COMMISSION REGULATION (EEC) No 72/77

of 13 January 1977

amending Regulation (EEC) No 1470/68 on the drawing and reduction of samples and the determination of oil content, impurities and moisture in oil seeds

THE COMMISSION OF THE EUROPEAN COMMUNITIES,

Having regard to the Treaty establishing the European Economic Community,

Having regard to Council Regulation No 136/66/EEC of 22 September 1966 on the establishment of a common organization of the market in oils and fats (1), as last amended by Regulation (EEC) No 1707/73 (2), and in particular Article 26 (3) thereof,

Whereas an appropriate method should be laid down in order to enable the erucic acid content in colza and rape seeds to be determined accurately; whereas Commission Regulation (EEC) No 1470/68 of 23 September 1968 on the drawing and reduction of samples and the determination of oil content, impurities and moisture in oil seeds (3), as last amended by Regulation (EEC) No 3025/75 (4), should consequently be amended;

Whereas the measures provided for in this Regulation are in accordance with the opinion of the Management Committee for Oils and Fats,

HAS ADOPTED THIS REGULATION:

Article 1

The following Article 2b is hereby added to Regulation (EEC) No 1470/68:

'The determination of the erucic acid content specified in Article 4 of Regulation No 282/67/EEC shall be carried out in accordance with the method set out in Annex VI to this Regulation.

The maximum content of erucic acid specified in the first indent of Article 3 (2) of Regulation No 282/67/EEC shall be considered to be present in colza and rape seeds where the oil extracted from them in accordance with the method given in Title I of Annex VI contains a maximum of 15 % erucic acid (C 22), calculated in accordance with the method set out in Titles II and III of the said Annex.'

Article 2

An Annex VI, as set out in the Annex hereto, is hereby added to Regulation (EEC) No 1470/68.

Article 3

This Regulation shall enter into force on the third day following its publication in the *Official Journal of the European Communities.*

This Regulation shall be binding in its entirety and directly applicable in all Member States.

Done at Brussels, 13 January 1977.

For the Commission

Finn GUNDELACH

Vice-President

(1) OJ No 172, 30. 9. 1966, p. 3025/66.
(2) OJ No L 175, 29. 6. 1973, p. 5.
(3) OJ No L 239, 28. 9. 1968, p. 2.
(4) OJ No L 300, 20. 11. 1975, p. 5.

ANNEX VI

COMMUNITY METHOD OF ANALYSIS TO BE USED FOR THE DETERMINATION OF THE ERUCIC ACID CONTENT OF SEEDS TAKEN OVER BY INTERVENTION AGENCIES

I. Preparation of samples

II. Preparation of the methyl esters of the fatty acids

III. Gas chromatography of the methyl esters of the fatty acids

I. PREPARATION OF THE SAMPLES

1. The drawing of the seed samples, the reduction of the laboratory samples to samples for analysis and the determination of the oil content of the product as such shall be carried out in accordance with the procedures respectively described in Annexes I, II and V to Regulation (EEC) No 1470/68.

2. Preparation of the sample of the oil extracted from the seeds subjected to the sampling procedure.

2.1. If the sample is liquid and perfectly clear, shake it as a precautionary measure before drawing the test portions.

2.2. If the sample is liquid but cloudy or has thrown down a deposit, place it in an oven kept at 50 °C. When the sample has reached this temperature, give it a vigorous shake and allow it to settle. Filter it through a paper in the oven with the temperature kept to 30 °C.
The sample must be clear.

2.3. If the sample is congealed, place it in the oven kept at a temperature 10 °C above the expected melting range. If the sample when melted is clear, proceed as under Section 2.1 ; if the sample when melted is cloudy or has thrown down a deposit, proceed as under Section 2.2.

II. PREPARATION OF THE METHYL ESTERS OF THE FATTY ACIDS

1. FOREWORD

The aim of the methods is the conversion of the oils and fats of animal or vegetable origin, or the fatty acids of any origin, into the methyl esters of the fatty acids.

The resultant methyl esters can be used in any method requiring such a conversion, including gas chromatography, thin-layer chromatography and infra-red spectrophotometry.

2. FIELD OF APPLICATION

The methods described are applicable to oils and fats of animal or vegetable origin. The methods described in Section 3 are applicable to the preparation of the methyl esters of the fatty acids with six or more atoms of carbon. If the C_6 and C_8 fatty acids are present and when the preparation of esters intended for gas chromatography is involved, it is essential not to drive off the solvent from the solution of the methyl esters.

The general methods in Section 3.1 may end in erroneous results if the following are present :

— compounds with secondary oxygen groupings (hydroxy, hydroperoxy, keto or expoxy groupings) ;

— compounds with cyclopropane or cyclopropene groupings ;

— conjugated polyunsaturated compounds and acetylene compounds ;

— waxes.

If so, it is preferable to use one of the methods described in Section 3.2. Oils and fats in which these groupings are present to a very limited extent (e.g., cottonseed oil) may nevertheless be analyzed in accordance with Section 3.1.

Phospholipids may be analyzed following saponification and esterification of the fatty acids.

Unsaponifiable matter is not eliminated and, if present to any appreciable extent, may interefere in the subsequant analyses (Note 1).

3. METHODS OF PREPARATION OF THE METHYL ESTERS OF THE FATTY ACIDS WITH SIX OR MORE CARBON ATOMS

The general method of preparation of the methyl esters using boron trifluoride is described in Section 3.1 and should be adopted in preference to all others. If it is impossible to use it, the alternatives described in Section 3.2 may be used.

3.1. **General method using boron trifluoride**

3.1.1. *Principle*

Saponification of the glycerides, liberation and esterification of the fatty acids in the presence of boron trifluoride.

3.1.2. *Apparatus*

— Flasks, 50 and 100 ml, with ground necks ;

— reflux condenser, effective length 20 to 30 cm, with ground joint to fit these flasks ;

— degreased boiling aid ;

— nitrogen bubbling tube ;

— graduated pipette, 10 ml capacity or more, and pipette bulb ; or an automatic pipette ;

— ground, stoppered test tubes ;

— separating funnels, 250 ml.

3.1.3. *Reagents*

— Methanol solution of sodium hydroxide, approximately 0·5N :

Dissolve 2 g of sodium hydroxide in 100 ml of methanol containing not more than 0·5 % (m/m) of water. If the solution has to be kept for use over a fair length of time, a little sodium carbonate forms as a white precipitate ; this has no affect whatsoever on the preparation of the methyl esters ;

— methanol solution of boron trifluoride, with a content ranging from 12 to 15 % (m/m). (Solutions with 14 and 50 % can be bought) (Note 2).

Warning. Boron trifluoride is toxic. The user is therefore not recommended to prepare the solution himself from boron trifluoride and methanol (Note 3) ;

— heptane, chromatography grade (Note 2, Note 4) ;

— redistilled petroleum ether (boilina from 40 to 60 °C) with a bromine value less than 1, residue free ; or hexane (Note 2) ;

— anhydrous sodium sulphate ;

— saturated solution of sodium chloride ;

— methyl red, 0·1 % (m/v) solution in 60 % (v/v) ethanol ;

— nitrogen with an oxygen content less than 5 mg per kg of gas.

3.1.4. *Procedure*

Owing to the toxicity of boron trifluoride, the operations concerned should be carried out in a fume cupboard. All glassware should be washed *immediately after use.*

If the oils or fatty acids contain acids with more than two double bonds, it is desirable to eliminate air from the methanol and flask by flushing with nitrogen for a few minutes. Prepare the sample in accordance with Title I, 'Preparation of the samples'. An exact weight is unnecessary. The size of the test portion need only be known to choose the right flask and quantities of reagents in accordance with this table :

Test portion (mg)	Flask (ml)	0·5 N NaOH (ml)	Solution of BF_3 in methanol (ml)
100- 250	50	4	5
250- 500	50	6	7
500- 750	100	8	9
750-1000	100	10	12

When the methyl esters are intended for gas chromatography analysis, the test portion should preferably be about 350 mg. If smaller, it is essential to take the precaution that the sample drawn is representative (Note 5).

3.1.4.1. *With oils and fats*

Place the prepared test portion in the flask. Add the correct quantity of methanol solution of sodium hydroxide and a boiling aid. Fit the reflux condenser to the flask. Raise to the boil under reflux until the oil or fat droplets disappear (this operation lasts 5 to 10 minutes but may be longer than this in some exceptional cases).

Using the graduated pipette fitted with a pipette bulb or the automatic pipette, introduce the correct quantity of methanol solution of boron trifluoride into the boiling mixture through the top of the reflux condenser. Continue to boil for two minutes. To the boiling mixture add 2 to 5 ml of heptane (Note 4) through the top of the reflux condenser (the quantity of heptane is immaterial to the reaction); continue to boil for 1 minute. Remove the source of heat and then the reflux condenser from the flask. Add a few ml of saturated sodium chloride solution and gently stir the flask by rotation.

Continue to add more saturated sodium chloride solution until the surface of the liquid reaches the neck of the flask. Transfer about 1 ml of the top layer (heptane solution) into a ground-neck test tube and add just enough anhydrous sodium sulphate to eliminate the traces of water. If the test portion was of 350 mg, the solution obtained contains about 5 to 10 % of methyl esters and can be injected directly into a gas chromatography column. If otherwise, dilute the heptane solution to obtain a concentration of 5 to 10 % in methyl esters (Note 6).

To obtain all the esters in a dry state, transfer the salt solution and the heptane phase into a 250 ml separating funnel. Separate. Extract the salt solution with 50 ml of petroleum ether (boiling from 40 to 60 °C) or hexane.

Repeat this extraction again. Combine the heptane phase and the two extracts and wash with 20 ml portions of wash until the acidity disappears (methyl red indicator). Dry with anhydrous sodium sulphate and evaporate the solvent on a water bath in a stream of nitrogen (Notes 6 and 7). If the test portion was of less than 500 mg, it is desirable to reduce the volumes of solvent and water proportionately.

3.1.4.2. *With fatty acids*

If the sample consists of fatty acids only, saponification is omitted.

Place the desired volume of prepared fatty acids in a flask. Add the prescribed quantity of methanol solution of boron trifluoride. Boil for two minutes. Then proceed as under Section 3.1.4.1 from the point where it says 'To the boiling mixture add ...'.

3.2. **Alternative methods without using boron trifluoride**

3.2.1. *With neutral oils and fats* (acid value < 2)

3.2.1.1. *Principle*

Methanolysis of the glycerides in an alkaline medium.

3.2.1.2. *Apparatus*

— high-speed stirrer and a suitable heater (e.g., a heating magnetic stirrer);

— conical or round-bottom flask, 100 ml, with ground neck;

— nitrogen bubbling tube;

— reflux condenser fitting the conical or round-bottom flask;

— degreased boiling aid;

— separating funnels, 125 ml;

— narrow neck conical flask, 50 ml.

3.2.1.3. *Reagents*

— methanol, containing not more than 0·5 % (m/m) of water;

— methanol solution of potassium hydroxide, about 1 N: dissolve 5·6 g of potassium hydroxide in 100 ml of methanol containing not more than 0·5 % (m/m) of water;

— heptane, chromatography grade (Note 2, Note 4);

— anhydrous sodium sulphate;

— nitrogen, containing less than 5 mg of oxygen per kg of gas.

3.2.1.4. *Procedure*

If the oils contain acids with more than two double bonds, it is desirable to eliminate air from the methanol and flask by flushing with nitrogen for a few minutes.

Prepare the sample.

Into the 100 ml conical or round-bottom flask place about 4 g (Note 5) of the prepared oil or fat.

Add about 40 ml of methanol, 0·5 ml of the potassium hydroxide solution and the boiling aid. Fit the reflux condenser, stir and bring to the boil. The solution should turn clear. The reaction is usually completed in 5 to 10 minutes. With oils of the 'castor oil' type, clearness is not the reaction criterion (Note 8).

Cool under running water and transfer into a 125 ml separating funnel. Rinse the conical or round-bottom flask with 20 ml of heptane (Note 4) and pour into the funnel.

Add about 40 ml of water, shake and allow to settle. The esters are contained in the upper heptane layer. Extract the aqueous layer once more with 20 ml of heptane. Combine the two extracts and wash twice with 20 ml of water. Separate and dry the solution of the esters over anhydrous sodium sulphate, and filter through cotton wool; if necessary, evaporate the solvent down to a volume of 20 ml with a stream of nitrogen in a 50 ml conical flask over a water-bath at boiling point (Notes 6 and 7).

3.2.2. *With acidic oils and fats (acid value > 2) and fatty acids*

3.2.2.1. *Principle*

Neutralization of the free fatty acids, alkaline methanolysis of the glycerides followed by the esterfication of the fatty acids in an acid medium in the case of acidic oils and fats. Esterification in an acid medium in the case of the fatty acids.

3.2.2.2. *Apparatus*

— high-speed stirrer and a suitable heater (e.g., a heating magnetic stirrer);

— conical or round-bottom flask, 250 ml, with ground neck;

— nitrogen bubbling tube;

— reflux condenser fitting the conical or round-bottom flask;

— degreased boiling aid;

— separating funnels, 250 ml;

— narrow neck conical flask, 100 ml.

3.2.2.3. *Reagents*

— sodium methylate solution, prepared by dissolving 1 g of sodium in 100 ml of methanol containing not more than 0·5 % (m/m) of water;

— methanol solution of anhydrous hydrogen chloride, about 1 N (see Remarks, Section 3.2.2.6 (a) and (b));

— heptane, chromatography grade (Notes 2 and 4);

— anhydrous sodium sulphate;

— nitrogen, containing less than 5 mg of oxygen per kg of gas.

3.2.2.4. *Procedure with acidic oils and fats*

If the oils contain acids with more than two double bonds, it is desirable to eliminate air from the methanol and flask by flushing with nitrogen for a few minutes.

Prepare the sample in accordance with Title I, 'Preparation of the samples'. Place 4 g (Note 5) of prepared oil or fat in a 250 ml conical or round-bottom flask.

Add 40 ml of sodium methylate solution (see Remarks, Section 3.2.2.6 (c)). Fit the reflux condenser to the flask and raise to the boil. Usually in about 10 minutes time the solution should become clear. In practice the reaction is completed in 15 minutes.

Then add not less than 50 ml of hydrogen chloride solution to the flask and continue boiling for 10 minutes (see Remarks, Section 3.2.2.6 (d)).

Cool under running water, add 100 ml of water to the flask and then pour the mixture into a 250 ml separating funnel and add 30 ml of heptane (Note 4). Shake vigorously and allow to settle until the two layers separate completely. Collect the heptane layer. Extract the aqueous layer for the second time with 30 ml of heptane. Combine the two heptane extracts and wash several times with water until neutral. Separate and dry over anhydrous sodium sulphate. Filter through cotton wool and, if necessary, evaporate the solvent down to a volume of 20 ml with a stream of nitrogen in a 100 ml conical flask over a water-bath kept at boiling point (Notes 6 and 7).

3.2.2.5. *Procedure with fatty acids*

It is essential with fatty acids to use a simplified procedure as follows:

If the fatty acids contain acids with more than two double bonds, it is desirable to eliminate the air from the methanol and flask by flushing with nitrogen for a few minutes.

Prepare the sample in accordance with Title I 'Preparation of the samples'. Place about 4 g (Note 5) of prepared oil or fat in a 250 ml conical or round-bottom flask.

Add 50 ml of hydrogen chloride solution and a boiling aid, fit the reflux condenser and then boil for 10 minutes. Cool under running water, add 100 ml of water to the flask, then pour the mixture into a 250 ml separating funnel and add 30 ml of heptane (Note 4). Shake vigorously and allow to settle until the two layers separate completely. Run off the aqueous layer and extract a second time with 30 ml of heptane. Combine the two heptane extracts and wash them repeatedly with water until neutral. Separate and dry over anhydrous sodium sulphate. Filter through cotton wool and evaporate the solvent down to a volume of 20 ml with a stream of nitrogen in a 100 ml conical flask over a water-bath kept at boiling point (Notes 6 and 7).

3.2.2.6. *Remarks*

(a) It is no trouble at all in the laboratory to prepare small quantities of hydrogen chloride by the simple expedient of displacing it from the commercial solution (s.g. 1·18) by the gradual addition of concentrated sulphuric acid (s.g. 1·84). The gas given off is simply dried by bubbling through sulphuric acid. As methanol has a great affinity for hydrogen chloride, it is desirable to take every normal precaution in bringing them together; for example, by passing in the gas through a small inverted funnel which just touches the surface of the methanol. It is moreover feasible to prepare large quantities of methanol solutions of hydrogen chloride in advance, which have excellent keeping properties in ground-stoppered bottles held in the dark.

(b) It is possible to use a methanol solution of about 1 N sulphuric acid as a substitute for the methanol solution of hydrogen chloride, but the esterification step lasts at least 20 minutes and the precipitated sodium sulphate impedes the boiling and calls for filtration or the use of a magnetic stirrer.

(c) It is also feasible to pour in 40 ml of methanol and add 0·4 g of sodium before placing in the test portion, thus preparing the sodium methylate solution on the spot.

(d) Having regard to the relatively large amount of sodium methylate, a sodium chloride precipitate occurs with very acidic oils and fats which may repeatedly give rise to bumping during the ensuing boiling. It is possible to filter off this precipitate but this action is usually unnecessary owing to the short duration advised for the heating.

4. METHOD SPECIFIC FOR THE PREPARATION OF THE METHYL ESTERS OF FATTY ACIDS WITH FOUR OR MORE ATOMS OF CARBON

4.1. Principle

The methyl esters are obtained by reacting the oil or fat with a methanol solution of potassium hydroxide in an intermediate step before saponification takes place.

4.2. Field of application

The method is intended mainly for the preparation of methyl esters entering gas chromatography analysis.

The method is ruled out for acidic oils and fats (acid value > 2).

4.3. Apparatus

— test tubes, about 20 ml, with ground stoppers;

— volumetric flasks, 50 and 100 ml;

— graduated pipettes, 1 ml (or over);

— measuring cylinders, 10 ml.

4.4. Reagents

— methanol solution of potassium hydroxide, about 2 N: dissolve 11·2 g of potassium hydroxide in 100 ml of methanol containing not more than 0·5 % (m/m) of water;

— heptane, chromatography grade (Notes 2 and 4);

— reference solution I: weigh to within 0·1 mg into a 50 ml volumetric flask about 1 g of methyl pentanoate and add heptane up to the mark;

— reference solution II: weigh to within 0·1 mg into a 100 ml volumetric flask about 200 mg of methyl pentanoate and add heptane up to the mark.

4.5. **Procedure**

When butyric acid needs to be subsequently determined by gas chromatography, it is essential to use a reference solution ; reference solution I where the presumed butyric acid content is more than 1 % and reference solution II when less.

If the fatty acid composition needs to be determined by gas chromatography, it is not essential to use any reference solution.

Prepare the sample in accordance with Title I, 'Preparation of the samples'. Weigh to within 1 mg into a test tube of about 20 ml with a ground stopper about 1 g of prepared sample. Add 10 ml of heptane from a measuring cylinder. Add 1·0 ml of the appropriate reference solution from a graduated pipette.

Note : If the butyric acid is not to be determined, it is not necessary to weigh the sample to within 1 mg or to add a reference solution.

Add 0·5 ml of about 2 N methanol solution of potassium hydroxide and mix the contents of the test tube by a series of see-saw movements until clear. This requires about 20 seconds. Almost immediately after becoming clear the solution again becomes cloudy owing to the separation of glycerol. The glycerol separates out rapidly.

Separate the top layer containing the methyl esters.

5. NOTES

1. If the unsaponifiable matter gives rise to interference, dilute with water the solution obtained after saponification and eliminate the unsaponifiable matter in the usual way by extraction with diethyl ether, hexane or petroleum ether.

   Acidify the aqueous soapy solution and filter off the fatty acids.

   Prepare their methyl esters in accordance with the procedures of Sections 3.1.4.2 or 3.2.3.5.

2. Certain reagents, especially methanol solutions of boron trifluoride, can give rise during the gas chromatography of the methyl esters to the appearance of spurious peaks (in the C_{20}-C_{22} region from methanol solutions of boron trifluoride). Each new batch of reagent must therefore be tested by preparing and chromatographing the methyl esters of pure oleic acid. The various reagents must not give rise to peaks interfering during gas chromatography with those of the methyl esters of the fatty acids.

3. If the methanol solution of boron trifluoride must unavoidably be prepared from boron trifluoride in gaseous form, proceed as follows : take a two litre flask containing one litre of methanol and cool in a bath of ice and water. Working in a fume cupboard with the flask kept in the bath, bubble BF_3 from a cylinder through a glass tube into the methanol until 125 g are absorbed. Maintain a flow of BF_3 in the glass tube before immersing it in the methanol and until it has been withdrawn to prevent the liquid from being sucked back into the gas regulator. The gas should not escape so quickly from the flask as to give rise to white fumes. The reagent has a useful shelf-life of two years.

4. Heptane (mixture of pure C_7 isomers tested by means of gas chromatography) may be replaced by hexane if fatty acids with 20 or more carbon atoms are absent.

5. If the test portion is not available in the requisite quantity, it may be cut to 10 mg or less providing that the volumes of the reagents and the size of the apparatus are reduced in proportion.

6. The methyl esters should preferably be analyzed as soon as possible. The heptane solution containing the methyl esters may, if necessary, be kept under an inert gas blanket in the refrigerator. It is desirable in the event of prolonged storage to protect the methyl esters against autoxidation by adding to the solution an oxidation inhibitor which will not interfere with subsequent analyses, e.g., 0·005 % (m/v) of B.H.T. (di-ter.butyl-2-6-methyl-4-phenol). Dry, solvent-free methyl esters may, if necessary, be kept for 24 hours under an inert gas blanket in a refrigerator, or for a longer period in a sealed evacuated tube in a deep-freezer.

7. There is a risk of losing some of the more volatile methyl esters if the evaporation of the solvent is protracted or if the nitrogen flush is too vigorous.

   For infra-red spectrophotometry purposes the solvent must be as completely eliminated as possible. For gas chromatography purposes it is desirable not to drive off the solvent.

8. With oils of the castor oil type, clearness is not the reaction criterion.

III. GAS CHROMATOGRAPHY OF THE METHYL ESTERS OF THE FATTY ACIDS

1. FOREWORD

The purpose of this method is to give general guidance for the determination by gas chromatography of the qualitative and quantitative composition of the mixture of methyl esters derived from the fatty acids in accordance with Title II. Polymerized fatty acids cannot be analyzed by this method. The instructions are for usual gas chromatographs with a packed column and flame-ionization detector (Note 1).

2. APPARATUS

2.1. **Gas chromatograph**

An apparatus is suitable if its efficiency and resolving power are as laid down in Section 4.1.2.

2.1.1. *Injector*

The dead space of the injector must be as small as possible. Unless its material properties say otherwise, its temperature will be 25 to 50 °C above that of the column.

2.1.2. *Oven*

The oven must be capable of heating the columns to a temperature of at least 220 °C and of holding the desired temperature to within 1 °C.

If programmed conditions are used, it is desirable to choose a twin apparatus.

2.1.3. *Column*

2.1.3.1. *Tube*

Made of a material inert to the oils and fats to be analyzed, e.g., glass or, failing which, stainless steel (Note 2). Length of 1 to 3 metres ; a relatively short column will be used if long-chain ($> C_{20}$) are present.

When determining C_4 and C_6 acids, it is desirable to use a two metre column.

Internal diameter of 2 to 4 mm.

2.1.3.2. *Packing*

Support : acid-washed and silanized diatomaceous earth or any other inert support with a close size analysis range (25 µm between 125 and 200 µm), the average value being governed by the internal diameter and the length of the column.

Stationary phase : a polar phase of the polyester type (e.g., diethyleneglycol polysuccinate, butan -di-ol polyadipate, etc.) or any other phase complying with the requirements specified hereinafter (e.g., cyanosilicones, etc.). The rate of impregnation will range from 5 to 20 %. Apolar phases are usable for some separations.

2.1.3.3. *Conditioning*

Disconnect the column, if possible, from the detector, raise the temperature of the apparatus oven to 10 °C above the working temperature and keep the column which has just been prepared under a stream of inert gas of 20 to 60 ml/min. at that temperature for at least 16 hours, then at 195 °C for two hours.

2.1.4. *Detector*

The detector should be capable of being taken to a higher temperature than the column.

The operating instructions given hereinafter refer to the use of a flame ionization detector (Note 1).

2.2. **Syringe**

This should be not more than 10 µl, divided into tenths of a µl.

2.3. **Recorder**

If the recorded curve is used for calculating the composition of the mixture analyzed, the recorder must be a highly accurate electronic apparatus compatible with the equipment used :

— speed of response of less than 1·5 seconds or, better still, one second (the speed of response is the time the pen of the recorder takes to travel from 0 to 90 % when a signal of 100 % is momentarily introduced) ;

— paper not less than 25 cm wide ;

— paper unwinds at a rate of 25 to 150 cm/h.

2.4. **Integrator or calculator** (optional extra)

An electronic integrator or a calculator, when used, makes calculating rapid and accurate. It must give a linear response, have adequate sensitivity and the correction of the deviation of the base line must be satisfactory.

3. REAGENTS

— Carrier gas : any inert gas (nitrogen, helium, argon, etc.), dried very thoroughly and containing less than 10 ppm of oxygen ;

— ancillary gases : hydrogen not less pure than 99·9 % and containing no organic matter, air or oxygen containing no organic matter ;

— standardization substances : mixtures of methyl esters or methyl esters from an oil of known composition close, if possible, to that of the oil and fat to be analyzed.

4. PROCEDURE

4.1. **Adjustment of the apparatus**

4.1.1. *Determination of optimum working conditions*

It is essential for choosing the working conditions to take account of the following variables :

length and diameter of the column ;
nature and quantity of the stationary phase ;
temperature of the column ;
flowrate of the carrier gas ;
resolution desired ;
size of the test portion ;
duration of the analysis.

The size of the test portion shall be chosen to be such that the detector and electrometer together give a linear response. In general, the values giving the desired results, i.e., the number of theoretical plates in the case of methyl stearate equal to not less than 2 000 and elution thereof in about 15 minutes, will be as follows :

Internal diameter of column (mm)	Flowrate of vector gas (ml/min.)
2	15-25
3	20-40
4	40-60
Stationary phase concentration (%)	**Temperature (°C)**
5	175
10	180
15	185
20	185

If the apparatus allows, the injector shall be at a temperature near to 200 °C and the detector at a temperature the same as or higher than the column.

Hydrogen usually flows through the flame ionization detector at half the rate of the carrier gas and the oxygen 5 to 10 times as fast as the hydrogen.

4.1.2. *Determination of efficiency and resolution*

Analyze a mixture of methyl stearate and oleate in about equivalent proportions (e.g., methyl esters from cocoa butter). The size of the test portion, the column temperature and the flowrate of the carrier gas shall be chosen to be such that the maximum of the methyl stearate peak is recorded about 15 minutes later than the solvent peak and that the peak in question is equivalent to about three-quarters of the total scale. Calculate n, the number of theoretical plates (efficiency), from the formula :

$$n = 16 \left(\frac{dR_1}{\omega 1}\right)^2$$

and R, the resolution, from the formula :

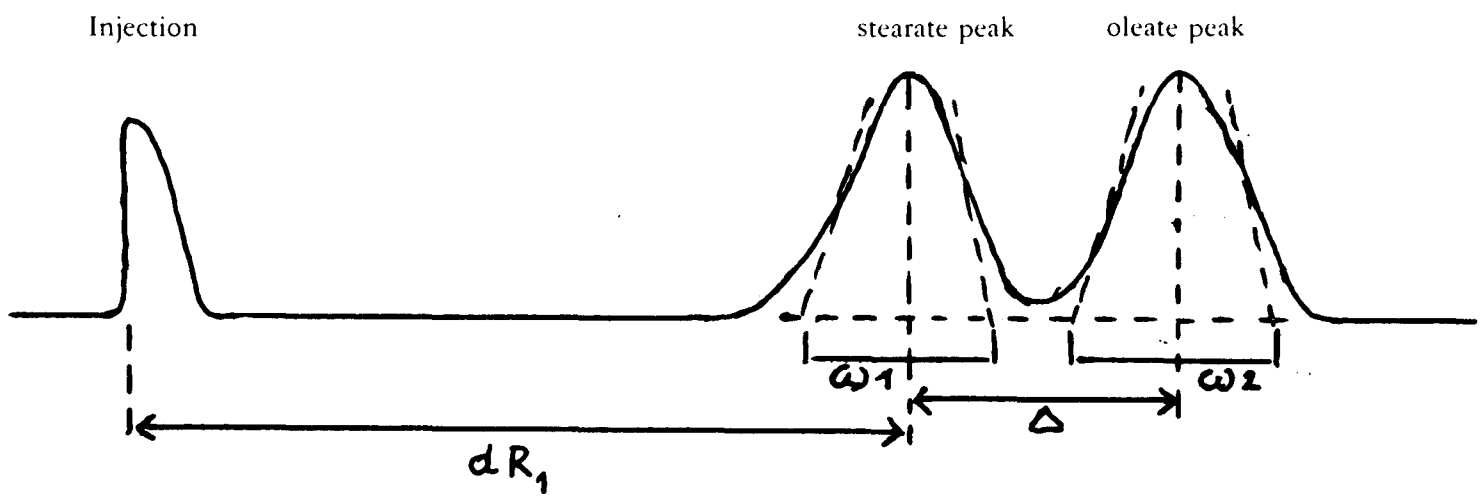
$$R = \frac{2\,\Delta}{\omega 1 + \omega 2}$$

where :

dR_1 is the retention distance from injection to the relative maximum of the methyl stearate peak, measured in mm.

ω_1 and ω_2 are the widths of the methyl stearate and oleate peaks measured in mm between the points at which the tangents to the points of inflexion in the curves intersect the base line.

Δ is the distance between the relative maxima of the methyl stearate and oleate peaks.



The operating conditions to be adopted shall be those which give for methyl stearate a number equal to or greater than 2 000 theoretical plates and a resolution of not less than 1·25. They must also be such that linoleic acid ($C_{18:3}$) is separated from arachidic ($C_{20:0}$) and gadoleic ($C_{20:1}$) acids.

4.2. Analysis

The test portion shall be 0·1 to 2 µl of the heptane solution of methyl esters obtained in accordance with Title II. If the esters are free of solvents, bring them into about 10 % solution with heptane and inject 0·1 to 1 µl of that.

The test portion may be increased (up to 10 times) in the usual cases ; the operating conditions will be those determined under Section 4.1.1 for the identification of constituents present as traces. It will nevertheless be possible to operate with a lower column temperature when it is necessary to determine fatty acids below C_{12} or higher when it is necessary to determine fatty acids above C_{20}.

It is possible, if desired, to operate in both these cases with programmed temperatures. For example, if the sample contains methyl esters of fatty acids with less than 12 atoms of carbon, inject the sample at 100 °C (or at 50 to 60 °C if there is butyric acid present) and programme immediately at 4 to 8 °C/min up to the optimum temperature.

The two processes can, in some cases, be combined, i.e., continue the elution at steady temperature after the temperature programming period until all constituents are eluted. If the apparatus cannot work with temperature programming, operate at two fixed temperatures between 100 and 195 °C.

It is desirable, where necessary, to carry out analyses on two fixed phases of opposite polarity in order to verify the absence of masked peaks, e.g., with fish oils or if conjugated $C_{18:3}$ and $C_{20:0}$ or $C_{18:3}$ and $C_{18:2}$ are present simultaneously.

5. EXPRESSION OF RESULTS

5.1. Qualitative analysis

Analyze the reference mixture, composition known, under operating conditions identical to those of the test and determine the retention distances (or retention times) for the constituent fatty acids. Plot graphs giving the logarithm of the retention distance (or retention time) against the number of carbon atoms ; graphs plotted under isothermal conditions and for straight-chain esters or a fixed degree of unsaturation on semi-logarithmic paper should be virtually parallel straight lines.

Identify the peaks for the test by referring to these straight lines or interpolating, if necessary.

It is essential to rule out operating conditions such that 'masked peaks' can exist, i.e., that two constituents are indistinguishable because of inadequate resolution.

5.2. Quantitative analysis

5.2.1. *Determination of composition*

Use (save in the case of exceptions) the internal standardization method, i.e., assume that the whole of the constituents present in the sample are depicted on the chromatogram and therefore that the sum of the areas under the peaks is equal to 100 % of the constituents (complete elution).

If the apparatus includes an integrator, use the results it gives. If not, determine the area under each peak by the method of triangulation, multiplying its height by its width at mid-height and allowing, when necessary, for the various attenuations used during the recording.

5.2.1.1. *General case*

If important constituents below C_8 are absent, calculate for each constituent its content expressed as a methyl ester percentage by determining the percentage equivalent to the corresponding peak over the sum of the areas under all the peaks :

$$\text{percentage (m/m) of compound i, expressed as methyl ester} = \frac{A_i}{\Sigma A_i} \times 100$$

where :

A_i = area under the peak corresponding to compound i

ΣA_i = sum of the areas under all the peaks.

5.2.1.2. *Use of correction factors*

Sometimes, especially when acids below C_8 or acids with secondary groupings are present or thermal conductivity detectors (katharometers) are used, it is necessary to apply correction factors to convert peak area percentages into the percentages of the constituents by weight.

Determine the correction factors from a chromatogram derived from a reference mixture of methyl esters of exactly known composition under identical conditions to the test.

For the reference mixture :

$$\text{percentage (m/m) of compound i} = \frac{B_i}{\Sigma B_i} \times 100$$

where :

B_i = the weight of compound i in the reference mixture ;

ΣB_i = the sum of the weights of the various constituents of the reference mixture.

From the chromatogram derived from the reference mixture it is possible to calculate :

$$\text{percentage (area/area) of compound i} = \frac{C_i}{\Sigma C_i} \times 100$$

C_i = area under the peak corresponding to compound i,

ΣC_i = sum of the areas under all peaks,

whence :

$$\text{correction factor } K_i = \frac{B_i \times \Sigma C_i}{C_i \times \Sigma B_i}$$

Customarily, correction factors are expressed in terms of $K_{16} = 1$ and the relative factors become :

$$K'_i = \frac{K_i}{K_{16}}$$

In the case of the sample, the content of each constituent is given by

$$\text{the percentage (m/m) of compound i} = \frac{K'_i \times A_i}{\Sigma(K'_i \times A_i)} \times 100$$

expressed as methyl esters

5.2.1.3. *Use of an internal standard*

It is essential in some cases (especially in the determination of the C_4 and C_6 acids and in the determination of the acids where not all the fatty acids are eluted) to use an internal standard (C_5, C_{15} or C_{17}, as the case may be) and then determine its correction factor :

$$\text{percentage (m/m) of compound i} = \frac{m_S \times K'_i \times A_i}{m \times K'_S \times A_S} \times 100$$

expressed as methyl esters,

where :

m_S = the weight of the internal standard in mg ;

m = the weight of sample in mg ;

K'_S = correction factor of the internal standard ;

A_S = area under the peak corresponding to the internal standard ;

A_i = area under the peak corresponding to compound i.

5.2.2. *Expression of results*

Give results to :

three significant figures when less than 10 % ;
two significant figures when between 1 and 10 % ;
one significant figure when below 1 % ;
meaning always with one digit after the decimal point.

5.2.3. *Repeatability*

The difference between the results of two determinations carried out on the same day on the same apparatus by the same operator and on the same esters and in respect of the constituents present to the extent of more than 5 % must not exceed 3 % in relative value of the determined value, the absolute ceiling being 1 %. In the case of the constituent present to the extent of less than 5 %, the repeatability rapidly deteriorates as the percentage content falls.

5.2.4. *Reproducibility*

The difference between the results found in two different laboratories for constituents present to the extent of more than 5 % must not exceed 10 % in relative value of the determined value, the absolute ceiling being 3 %. In the case of constituents present to the extent of less than 5 %, there producibility rapidly deteriorates as the percentage content falls.

6. NOTES

1. A gas chromatograph with a thermal conductivity detector (katharometer) may be used. The conditions described must then be modified as follows :

Tube :	length of two to four metres, with an internal diameter of 4 mm ;
Support :	size analysis within the range of 160 to 200 µm ;
Degree of impregnation :	15 to 25 % ;
Carrier gas :	helium or, failing which, hydrogen with as low an oxygen content as possible. No ancillary gas ;
Injector temperature :	40 to 60 °C higher than the column temperature ;
Column temperature :	in the range of 180 to 200 °C ;
Carrier gas flowrate :	usually in the range of 60 to 80 ml/min. ;
Quantities injected :	usually in the range of 0·5 to 2 µl.

Take account, in the quantitative analysis, of the correction factors defined in Section 5.2.1.2.

2. If polyunsaturated constituents with more than three double bonds are present, a stainless steel tube may cause them to decompose.