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METHOD OF ANALYSIS

DETERMINATION OF *TRANS* UNSATURATED FATTY ACIDS BY CAPILLARY COLUMN GAS CHROMATOGRAPHY

1. Scope and field of application

This standard describes a procedure for the determination of the *trans* fatty acid content of vegetable fats and oils by capillary column gas chromatography. The method is applicable to oils and fats containing fatty acids with between 10 and 24 carbon atoms. It is not applicable to vegetable fats that have undergone any hydrogenation process.

WARNING: This standard may require using dangerous apparatus and substances or performing operations that involve some risk. The method does not make provision for all the precautions connected with its application. Any person using this method is therefore responsible for ascertaining and fixing beforehand whatever safety measures are appropriate and for taking any measures required by law.

2. Principle

Preparation of the fatty acid methyl esters from the fat, and analysis by capillary column gas chromatography. Identification of the fatty acids from their retention times. Quantification by determination of the ratio of the area under the relevant peak to the sum of the areas under all the peaks for the fatty acids.

3. Purpose

Determination of the *trans* unsaturated fatty acids with 18 carbon atoms that may be present at specific concentrations in natural vegetable fats and oils and in such oils that have undergone refining.

4. Definitions

According to this method, the *trans* fatty acid content of a vegetable fat or oil is given by the sum of the following acid contents relative to the total fatty acids:

- *trans*-octadecenoic (T 18:1)
- *trans, trans*-octadecadienoic (TT 18:2)
- *cis-trans* and *trans-cis* octadecadienoic [(CT + TC) 18:2]
- *trans-cis-trans*, *cis-cis-trans*, *cis-trans-cis* and *trans-cis-cis* octadecatrienoic [(TCT + CCT + CTC + TCC) 18:3].

5. Apparatus

5.1. Gas chromatograph suitable for use with a capillary column, equipped with a splitting system consisting of:

5.1.1. Thermostatic chamber for the column, capable of maintaining the desired temperature to within 0.1°C.

5.1.2. Temperature-controlled injection unit.

5.1.3. Flame ionisation detector and converter-amplifier.

5.1.4. Flow meters for carrier and auxiliary gases.

5.2. Glass or fused silica capillary column, internal diameter 0.25-0.32 mm, length 50 m, coated with cyanopropylsilicone **(1)** to a thickness of 0.1-0.3 μm.

5.3. Recorder-integrator suitable for use with a converter-amplifier, with a response time of not more than one second and a variable paper speed.

5.4. Microsyringe for gas chromatography, 10 μl delivery, with a hardened needle.

5.5. Rotary evaporator.

5.6. Apparatus needed to prepare the methyl esters.

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(1) Commercially prepared chromatographic columns are available on the market (e.g. SP 2340, CP Sil 88, Silar 10, etc.).

6. Reagents

6.1. Carrier gas: Pure helium or hydrogen, gas chromatography quality (caution: see appendices).

6.2. Auxiliary gas: air and hydrogen, gas chromatography quality (caution: see appendices).

6.3. Reference samples: methyl esters of pure fatty acids, in particular *cis* and *trans* isomers of octadecenoic **(1),** octadecadienoic **(2)** and octadecatrienoic **(1)** acid.

6.4. n-hexane (caution: see appendices).

7. Procedure

7.1. Preparation of the methyl esters: Use a procedure that involves basic catalysis.

Fats and oils with a free acidity of more than 3 % should be neutralised beforehand.

7.2. Checking of the gas chromatograph and conditioning of the column

Carry out the preliminary checks of the injection unit and recorder. Fit the column in the gas chromatograph without connecting the outlet end to the detector. Run a light flow of carrier gas (1-2 ml/minute) through the column and gradually heat to 210°C in no less than 4 hours. Hold the column at this temperature for at least one night and then allow to cool. Connect the outlet end to the detector, check the stability of the connection and bring the injection unit to the conditioning temperature. Connect the detector and recorder and after 3-4 hours check that the linearity and drift of the base line are satisfactory, operating at a sensitivity at least four times greater than that intended for the analysis (a positive drift of more than 5% of the full-scale value/hour indicates that the column has not been properly conditioned).

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(1) Methyl esters of octadecenoic and octadecatrienoic acid are available on the market (e.g. from firms like Alltech, Supelco, etc.)

(2) Isomers of octadecadienoic acid can be prepared in the laboratory by isomerisation of pure linoleic acid with the aid of selenium (see Strocchi A. *et al.,* Rivista Italiana delle Sostanze Grasse, 45 (8) 607, 1968; Schonfield *et al.,* Journal of American Oil Chemists Society, 38, 208, 1961).

7.3. Choice of operating conditions and checking of column efficiency

7.3.1. Operating conditions

General operating conditions are as follows:

- column temperature, 150-200°C, if possible by temperature programming (e.g. 165°C for 15 minutes, then raising the temperature by 5°C/min. to 200°C).
- injector temperature: 250°C.
- detector temperature: 260-280°C.
- volume of carrier gas (helium or hydrogen): 1.2 ml/minute.
- quantity of substance injected: 1 µl of solution at 2% in hexane.

These conditions may be varied in the light of the characteristics of the column and gas chromatographic conditions in order to ensure a gas chromatographic profile similar to that shown in Figure 1. Check that the linolenic acid (C18:3) peak appears as a resolved peak just before the eicosenoic acid (C20: 1) peak. If it does not, two steps can be taken: change the oven temperature and/or use a column of different polarity.

7.3.2. Column efficiency

7.3.2.1. The efficiency of a column is determined by its separating and resolving power. It must therefore provide chromatograms meeting the following requirements if the analyses are to be performed correctly:

- separating power: the peaks for the methyl esters of all the fatty acids present must be quite distinct;
- resolving power: the peaks must be completely resolved, i.e. the peak trace should return to the base line before leaving for the next peak. For some typical pairs, for instance the *trans* C18: 1/*cis* 18:1 acids, incomplete resolution is tolerated. In such cases the column is considered satisfactory if the resolution index is more than 2. The resolution index between two peaks is defined by the ratio:

$$\frac{a}{b}$$

where:

a = distance from the base line to the tip of the smallest peak;

b = distance from the base line to the lowest point of the trace line between two peaks.

7.3.2.2. To check that the above requirements are satisfied, inject equal quantities of the test portion of the methyl esters, doing so several times, and adjust the temperature and flow of the carrier gas until the best separation results are obtained. Also check the resolving power by reducing the test portion, if necessary, and increasing the sensitivity until the best peak resolution is obtained.

If all the fatty acids present are separated and the resolution index is more than 2, the column is suitable and the operating conditions selected should be maintained in all the successive determinations. If not, the column is not efficient.

7.4. Analytical Procedure. Using the 10 µl micro-syringe, take up 1 µl of hexane, draw in 0.5 µl of air and then 0.5 + 1 µl of the sample solution. Pull back the plunger of the syringe further so the needle is emptied. Push the needle through the membrane of the injection unit and after one to two seconds inject rapidly, then slowly removing the needle after five seconds. Continue recording until the methyl esters present are completely eluted. The base line must always satisfy the requirements specified in 7.3.2.1.

7.5. Peak identification. The order in which the fatty acid methyl esters appear on the chromatogram is a direct function of their number of carbon atoms. The unsaturated esters are eluted after the corresponding saturated esters and their elution is a direct function of the number of double bonds. The *trans* fatty acid esters are eluted before the corresponding *cis* isomers.

The individual methyl esters are then identified by their retention times, which are compared with the retention times of the reference portions. A specimen chromatogram is given in Figure 1.

7.6. Quantitative analysis

7.6.1. Calculate the areas of each peak using the electronic integrator. To ensure the correct analytical sensitivity, the height of the peak corresponding to the methyl ester of arachidic acid must not be less than 20% of the full-scale value. In the case of groundnut oil, the height must not be less than 50%.

7.6.2. The percentage for each fatty acid is calculated from the ratio of the area under the corresponding peak to the sum of the areas under all the peaks using the formula:

$$100 \ \frac{A_x}{\sum A}$$

where:

A_x = peak area for fatty acid x;

ΣA = total of all the peak areas.

8. Expression of the results

8.1. Record the "total *trans* fatty acids, %" as the sum of the percentage contents of the following acids:

- *trans*-octadecenoic;
- *trans-trans* octadecadienoic;
- *cis-trans* and *trans-cis* octadecadienoic;
- *trans-cis-trans*, *cis-cis-trans*, *cis-trans-cis* and *trans-cis-cis* octadecatrienoic.

Express the result to two decimal points.

Note

Certain precautions are required to ensure proper analytical evaluation of the results. Small quantities of *trans* oleic acid could be formed when the sample is introduced into the gas chromatographic injector. To prevent this from occurring it is important to check procedure in the following manner:

A sample of methyl esters of an extra virgin olive oil of known origin (or a methyl oleate standard or a mixture with fatty acid methyl esters with a minimum oleic acid content of 60% that are certified free of *trans* fatty acids) is prepared as described in section 7.1. and analysed under the conditions specified in the method. The percentage of *trans* octadecenoic fatty acids is then measured. Normally, no *trans* fatty acids are present. However, the presence of 0.01% *trans* octadecenoic fatty acids is tolerated.

If higher values are recorded, the injector insert should be replaced by a perfectly clean, deactivated insert and the analysis should be repeated.

A further precaution has to be taken in the case of injector inserts packed with a variety of materials (glass wool, diatomaceous earth, etc.) since the presence of such materials may catalyse the formation of *trans* fatty acids.

Certain precautions therefore have to be taken.

Analyse a sample of extra virgin olive oil of known origin, as in the preceding case. Measure the percentage of *trans* octadecenoic fatty acids. If the values are above 0.01%, proceed as follows:

(1) Replace the injector insert by a clean, deactivated insert.

(2) Lower the injector temperature by 30-50°C and repeat the test until the values obtained for the *trans* octadecenoic fatty acids are equal to or below the above-mentioned limit.

(3) Remove the packing material from the injector*.

It is wise to take one further precaution when analysing extra virgin olive oils. When authentic oils are analysed, a peak forms whose retention time is almost identical to that of *cis-trans* linoleic. This peak, the value of which varies around 0.01%, should not be taken into consideration in the calculation when it appears on its own in the chromatogram. It should, however, be taken into account if it appears on the chromatogram together with *trans-cis* linoleic.

***** It is necessary to use packing material (glass wool, diatomaceous earth, etc.) if automatic samplers are employed. If injection is manual, the packing material may be removed.

PRECISION VALUES OF THE METHOD

1. Analysis of the collaborative test results

The precision values of the method are given in the table overleaf.

Nineteen laboratories holding IOOC recognition at the time took part in the collaborative test arranged by the Executive Secretariat in 1999. The laboratories were from eight countries.

The test was performed on five samples:

A: extra virgin olive oil
B: virgin olive oil + refined sunflower oil
C: virgin olive oil + refined olive-pomace oil
D: virgin olive oil + refined soybean oil + refined sunflower oil
E: refined olive oil + refined olive-pomace oil + refined soybean oil + lampante virgin olive oil

The results of the collaborative test organised by the IOOC Executive Secretariat have been statistically processed according to the rules laid down in the international standards ISO 5725 **Accuracy (trueness and precision) of measurement methods and results.** Outliers were examined by applying Cochran's and Grubbs' test to the laboratory results for each determination (replicates a and b) and each sample.

The table lists:

n number of participating laboratories

outliers number of laboratories with outlying values

mean mean of the accepted results

r value below which the absolute difference between two single independent test results obtained with the same method on identical test material in the same laboratory by the same operator using the same equipment within short intervals of time may be expected to lie with a probability of 95%

S_r Repeatability standard deviation

RDS_r (%) Repeatability coefficient of variation (S_r x 100/mean)

R value below which the absolute difference between two single test results obtained with the same method on identical test material in different laboratories with different operators using different equipment may be expected to lie with a probability of 95%

S_R Reproducibility standard deviation

RDS_R (%) Reproducibility coefficient of variation (S_R x 100/mean)

***Trans* fatty acid composition (%): C18:1T**

	A	B	C	D	E
No. labs	17	17	17	17	17
Outliers	5	2	4	0	0
Mean	0,012	0,020	0,020	0,021	0,051
R	0,007	0,009	0,010	0,010	0,020
S_r	0,003	0,003	0,004	0,004	0,007
RSD_r (%)	22,610	20,160	32,110	22,001	14,080
R	0,010	0,021	0,021	0,023	0,031
S_R	0,004	0,008	0,008	0,008	0,011
RSD_R (%)	30,960	46,580	32,110	50,003	21,741

***Trans* fatty acid composition (%): C18:2T+ C18:3T**

	A	B	C	D	E
No. labs	16	17	16	17	17
Outliers	5	2	2	4	0
Mean	0,014	0,032	0,011	0,044	0,303
R	0,011	0,010	0,007	0,010	0,043
S_r	0,004	0,004	0,003	0,004	0,015
RSD_r (%)	34,920	13,361	18,983	9,852	5,061
R	0,016	0,020	0,017	0,023	0,131
S_R	0,006	0,007	0,006	0,008	0,047
RSD_R (%)	47,702	25,643	42,801	22,413	15,392

2. Normative references

ISO 5725-1: 1994 Accuracy (trueness and precision) of measurement methods and results – Part 1: General principles and definitions

ISO 5725-2: 1994 Accuracy (trueness and precision) of measurement methods and results – Part 2: Basic method for the determination of the repeatability and reproducibility of a standard measurement method

ISO 5725-5: 1994 Accuracy (trueness and precision) of measurement methods and results – Part 5: Alternative methods for the determination of the precision of a standard measurement method

ISO 5725-6: 1994 Accuracy (trueness and precision) of measurement methods and results – Part 6: Use in practice of accuracy values

APPENDICES

A. Operating precautions

A.1. Bottled, compressed air. Compressed gas at high pressure. Use with caution in the presence of combustible substances since the self-ignition temperature of most of the organic compounds in the air is considerably lower under high pressure. Make sure the bottle valve is always closed when not in use. Always use a pressure reducer. Release the tension of the reducer spring before opening the bottle valve. Do not stand in front of the bottle outlet when opening the valve. Do not switch the gas from one bottle to another. Do not mix gas in the bottle. Make sure the bottles cannot be knocked over. Keep them away from sunlight or sources of heat. Store in a corrosion-free environment. Do not use damaged or unlabelled bottles. Air intended for technical purposes must not be used for inhaling or for respiratory apparatus.

A.2. Bottled, compressed helium. Compressed gas at high pressure. It reduces the amount of oxygen available for breathing. Keep the bottle shut. Ensure proper ventilation during usage. Do not enter storage areas unless they are properly ventilated. Always use a pressure reducer. Release the tension of the reducer spring before opening the bottle valve. Do not transfer gas from one bottle to another. Do not mix gas in the bottle. Make sure the bottles cannot be knocked over. Do not stand in front of the bottle outlet when opening the valve. Keep the bottles away from sunlight and sources of heat. Store in a corrosion-free environment. Do not inhale. Use solely for technical purposes.

A.3. Bottled, compressed hydrogen. Highly inflammable, under pressure. Keep away from sources of heat, sparks, naked flames or electric apparatus not manufactured from non-inflammable material. Make sure the bottle valve is shut when not in use. Always use with a pressure reducer. Release the tension of the reducer spring before opening the bottle valve. Do not stand in front of the bottle outlet when opening the valve. Ensure proper ventilation during usage. Do not transfer hydrogen from one bottle to another. Do not mix gas in the bottle. Make sure the bottles cannot be knocked over. Keep them away from sunlight and sources of heat. Store in a corrosion-free environment. Do not use damaged or unlabelled bottles.

A.4. n-hexane. Highly inflammable. Fumes may ignite. Keep away from sources of heat, sparks, or naked flames. Make sure the bottles are always properly closed. Ensure proper ventilation during usage. Avoid build-up of fumes and remove any possible fire risk, such as heaters or electric apparatus not manufactured from non-inflammable material. It is pernicious if inhaled since it can damage the cells of the nervous system. Avoid breathing in the fumes. Use a suitable respiratory apparatus if necessary. Avoid contact with eyes and skin.

Fig. 1. Specimen chromatogram obtained from capillary column analysis of the *trans* fatty acids.