the chromatogram obtained with the reference solution, verify the absence in the chromatogram obtained with the test solution of a spot with an R_f of about 0.25 (erucic acid).

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2.4.22. COMPOSITION OF FATTY ACIDS BY GAS CHROMATOGRAPHY

The test for foreign oils is carried out on the methyl esters of the fatty acids contained in the oil to be examined by gas chromatography (*2.2.28*).

METHOD A

This method is not applicable to oils that contain glycerides of fatty acids with an epoxy-, hydroepoxy-, cyclopropyl or cyclopropenyl group, or those that contain a large proportion of fatty acids of chain length less than 8 carbon atoms or to oils with an acid value greater than 2.0.

Test solution. When prescribed in the monograph, dry the oil to be examined before the methylation step. Weigh 1.0 g of the oil into a 25 ml round-bottomed flask with a ground-glass neck fitted with a reflux condenser and a gas port into the flask. Add 10 ml of *anhydrous methanol R* and 0.2 ml of a 60 g/l solution of *potassium hydroxide R* in *methanol R*. Attach the reflux condenser, pass *nitrogen R* through the mixture at a rate of about 50 ml/min, shake and heat to boiling. When the solution is clear (usually after about 10 min), continue heating for a further 5 min. Cool the flask under running water and transfer the contents to a separating funnel. Rinse the flask with 5 ml of *heptane R* and transfer the rinsings to the separating funnel and shake. Add 10 ml of a 200 g/l solution of *sodium chloride R* and shake vigorously. Allow to separate and transfer the organic layer to a vial containing *anhydrous sodium sulphate R*. Allow to stand, then filter.

Reference solution (a). Prepare 0.50 g of the mixture of calibrating substances with the composition described in one of the tables 2.4.22, as prescribed in the individual monograph (if the monograph does not mention a specific solution, use the composition described in Table 2.4.22.-1). Dissolve in *heptane R* and dilute to 50.0 ml with the same solvent.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 10.0 ml with *heptane R*.

Reference solution (c). Prepare 0.50 g of a mixture of fatty acid methyl esters[(1)], which corresponds in composition to the mixture of fatty acids indicated in the monograph of the substance to be examined. Dissolve in *heptane R* and dilute to 50.0 ml with the same solvent. Commercially available mixtures of fatty acid methyl esters may also be used.

Column:

- *material*: fused silica, glass or quartz,
- *size*: *l* = 10-30 m, Ø = 0.2-0.8 mm,
- *stationary phase*: *poly[(cyanopropyl)(methyl)][(phenyl)(methyl)]siloxane R* or *macrogol 20 000 R* (film thickness 0.1-0.5 µm) or some other suitable stationary phase.

Carrier gas: *helium for chromatography R* or *hydrogen for chromatography R*.

Flow rate: 1.3 ml/min (for a column Ø = 0.32 mm).

Split ratio: 1:100 or less, according to the internal diameter of the column used (1:50 when Ø = 0.32 mm).

Temperature:

- *column*: 160-200 °C, according to the length and type of column used (200 °C for a column 30 m long and coated with a layer of *macrogol 20 000 R*); if necessary, or where prescribed, raise the temperature of the column at a rate of 3 °C/min from 170 °C to 230 °C (for the *macrogol 20 000 R* column),
- *injection port*: 250 °C,
- *detector*: 250 °C.

Detection: flame ionisation.

Injection: 1 µl.

Sensitivity: the height of the principal peak in the chromatogram obtained with reference solution (a) is 50 per cent to 70 per cent of the full scale of the recorder.

System suitability when using the mixture of calibrating substances in Table 2.4.22.-1 or 2.4.22.-3:

- *resolution*: minimum 1.8 between the peaks due to methyl oleate and methyl stearate in the chromatogram obtained with reference solution (a),
- *signal-to-noise ratio*: minimum 5 for the peak due to methyl myristate in the chromatogram obtained with reference solution (b),
- *number of theoretical plates*: minimum 30 000 calculated for the peak due to methyl stearate in the chromatogram obtained with reference solution (a).

System suitability when using the mixture of calibrating substances in Table 2.4.22.-2:

- *resolution*: minimum 4.0 between the peaks due to methyl caprylate and methyl caprate in the chromatogram obtained with reference solution (a),
- *signal-to-noise ratio*: minimum 5 for the peak due to methyl caproate in the chromatogram obtained with reference solution (b),
- *number of theoretical plates*: minimum 15 000 calculated for the peak due to methyl caprate in the chromatogram obtained with reference solution (a).

ASSESSMENT OF CHROMATOGRAMS

Avoid working conditions tending to give masked peaks (presence of constituents with small differences between retention times, for example linolenic acid and arachidic acid).

Qualitative analysis. Identify the peaks in the chromatogram obtained with reference solution (c); the peaks may also be identified by drawing calibration curves using the chromatogram obtained with reference solution (a) and the information given in Tables 2.4.22.-1, 2.4.22.-2 and 2.4.22.-3:

a) using isothermal operating conditions giving the logarithms of reduced retention times as a function of the number of carbon atoms of the fatty acid; identify the peaks by means of the straight line thus obtained and the "equivalent chain lengths" of the different peaks. The calibration curve of the saturated acids is a straight line. The logarithms of reduced retention times of unsaturated acids are situated on this line at points corresponding to non-integer values of carbon atoms known as "equivalent chain lengths";

b) using linear temperature programming giving the retention time according to the number of carbon atoms of the fatty acid; identify by reference to the calibration curve.

Quantitative analysis. In general, the normalisation procedure is used in which the sum of the areas of the peaks in the chromatogram, except that of the solvent, is set at 100 per cent. The content of a constituent is calculated

(1) The fatty acid methyl esters used show a quality at least as good as that guaranteed by the BCR (Community Bureau of Reference) of the European Union.

by determining the area of the corresponding peak as a percentage of the sum of the areas of all the peaks. Disregard any peak with an area less than 0.05 per cent of the total area.

In certain cases, for example in the presence of fatty acids with 12 or less carbon atoms, correction factors can be prescribed in the individual monograph to convert peak areas in per cent *m/m*.

METHOD B

This method is not applicable to oils that contain glycerides of fatty acids with an epoxy-, hydroepoxy-, cyclopropyl or cyclopropenyl group or to oils with an acid value greater than 2.0.

Test solution. Introduce 0.100 g of the substance to be examined in a 10 ml centrifuge tube with a screw cap. Dissolve with 1 ml of *heptane R* and 1 ml of *dimethyl carbonate R* and mix vigorously under gentle heating (50-60 °C). Add, while still warm, 1 ml of a 12 g/l solution of *sodium R* in *anhydrous methanol R*, prepared with the necessary precautions and mix vigorously for about 5 min. Add 3 ml of *distilled water R* and mix vigorously for about 30 s. Centrifuge for 15 min at 1500 *g*. Inject 1 µl of the organic phase.

Reference solutions and assessment of chromatograms. Without specific prescription in the individual monograph, proceed as described under Method A.

Column:

- *material*: fused silica,
- *size*: *l* = 30 m, Ø = 0.25 mm,
- *stationary phase*: *macrogol 20 000 R* (film thickness 0.25 µm),

Carrier gas: *helium for chromatography R.*

Flow rate: 0.9 ml/min.

Split ratio: 1:100.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 15	100
	15 - 36	100 → 225
	36 - 61	225
Injection port		250
Detector		250

Detection: flame ionisation.

Injection: 1 µl.

METHOD C

This method is not applicable to oils that contain glycerides of fatty acids with epoxy-, hydroperoxy-, aldehyde, ketone, cyclopropyl and cyclopropenyl groups, and conjugated polyunsaturated and acetylenic compounds because of partial or complete destruction of these groups.

Test solution. Dissolve 0.10 g of the substance to be examined in 2 ml of a 20 g/l solution of *sodium hydroxide R* in *methanol R* in a 25 ml conical flask and boil under a reflux condenser for 30 min. Add 2.0 ml of *boron trifluoride-methanol solution R* through the condenser and boil for 30 min. Add 4 ml of *heptane R* through the condenser and boil for 5 min. Cool and add 10.0 ml of *saturated sodium chloride solution R*, shake for about 15 s and add a quantity of *saturated sodium chloride solution R* such that the upper phase is brought into the neck of the flask. Collect 2 ml of the upper phase, wash with 3 quantities, each of 2 ml, of *water R* and dry over *anhydrous sodium sulphate R*.

Reference solutions, chromatographic procedure and assessment of chromatograms. Without specific prescription in the individual monograph, proceed as described under Method A.

Table 2.4.22.-1. – *Mixture of calibrating substances*[2]

Mixture of the following substances	Equivalent chain length[3]	Composition (per cent *m/m*) Iso-thermal	Linear temperature programme
Methyl laurate R	12.0	5	10
Methyl myristate R	14.0	5	15
Methyl palmitate R	16.0	10	15
Methyl stearate R	18.0	20	20
Methyl arachidate R	20.0	40	20
Methyl oleate R	18.3	20	20

Table 2.4.22.-2. – *Mixture of calibrating substances*[2]

Mixture of the following substances	Equivalent chain length[3]	Composition (per cent *m/m*) Iso-thermal	Linear temperature programme
Methyl caproate R	6.0	5	10
Methyl caprylate R	8.0	5	35
Methyl caprate R	10.0	10	35
Methyl laurate R	12.0	20	10
Methyl myristate R	14.0	40	10

Table 2.4.22.-3. – *Mixture of calibrating substances*[2]

Mixture of the following substances	Equivalent chain length[3]	Composition (per cent *m/m*) Iso-thermal	Linear temperature programme
Methyl myristate R	14.0	5	15
Methyl palmitate R	16.0	10	15
Methyl stearate R	18.0	15	20
Methyl arachidate R	20.0	20	15
Methyl oleate R	18.3	20	15
Methyl eicosenoate R	20.2	10	10
Methyl behenate R	22.0	10	5
Methyl lignocerate R	24.0	10	5

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2.4.23. STEROLS IN FATTY OILS

SEPARATION OF THE STEROL FRACTION

Prepare the unsaponifiable matter and then isolate the sterol fraction of the fatty oil by thin-layer chromatography (*2.2.27*), using *silica gel G R* in a 0.3 mm to 0.5 mm layer as the coating substance.

(2) For GC with capillary column and split inlet system, it is recommended that the component with the longest chain length of the mixture to be examined be added to the calibration mixture, when the qualitative analysis is done using calibration curves.
(3) This value, which is to be calculated using calibration curves, is given as an example for a column of *macrogol 20 000 R*.