



## DRAFT TANZANIA STANDARD

Edible coconut oil – Specification

TANZANIA BUREAU OF STANDARDS

## Edible coconut oil – Specification

### 0 Foreword

Coconut oil is extracted from the kernel or meat of mature coconuts harvested from the coconut palm (*Cocos nucifera* L.). It has various applications including cooking since the higher saturated fat content allows for greater stability at higher temperatures and better shelf life. It is one of traded edible vegetable oils in Tanzania.

This Tanzania Standard has been prepared to ensure the safety and quality of edible coconut 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 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 the methods of sampling and test for edible coconut oil.

### 2 Reference

The following referenced documents are indispensable for the application of this Tanzania Standard. 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 fats and oils — Specification*

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

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 ° C– Reference method*
- TZS 1329, *Animal and vegetable fats and oils – Determination of refractive index*
- 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 following terms and 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 coconut oil

edible oil derived from the kernel of *Cocos nucifera* Linn.

#### 3.4 virgin coconut oil

coconut 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 coconut oil

coconut oil obtained, by mechanical procedures and/or solvent extraction and subjected to refining processes

### 4 Requirements

#### 4.1 General requirements

Edible coconut 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.

#### 4.2 Specific requirements

Edible coconut 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 coconut oil**

S. No.	Parameter	Requirement	Test method
i.	Relative density( at 40 °C/water at 20 °C)	0.908-0.921	TZS 1328
ii.	Refractive index,( ND 40 ° C )	1.448 - 1.450	TZS 1329
iii.	Saponification value, mg KOH/g oil	248 - 265	TZS 1325
iv.	Iodine value (Wij's), g/100	6.3– 10.6	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 ° C, % m/m, max.	0.2	TZS 1326
viii.	Insoluble impurities, % m/m, max.	0.05	TZS 1336
ix.	Free Fatty Acids(FFA) as lauric acid, % max.	Refined oils: 0.2 Cold pressed and virgin oils: 1.5	TZS 1331
x.	Peroxide value, mEq peroxide- oxygen/kg oil, max	Refined oils :10 Cold pressed and virgin oils: 15	TZS 1324
xi.	Total sterols(mg/kg)	400-1200	TZS 1371
xii.	Reichert values	6 - 8.5	See Annex B
xiii.	Polenske values	13 - 18	See Annex B

#### 4.3 Food additives

##### 4.3.1 General

**4.3.1.1** Food additives may be used during processing of edible coconut 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.

**Table 2 – Requirements for antioxidant in edible coconut 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- <i>alpha</i> - , mg/kg, max	300 (singly or in combination)	TZS 1370
307b	Tocopherol concentrate, mixed , mg/kg, max		
307c	Tocopherol, dl- <i>alpha</i> , 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	

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		

#### 4.3.4 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 coconut oil may be fortified in accordance with TZS 1313.

## 5 Hygiene

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

## 6 Contaminants

### 6.1 Pesticide residues

Edible coconut oil shall comply with relevant maximum pesticide residue limits established by the Codex Alimentarius Commission.

### 6.2 Soap content

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

### 6.3. Metals contaminants

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

## 7 Sampling and Tests

### 7.1 Sampling

Edible coconut 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, pure analytical grade chemicals and distilled water shall be used in tests.

## 8 Packaging, marking and labeling

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

### 8.1 Packaging

Edible coconut oil shall be packaged in containers made from food grade packaging material and sealed in a manner that will safeguard the hygienic, nutritional and organoleptic properties of the product throughout the shelf life of the product.

### 8.2 Marking and labeling

**8.2.1** Containers of edible coconut oil 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(optional)

**8.2.2** The containers shall also be marked with the TBS Standards 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-0.7	TZS 288(Part 1 &2)	
C8:0	4.6-10.0		
C10:0	5.0-8.0		
C12:0	45.1-53.2		
C14:0	16.8-21.0		
C16:0	7.5-10.2		
C16:1	ND		
C17:0	1.0-3.0		
C17:1	12.0-19.0		
C18:0	1.0-3.5		
C18:1	ND-0.2		
C18:2	ND-0.2		
C18:3	ND-0.2		
C 20:0	ND-0.2		
C 20:1	ND-0.2		
C20:2	ND		
C22:0	ND		
C22:1	ND		
C22:2	ND		
C24:0	ND		
C24:1	ND		
<p><b>NOTE-</b> Free fatty acid composition is expressed as % of total fatty acids ND-none detectable,defined as ≤0.05</p>			

**ANNEX B  
(normative)**

**Determination of Reichert-Meissl and Polenske Value**

**Principle**

The material is saponified by heating with glycerol sodium hydroxide solution and then split by treatment with dilute sulfuric acid. The volatile acids are immediately steam distilled. The soluble volatile acid in the distillate are filtered out and estimated by titration with standard sodium hydroxide solution.

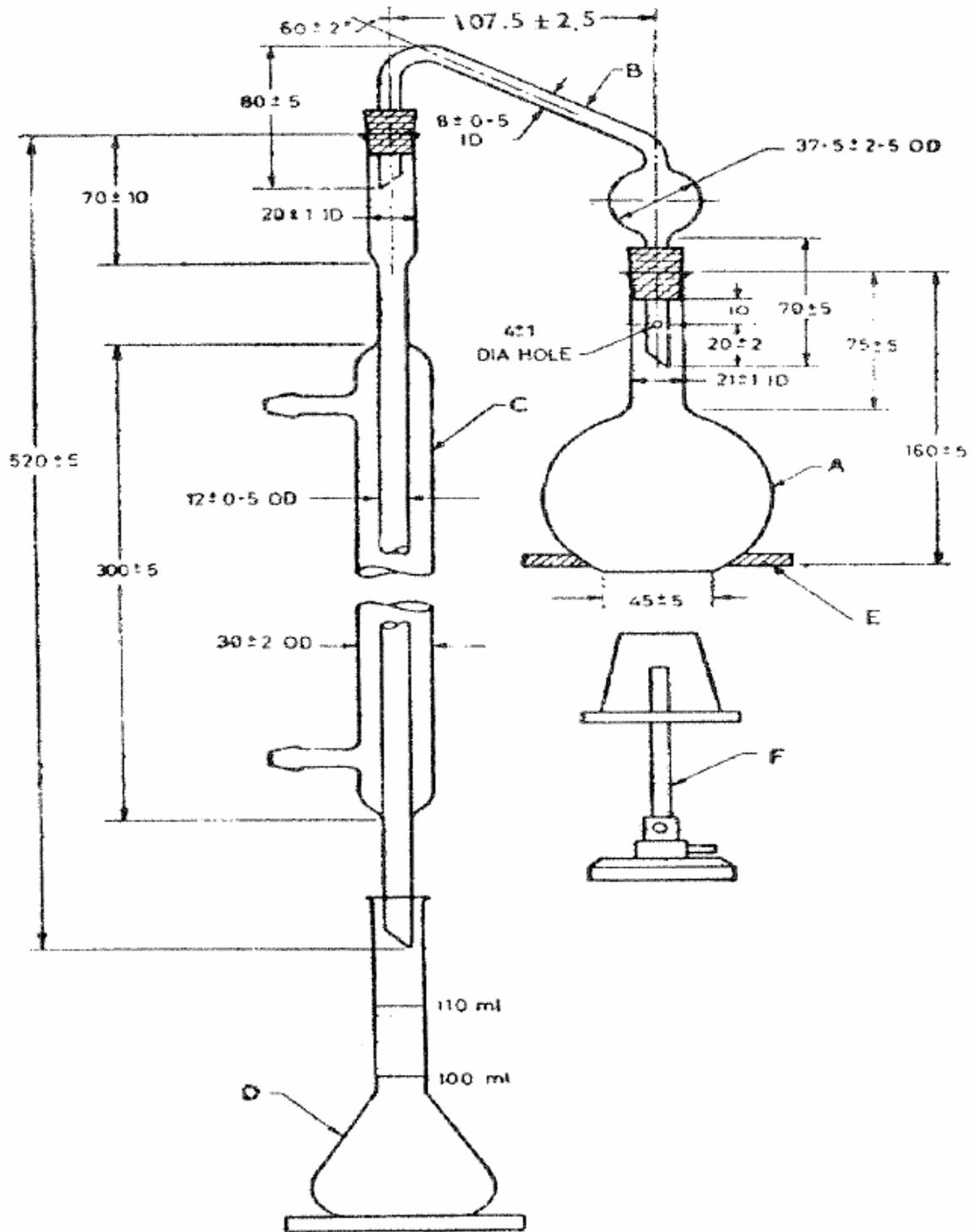
**Analytical Importance**

These determinations have been used principally for analysis of butter and margarines. Butter fat contains mainly butyric acid glycerides. Butyric acid is volatile and soluble in water.

No other fat contains butyric acid glycerides, and therefore, the Reichert-Meissl value of the butter fat is higher than that for any other fat. Coconut oil and palm kernel oil contain appreciable quantities of caprylic capric and lauric acid glycerides. These fatty acids are steam volatile but not soluble in water, and hence give high Polenske value.

**Apparatus**

- a. An **all-glass distillation** assembly conforming to specifications given in AOCS Official Methods Cd 5-40 or Methods of Analysis, AOAC- 17th Edn.,2000 Figure 925.41, chapter 41 page 14 or distillation apparatus as shown in the diagram below;
- b. **25 ml beaker**
- c. **100 ml graduated cylinder**
- d. **100 ml pipette**
- e. **Graduated burette**
- f. **Asbestos board with a hole about 65 mm dia** for supporting the flask over the burner. During distillation the flask shall fit snugly into the hole of the board to prevent the flame from impinging on the surface of the flask above the hole.
- g. **Bunsen burner** sufficiently large to allow completion of distillation in the prescribed time.



All dimensions in millimetres.

Figure 1: Reichert-Meissl Distillation Apparatus

## Reagents

- a. **Glycerine**
- b. **Concentrated sodium hydroxide solution:** 50 % (w /w) Dissolve Sodium Hydroxide in equal wt of water and store solution in a bottle. Use clear solution free from deposit.
- c. **Pumice stone grains**
- d. **Dilute sulfuric acid solution:** Approximately 1.0 N
- e. **Sodium hydroxide solution:** 0.1N solution in water, accurately standardised
- f. **Phenolphthalein indicator:** Dissolve 0.1 g of phenolphthalein in 100 ml of ethyl alcohol
- g. **Ethyl alcohol:** 90% by volume and neutral to phenolphthalein

## Procedure

1. Weigh accurately  $5 \pm 0.1$ g of filtered oil or fat sample into a clean, dry, 300ml distilling flask.
2. Add 20 ml of glycerine and 2 ml of concentrated sodium hydroxide solution, and heat with swirling over a flame until completely saponified, as shown by the mixture becoming perfectly clear.
3. Cool the contents slightly and add 90 ml of boiling distilled water, which has been vigorously boiled for about 15 min. After thorough mixing the solution should remain clear. If the solution is not clear (indicating incomplete saponification) or is darker than light yellow (indicating over-heating), repeat the saponification with a fresh sample of the oil or fat. If the sample is old, the solution may sometimes be dark and not clear.
4. Add about 0.6 - 0.7 gm of pumice stone grains, and 50 ml of dilute sulfuric acid solution.
5. Immediately connect the flask to the distillation apparatus. Place the flask on asbestos board so that it fits snugly into the aperture. This will prevent the flame from impinging on the surface of the flask above the level of the liquid and avoid super heating.
6. Heat very gently until the liberated fatty acids melt and separate. Then set the flame so that 110 ml of distillate shall be collected within 19 to 21 min. The beginning of the distillation is to be taken as the moment when the first drop of the distillate falls from the condenser in the receiving flask. Keep the water in the condenser flowing at a sufficient speed to maintain the temperature of the outgoing water from the condenser between 15 and 20°C. Collect the distillate in a graduated flask.
7. When the distillate exactly reaches the 110 ml mark on the flask, remove the flame and quickly replace the flask by a 25 ml measuring cylinder. Stopper the graduated flask and without mixing placed it in a water bath maintained at 15°C for 10 min so that the 110 ml graduation mark is 1 cm below the water level in the bath. Swirl round the contents of the flask from time to time.
8. Remove the graduated flask from the cold water bath, dry the outside and mix the content gently by inverting the flask 4 to 5 times without shaking. Avoid wetting the stopper with the insoluble acids.
9. Filter the liquid through a dry, 9 cm Whatman No. 4 filter paper. Reject the first 2-3 ml of the filtrate and collect the rest in a dry flask. The filtrate should be clear.
10. Pipette 100 ml of the filtrate and add 5 drops of the phenolphthalein solution, and titrate against standard 0.1N sodium hydroxide solution.
11. Run a Blank Test without the fat, but using the same quantities of the reagents.

### Calculation

Reichert-Meissl Value =  $(A - B) \times N \times 11$

where,

A = Volume in ml of standard sodium hydroxide solution required for the test;

B = Volume in ml in standard sodium hydroxide solution required for the blank; and

N = Normality of standard sodium hydroxide solution.

### Determination of Polenske Value

After titrating the soluble volatile acids, detach the still head and rinse the condenser with three successive 15 ml portions of cold distilled water passing each washing separately through the measuring cylinder, 110 ml graduated flask and the filter paper and allow all of it to pass through.

Discard all the washings. Place the funnel on a clean conical flask. Dissolve the insoluble fatty acids by three similar washings of the condenser, the measuring cylinder, the 110 ml flask with stopper, and the filter paper with 15 ml portions of ethyl alcohol.

Combine the alcoholic washings in a clean flask, add 5 drops of phenolphthalein indicator solution, and titrate with standard (0.1N) sodium hydroxide solution.

$$\text{Polenske value} = 10 \times V \times N$$

where,

V = Volume in ml of standard sodium hydroxide solution required for the test; and

N = Normality of the standard sodium hydroxide solution.

Note:- Unless the directions are followed in every detail reproducible results cannot be obtained.

**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,  $\varphi(\text{CH}_3\text{COOH}) \geq 98\%$ .**

**2.5 Tween-20.**

**2.6 Sodium bicarbonate ( $\text{NaHCO}_3$ ).**

**2.7 Disodium hydrogen phosphate ( $\text{Na}_2\text{HPO}_4$ ).**

**2.8 Potassium dihydrogen phosphate ( $\text{KH}_2\text{PO}_4$ ).**

**2.9 Potassium chloride (KCl).**

**2.10 Hydrochloric acid,  $c(\text{HCl}) = 12 \text{ mol/l}$ .**

**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  $\text{Na}_2\text{HPO}_4$  (2.7), 0.2 g  $\text{KH}_2\text{PO}_4$  (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  $\text{NaHCO}_3$  (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  $\mu\text{g/ml}$  to 30  $\mu\text{g/ml}$  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,  $\rho_{\text{OTA}}$ , in micrograms per millilitre of solution using Formula (1):

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

where

- $A_{\text{max}}$  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);
- $\kappa$  is the molar absorption coefficient of ochratoxin A, in solvent mixture (here: 544 m<sup>2</sup>/mol);
- $\delta$  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, $\rho_{\text{OTA}} = 1$ 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, $\rho(\text{NaOCl}) = 4$ g/100 ml.

#### 2.22 Boron trifluoride.

#### 2.23 Boron trifluoride in methanol solution, $\rho(\text{BF}_3) = 14$ 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  $\mu\text{m}$ .

**3.6 Immunoaffinity column**, which shall contain antibodies raised against ochratoxin A, ToxinFast® Ochratoxin A Immunoaffinity Column (Huaan Magnech)<sup>1</sup>) 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  $\pm 2$  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  $\mu\text{l}$ .

**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<sub>18</sub>, 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  $\mu\text{m}$

## 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*<sub>1</sub>). Cover and shake for 30 min or blend for 3 min. The extract is centrifuged at 8 000g for 5 min or filtered through folded filter paper [3.3 a)].

#### 4.4.2 Dilution

Pipet 4.0 ml (*V*<sub>2</sub>) filtered extract into 50 ml centrifuge tube (3.4), and dilute with 26.0 ml (*V*<sub>3</sub>) 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*<sub>2</sub>) filtered extract into 50 ml centrifuge tube (3.4), and dilute with 39.0 ml (*V*<sub>3</sub>) 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 Immunoaffinity column cleanup

Pass all extract A or 30.0 ml extract B (*V*<sub>4</sub>) 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<sub>2</sub> cautiously without exceeding 40°C. Redissolve in 0.5 ml (*V*<sub>5</sub>) 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):

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

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.

DRAFT FOR STAKEHOLDER'S COMMENTS