TECHNICAL SPECIFICATION

ISO/TS 23647

First edition
2010-10-01

Vegetable fats and oils — Determination of wax content by gas chromatography

Corps gras d'origine végétale — Détermination de la teneur en cires par chromatographie en phase gazeuse

Reference number
ISO/TS 23647:2010(E)

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Case postale 56 • CH-1211 Geneva 20
Tel. + 41 22 749 01 11
Fax + 41 22 749 09 47
E-mail copyright@iso.org
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Published in Switzerland

Contents

Page

Foreword

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International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

In other circumstances, particularly when there is an urgent market requirement for such documents, a technical committee may decide to publish other types of document:

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An ISO/PAS or ISO/TS is reviewed after three years in order to decide whether it will be confirmed for a further three years, revised to become an International Standard, or withdrawn. If the ISO/PAS or ISO/TS is confirmed, it is reviewed again after a further three years, at which time it must either be transformed into an International Standard or be withdrawn.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

ISO/TS 23647 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 11, *Animal and vegetable fats and oils*.

Introduction

Vegetable fats and oils consist predominantly of triacylglycerols, but they also contain small amounts of non-glyceridic substances often called minor components. The composition of these minor constituents (e.g. sterols, steryl esters, triterpene dialcohols, and waxes) provides highly characteristic information about the identity of the oils. Since the physical and chemical steps in the processing of vegetable fats and oils can cause alteration in their composition and content, analysis of these minor components can be conveniently applied to characterize the processing steps through which an oil was obtained.

Waxes are natural compounds occurring in various vegetable oils and can easily crystallize at low temperatures resulting in cloudiness. Wax removal is very often a part of the vegetable oil-refining process (e.g. sunflower, rice bran, corn oil) and its efficiency requires measurement.

This Technical Specification does not cover the analysis of waxes of olive oil.

With the exception of olive oil, there is currently no reliable official method to measure the wax content and composition of vegetable fats and oils. Cold tests give neither qualitative nor quantitative results; methods developed for olive oils are not applicable to seed oils and can cause a lot of problems in the interpretation of results. There is an industrial and commercial need for an International Standard which is applicable to crude, degummed, pre-dewaxed, winterized, and fully refined vegetable fats and oils.

Waxes from different vegetable oils and fats are separated from triacylglycerols and from other non-glyceridic compounds containing double bonds by column chromatography using a mixed column packing consisting of silica gel and silica gel impregnated with $AgNO_3$.

The wax fraction is further analysed by capillary gas chromatography. The method also gives information about the total content of waxes and their composition. The information given by the method can be easily used to check oil quality and to track the efficiency of the dewaxing process.

Vegetable fats and oils — Determination of wax content by gas chromatography

1 Scope

This Technical Specification specifies a gas chromatographic method for determining the wax content of crude, degummed, neutralized, winterized, and fully refined vegetable oils, such as sunflower, soybean, rapeseed, corn, and rice bran oils. It is not applicable to olive oils or olive pomace oils.

Waxes are esters of long chain fatty acids and fatty alcohols (having C_{20} or longer saturated carbon chain).

The wax content is expressed in milligrams per kilogram of 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.

ISO 661, *Animal and vegetable fats and oils — Preparation of test sample*

3 Terms and definitions

For the purpose of this document, the following terms and definitions apply.

3.1
wax content
mass fraction of those substances in the sample, which are determined under the conditions specified in this Technical Specification

NOTE The wax content is expressed in milligrams per kilogram of oil.

4 Principle

The waxes are separated by column chromatography using a mixed column packing consisting of silica gel and silica gel impregnated with $AgNO_3$. Determination of the waxes is carried out using capillary gas chromatography (GC), applying an internal standard, previously added to the oil.

5 Reagents and materials

WARNING — Attention is drawn to the regulations which specify the handling of dangerous matter. Technical, organizational and personal safety measures shall be followed.

During the analysis, unless otherwise stated, use only reagents of recognized analytical grade suitable for HPLC column chromatography and distilled or demineralized water or water of equivalent purity.

5.1 ***n*-Hexane**.

5.2 **Dichloromethane**.

5.3 ***n*-Heptane**.

5.4 **Chloroform**.

5.5 **Silica gel 60**, of particle size 0,063 mm to 0,200 mm (70 mesh to 230 mesh), such as Merck No. 107734[1)].

5.6 **Silver nitrate** ($AgNO_3$).

5.7 **Hexatriacontane**[2)] (C_{36} paraffin).

5.7.1 **Internal standard solution**, mass concentration 0,1 mg/ml in *n*-heptane.

5.7.2 **Standard for determination of response factor**, mass concentration 1 mg/ml in *n*-heptane.

5.8 **Standards for peak identification in the gas chromatogram**, pure waxes[3)], e.g. C_{40}, C_{44}.

5.8.1 **Standard for determination of response factor**, stearyl stearate or stearic acid stearyl ester (C_{36} wax), mass concentration 1 mg/ml in chloroform.

5.9 **Crystallized sunflower wax**, prepared from winter cake or from crude sunflower oil using crystallization. For the preparation, see Annex C.

5.10 **Cotton wool**, surgical quality, non-absorbent.

6 Apparatus

6.1 **Chromatographic column**, made of glass, of internal diameter 30 mm and length 450 mm, equipped with a polytetrafluoroethylene (PTFE) tap.

6.2 **Glass rod**, about 600 mm in length.

6.3 **Pasteur pipette**, ISO 7712[4].

6.4 **Vacuum rotary evaporator**.

6.5 **Round-bottomed flask**, capacity 250 ml (to collect wax fraction).

6.6 **Pear-shaped flask**, capacity 25 ml (to collect the concentrated wax fraction).

6.7 **Gas chromatograph**, equipped with flame ionization detector, split/splitless injector and integrator or data acquisition system.

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1) Merck No. 107734 is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product. Equivalent products may be used if they can be shown to lead to the same results.

2) Hexatriacontane is available from Sigma-Aldrich with a purity of min. 99 % mass fraction. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this supplier.

3) Pure waxes e.g. behenic acid stearyl ester (C_{40}), stearic acid stearyl ester (C_{36}) and behenic acid behenyl ester (C_{44}) are available from Sigma-Aldrich, purity approximately 99 % mass fraction. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this supplier.

6.8 Fused silica capillary column, length 25 m, internal diameter 0,32 mm or preferably 0,20 mm, coated with dimethylpolysiloxane (e.g. HP-1 or OV-1 or equivalent), film thickness 0,11 µm.

7 Sampling

Sampling is not part of the method specified in this Technical Specification. A recommended sampling method is given in ISO 5555[1].

It is important the laboratory receive a truly representative sample which has not been damaged or changed during transport or storage.

8 Preparation of the test sample

Prepare the test sample in accordance with ISO 661.

Heat the laboratory sample up to 130 °C. Mix it so as to melt completely the crystallized waxes and remove any moisture (a magnetic stirrer or microwave oven may be used).

9 Procedure

9.1 Preparation of silver nitrate-impregnated silica gel 60

Place 100 g of silica gel 60 (5.5) into a ceramic bowl. Pour a silver nitrate solution, containing 5 g $AgNO_3$ (5.6) dissolved in 240 ml water on to the silica gel and, after thorough mixing, make its surface flat. Put this suspension into a cold electric oven, heat to 170 °C and activate the gel overnight. Allow to cool slowly to 50 °C in the electric oven (in the dark). Keep this column packing in the dark in a well-sealed bottle. If necessary, remove the dark particles from the impregnated silica gel surface.

The gel contains 5 % mass fraction $AgNO_3$.

9.2 Column packing

Using the glass rod (6.2) place a wad of the cotton wool (5.10) in the lower part of the column (6.1) and press it down. Pour about 30 ml of *n*-hexane (5.1) into the column and remove air by pressing the cotton wool down with the rod. Allow the solvent layer to rise approximately 2 mm to 3 mm above the cotton wool before putting the silica gel into the column.

Introduce 3 g of impregnated silica gel (9.1) into the glass column (6.1), remove any entrapped air, and flatten the surface. Add 12 g of silica gel 60 (5.5) on the top of the previous packing, remove any air, and protect the packing against light. Wash the column packing with approximately 90 ml to 100 ml of *n*-hexane, removing any air bubbles by slight tapping if necessary. The tap should be open when adding the solvent for washing. Allow approximately 5 mm of solvent layer above the column packing before adding the sample solution.

9.3 Isolation of waxes

9.3.1 Sample preparation

Solvent mixture A: *n*-hexane (5.1) and dichloromethane (5.2), volume fraction of hexane, $\varphi_{C_6H_{14}}$ = 95 ml/100 ml and of dichloromethane, $\varphi_{CH_2Cl_2}$ = 5 ml/100 ml.

Weigh the sample according to the expected wax content (sunflower oil: 3 g from crude oil and neutralized oil, and 4 g to 4,5 g from winterized and fully refined oil, other oils: 4 g to 4,5 g). Add 3 ml of internal standard solution (5.7.1) and add 7 ml of solvent mixture A to the sample. Mix thoroughly and transfer approximately 2 ml of this solution by means of a Pasteur pipette (6.3) into the column.

Adjust the flow rate to 1,5 ml/min to 2 ml/min and wash the inner surface of the glass column three times with approximately 3 ml of the solvent mixture A. Do not let the column run dry, allow approximately 5 mm of solvent layer above the column packing before adding the next portion of the solvent.

9.3.2 Column chromatography

Solvent mixture B: *n*-hexane (5.1) and dichloromethane (5.2), volume fraction of hexane, $\varphi_{C_6H_{14}}$ = 80 ml/100 ml and of dichloromethane, $\varphi_{CH_2Cl_2}$ = 20 ml/100 ml.

Elute the waxes with 190 ml of Solvent B. Adjust the flow rate to approximately 3 ml/min.

Collect all material eluting from the column in a round-bottomed flask (6.5), and evaporate the solvent by means of a vacuum rotary evaporator (6.4). Dissolve the residue in a small quantity of chloroform (5.4) transfer the solution into a pear-shaped flask (6.6), evaporate the solvent again and re-dissolve the waxes in approximately 1 ml of chloroform. The GC analysis is carried out using this solution.

9.4 Gas chromatographic determination of waxes

9.4.1 Recommended gas chromatographic conditions

Column: HP-1 (25 m × 0,20 mm, 0,11 µm film)

Oven: 170 °C (0,1 min); 170 °C to 350 °C (6 °C/min); 350 °C isotherm (20 min)

Carrier gas: H_2

Flow rate: 1,4 ml/min

Injector: 355 °C, split ratio: 1:30

Detector: 360 °C (FID)

These conditions may be adjusted in accordance with the characteristics of the gas chromatograph apparatus and the column to give good separation for the wax compounds.

9.4.2 Determination of response factor

Mix equal volumes of the standard solutions (5.7.2 and 5.8.1), and inject 1 µl of the mixture into the GC to determine the response factor, F_r

$$F_r = \frac{A_{IS}\ \rho}{A\ \rho_{IS}}$$

where

A_{IS} is the area of the hexatriacontane (5.7.2) peak;

A is the area of the stearyl stearate peak;

ρ is the mass concentration of stearyl stearate (5.8.1);

ρ_{IS} is the mass concentration of hexatriacontane (5.7.2).

The value of F_r should be between 1,1 and 1,2. Keep this solution in a sealed vial in the dark and repeat the determination as required, normally once a week.

9.4.3 Calculation of wax content

Inject 1,5 µl to 2 µl of solution of waxes (isolated according to 9.3) into the GC. The elution order is the following: solvent, hydrocarbons, internal standard (hexatriacontane), waxes.

If the isolation (9.3) is done properly, the wax fraction (especially the sunflower wax) should show a typical pattern: continuously decreasing peak area after that for C_{48} (see Figure A.1 and Table C.1). However, overlapping peaks or big peaks (e.g. sterol esters and triglycerides) at the end of the wax region can make data analysis impossible. In this case, repeat the column chromatography (see Figure A.4).

9.4.3.1 Sunflower oil

The main components of sunflower wax are: C_{44}, C_{46}, C_{48} waxes. The sunflower wax contains mainly waxes with an even number of carbon atoms e.g. C_{44}, C_{46}, C_{48}, C_{50}, C_{52}, C_{54}. Among these major peaks, minor peaks with an odd carbon number can also be found. All the peaks (minor and major peaks) are used in the calculation.

The calculation is based on a series of previous measurements and the literature (References [5][6][7]). The wax content, w_t, expressed as a mass fraction in milligrams per kilogram oil, is given by:

$$w_t = \frac{F_r \left[A_{>C_{44}} + (1+k)\, A_{C_{44}} \right] m_{IS}}{A_{IS}\; m}$$

where

F_r is the response factor (9.4.2);

$A_{>C_{44}}$ is the total area of peaks due to all waxes having more than C_{44};

$A_{C_{44}}$ is the area of the C_{44} wax peak;

A_{IS} is the area of the hexatriacontane peak;

k is an empirical factor (k = 1,0 for refined oils and winterized oils; k = 0,5 for non-refined oils, see Reference [5]);

m_{IS} is the mass, in micrograms, of internal standard added to the oil;

m is the mass, in grams, of the oil.

9.4.3.2 Other oils

The main wax components of vegetable oils (soybean, rapeseed, and corn) are: C_{40}, C_{41}, C_{42}, C_{44}, C_{46} waxes. Their content is generally below 20 mg/kg in a well-refined deodorized oil. The exception is crude rice bran oil, which can have 1 500 mg/kg to 2 000 mg/kg wax content with a similar composition to sunflower.

9.4.3.2.1 Rice bran oil

The wax content, w_t, expressed as a mass fraction in milligrams per kilogram oil, is given by:

$$w_t = \frac{F_r\, (A_{\geqslant C_{42}}\; m_{IS})}{A_{IS}\; m}$$

where

F_r is the response factor (9.4.2);

$A_{\geqslant C_{42}}$ is the total area of peaks due to all waxes with C_{42} and above;

A_{IS} is the area of the hexatriacontane peak;

m_{IS} is the mass, in micrograms, of internal standard added to the oil;

m is the mass, in grams, of the oil.

9.4.3.2.2 Other oils (soybean, rapeseed, and corn)

The wax content, w_t, expressed as a mass fraction in milligrams per kilogram oil, is given by

$$w_t = \frac{F_r \left[A_{>C_{44}} + (1+k)\, A_{C_{44}} \right] m_{IS}}{A_{IS}\, m}$$

where

F_r is the response factor (9.4.2);

$A_{>C_{44}}$ is the total area of peaks due to all waxes having more than C_{44};

$A_{C_{44}}$ is the area of the C_{44} wax peak;

A_{IS} is the area of hexatriacontane peak;

k is an empirical factor, k = 0,5 (see Reference [5]);

m_{IS} is the mass, in micrograms, of internal standard added to the oil;

m is the mass, in grams, of the oil.

NOTE For soybean oil, the sum of all wax peaks can result in a very high wax content and cause inconvenience.

10 Precision

10.1 Results of interlaboratory test

Details of an interlaboratory test on the precision of the method are summarized in Annex B. The values derived from this interlaboratory test may not be applicable to ranges and matrices other than those given.

10.2 Repeatability

The absolute difference between two independent single test results, obtained using the same method on identical test material in the same laboratory by the same operator using the same equipment within a short interval of time, will in not more than 5 % of cases exceed the values in Table B.1.

10.3 Reproducibility

The absolute difference between two single test results, obtained using the same method on identical test material in two different laboratories with different operators using different equipment, will in not more than 5 % of cases exceed the values in Table B.1.

11 Test report

The test report shall contain at least the following information:

a) all information necessary for the complete identification of the sample;

b) the sampling method used, if known;

c) the test method, with reference to this Technical Specification (ISO/TS 23647:2010);

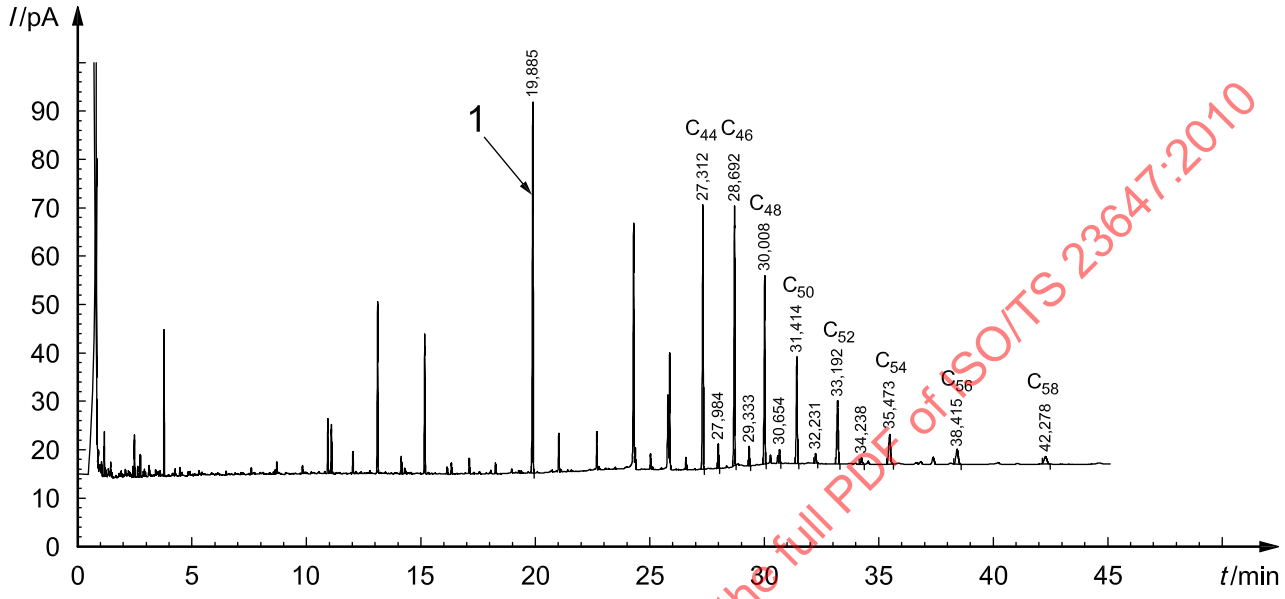
d) all operating details not specified in this Technical Specification, or regarded as optional, together with details of any incidents which may have influenced the results;

e) the test results obtained;

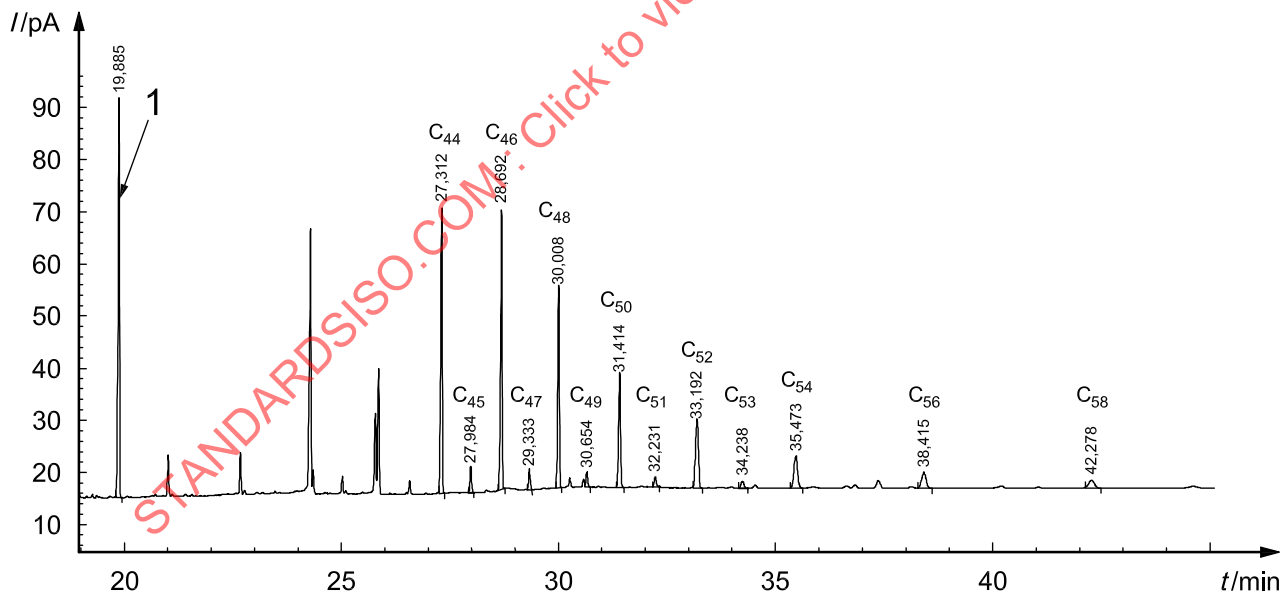
f) if the repeatability has been checked, the final quoted result obtained.

Annex A
(informative)

Chromatograms



a) **Chromatogram A — Full gas chromatographic profile**



b) **Chromatogram B — Enlargement**

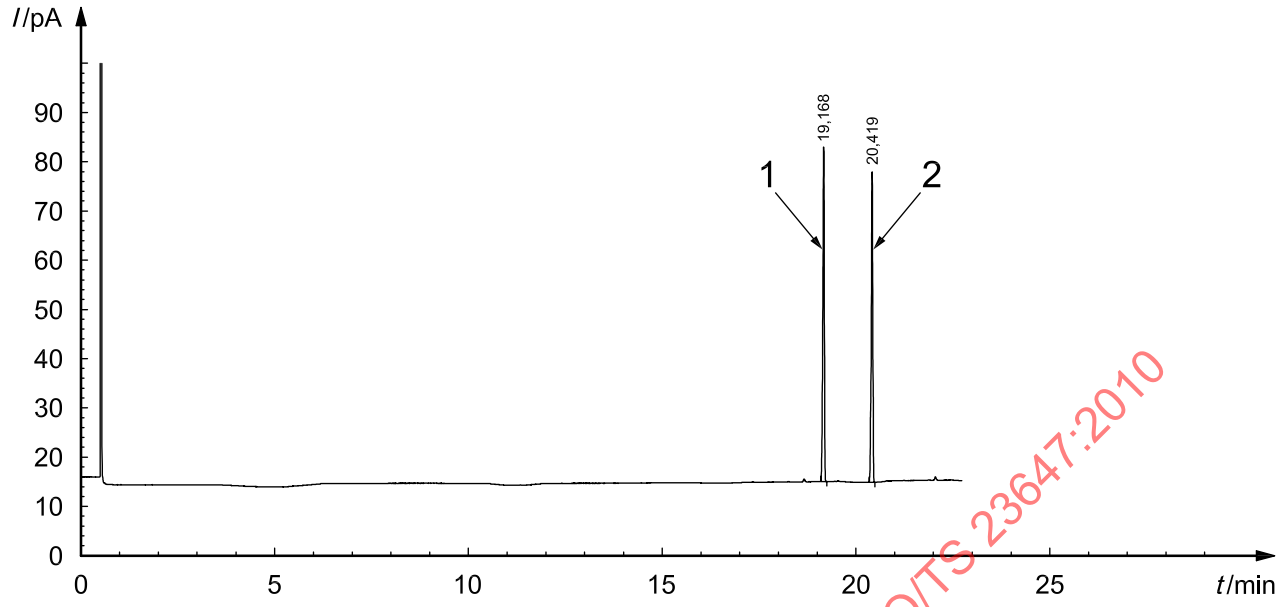
Key

I response

t time

1 hexatriacontane internal standard solution, 0,1 mg/ml in *n*-heptane

Figure A.1 — Example of a typical chromatograms of crude sunflower oil wax composition



Key

I response

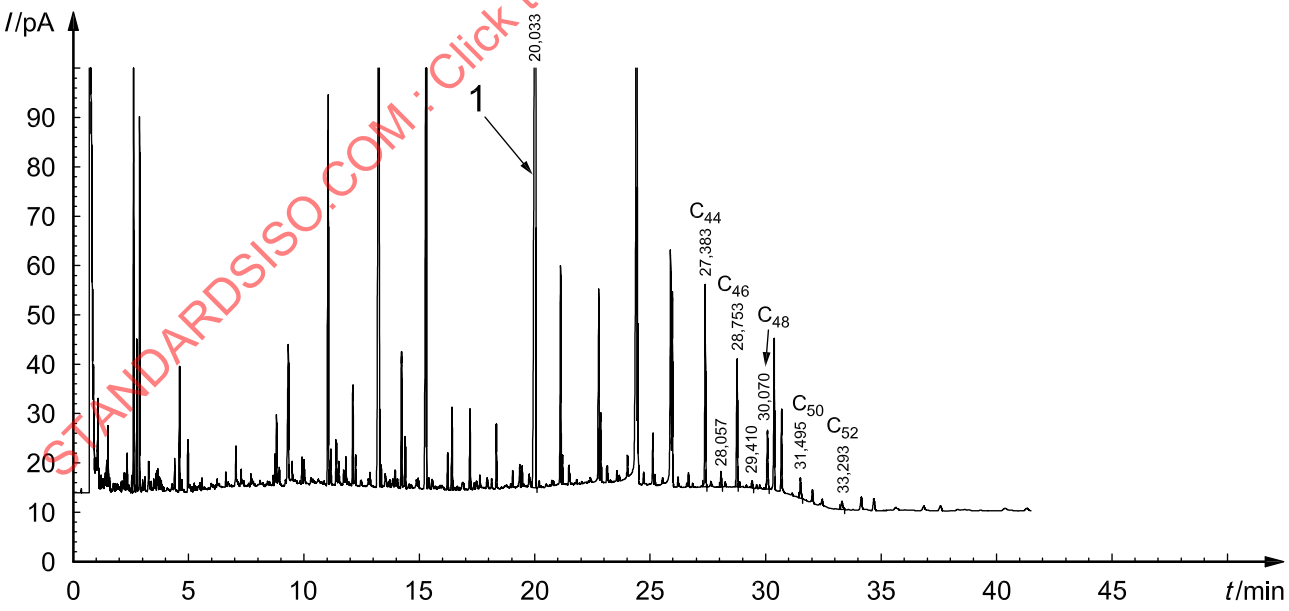
t time

1 hexatriacontane internal standard solution, 1 mg/ml in *n*-heptane

2 stearyl stearate 1,032 mg/ml in chloroform

NOTE In this case, the response factor, F_r = 1,09.

Figure A.2 — Typical chromatogram showing response factor determination



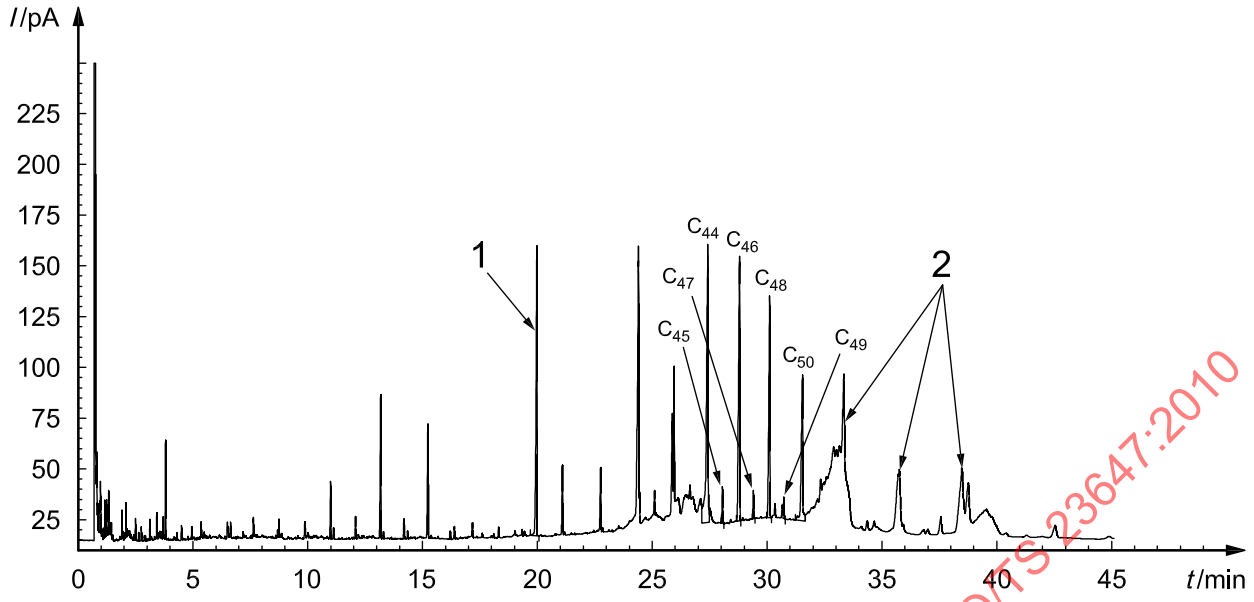
Key

I response

t time

1 hexatriacontane internal standard solution, 1 mg/ml in *n*-heptane

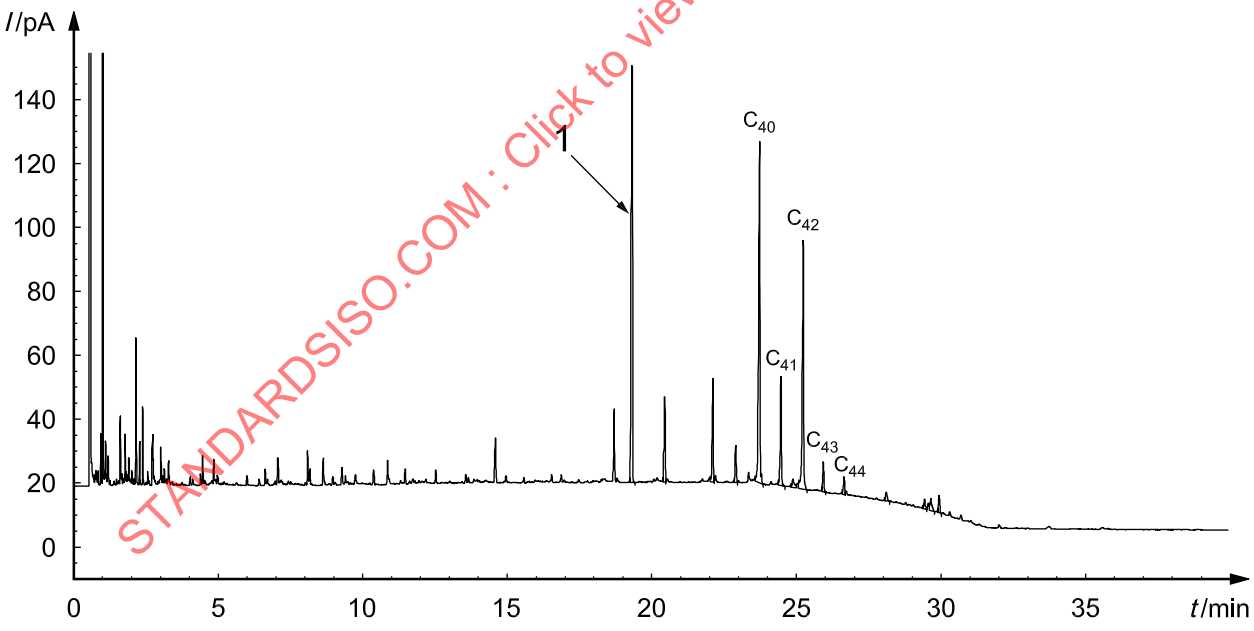
Figure A.3 — Typical chromatogram of winterized, fully refined sunflower oil wax composition



Key

I response
t time
1 hexatriacontane internal standard solution, 1 mg/ml in *n*-heptane
2 triglycerides

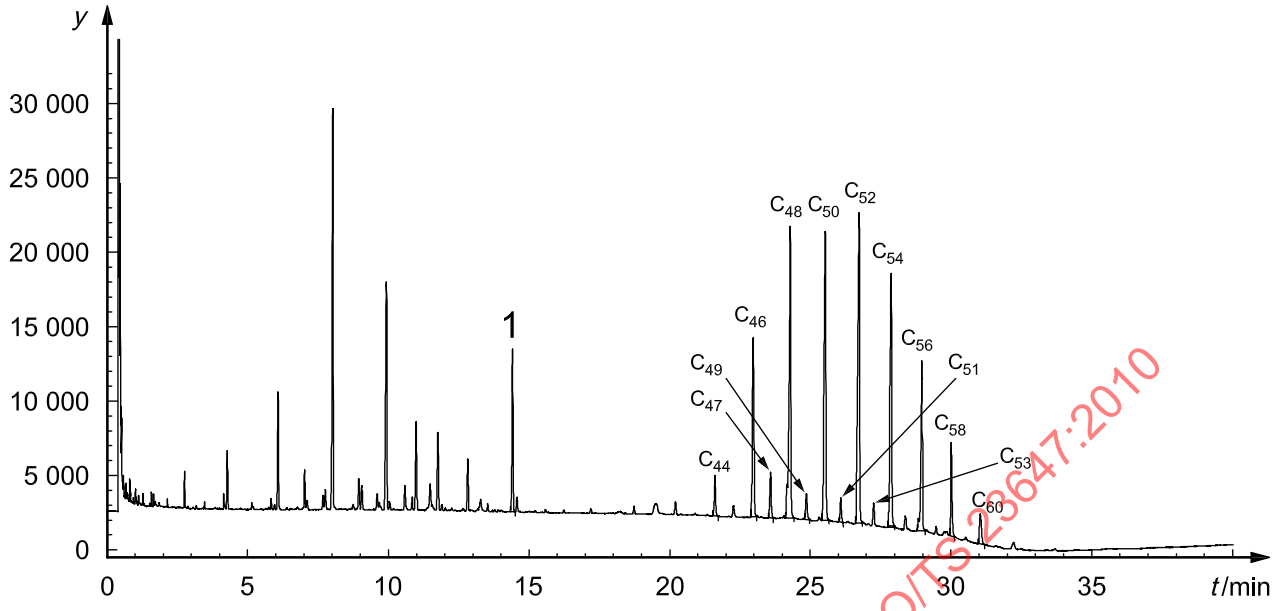
Figure A.4 — Typical chromatogram of a crude sunflower wax co-eluted with triglycerides



Key

I response
t time
1 hexatriacontane internal standard solution, 1 mg/ml in *n*-heptane

Figure A.5 — Typical chromatogram of soybean oil wax composition



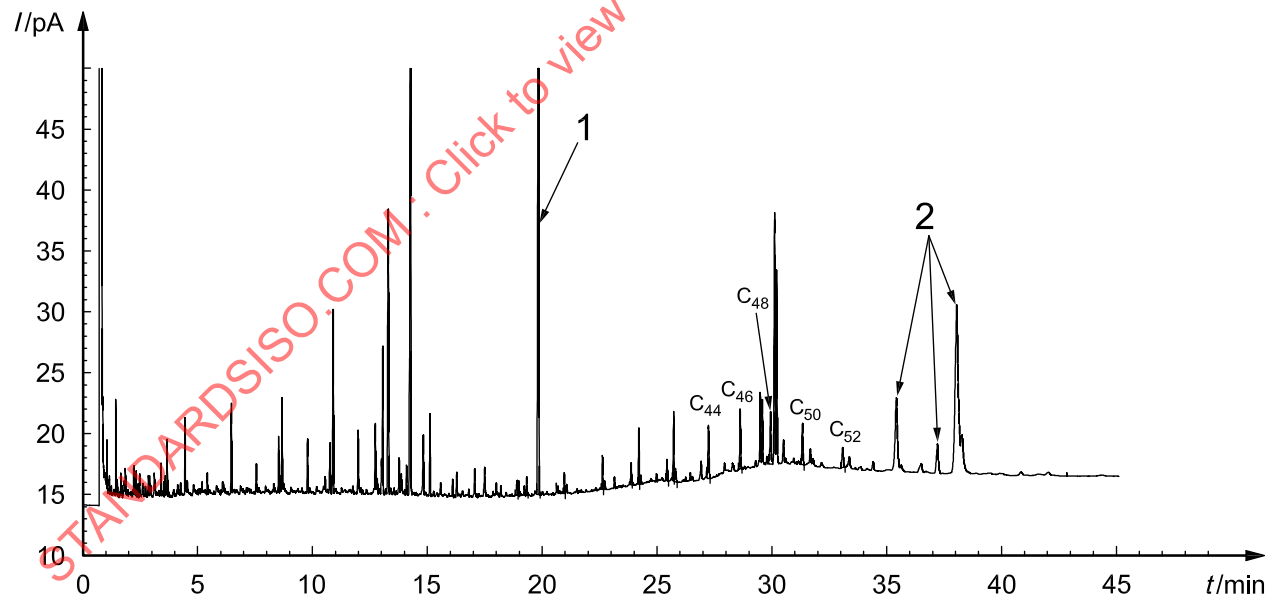
Key

y response

t time

1 hexatriacontane internal standard solution, 1 mg/ml in *n*-heptane

Figure A.6 — Typical chromatogram of rice bran oil wax composition



Key

I response

t time

1 hexatriacontane internal standard solution, 1 mg/ml in *n*-heptane

2 triglycerides

Figure A.7 — Typical chromatogram of corn oil wax composition

Annex B
(informative)

Results of interlaboratory tests

An international collaborative test involving seven laboratories in four countries was carried out on eight samples (four duplicate samples).

The results obtained were subjected to statistical analysis in accordance with ISO 5725-1[2] and ISO 5725-2[3] to give the precision data shown in Table B.1.

Table B.1 — Statistical evaluation of the collaborative trial

Parameter	Samples of different sunflower oils							
	Crude oil, not winterized		Refined and 10 % crude oil, winterized		Bleached and crude oil, not winterized		Refined oil winterized	
Number of laboratories participating	7	7	7	7	7	7	7	7
Number of laboratories after eliminating outliers	7	7	7	7	7	7	7	7
Number of test results from remaining laboratories	2	2	2	2	2	2	2	2
Mean value, $\bar{w}_t$, mg/kg	406,9	379,6	107,8	100,4	241,0	241,6	39,6	37,0
Repeatability standard deviation, s_r, mg/kg	21,4	9,9	17,2	6,8	9,09	9,1	1,9	3,2
Coefficient of variation of repeatability, $C_{V,r}$, %	5	3	16	7	4	4	5	9
Repeatability limit, r, (2,8 s_r) mg/kg	59,8	27,6	48,3	19,1	24,4	25,6	5,4	8,9
Reproducibility standard deviation, s_R, mg/kg	66,6	61,0	22,6	26,7	49,9	30,6	12,1	10,9
Coefficient of variation of reproducibility, $C_{V,R}$, %	16	16	21	27	21	13	31	29
Reproducibility limit, R, (2,8 s_R), mg/kg	186,5	170,8	63,2	74,7	139,6	85,8	33,8	30,4

Annex C
(informative)

Preparation of crystalline sunflower wax standard

C.1 Reagents and materials

During the analysis, unless otherwise stated, use only reagents of recognized analytical grade suitable for HPLC column chromatography.

C.1.1 ***n*-Hexane**.

C.1.2 **Dichloromethane**.

C.1.3 **Acetone**.

C.1.4 **Filter paper**, quantitative, 80 g/m^2.

C.1.5 **Sunflower winter cake**, filter aid together with oil and wax, oil mass fraction is 50 % to 70 %.

C.1.6 **Dried, degummed sunflower oil**, with at least 500 mg/kg of wax.

C.1.7 **Filter aid**, Clarcel[4] , Perfil[4] or any other similar type.

C.1.8 **Bleaching clay**, acid activated.

C.2 Apparatus

C.2.1 **Magnetic stirrer**.

C.2.2 **Glass funnel**, diameter 120 mm.

C.2.3 **Round-bottomed flask**, capacity 500 ml.

C.2.4 **Round-bottomed flask**, capacity 2 000 ml.

C.2.5 **Beaker**, capacity 1 000 ml.

C.2.6 **Erlenmeyer flask**, capacity 500 ml.

C.2.7 **Vacuum rotary evaporator**.

C.2.8 **Büchner funnel**, internal diameter 80 mm.

4) Product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.