test solution. The fatty acids may also be obtained from the soap solution prepared during the determination of the unsaponifiable matter.

Test solution. Dissolve 40 mg of the mixture of fatty acids obtained from the substance to be examined in 4 ml of *chloroform R*.

Reference solution. Dissolve 40 mg of the mixture of fatty acids obtained from a mixture of 19 volumes of *maize oil R* and 1 volume of *rapeseed oil R* in 4 ml of *chloroform R*.

Apply to the plate 3 µl of each solution. Develop over a path of 8 cm using a mixture of 10 volumes of *water R* and 90 volumes of *glacial acetic acid R*. Dry the plate at 110 °C for 10 min. Allow to cool and, unless otherwise prescribed, place the plate in a chromatographic chamber, with a tightly fitting lid, that has previously been saturated with iodine vapour by placing *iodine R* in an evaporating dish at the bottom of the chamber. After some time brown or yellowish-brown spots become visible. Remove the plate and allow to stand for a few minutes. When the brown background colour has disappeared, spray with *starch solution R*. Blue spots appear which may become brown on drying and again become blue after spraying with *water R*. The chromatogram obtained with the test solution always shows a spot with an R_F of about 0.5 (oleic acid) and a spot with an R_F of about 0.65 (linoleic acid) corresponding to the spots in the chromatogram obtained with the reference solution. With some oils a spot with an R_F of about 0.75 may be present (linolenic acid). By comparison with the spot in 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-, hydroperoxy-, 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 2.4.22 tables, 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 that 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*: *macrogol 20 000 R* (film thickness 0.1-0.5 µm) or another 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*: in isothermal conditions, 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 a linear temperature programming is necessary, raise the temperature of the column at a rate of 3 °C/min from 170 °C to 230 °C, for example;
- *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 50-70 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 Table 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) (isothermal operating conditions or linear temperature programming).

When using isothermal operating conditions, 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 or 2.4.22.-3.

Table 2.4.22.-1. – *Mixture of calibrating substances (for gas chromatography 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)*

Mixture of the following substances	Composition (per cent *m/m*)
Methyl laurate R	5
Methyl myristate R	5
Methyl palmitate R	10
Methyl stearate R	20
Methyl arachidate R	40
Methyl oleate R	20

Table 2.4.22.-2. – *Mixture of calibrating substances (for gas chromatography 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)*

Mixture of the following substances	Composition (per cent *m/m*)
Methyl caproate R	10
Methyl caprylate R	10
Methyl caprate R	20
Methyl laurate R	20
Methyl myristate R	40

Table 2.4.22.-3. – *Mixture of calibrating substances (for gas chromatography 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)*

Mixture of the following substances	Composition (per cent *m/m*)
Methyl myristate R	5
Methyl palmitate R	10
Methyl stearate R	15
Methyl arachidate R	20
Methyl oleate R	20
Methyl eicosenoate R	10
Methyl behenate R	10
Methyl lignocerate R	10

Measure the reduced retention time (t'_R) of each peak in the chromatogram obtained with reference solution (a). t'_R is the retention time measured from the solvent peak and not from the time of injection. Plot the straight line:

$$\log (t'_R) = f \text{ (equivalent chain length)}$$

The logarithms of t'_R of unsaturated acids are situated on this line at points corresponding to non-integer values of carbon atoms known as 'equivalent chain lengths'; the equivalent chain length is the length of the theoretical saturated chain that would have the same t'_R as the fatty acid to be identified. For example, linoleic acid has the same t'_R as the theoretical saturated fatty acid having 18.8 carbon atoms.

Identify the peaks in the chromatogram obtained with the test solution by means of the straight line and the reduced retention times. Equivalent chain lengths are given in Table 2.4.22.-4.

Table 2.4.22.-4. – *Equivalent chain lengths (this value, which is to be calculated using calibration curves, is given as an example for a column of macrogol 20 000 R)*

Fatty acid	Equivalent chain length
Caproic acid	6.0
Caprylic acid	8.0
Capric acid	10.0
Lauric acid	12.0
Myristic acid	14.0
Palmitic acid	16.0
Palmitoleic acid	16.3
Margaric acid	17.0
Stearic acid	18.0
Oleic acid	18.3
Linoleic acid	18.8
Gamma-linolenic acid	19.0
Alpha-linolenic acid	19.2
Arachidic acid	20.0
Eicosenoic acid	20.2
Arachidonic acid	21.2
Behenic acid	22.0
Erucic acid	22.2
12-Oxostearic acid	22.7
Ricinoleic acid	23.9
12-Hydroxystearic acid	23.9
Lignoceric acid	24.0
Nervonic acid	24.2

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 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-, hydroperoxy-, 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 into 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. Where there is no 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-, hydroepoxy-, 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. Where there is no specific prescription in the individual monograph, proceed as described under Method A.

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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 a *TLC silica gel plate R* with a 0.2 mm to 0.5 mm layer.

Test solution (a). In a 150 ml flask fitted with a reflux condenser, place a volume of a 2 g/l solution of *betulin R* in *methylene chloride R* containing betulin corresponding to about 10 per cent of the sterol content of the sample used for the determination (e.g. in the case of olive oil add 500 µl, in the case of other vegetable oils add 1500 µl of the betulin solution). If the monograph requires the percentage content of the individual sterols in the sterol fraction, the addition of betulin may be omitted. Evaporate to dryness under a current of *nitrogen R*. Add 5.00 g (*m*) of the substance to be examined. Add 50 ml of *2 M alcoholic potassium hydroxide R* and heat on a water-bath for 1 h, swirling frequently. Cool to a temperature below 25 °C and transfer the contents of the flask to a separating funnel with 100 ml of *water R*. Shake the liquid carefully with 3 quantities, each of 100 ml, of *peroxide-free ether R*. Combine the ether layers in another separating funnel containing 40 ml of *water R*, shake gently for a few minutes, allow to separate and reject the aqueous phase. Wash the ether phase with several quantities, each of 40 ml, of *water R*, until the aqueous phase is no longer alkaline to phenolphthalein. Transfer the ether phase to a tared flask, washing the separating funnel with *peroxide-free ether R*. Distil off the ether with suitable precautions and add 6 ml of *acetone R* to the residue. Carefully remove the solvent in a current of *nitrogen R*. Dry to constant mass at 100-105 °C. Allow to cool in a desiccator and weigh. Transfer the residue to a small test tube with *methylene chloride R*. Evaporate under a stream of *nitrogen R* to a volume of about 1 ml. Depending on the insaponifiable content of the oil, adapt the final concentration of the solution to 25-50 mg/ml.

Test solution (b). Treat 5.00 g of *rapeseed oil R* as prescribed for the substance to be examined, beginning at the words "Add 50 ml of *2 M alcoholic potassium hydroxide R*".

Test solution (c). Treat 5.00 g of *sunflower oil R* as prescribed for the substance to be examined, beginning at the words "Add 50 ml of *2 M alcoholic potassium hydroxide R*".

Reference solution. Dissolve 25 mg of *cholesterol R* and 10 mg of *betulin R* in 1 ml of *methylene chloride R*.

Use a separate plate for each test solution. Apply as a band of 10 mm, at 20 mm from the base and 10 mm from the left edge, 10 µl of the reference solution and as bands of 150 mm, at 20 mm from the base, 0.5 ml of test solutions (a), (b) or (c). Develop over a path of 17 cm using a mixture of 35 volumes of *ether R* and 65 volumes of *hexane R*. Dry the plates in a current of *nitrogen R*. Spray the plates with a 2 g/l solution of *dichlorofluorescein R* in *ethanol R* and examine in ultraviolet light at 254 nm. The chromatogram obtained with the reference solution shows bands corresponding to cholesterol and betulin. The chromatograms obtained with the test solutions show bands with similar R_F values due to sterols. From each of the chromatograms, remove an area of coating corresponding to the area occupied by the sterol bands and additionally the area of the zones 2-3 mm above and below the visible zones corresponding to the reference solution. Place separately in three 50 ml flasks. To each flask add 15 ml of *methylene chloride R* and heat under reflux with stirring, for 15 min. Filter each solution through a sintered-glass filter (40) (*2.1.2*) or suitable filter