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DRAFT TANZANIA STANDARD

Edible olive oil and edible olive pomace oil — Specification

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TANZANIA BUREAU OF STANDARDS

Edible olive oil and edible olive pomace oil — Specification

0 Foreword

Olive oil is produced by pressing whole olives. Once the mechanical oil extraction of olive oil is complete, approximately 5-8% of the oil remains in the pulp, which then needs to be extracted with the help of solvents. Olive pomace oil is extracted from olive pulp after the first press. Edible olive oil and edible olive pomace oil are commonly used in cooking, whether for frying or as a salad dressing. They are one of the traded edible vegetable oils in Tanzania.

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

In the preparation of this Tanzania Standard considerable help was derived from:

Codex Stan 33-1981 Amended in 2013*, Codex standard for olive oils and olive pomace oils* published by the Codex Alimentarius Commission,

International Olive Council COI/T.20/Doc. No 11 /Rev. 3 of 2017, *method for the determination of stigmastidienes in vegetable oils* published by International Olive Council

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 the 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 test for edible olive oil and edible olive pomace oil intended for direct human consumption.

2. Normative references

The following referenced standards are indispensable for the application of this standard. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced standard (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 spectrophotometric 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 esters of fatty acids*

TZS 538, *Packaging and labeling of foods*

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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 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 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 1368, *Animal and vegetable fats and oils — Determination of ultraviolet absorbance expressed as specific UV extinction*

TZS 1370, *Animal and vegetable fats and oils — Determination tocopherol and tocotrienol content by HPLC*

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

TZS 1372, *Olive oils and olive pomace oils — Determination of wax content by capillary gas chromatography*

TBS/AFDC 4(6108), *Oils and Fats — Determination of the difference between actual and theoretical content of triacyglycerols with ECN 42 in olive oils*

TBS/AFDC 4(6109), *Animal and vegetable fats and oils — Determination of the composition of fatty acids in the 2-position of the triglyceride molecules*

TBS/AFDC 25(5981)/ISO 5496, *Sensory analysis -- Methodology -- Initiation and training of assessors in the detection and recognition of odours*

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TBS/AFDC 25(5982)/ISO 11037, *Sensory analysis -- Guidelines for sensory assessment of the colour of products*

TBS/AFDC 25(6121)/ISO 3972, *Sensory analysis -- Methodology -- Method of investigating sensitivity of taste*

3. Terms and definitions

For the purpose of this Tanzania Standard the following terms and definitions 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 olive oil
edible oil obtained solely from the fruit of the olive tree (*Olea europaea* L.), to the exclusion of oils obtained using solvents or re-esterification processes and of any mixture with oils of other kinds.

3.4 extra virgin olive oil
virgin olive oil with a free acidity, expressed as oleic acid, of not more than 0.8 grams per 100 grams and whose other characteristics correspond to those laid down for this category

3.5 virgin olive oils
oils obtained from the fruit of the olive tree (*Olea europaea* L.) solely by mechanical or other physical means under conditions, particularly thermal conditions, that do not lead to alterations in the oil, and which have not undergone any treatment other than washing, decanting, centrifuging and filtration.

3.6 olive-pomace oil
olive oil that is extracted from olive pulp after the first press treated with solvents or other physical treatments, to the exclusion of oils obtained by re-esterification processes and of any mixture with oils of other kinds.

3.7 refined olive oil
olive oil obtained from virgin olive oils by refining methods which do not lead to alterations in the initial glyceridic structure.

3.8 refined olive-pomace oil
oil obtained from crude olive-pomace oil by refining methods which do not lead to alterations in the initial glyceridic structure.

4. Requirements

4.1 General requirements
Edible olive oil and edible olive pomace 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.

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4.1.1 Edible olive oil and edible olive pomace oil shall have sensory characteristics as shown in Table 1.

Table 1–Sensory characteristics

S/N	Characteristic	Refined olive oil	Olive oil	Refined olive-pomace oil	Olive-pomace oil	Test method
1	Odour	Acceptable	Good	Acceptable	Acceptable	TBS/AFDC 25(5981
2	Taste	Acceptable	Good	Acceptable	Acceptable	TBS/AFDC 25(6121)
3	Colour	light yellow	light, yellow to green	light, yellow to brownish yellow	light, yellow to green	TBS/AFDC 25(5982)

4.1.2 The clarity of the product shall be judged by the absence of turbidity after keeping the filtered sample at 20 °C for 24 h.

4.2 Specific requirements

4.2.1 Edible olive oil and edible olive pomace oil shall have specific requirements as shown in Table 2.

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Table 2 — Specific requirements of edible olive oil and edible olive- pomace oil

S/N	Characteristic	Requirements for types					Test method
		Olive pomace oil	**Refined olive pomace oil**	**Virgin olive oil**	**Refined olive oil**	**Olive oil**	
1	Refractive index (ND 20 °C)	1.4680-1.4707		1.4677-1.4705			TZS 1329
2	Relative density at 20 °C/Water 20ºC	0.910 - 0.916					TZS 1328
3	Saponification value, mg KOH/g	182-193		184-196			TZS 1325
4	Iodine value (Wijs), g/100	75-92		75-94			TZS 1327
5	Unsaponifiable matter, g/kg, max	30		15			TZS 1332
6	Moisture and volatile matter at 105 °C, % m/m, max.	0.1		0.2	0.1	0.1	TZS 1326
7	Insoluble impurities, % m/m, max	0.05		0.1	0.05		TZS 1336
8	Peroxide value, mEq oxygen/kg, max.	15	5	20	5	15	TZS 1324
9	Free fatty acid as (oleic acid) ,%max.	0.5	0.15	1.0	0.15	0.5	TZS 1331
10	Test for presence of olive residue (pomace) oil in olive oil	Positive	Not applicable	Negative			ANNEX A
11	Total sterols, mg/kg, min.	1600	1800	1000			TZS 1371
12	Erythrodiol and uvaol (% total sterols), max.	Not applicable		4.5			TZS 1371
13	Wax content, mg/kg	>350		≤250	≤350		TZS 1372
14	Absorbency in UV at 270nm, max.	1.70	2.00	0.25	1.10	0.90	TZS 1368
15	Soap content, % m/m, max.	0.005					TZS 1322

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16	Stigmastadiene content, mg/kg, max	Not applicable	Not applicable	0.15	Not applicable	Not applicable	ANNEX B
17	Difference between actual and theoretical ECN 42 Triglyceride content, max.	0.5		0.2	0.3		TBS/ AFDC 4(6108)

4.2.2 Absorbency in ultra-violet at 232 nm as determined by TZS 1368 shall be **2.50[4] max f**or Extra virgin olive oil and **2.60[4] max** for Virgin olive oil

4.2.3 Absorbency in ultra-violet at 270 nm for Extra virgin olive oil shall be **0.22 max** as determined by TZS 1368

4.2.4 Fatty acid composition as determined by TZS 288 (Part 1& 2) (% total fatty acids) shall be as shown in Table 3.

Table 3 – **Fatty acid composition**

Fatty acid	Virgin olive oils	Olive oil and refined olive oil	Olive-pomace oil and refined olive pomace oil
C14:0	0.0 – 0.05	0.0 – 0.05	0.0 – 0.05
C16:0	7.5 – 20.0	7.5 – 20.0	7.5 – 20.0
C16:1	0.3 – 3.5	0.3 – 3.5	0.3 – 3.5
C17:0	0.0 – 0.3	0.0 – 0.3	0.0 – 0.3
C17:1	0.0 – 0.3	0.0 – 0.3	0.0 – 0.3
C18:0	0.5 – 5.0	0.5 – 5.0	0.5 – 5.0
C18:1	55.0 – 83.0	55.0 – 83.0	55.0 – 83.0
C18:2	3.5 – 21.0	3.5 – 21.0	3.5 - 21.0
C18:3[3]	-	-	-
C20:0	0.0 – 0.6	0.0 – 0.6	0.0 - 0.6
C20:1	0.0 – 0.4	0.0 – 0.4	0.0 - 0.4
C22:0	0.0 – 0.2	0.0 – 0.2	0.0 - 0.3
C24:0	0.0 – 0.2	0.0 – 0.2	0.0 - 0.2

4.2.5 Saturated fatty acids at the 2-position in the triglyceride (sum of palmitic & stearic acids) as determined by TBS/AFDC4(6109) shall be as shown in the table 4.

Table 4 – **Composition Characteristics**

Type of oil	Requirement (% Max)
Virgin olive oils	1.5
Refined olive oil	1.8
Olive oil	1.8
Refined olive-pomace oil	2.2
Olive-pomace oil	2.2

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4.3 Food additives

4.3.1 Edible olive oil and edible olive-pomace oil may contain alpha-tocopherols d-*alpha* tocopherol, mixed tocopherol concentrate, dl-*alpha*-tocopherol, to restore natural tocopherol lost during the refining process. The concentration of alpha-tocopherol in the final product shall not exceed 200 mg/kg when tested in accordance with TZS 1370.

4.3.2 Edible olive oil and edible olive-pomace oil may also contain food additives which are prescribed in Codex Stan 192. In addition, food additives shall not be used in virgin or cold pressed oils.

4.4 Fortification
Edible olive oil and edible olive pomace oil may be fortified in accordance with TZS 1313.

5. Hygiene

Edible olive oil and edible olive pomace oil shall be produced, processed and handled in accordance with TZS 109.

6. Contaminants

6.1 Metal contaminants
The level of metal contaminants in edible olive oil and edible olive pomace oil shall conform to the limits specified in Table 5.

Table 5 — Limits for metal contaminants in edible olive oil and edible olive pomace oil

S/N	Contaminant	Maximum level	Test method
1	Iron, mg/kg	3	TZS 1335
2	Copper, mg/kg	0.1	TZS 1335
3	Lead, mg/kg	0.1	TZS 268
4	Arsenic, mg/kg	0.1	TZS 76
5	Nickel, mg/kg	0.1	TZS 1335

6.2 Pesticide residues
The maximum allowable pesticide residue limits in edible olive oil and edible olive pomace oil shall be as prescribed in the relevant Codex Alimentarius Standard.

6.3 Mycotoxins

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

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

6.4.3 Edible olive oil and edible olive pomace oil shall be free from Poly-Hydroxy alkanoates (PHA).

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7. Sampling and test methods

7.1 Sampling

Edible olive oil and edible olive pomace oil shall be sampled as prescribed in TZS 54.

7.2 Test method

7.2.1 Unless specified otherwise, analytical grade chemicals and distilled water shall be used in tests.

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

8 Packaging, marking and labeling

8.1 Edible olive oil and edible olive pomace oil shall be supplied in suitably sealed and closed food grade containers of material protecting the product from spoilage or contamination without adversely affecting the physical, chemical and sensory quality of the product.

8.2 Edible olive oil and edible olive pomace oil shall be marked and labeled in accordance with TZS 538. In addition, each container of edible olive oil and edible olive pomace 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)

8.3 The containers shall also be marked with the TBS Standards Mark of Quality.

NOTE — The TBS Standards Mark of Quality may be used by manufacturers only under licence from TBS. Particulars of conditions under which the licences are granted, may be obtained from TBS.

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ANNEX A
(Normative)
Test for presence of olive residue (pomace) oil in olive oil

A.1 Principle

The test is based on the temperature of precipitation of salts of fatty acids after saponification.

A.2 Preparation of sample

The sample is filtered through paper at a temperature slightly above the melting point of certain solid constituents which could separate from the fluid fatty matter.

A.3 Procedure

A.3.1. Saponify 1 g of oil by boiling for 10 min with 5 ml alcoholic KOH (42.5 gm KOH in 72 ml water made up to 500 ml with 95 % ethyl alcohol).

A.3.2. After cooling add 1.5 ml aqueous acetic acid (1+ 2 by volume such that 1.5 ml exactly neutralizes 5 ml of aqueous alcoholic KOH) and 50 ml of 70 % ethanol warmed to 50 °C.

A.3.4 Mix, insert a thermometer and allow to cool.

A.4 Results

If a precipitate forms above 40 °C, the test for the presence of olive residue oil is positive. Allow to cool to ambient temperature for 12 h. Observe solution again. The formation of a flocculent precipitate floating in the middle of the liquid also indicates that the test is positive. A cloudiness not forming into flakes does not indicate the presence of olive residue oil.

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ANNEX B
(Normative)
Determination of stigmastadienes in vegetable oils

1. Principle

Isolation of unsaponifiable matter. Separation of steroidal hydrocarbon fraction by column chromatography on silica gel and analysis by capillary gas chromatography.

2. Apparatus

2.1. 250 ml **flat-bottomed flasks**

2.2 Reflux condenser.

2.3. 500 ml **separating funnels**.

2.4. 100 ml **round-bottomed flasks**.

2.5. Rotary evaporator.

2.6. Glass chromatography column (1.5 cm i.d. by 50 cm length) with Teflon stopcock and a plug of glass wool fibre at the bottom. To prepare the silica gel column, pour hexane into the chromatography column to a depth of approximately 5 cm and then fill with a slurry of silica gel in hexane (15 g in 40 ml) with the help of hexane portions. Allow to settle and finish settling by applying slight vibration. Add anhydrous sodium sulphate to a height of 0.5 cm, finally elute the excess hexane.

2.7 Gas chromatograph with flame ionisation detector, split or on-column injector and oven programmable to within ± 1ºC.

2.8. **Fused silica capillary columns** for gas chromatography (0.25 or 0.32 mm i.d. by 25 m length) coated with 5% phenylmethylsilicone phase, 0.25 µm film thickness.

Note 1 — Other columns of similar or lower polarity can be used.

2.9. **Integrator-recorder** with possibility of valley-valley integration mode.

2.10. 5-10 µl **microsyringe** for gas chromatography with hardened needle.

3. Reagents

All reagents should be of analytical grade unless otherwise specified. The water used should be distilled water, or water of at least equivalent purity.

3.1. **Hexane** or **mixture of alkanes** of b.p. interval 65-70ºC**,** distilled with rectifying column.

Note 2 — The hexane must be distilled to remove impurities.

3.2. 96 v/v **ethanol.**

3.3. Anhydrous sodium sulphate.

3.4. Alcoholic potassium hydroxide solution at 10% w/v. Add 10 ml of water to 50 g potassium hydroxide, stir, and then dissolve the mixture in ethanol to 500 ml.

Note 3 — Alcoholic potash turns brown on standing. It should be prepared freshly each day and kept in well-stoppered dark glass bottles.

3.5. Silica gel 60 for column chromatography, 70-230 mesh (Merck ref. 7734 or similar).

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Note 4 — Usually, silica gel can be used directly from the original container without any treatment. However, some batches of silica may show low activity resulting in bad chromatographic separations. Under these circumstances, the silica gel should be treated in the following way: deactivate the silica gel by heating for a minimum of four hours at 550 ºC. After heating, place the silica gel in a desiccator while the gel is cooling and then transfer the silica gel to a stoppered flask. Add 2% of water and shake until no lumps can be seen and the powder flows freely.

3.6. Stock solution (200 ppm) of cholesta-3,5-diene (Sigma, 99% purity) in hexane (10 mg in 50 ml).

3.7. Standard solution of cholesta-3,5-diene in hexane at a concentration of 20 ppm, obtained by dilution of the stock solution (5.6).

Note 5 — If kept at under 4 ºC, solutions 3.6 and 3.7 will not deteriorate over a period of at least 4 months.

3.8. **Carrier gas for chromatography:** N-50 helium or hydrogen.

3.9. **Auxiliary gases** for flame ionisation detector: N-50 hydrogen and purified air.

3.10 Solution of n-nonacosane in hexane at a concentration of approx. 100 ppm.

4. Procedure

4.1. Preparation of unsaponifiable matter:

4.1.1. Weigh 20 ± 0.1 g of oil into a 250-ml flask, add 1 ml of the standard solution of cholesta-3,5 diene (20 mg) and 75 ml of alcoholic potash at 10%, fit reflux condenser, and heat to slight boiling for 30 minutes. Remove the flask containing the sample from the heat and allow the solution to cool slightly (do not allow to cool completely as the sample will set). Add 100 ml of water and transfer the solution to a separating funnel with the aid of 100 ml of hexane. Shake the mixture vigorously for 30 seconds and leave to stratify.

Note 6 — If an emulsion is produced, wait as it disappears rapidly or add small quantities of ethanol.

4.1.2. Transfer the aqueous phase beneath to a second separating funnel and extract again with 100 ml of hexane. Once more run off the lower phase and wash the hexane extracts (combined in another separating funnel) three times with 100 ml each time of a mixture of ethanol-water (1:1) until neutral pH is reached.

4.1.3. Pass the hexane solution through anhydrous sodium sulphate (50 g), wash with 20 ml hexane and evaporate in a rotary evaporator at 30 ºC and low pressure until dryness.

4.2. Separation of steroidal hydrocarbon fraction:

4.2.1. Take the residue to the fractionating column with the aid of two 1-ml portions of hexane, run the sample onto the column by allowing the solution level to drop to the top of the sodium sulphate and start chromatographic elution with hexane at a flow rate of 1 ml/min. approx. Discard the first 25-30 ml of the elution and then collect the following 40 ml fraction

Note 7 — The first fraction contains saturated hydrocarbons (Figure 1 a) and the second fraction steroidal hydrocarbons. Further elution provides squalene and related compounds. To achieve a good separation between the saturated and steroidal hydrocarbons, the fraction volumes have to be optimised. To do so, the volume of the first fraction should be adjusted so that when the second fraction is analysed the peaks representing the saturated hydrocarbons are low (see Figure 1c); if they do not appear but the intensity of the standard peak is low, the volume should be reduced. Anyway, a complete separation between the components of the first and second fractions is unnecessary, as there is no overlapping of peaks during GC analysis. The optimisation of the volume of the second fraction is generally not needed as a good separation exists with the further components. Nevertheless, the presence of a large peak at a retention time approx. 1.5 min lower than the standard is due to squalene, and it is indicative of a bad separation.

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4.2.2. Evaporate the second fraction in an evaporator at 30 ºC and low pressure until dryness, and immediately dissolve the residue in 0.2 ml of hexane. Keep the solution in the refrigerator until analysis.

Note 8 — Residues 4.1.3. and 4.2.2 should not be kept dry or at room temperature. As soon as they are obtained, the solvent should be added and the solutions should be kept in the refrigerator.

4.3. Gas chromatography

4.3.1. Working conditions

- Injector temperature: 300 ºC.
- Column temperature: 320 ºC.
- Integrator-recorder: The parameters for integration should be fixed so as to give a correct assessment of the areas.
  Valley-valley integration mode is recommended.
- Sensitivity: About 16 times the minimum attenuation.
- Amount of solution injected: 1 ml.
- Oven programming temperatures: Initial 235 ºC for 6 min. and then rising at 2 ºC/min. up to 285 ºC.
- Injector with 1:15 flow divider.
- Carrier: Helium or hydrogen at about 120 kPa pressure.

These conditions may be adjusted in accordance with the characteristics of the chromatograph and the column to give chromatograms meeting the following requirements: internal standard peak within approx. 5 min. of the time given in 4.3.2; the internal standard peak should be at least 80% of the full scale.

The gas chromatographic system must be checked by injecting a mixture of the stock solution of cholestadiene (3.6) and n-nonacosane solution (3.10). The cholesta-3,5-diene peak has to appear before the n-nonacosane (Fig. 1c); if this does not occur, two steps can be taken: to bring down the initial oven temperature or to replace the GC-column by a less polar one.

4.3.2 Peak identification

The internal standard peak appears at approx. 19 min. and the stigmasta-3,5-diene at a relative retention time of 1.29 (see Figure 1b).

The stigmasta-3,5-diene goes with small quantities of an isomer, and usually both originate a single chromatographic peak. Nevertheless, if the column is too polar or shows a great resolving power, the isomer can appear as a small peak before and close to that of stigmasta-3,5-diene. In this case, the two areas have to be summed (Figure 2). It is advisable to eliminate the stigmastadiene split by replacing the column by one which is a less polar or has a wider internal diameter.

Note 9 — Stigmastadienes for reference can be obtained from the analysis of a refined vegetable oil by applying the method for the determination of steroidal hydrocarbons. Stigmastadienes originate a significant and easily identifiable peak.

Note 10 — Care must be taken that there is no overlap between the internal standard and stigmastadienes and any of the peaks which appear in the first fraction eluted from the silica gel column.

4.3.3 Quantitative analysis

The stigmastadienes content is determined according to the formula:

$$\text{mg/kg of stigmastadienes} = \frac{As \times Mc}{Ac \times Mo}$$

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where: As = area of stigmastadienes peak (if the peak is split up, sum of the areas of the two isomers)

Ac= area of internal standard (cholestadiene)

Mc = mass of standard added, in micrograms

Mo = mass of oil taken, in grams.

Detection limit: About 0.01 mg/kg.

Note 11 — When stigmastadienes appear in concentrations of more than 4 mg/kg, if quantifying is required, the method for determination of steroidal hydrocarbons must be applied.

Figure 1. Gas chromatograms obtained from olive oil samples analysed on a fused silica capillary column (0.25 mm i.d. by 25 m length) coated with 5%- phenylmethylsilicone, 0.25 mm film thickness.

a) First fraction (30 ml) from a virgin oil, spiked with standard.
b) Second fraction (40 ml) from an olive oil containing 0.10 mg/kg of stigmastadienes.
c) Second fraction (40 ml) containing a small proportion of the first fraction.

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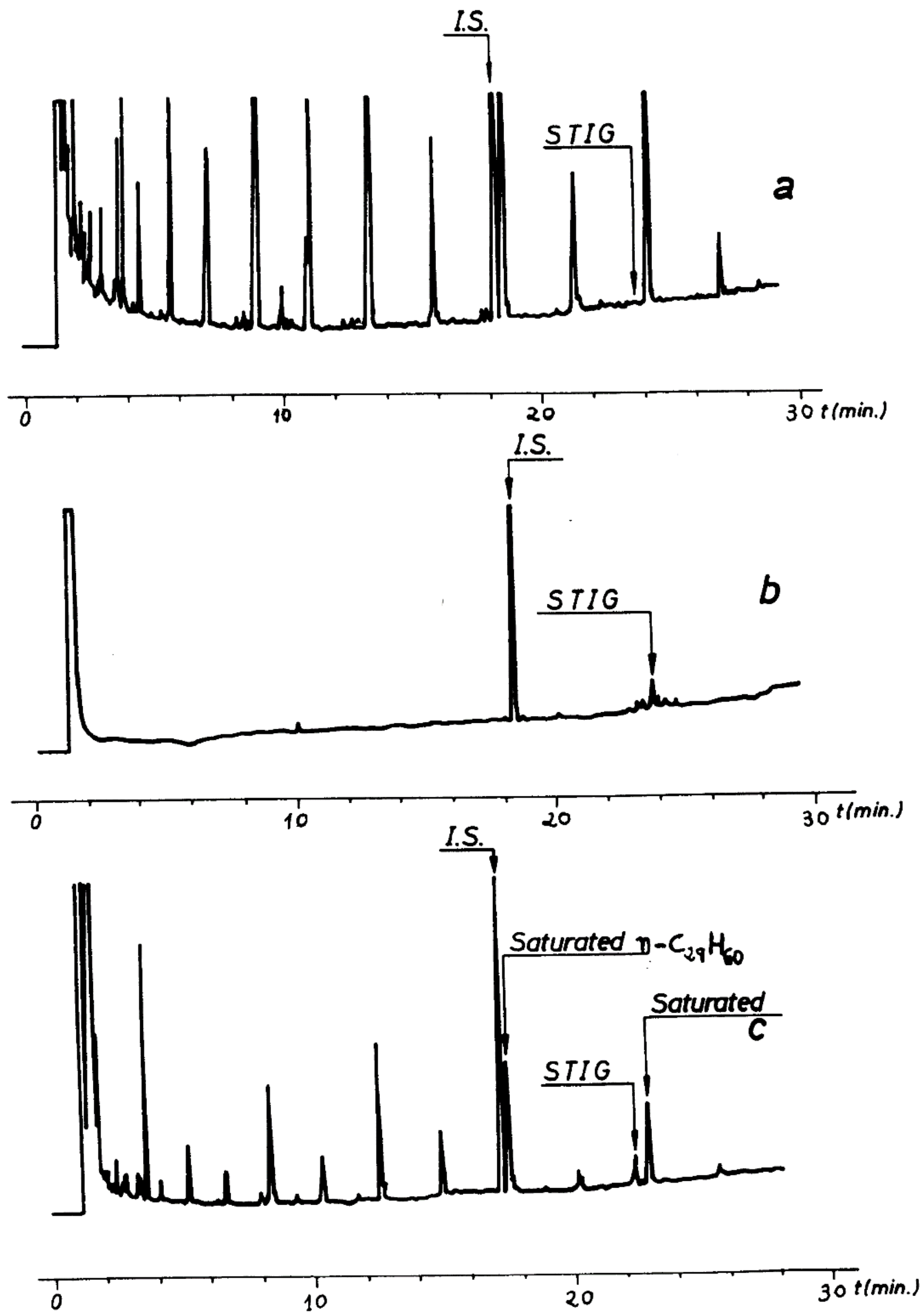

Figure 1

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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, φ(CH_3COOH) ≥ 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.

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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):

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

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.

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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

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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

Pipet 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.

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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.

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

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