TC 34

INTERNATIONAL STANDARD ISO 5508

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Animal and vegetable fats and oils — Analysis by gas-liquid chromatography of methyl esters of fatty acids

Corps gras d'origines animale et végétale — Analyse par chromatographie en phase gazeuse des esters méthyliques d'acides gras

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FOREWORD

ISO (the International Organization for Standardization) is a worldwide federation of national standards institutes (ISO member bodies). The work of developing International Standards is carried out through ISO technical committees. Every member body interested in a subject for which a technical committee has been set up 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.

Draft International Standards adopted by the technical committees are circulated to the member bodies for approval before their acceptance as International Standards by the ISO Council.

International Standard ISO 5508 was developed by Technical Committee ISO/TC 34, *Agricultural food products*, and was circulated to the member bodies in July 1976.

It has been approved by the member bodies of the following countries :

Australia	Hungary	Portugal
Austria	Iran	South Africa, Rep. of
Bulgaria	Israel	Spain
Canada	Korea, Rep. of	Thailand
Czechoslovakia	Netherlands	Turkey
France	New Zealand	United Kingdom
Germany, F.R.	Poland	Yugoslavia

The member body of the following country expressed disapproval of the document on technical grounds :

Chile

Printed in Switzerland

Animal and vegetable fats and oils — Analysis by gas-liquid chromatography of methyl esters of fatty acids

1 SCOPE AND FIELD OF APPLICATION

This International Standard gives general guidance for the application of gas-liquid chromatography to determine the qualitative and quantitative composition of a mixture of fatty acid methyl esters obtained according to ISO 5509.

The method is not applicable to polymerized fatty acids.

2 REFERENCE

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

3 PRODUCTS REQUIRED

3.1 Carrier gas

Inert gas (nitrogen, helium, argon, etc.), thoroughly dried and with an oxygen content less than 10 mg/kg.

3.2 Auxiliary gases

3.2.1 Hydrogen (purity ⩾ 99,9 %), free from organic impurities.

3.2.2 Air or **oxygen,** free from organic impurities.

3.3 Reference standards

A mixture of methyl esters, or the methyl esters of an oil of known composition, preferably similar to that of the fatty matter to be analysed.

4 APPARATUS

The instructions given relate to the ordinary equipment used for gas liquid chromatography, employing a packed column and a flame-ionization detector. Any apparatus giving the efficiency and resolution defined in 5.1.2 is suitable.

4.1 Gas chromatograph

4.1.1 *Injection system*

The injection system shall have the least dead space possible. Unless materially impossible, it shall be capable of being heated to a temperature 20 to 50 °C higher than that of the column.

4.1.2 *Oven*

The oven shall be capable of heating the column to a temperature of at least 220 °C and of maintaining the desired temperature to within ± 1 °C.

If programmed heating is to be used, an apparatus with a twin column is recommended.

4.1.3 *Packed column*

4.1.3.1 COLUMN

The column shall be constructed of a material inert to the substances to be analysed (glass or stainless steel).

NOTE — If polyunsaturated components with more than 3 double bonds are present, they may be decomposed in a stainless steel column.

– Length : 1 to 3 m. A relatively short column should be used when long-chain fatty acids (above C_{20}) are present. When analysing acids with 4 or 6 carbon atoms, it is recommended that a 2 m column be used.

– Internal diameter : 2 to 4 mm.

4.1.3.2 Packing

– Support : Acid-washed and silanized diatomaceous earth, or other suitable inert support with a narrow range of grain size (25 µm between the limits 125 µm to 200 µm), the average grain size being related to the internal diameter and length of the column.

– Stationary phase : Polyester type of polar liquid (for example, diethylene glycol polysuccinate, butanediol polysuccinate, ethyleneglycol polyadipate, etc.), cyanosilicones or any other liquid permitting the chromatographic separation required (see clause 5). The stationary phase should amount to 5 to 20 % of the packing. A non-polar stationary phase can be used for certain separations.

4.1.3.3 Conditioning of the column

With the column disconnected, if possible, from the detector, gradually heat the oven to 185 °C and pass a current of inert gas through the freshly prepared column at a rate of 20 to 60 ml/min for at least 16 h at this temperature, and for a further 2 h at 195 °C.

4.1.4 *Detector*

The detector should preferably be capable of being heated to a temperature above that of the column.

4.2 Syringe

Maximum capacity 10 µl, graduated in 0,1 µl.

4.3 Recorder

If the recorded curve is to be used to calculate the composition of the mixture analysed, an electronic recorder of high precision, compatible with the apparatus used, is required. The recorder shall have the following characterristics :

– rate of response below 1,5 s, preferably 1 s;
(The rate of response is the time taken for the recording pen to pass from 0 to 90 % following the sudden introduction of a 100 % signal.)

– width of the paper : 25 cm minimum;

– paper speed : adjustable to values between 0,4 and 2,5 cm/min.

4.4 Integrator or calculator (optional)

Rapid and accurate calculation can be performed with the help of an electronic integrator or calculator. This shall give a linear response with adequate sensitivity, and the correction for deviation of the base line shall be satisfactory.

5 PROCEDURE

The operations described below relate to the use of a flame-ionization detector.

NOTE – A gas-liquid chromatograph employing a catharometer detector (working on thermal conductivity changes) may be used. Operating conditions are then modified as indicated in clause 7.

5.1 Test conditions

5.1.1 *Selection of optimum operating conditions*

In selecting the test conditions, the following variables should be taken into account :

– length and diameter of the column;

– nature and amount of the stationary phase;

– temperature of the column;

– carrier gas flow;

– resolution required;

– size of the test portion, selected in such a way that the assembly of the detector and electrometer gives a linear response;

– duration of analysis.

Generally, the following values will lead to the desired results, i.e. at least 2 000 theoretical plates for methyl stearate and its elution within about 15 min.

Internal diameter of column	Carrier gas-flow
mm	ml/min
2	15 to 25
3	20 to 40
4	40 to 60

Concentration of stationary phase	Temperature
%	°C
5	175
10	180
15	185
20	185

Where the apparatus allows it, the injector should be at a temperature of about 200 °C and the detector at a temperature equal to or higher than that of the column.

As a rule, the ratio of the flow rate of the hydrogen supplied to the flame-ionization detector to that of the carrier gas varies from 1 : 2 to 1 : 1 depending on column diameter. The flow of oxygen is about 5 to 10 times that of the hydrogen.

5.1.2 *Determination of efficiency and resolution*

Carry out the analysis of a mixture of methyl stearate and oleate in about equivalent proportions (for example methyl esters from cocoa butter).

Choose the size of the test portion, the temperature of the column and the carrier gas flow so that the maximum of the methyl stearate peak is recorded about 15 min after the solvent peak, and occupies about 3/4 of the full scale.

Calculate the number of theoretical plates, n (efficiency), using the formula :

$$n = 16 \left(\frac{dR_1}{\omega_1} \right)^2$$

and the resolution, R, using the formula :

$$R = \frac{2\Delta}{\omega_1 + \omega_2}$$

where

dR_1 is the retention distance, measured in millimetres, from the start to the maximum of the peak for methyl stearate;

ω_1 and ω_2 are the widths, in millimetres, of the peaks for methyl stearate and methyl oleate respectively, measured between the points of intersection of the tangents at the points of inflexion of the curve with the base line;

Δ is the distance between the respective peak maxima for methyl stearate and methyl oleate.

(See the diagram below.)

Operating conditions to be selected are those which will afford at least 2 000 theoretical plates for methyl stearate and a resolution of at least 1,25.

5.2 Test portion

Using the syringe (4.2), take 0,1 to 2 µl of the solution of methyl esters prepared according to ISO 5509. In the case of esters not in solution, prepare a solution, in heptane of chromatographic quality, of about 100 mg/ml and inject 0,1 to 1 µl of this solution.

If the analysis is for constituents present only in trace amounts, the size of the test portion may be increased (up to ten-fold).

5.3 Analysis

Generally, the operating conditions shall be those defined in 5.1.1. Nevertheless, it is possible to work with a lower column temperature when the determination of fatty acids with fewer than 12 carbon atoms is required, or at a higher temperature when determining fatty acids with more than 20 carbon atoms.

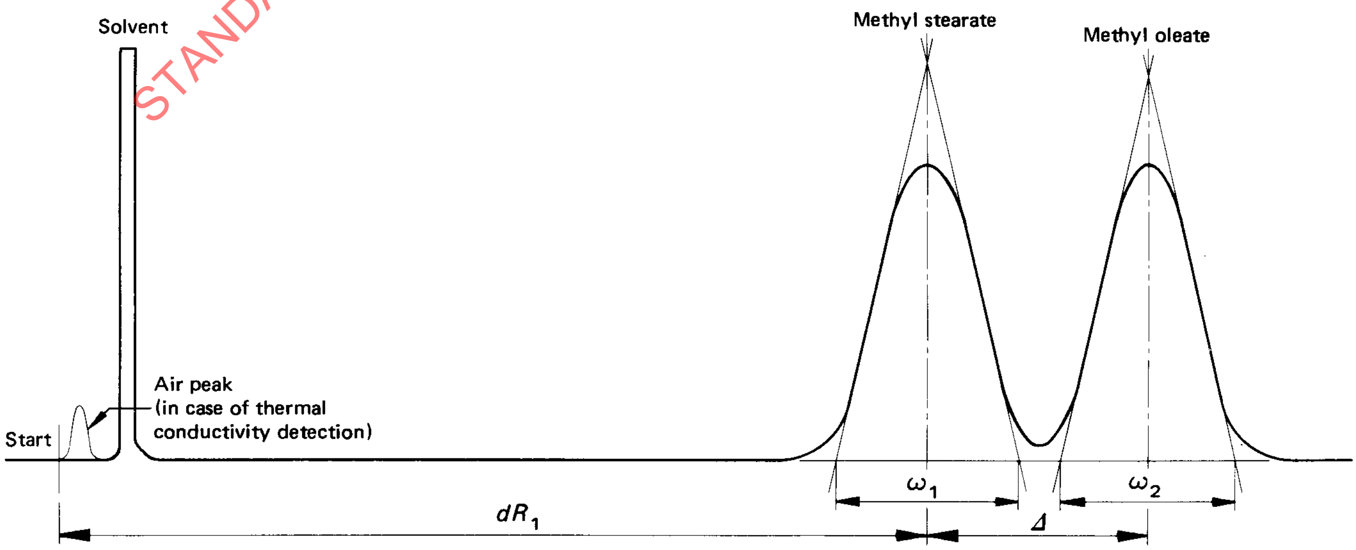
On occasion, it is possible to employ temperature programming in both the previous cases. For example, if the sample contains the methyl esters of fatty acids with fewer than 12 carbon atoms, inject the sample at 100 °C (or at 50 to 60 °C if butyric acid is present) and immediately raise the temperature at 4 to 8 °C/min to the optimum. In certain cases, the two procedures can be combined. After the programmed heating, continue the elution at a constant temperature until all the components have been eluted. If the instrument does not work with programmed heating, work at two fixed temperatures between 100 °C and 195 °C.

If necessary, it is recommended that an analysis be carried out on two fixed phases with different polarities to verify the absence of masked peaks, for example for fish oils or in the case of the simultaneous presence of $C_{18:3}$ and $C_{20:0}$ or $C_{18:3}$ and $C_{18:2}$ conjugated.

6 EXPRESSION OF RESULTS

6.1 Qualitative analysis

Analyse the reference standard mixture (see 3.3), using the same operating conditions as those employed for the sample, and measure the retention times or retention distances for the constituent fatty acids. Construct on semi-



logarithmic paper, for any degree of unsaturation, the graphs showing the logarithm of the retention time or distance as a function of the number of carbon atoms; in isothermal conditions, the graphs for straight-chain acids of the same degree of unsaturation should be straight lines. These lines should be approximately parallel.

Identify the peaks for the sample from these graphs, if necessary by interpolation.

It is necessary to avoid conditions such that "masked peaks" exist, i.e. where the resolution is insufficient to separate two constituents.

6.2 Quantitative analysis

6.2.1 *Determination of the composition*

Apart from exceptional cases, use the internal normalization method, i.e. assume that the whole of the components of the sample are represented on the chromatogram, so that the total of the areas under the peaks represents 100 % of the constituents (total elution).

If the equipment includes an integrator, use the figures obtained therefrom. If not, determine the area under each peak by multiplying the height of the peak by its width at mid-height, and where necessary take into account the various attenuations used during the recording.

6.2.2 *Method of calculation and formulae*

6.2.2.1 General case

Calculate the content of a given constituent, expressed as a percentage by mass of methyl esters, by determining the percentage represented by the area of the corresponding peak relative to the sum of the areas of all the peaks as follows :

percentage by mass of component i, expressed as methyl esters,

$$= \frac{A_i}{\Sigma A_i} \times 100$$

where

A_i is the area under the peak corresponding to component i;

ΣA_i is the sum of the areas under all the peaks.

Give the result to one decimal place.

NOTE — In this general case, the result of the calculation based on relative areas is considered to represent a percentage by mass. For the cases in which this assumption is not allowed, see 6.2.2.2.

6.2.2.2 Use of correction factors

In certain cases, particularly in the presence of fatty acids with fewer than 8 carbon atoms or of acids with secondary groups, when using thermal conductivity detectors or where the highest degree of accuracy is particularly required, correction factors should be used to convert the percentages of peak areas into mass-percentages of the components.

Determine the correction factors with the help of a chromatogram derived from the analysis of a reference mixture of methyl esters of known composition under operating conditions identical with those used for the sample.

For this reference mixture :

percentage by mass of component i

$$= \frac{m_i}{\Sigma m_i} \times 100$$

where

m_i is the mass of component i in the reference mixture;

Σm_i is the total of the masses of the various components of the reference mixture.

From the chromatogram of the reference mixture, calculate :

percentage (area/area) for component i

$$= \frac{A_i}{\Sigma A_i} \times 100$$

where

A_i is the area under the peak corresponding to component i;

ΣA_i is the sum of the areas under all the peaks.

Whence, correction factor

$$K_i = \frac{m_i \times \Sigma A_i}{A_i \times \Sigma m_i}$$

Commonly, the correction factors are made relative to K_{C16}, so that the relative factors become :

$$K'_i = \frac{K_i}{K_{C16}}$$

For the sample, the content of each component is given by :

percentage by mass of component i, expressed as methyl esters,

$$= \frac{K'_i \times A_i}{\Sigma (K'_i \times A_i)} \times 100$$

Give the results to one decimal place.

6.2.2.3 Use of an internal standard

In certain cases (notably the assay of C_4 and C_6 acids and the determination of the acids when all the fatty acids are not eluted) an internal standard should be used (C_5 and C_{15} or C_{17} respectively) and the correction factor for the internal standard should be determined.