# INTERNATIONAL STANDARD

# ISO 15304

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# Animal and vegetable fats and oils — Determination of the content of *trans* fatty acid isomers of vegetable fats and oils — Gas chromatographic method

*Corps gras d'origines animale et végétale — Détermination de la teneur en isomères* trans *d'acides gras de corps gras d'origine végétale — Méthode par chromatographie en phase gazeuse*



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## Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 3.

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.

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

ISO 15304 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 11, *Animal and vegetable fats and oils*.

Annexes A to E of this International Standard are for information only.

In this corrected version, the identification of the main C18:2 *cis* isomer peak in Figure B.2 (the central peak in the figure) has been corrected from

C18:1 12*cis*

to

C18:2 9*cis*, 12*cis*

# Animal and vegetable fats and oils — Determination of the content of *trans* fatty acid isomers of vegetable fats and oils — Gas chromatographic method



## 1 Scope

This International Standard specifies a gas chromatographic method using capillary columns for the determination of the content of *trans* fatty acid isomers of vegetable oils and fats.

The method is specially designed to evaluate, by a single capillary gas chromatographic (GC) procedure, the level of *trans* isomers as formed during (high temperature) refining, or during hydrogenation of vegetable oils or fats.

The method may also be used to report all other fatty acids (e.g. to obtain the full fatty acid composition and total amounts of saturated fatty acids, mono-unsaturated fatty acids and poly-unsaturated fatty acids) from the same sample and same analysis.

NOTE 1 The *trans*-isomer content as obtained with this method may not agree with the *trans*-isomer content as obtained using other methods.

NOTE 2 During (high temperature) refining (deacidification and deodorization), only geometrical isomers are formed of the mono- and poly-unsaturated fatty acids; i.e. the double bond(s) remain(s) at the same natural position. During hydrogenation, both positional and geometrical isomers are formed.

NOTE 3 For some specific *cis*- and *trans*-isomers formed during hydrogenation, co-elution is possible. This could influence the accuracy of the result. The level of these isomers is usually negligible in normal partially hydrogenated oils and fats.

## 2 Normative references

The following normative documents contain provisions which, through reference in this text, constitute provisions of this International Standard. For dated references, subsequent amendments to, or revisions of, any of these publications do not apply. However, parties to agreements based on this International Standard are encouraged to investigate the possibility of applying the most recent editions of the normative documents indicated below. For undated references, the latest edition of the normative document referred to applies. Members of ISO and IEC maintain registers of currently valid International Standards.

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

ISO 5509, *Animal and vegetable fats and oils — Preparation of methyl esters of fatty acids*

## 3 Terms and definitions

For the purposes of this International Standard, the following terms and definitions apply.

**3.1**
**content of *trans* fatty acid isomers of (high temperature) refined oils and fats**
sum of the C18:1 *trans*, C18:2 *trans* and C18:3 *trans* fatty acid methyl esters, expressed as a mass fraction of all fatty acid methyl esters

**3.2**
**content of *trans* fatty acid isomers of partially hydrogenated oils and fats**
sum of all *trans* double-bond-containing fatty acid methyl esters, expressed as a mass fraction of all fatty acid methyl esters

NOTE The content of *trans* fatty acid isomers is expressed in percent.

## 4 Principle

The methylated fatty acids of the sample are separated on a capillary gas chromatography column with a high polar stationary phase, with respect to their chain length, degree of (un)saturation and geometry and position of the double bonds.

## 5 Reagents and materials

Use only reagents of recognized analytical grade, unless otherwise specified.

**5.1 Carrier gas**, preferentially helium or hydrogen, or otherwise nitrogen, of gas chromatographic quality, dried and with oxygen removed by suitable filters.

**WARNING — Hydrogen, which is used only with capillary columns, can double the speed of the analysis (in comparison with helium) but is hazardous. Safety devices are available and it is essential that a suitable device be incorporated into the apparatus.**

**5.2 Certified Reference Material** (CRM), BCR 162 (soya/maize blend), European Commission, Community Bureau of Reference.[1)]

NOTE In addition to the use of CRM from the EC, the use of other calibration standards from reputable suppliers such as Supelco, Larodan, Nuchek and Sigma may be accepted. Perhaps different standards will be necessary for different hydrogenated oils (e.g. lauric, non-lauric oils and palm oil).

## 6 Apparatus

Usual laboratory equipment and, in particular, the following.

**6.1 Gas chromatograph**, equipped with a capillary injection system (preferred split mode, operated at a split ratio of approximately 1:100) and flame ionization detector (FID).

**6.2 Capillary column**, with a high polar stationary phase (e.g. CP™-Sil 88[2)] , SP-2340[3)] , BPX-70[4)] or similar highly polar cyanopropyl phases such as SP-2380 and SP-2560 which can give similar resolution of the various geometrical isomers).

NOTE For improved separations, a 100 m SP-2560 or CP™-Sil 88 column and hydrogen as carrier gas are recommended.

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1) European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (IRMM), Geel, Belgium.

2) Available from Chrompack, Middelburg, The Netherlands.

3) Available from Supelco, Bellafonte, PA, USA.

4) Available from SGE Inc., Austin, Texas, USA.

These types of columns are examples of suitable products which are available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of these products.

Examples of dimensions:

— CP™-Sil 88, (50 to 100) m × 0,25 mm i.d., 0,20 µm film;

— SP-2360, (50 to 100) m × 0,25 mm i.d., 0,20 µm film;

— SP-2340, 60 m × 0,25 mm i.d., 0,20 µm film;

— BPX-70, 50 m × 0,22 mm i.d., 0,25 µm film.

Optimum conditions shall be defined by the user following the instructions in 10.3. (See also annex A.)

## 7 Sampling

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

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

## 8 Preparation of test sample

Prepare the test sample in accordance with ISO 661.

Before taking the test portion from the sample, mix the sample thoroughly. Melt solid samples completely to ensure proper mixing.

## 9 Preparation of methyl esters

Prepare the methyl esters from the triglycerides of the prepared test sample in accordance with ISO 5509.

Methods specified in AOCS Official Method Ce 2-66 [2] or IUPAC 2.301 [3] may also be used.

For *trans*-isomer determination in, for example, virgin olive oils, the *trans*-esterification routine as specified in ISO 5509 is recommended to avoid any heating of the samples.

## 10 Procedure

### 10.1 General

In conjunction with the analysis of the test sample (or a series of test samples), analyse a blank sample (*n*-heptane) and a reference sample of CRM (5.2).

### 10.2 GC conditions

**10.2.1** Set up the gas chromatograph with one of the recommended combinations of temperature and column as described in Table 1.

Measure the average carrier gas linear velocity as indicated in Table 1, with a split ratio of approximately 1:100.

See annex B for typical chromatograms obtained with the conditions given in Table 1.

**Table 1 — Recommended GC conditions for identification and quantification of trans isomers in refined and hydrogenated vegetable oil samples**

| Parameter | Proposed optimum conditions | | |
|---|---|---|---|
| Stationary phase | SP-2340 | CP™-Sil 88 | BPX-70 |
| Temperature conditions, °C | isotherm 192 | isotherm 175 | isotherm 198 |
| Column head pressure, kPa | 125 | 130 | 155 |
| Linear velocity of carrier gas (helium), cm/s | 15 | 19 | 17 |
| **Parameter** | **Proposed optimum conditions for 100 m columns** | | |
| Stationary phase | SP-2560 | CP™-Sil 88 | CP™-Sil 88 |
| Temperature conditions, °C | (120 to 240) °C with 4 °C/min | isotherm 150 | isotherm 175 |
| Column head pressure, kPa | 220 | 170 | 160 |
| Linear velocity of carrier gas (hydrogen), cm/s | 30 | 30 | 30 |

**10.2.2** The temperature of the injection port and detector shall be 250 °C.

## 10.3 Performance check

Inject 0,5 µl to 1,0 µl of the methyl esters (concentration approximately 7 mg/ml in *n*-heptane) from the test sample into the gas chromatograph. Compare the result of a similar type of sample with the typical chromatograms given in annex B.

If the separation obtained is not identical to the example chromatograms, small changes in oven temperature may be required. Decrease or increase the oven temperature with subsequent steps of 1 °C until good separation is obtained. These small corrections might be required to correct for batch differences between columns and instrument temperature control, and generally fall within a range of only a few degrees (plus or minus) at maximum from the indicated value.

The C20:1 *cis* peak will elute earlier, relative to linolenic acid C18:3 9*cis*,12*cis*,15*cis* (*ccc*) if the oven temperature is increased (see reference [4]).

NOTE 1 The properties of the BPX-70 stationary phase are somewhat different, resulting in elution of the C20:1 cis peak after the C18:3 9*cis*,12*cis*,15*cis* peak, using these conditions (compare annex B).

If the GC system is set up properly, the separation obtained should allow identification of the small amount of the naturally present C18:1 11*cis* isomer next to the C18:1 9*cis* peak in (high temperature) refined oils, e.g. soyabean oil. The two C18:1 *cis* isomers should be clearly separated (see annex B).

NOTE 2 Hydrogenated marine oils can give rise to a much wider range of *trans* isomers, making identification and quantification more difficult.

The C20:1 *cis* natural isomer should be positioned exactly amidst the last eluting *trans* isomer C18:3 (*tcc)* and the C18:3 *ccc* peak (linolenic acid) in (high temperature) refined oils.

If the separation is sufficient for this type of analysis, in (high temperature) refined oils there should be a small peak for the C18:1 *trans* isomer, two approximately equally sized C18:2 *trans* isomers, and four (sometimes five) C18:3 *trans* isomers.

For partially hydrogenated oils and fats, the separation of the C18:1 13*trans* and the C18:1 9*cis* isomers should be visible on the chromatogram. This is required for an accurate peak-split between *cis* and *trans* isomers (see annex B).

The 18:1 13*trans* isomer always elutes with the 18:1 14*trans* isomer. Therefore, the peak for these two isomers should be identified as 18:1 (13+14)*trans*.

### 10.4 Peak identification

For (high temperature) refined oils and fats, the *trans* isomers are limited in number, as only geometrical isomers with the double bond(s) at the same natural position are formed. These specific isomers are for the C18 type of fatty acids: C18:1 9*trans*, C18:2 9*c*12*t* and C18:2 9*t*12*c* and for C18:3 the *tct*, *cct*, *ctc* and *tcc* 9,12,15 isomers (in some samples the C18:2 9*t*12*t* and C18:3 *ttc* isomers are found as well in very small amounts).

For partially hydrogenated oils and fats, the *trans* double-bond-containing isomers are identified using the equivalent chain length (ECL) concept (see references [5, 6]). For accurate peak identification with this system, the ECL values have to be determined after suitable calibration with available *cis* and *trans* fatty acid isomer standards[5)] (see also annex C).

The first sample in an analysis batch is always a blank (*n*-heptane). No peaks should be detected in the blank run. Repeat this test after every ten samples.

Per analysis batch (i.e. methylation performed in one batch) at least one reference sample (5.2) is analysed to check the performance of the methylation and GC analysis. The methylated fatty acids of the reference material are injected after each set of ten samples.

## 11 Calculations

### 11.1 General

The relative mass fraction of each component is calculated by determining the corrected area of the corresponding peak relative to the sum of the corrected areas of all peaks. Correction is required to compensate for the FID response for each component.

To determine the individual correction factors, use the BCR standard (see 5.2).

### 11.2 Calculation of the FID response factor

Calculate the FID response factor for each component by the equation:

$$F_x = \frac{M_x}{(n_x - 1) A_C}$$

where

$F_x$ is the FID response factor for component $x$;

$M_x$ is the relative molecular mass of component $x$;

$n_x$ is the number of carbon atoms of methylated fatty acid component $x$;

$A_C$ is the relative atomic mass of carbon ($A_C$ = 12,01).

In this case the calculation gives a theoretical response factor.

5) Fatty acid isomer standards are available from many chemicals suppliers (e.g. Nu-Check Prep Inc., US; Sigma, US; Larodan, Sweden).

### 11.3 Calculation of the FID correction factor

Calculate the FID correction factor for each component by the equation:

$$f_x = \frac{F_x}{F_r}$$

where

$f_x$ is the correction factor for component $x$;

$F_x$ is the FID response factor for component $x$;

$F_r$ is the FID response factor for C16:0 ($F_r$ = 1,407).

The FID response factor for C16:0 ($F_r$ = 1,407) is regarded as the reference ($f_x$ = 1,00). All other corrected FID response factors used in the calculation are relative to this value. For example, the corrected response factor for C10:0 becomes 1,10. See annex D for a list of FID factors.

### 11.4 Calculation of the relative mass fraction

Calculate the relative mass fraction of each component by the equation:

$$w_x = \frac{A_x \times f_x \times 100\ \%}{A_t}$$

where

$w_x$ is the relative mass fraction of component $x$, in percent by peak area;

$A_x$ is the area of the peak corresponding to component $x$, in area units;

$A_t$ is the sum of the corrected areas of all peaks, excluding the solvent peak, in area units;

$f_x$ is the correction factor for component $x$.

### 11.5 Calculation of the content of *trans* fatty acid isomers

#### 11.5.1 (High temperature) refined oils and fats

Calculate the *trans* fatty acid isomers content of (high temperature) refined oils and fats as the sum of the relative mass fractions of the C18:1 *trans*, C18:2 *trans* and C18:3 *trans* fatty acid methyl esters, relative to all fatty acid methyl esters. The maximum possible peaks which may be formed are: C18:1 *trans* (1 peak), C 18:2 *trans* (2 peaks) and C18:3 *trans* (4 peaks). See also Figures A.4 and A.5.

Report the result to the nearest 0,01 % (mass fraction).

#### 11.5.2 Partially hydrogenated oils and fats

Calculate the content of *trans* fatty acid isomers of partially hydrogenated oils and fats as the sum of the relative mass fractions of all *trans* double-bond-containing fatty acid methyl esters, relative to all fatty acid methyl esters.

Report the result to the nearest 0,1 % (mass fraction).

# 12 Precision

## 12.1 Interlaboratory test

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

NOTE Some partially hydrogenated oils may have *trans* fatty acid levels in excess of the range obtained from the interlaboratory trial.

## 12.2 Repeatability

The absolute difference between two independent single test results, obtained with 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 value of $r$ given in Table 2.

## 12.3 Reproducibility

The absolute difference between two single test results, obtained with the same method on identical test material in different laboratories by different operators using different equipment, will in not more than 5 % of cases exceed the value of $R$ given in Table 2.

**Table 2 — Repeatability limit ($r$) and reproducibility limit ($R$)**

| Sample | Mean *trans* fatty acid isomers content<br>% (mass fraction) | $r$<br>% (mass fraction) | $R$<br>% (mass fraction) |
|---|---|---|---|
| Sunflowerseed oil | 0,34 | 0,08 | 0,21 |
| Soyabean oil | 0,78 | 0,13 | 0,31 |
| Rapeseed oil | 1,09 | 0,13 | 0,40 |

# 13 Test report

The test report shall specify:

— all information necessary for the complete identification of the sample;

— the sampling method used, if known;

— the test method used, with reference to this International Standard;

— all operating details not specified in this International Standard, or regarded as optional, together with details of any incidents occurred when performing the method, which may have influenced the test result(s);

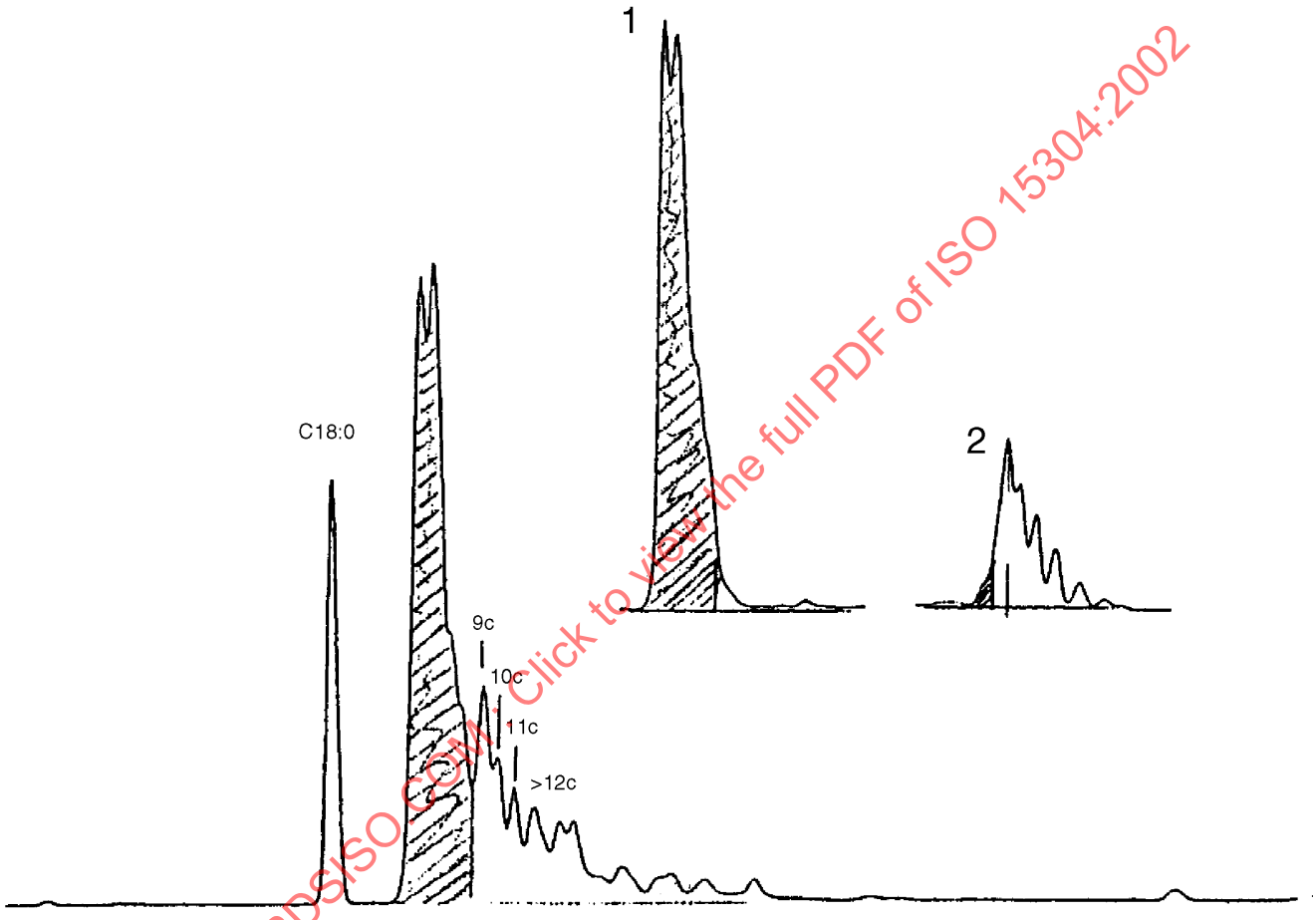
— the test result obtained;

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

# Annex A
(informative)

# Optimum conditions

See Figures A.1 to A.3.

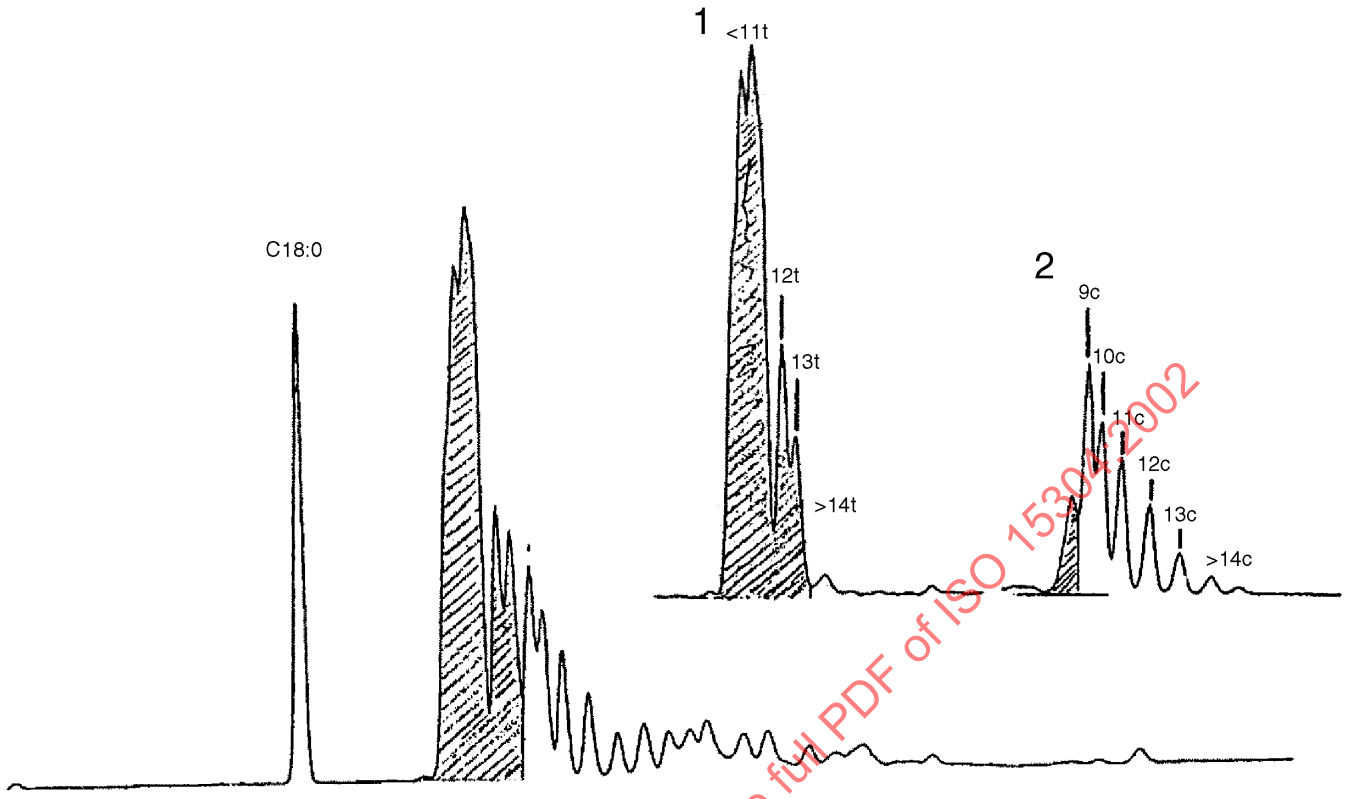



**Peak identification**

1 *trans*-monoenoic

2 *cis*-monoenoic

**Figure A.1 — Optimum conditions for a BPX–70 column, at 198 °C (isotherm), sample BO35**

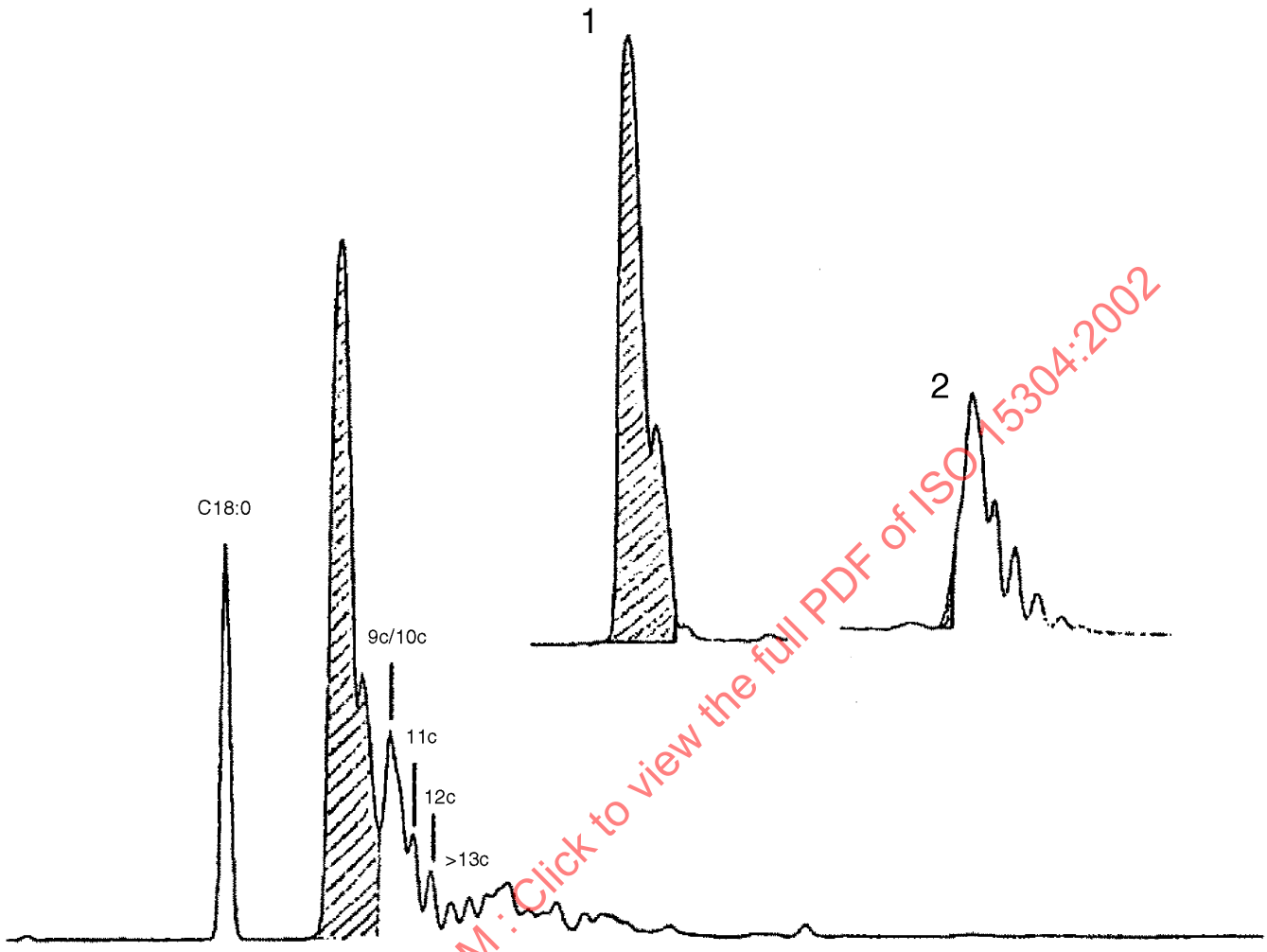



**Peak identification**

1 *trans*-monoenoic

2 *cis*-monoenoic

**Figure A.2 — Optimum conditions for a CP-Sil 88 column, at 175 °C (isotherm), sample BO35**




**Peak identification**

1 *trans*-monoenoic

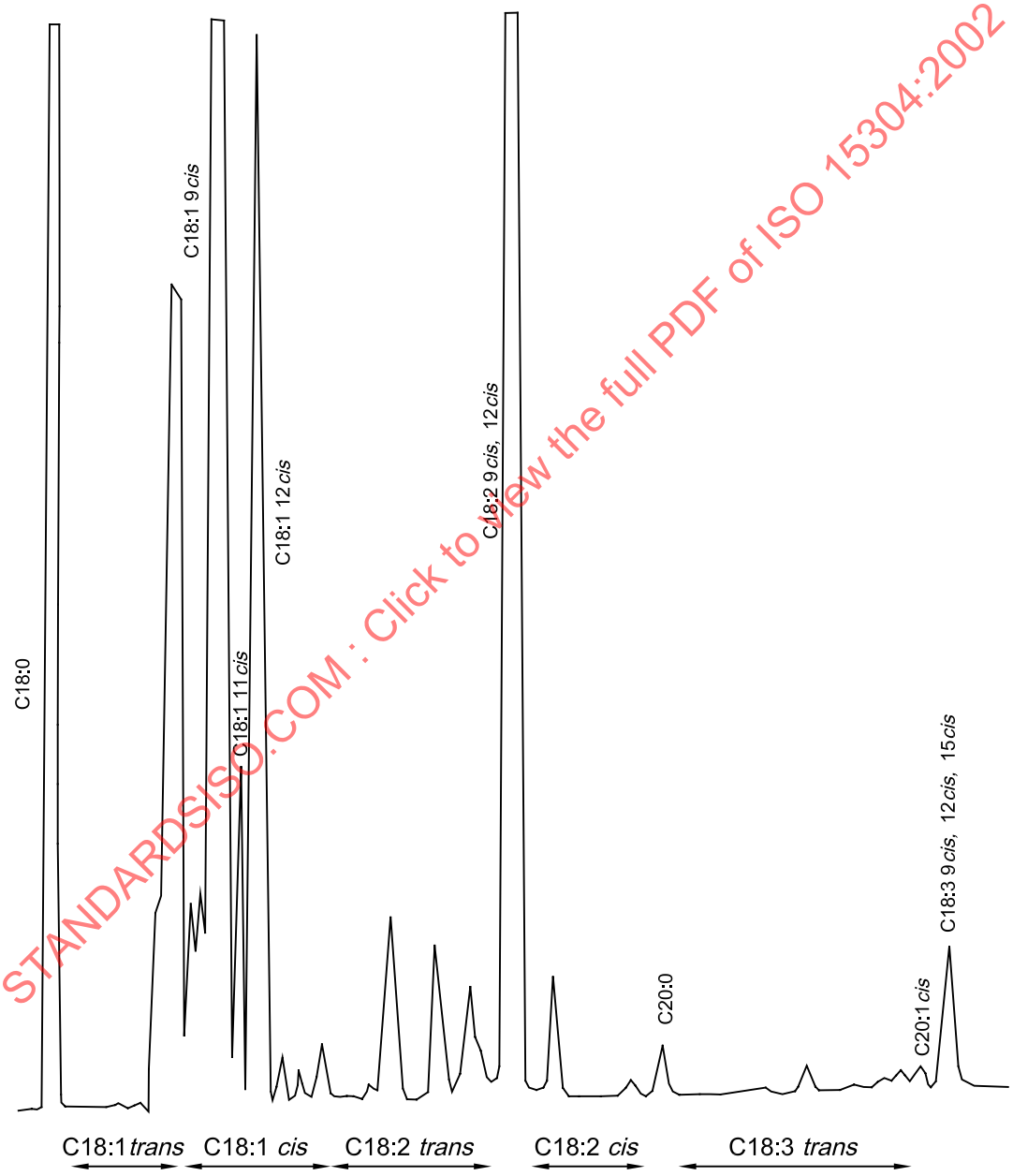
2 *cis*-monoenoic

**Figure A.3 — Optimum conditions for a SP-2340 column, at 192 °C (isotherm), sample BO35**

# Annex B
(informative)

# Examples of typical chromatograms obtained under the recommended conditions

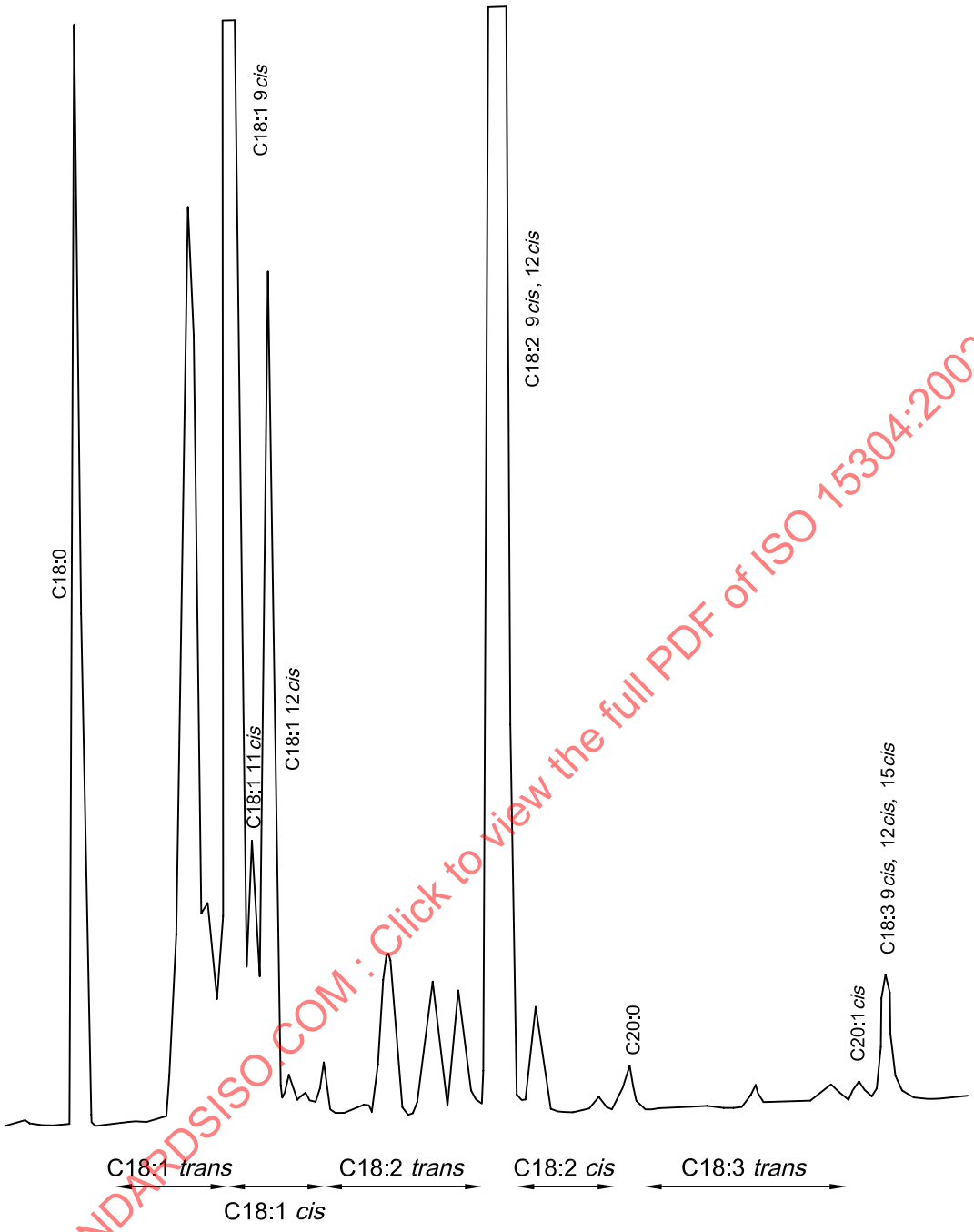
See Figures B.1 to B.5.






NOTE Obtained using a 50 m × 0,25 mm × 0,20 µm CP™-Sil 88 column (Chrompack) at 175 °C (isotherm). The *cis* and *trans* fatty acid isomer retention areas are indicated on the chromatogram.

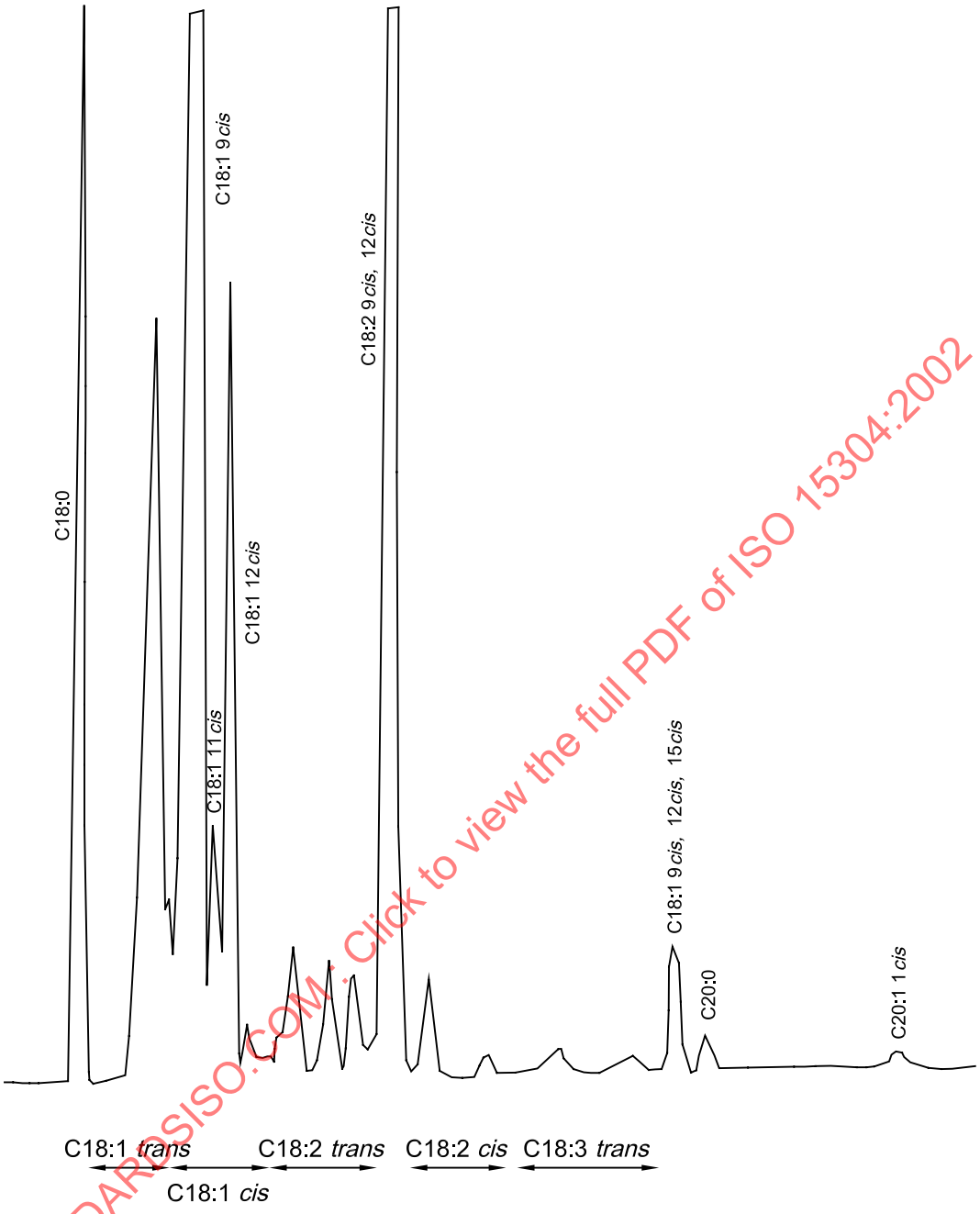
**Figure B.1 — Chromatogram of methyl esters from a partially hydrogenated soyabean oil sample**






NOTE Obtained using a 60 m × 0,25 mm × 0,20 µm SP-2340 column (Supelco) at 190 °C (isotherm). The *cis* and *trans* fatty acid isomer retention areas are indicated on the chromatogram.

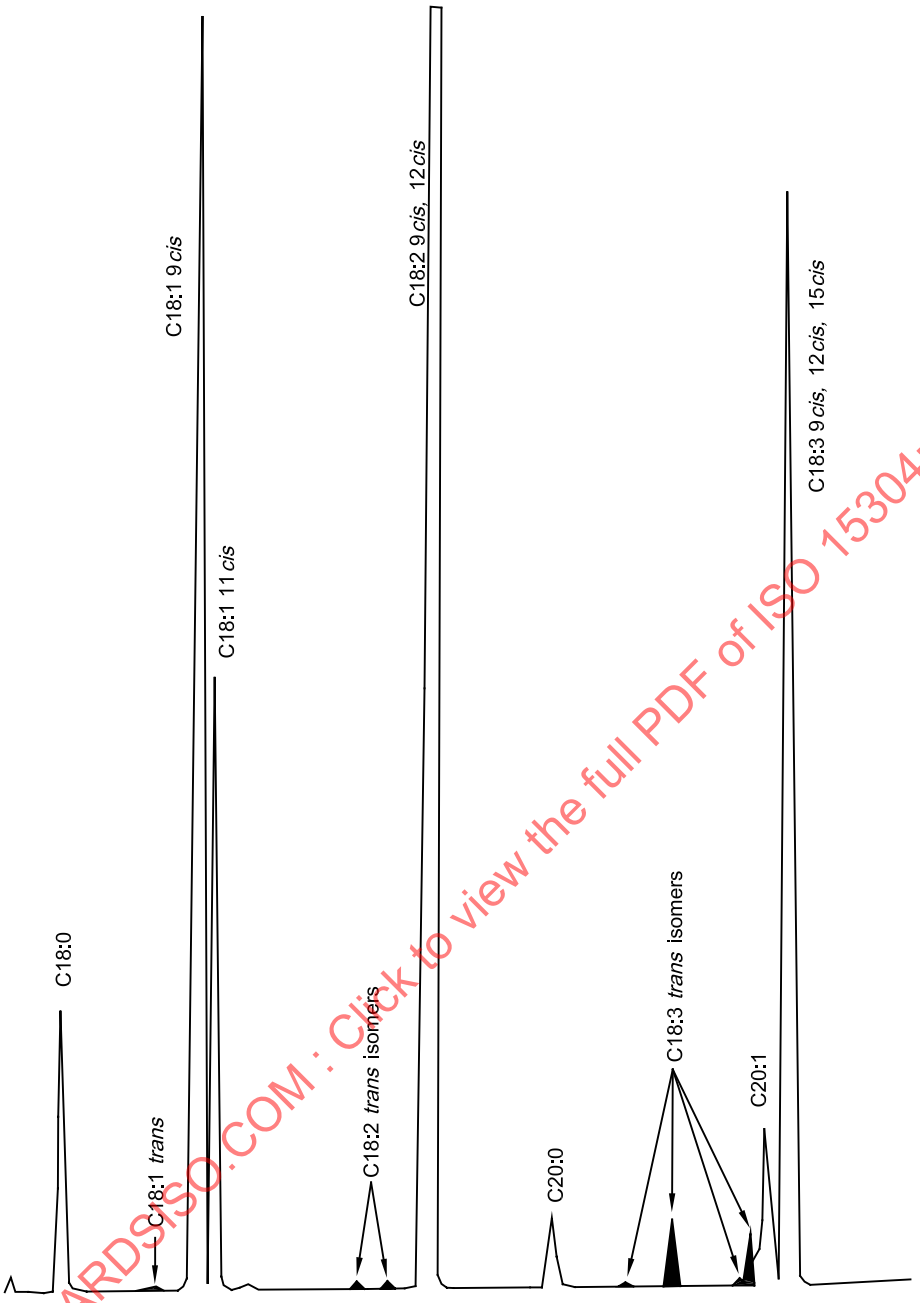
**Figure B.2 — Chromatogram of methyl esters from a partially hydrogenated soyabean oil sample**





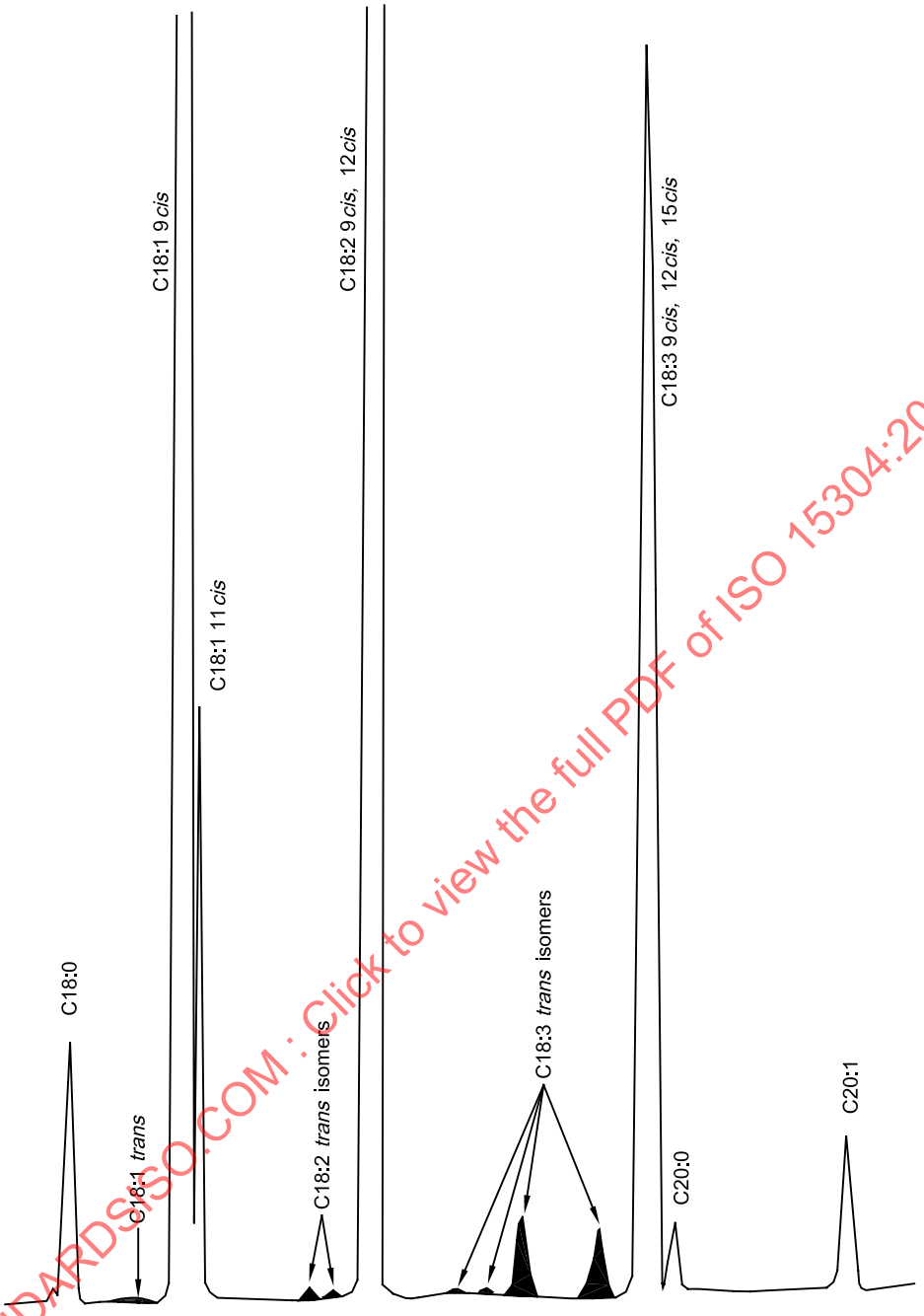

NOTE Obtained using a 50 m × 0,22 mm × 0,25 μm BPX-70 column (SGE) at 198 °C (isotherm). The *cis* and *trans* fatty acid isomer retention areas are indicated on the chromatogram.

**Figure B.3 — Chromatogram of methyl esters from a partially hydrogenated soyabean oil sample**




NOTE Obtained using a 50 m × 0,25 mm × 0,20 µm CP™-Sil 88 column (Chrompack) at 175 °C (isotherm). The *trans* fatty acid isomers are shaded.

**Figure B.4 — Chromatogram of methyl esters from a physically refined rapeseed oil sample**






NOTE Obtained using a 50 m × 0,22 mm × 0,25 µm BPX-70 column (SGE) at 198 °C (isotherm). The *trans* fatty acid isomers are shaded.

**Figure B.5 — Chromatogram of methyl esters from a high-temperature-refined rapeseed oil sample**

# Annex C
(informative)

## Equivalent chain length (ECL) values

| C18 isomer | | Stationary phase and temperature | | |
|---|---|---|---|---|
| | | SP-2340<br>192 °C | CP™-Sil 88<br>175 °C | BPX-70<br>198 °C |
| C18:1 | 6*cis* | | 18,58 | |
| | 7*cis* | | 18,58 | |
| | 9*cis* | 18,68 | 18,66 | 18,46 |
| | 10*cis* | | | |
| | 11*cis* | 18,76 | 18,74 | 18,53 |
| | 12*cis* | | 18,80 | |
| | 13*cis* | | 18,87 | |
| | 15*cis* | | 19,00 | |
| | 6*trans* | | 18,41 | |
| | 7*trans* | | 18,42 | |
| | 9*trans* | 18,49 | 18,46 | 18,28 |
| | 10*trans* | | 18,49 | |
| | 11*trans* | | 18,52 | |
| | 12*trans* | | 18,57 | |
| | 13*trans* | | 18,61 | |
| | 15*trans* | | 18,66 | |
| C18:2 | 9*c*12*c* | 19,62 | 19,63 | 19,14 |
| | 9*c*12*t* | 19,40 | 19,40 | 18,94 |
| | 9*t*12*c* | 19,48 | 19,49 | 19,02 |
| | 9*t*12*t* | 19,26 | 19,20 | 18,69 |
| | 12*c*15*c* | | 19,92 | |
| C18:3 | 6*c* 9*c*12*c* | | 20,28 | |
| | 9*t*12*t*15*t* | | 20,04 | |
| | 9*t*12*c*15*t* | 20,23 | 20,26 | 19,42 |
| | 9*c*12*c*15*t* | 20,35 | 20,36 | 19,61 |
| | 9*c*12*t*15*c* | | 20,53 | 19,51 |
| | 9*t*12*c*15*c* | 20,57 | 20,56 | 19,82 |
| | 9*c*12*c*15*c* | 20,68 | 20,67 | 19,93 |

NOTE 1 Determined at Unilever Research Vlaardingen for the most important fatty acid isomers, on the three highly polar stationary phases and proposed optimum conditions.

NOTE 2 For literature data, see references [5] and [6].