100 ml.

3.16 Microsyringe, of capacity 500 µl.

3.17 HPLC apparatus, comprising

a) **high performance liquid chromatograph**, eluent reservoir, a pump, an injection system, a fluorescence detector with variable wavelength setting and a data processing, e.g. an integrator with plotter, and

b) **analytical reverse-phase HPLC separating column**, C_{18}, which ensures a baseline resolved resolution of the ochratoxin A peak from all other peaks.

length:	150 mm
internal diameter:	4.6 mm
spherical particles of size:	5 µm

4. Procedure

4.1 General

The whole analytical procedure should be performed in one working day. If several samples are processed at the same time all samples should be analysed during the following night using an automatic sample injector.

4.4 Extraction of ochratoxin A from the sample

4.4.1 Extraction

Place 25 g (*m*), weighed to the nearest 0.1 g, of the sample into a conical flask or a blender (3.2), add 100 ml extraction solvent (2.12) (V_1). Cover and shake for 30 min or blend for 3 min. The extract is centrifuged at 8 000*g* for 5 min or filtered through folded filter paper [3.3 a)].

4.4.2 Dilution

Pipete 4.0 ml (V_2) filtered extract into 50 ml centrifuge tube (3.4), and dilute with 26.0 ml (V_3) PBS solution (2.13). The diluted extract is centrifuged at 8000 *g* for 5min, and then collected as extract A.

Alternatively, pipet 6.0 ml (V_2) filtered extract into 50 ml centrifuge tube (3.4), and dilute with 39.0 ml (V_3) PBS solution (2.13). The diluted extract is filtered through a glass microfibre filter [3.3 b)], and then collected as extract B.

4.5 Immunoaffnity column cleanup

Pass all extract A or 30.0 ml extract B (V_4) through the OTA immunoaffinity column at a flow-rate of about 1 to 2 drops per second, followed by 10 ml washing solution (2.14) and 10 ml distilled water at 2 drops per second. Elute OTA with 1.5 ml elution solvent (2.26). Evaporate eluate to dryness over steam bath under N_2 cautiously without exceeding 40°C. Redissolve in 0.5 ml (V_5) mobile phase (2.15). Transfer to liquid chromatography (LC) vial. If necessary, the sample can be filtered through PTFE membrane (3.5) before analysis by LC. Elution of ochratoxin A and the subsequent steps in the procedure described in this clause can depend on the type of immunoaffinity columns used. The elution volume, for example, should be checked to ensure it is appropriate for the type of column used.

Be careful not to overload the immunoaffinity column.

4.6 HPLC operating conditions

When the column [3.17 b)] and the mobile phase (2.15) are used the following settings have been found to be appropriate.

Flow rate:	1 ml/min	
Fluorescence detection:	Excitation wavelength:	333 nm
Emission wavelength:	460 nm	
Injection volume:	50 µl	
Column temperature:	35 °C	

4.7 Calibration graph

Prepare a calibration graph at the beginning of the analysis and whenever the chromatographic conditions are changed.

Inject at least four calibration solutions of different suitable concentrations (see 2.20). Plot the fluorescence values (peak height or peak area) of the ochratoxin A calibration solutions (2.20) against the ochratoxin A mass concentrations in nanograms. Ensure that the linearity check is carried out [5].

4.8 Identification

Identify ochratoxin A by comparing the retention time of the sample with that of the standard substance. Sometimes it can be necessary to identify the ochratoxin A peak by simultaneous injection of sample test solution and standard solution.

4.9 Determination

Chromatograph the sample. To carry out the determination by the external standard method, integrate the peak area or determine the peak height, and compare the results with the corresponding values for the standard substance with the nearest peak area/height, or use a calibration graph. In the case of a calibration graph, additional solutions with concentrations within the linear range may be prepared for the calibration graph.

Inject equal volumes of sample test solution and standard solution used for the calibration graph.

Read off the mass of ochratoxin A, in nanograms, corresponding to the fluorescence of the sample test solution from the calibration graph.

If the ochratoxin A response of the sample is outside the calibration graph, adjust the amount of sample injected by concentrating or diluting the sample test solution.

4.10 Confirmation

If necessary, confirm the identity by disappearance of the peak at the retention time for ochratoxin A and appearance of a new peak at the same retention time as that of standard methyl ester of ochratoxin A.

Take 200 µl of the sample test solution prepared as in 4.5, transfer into a pear-shaped flask and evaporate to dryness in a rotary evaporator (3.9). Take up the residue in 0.5 ml of dichloromethane (2.24), and add 1 ml of boron trifluoride methanol solution (2.23).

Stopper the flask tightly and heat it in a water bath at 50 °C to 60 °C for 15 min. After cooling, transfer the solution into a 50 ml separating funnel containing 15 ml of water, shake three times with 5 ml of dichloromethane each time for 30 s. Combine the organic phases in a second 50 ml separating funnel, add 10 ml of water for washing and shake for 30 s.

Subsequently filter the dichloromethane phase through sodium sulfate (2.25) into a pear-shaped flask, evaporate to dryness, take up in 500 µl of mobile phase (2.15) and subject this solution to chromatographic separation under the conditions as described in 4.6. The completeness of derivatization can be checked from the chromatograms. It is possible with this procedure to verify mass fractions of ochratoxin A of not less than 0.4 µg/kg.

An adequate standard solution (2.20) should be treated separately to check the retention times of the ochratoxin A methyl ester and the completeness of the derivatization.

9 Calculation

Calculate the mass fraction X_1 of ochratoxin A in micrograms per kilogram using Formula (2) (external standard method):

$$X_1 = \frac{c \times V_1 \times (V_2 + V_3) \times V_5}{m \times V_2 \times V_4} \qquad (2)$$

where

V_1 is the volume of the solvent used for extraction, in millilitres (V_1 = 100 ml);

V_2 is the volume of the extract used for dilution, in millilitres (extract A V_2 = 4 ml, or extract B V_2 = 6 ml);

V_3 is the volume of the PBS solution used for dilution, in millilitres(extract A V_3 = 26 ml, or extract B V_3 = 39 ml);

V_4 is the volume of the diluted extract used for cleanup, in millilitres (V_4 = 30 ml);

V_5 is the volume of the diluted extract used for cleanup, in millilitres (V_4 = 30 ml);

c is the concentration of OTA in final solution used for LC determination, in µg/l;

m is the mass of the test portion, in grams.

Report the result in the relevant format and after rounding to two decimal places.

NOTE Relevant legislation can apply.

Indicate whether or not a correction for recovery has been applied.