

Drying: in a current of cold air for not more than 1 min.

Second development: in an unsaturated tank, over 2/3 of the plate; use freshly prepared mobile phase.

Drying: in a current of cold air for not more than 1 min.

Detection: spray thoroughly with *dimethylaminobenzaldehyde solution R7* and dry in a current of hot air until the spot in the chromatogram obtained with reference solution (d) is clearly visible.

System suitability: test solution:

- the chromatogram shows at least 3 separated secondary spots.

Limits:

- **any impurity:** any spots, apart from the principal spot, are not more intense than the spot in the chromatogram obtained with reference solution (a) (0.3 per cent); not more than 4 such spots are more intense than the spot in the chromatogram obtained with reference solution (c) (0.1 per cent) and 2 of these may be more intense than the spot in the chromatogram obtained with reference solution (b) (0.2 per cent).

Loss on drying (2.2.32): maximum 5.0 per cent, determined on 0.500 g by drying at 120 °C under high vacuum.

ASSAY

Dissolve 0.500 g in 60 ml of *pyridine R*. Pass a stream of *nitrogen R* over the surface of the solution and titrate with 0.1 M *tetrabutylammonium hydroxide*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *tetrabutylammonium hydroxide* is equivalent to 68.04 mg of codergocrine mesilate (average $M_r = 680$).

STORAGE

Protected from light.

01/2009:2398

COD-LIVER OIL, FARMED

Iecoris aselli oleum domesticum

DEFINITION

Purified fatty oil obtained from the fresh livers of farmed cod, *Gadus morhua* L., solid substances being removed by cooling and filtering.

Content:

- **sum of the contents of EPA and DHA (expressed as triglycerides):** 10.0 per cent to 28.0 per cent;
- **vitamin A:** 50 IU (15 µg) to 500 IU (150 µg) per gram;
- **vitamin D₃:** maximum 50 IU (1.3 µg) per gram.

Authorised antioxidants in concentrations not exceeding the levels specified by the competent authority may be added.

PRODUCTION

The fish shall only be given feed with a composition that is in accordance with the relevant EU or other applicable regulations.

CHARACTERS

Appearance: clear, pale yellowish liquid.

Solubility: practically insoluble in water, slightly soluble in alcohol (96 per cent), miscible with light petroleum.

IDENTIFICATION

A. Examine the ¹³C NMR spectra obtained in the test for positional distribution (β(2)-acyl) of fatty acids (see Tests). The spectra contain peaks between 172 ppm and 173 ppm with shifts similar to those in the spectrum shown in Figure 2398-1.

The positional distribution (β(2)-acyl) for cervonic (docosahexaenoic) acid (C22:6 n-3; DHA), timnodonic (eicosapentaenoic) acid (C20:5 n-3; EPA) and moroctic acid (C18:4 n-3) complies with the limits.

B. Linoleic acid (see Tests).

TESTS

Acid value (2.5.1): maximum 2.0.

Anisidine value (2.5.36): maximum 10.0.

Peroxide value (2.5.5, Method B): maximum 5.0.

Unsaponifiable matter (2.5.7): maximum 1.5 per cent, determined on 2.0 g, and extracting with 3 quantities, each of 50 ml, of *peroxide-free ether R*.

Stearin. Heat at least 10 ml to 60-90 °C then allow to cool for 3 h in a bath of iced water or a thermostatically controlled bath at 0 ± 0.5 °C. If necessary, to eliminate insoluble matter, filter the sample after heating. The sample remains clear.

Positional distribution (β(2)-acyl) of fatty acids. Nuclear magnetic resonance spectrometry (2.2.33).

Test solution. Dissolve 190-210 mg of the substance to be examined in 500 µl of *deuterated chloroform R*. Prepare at least 3 samples and examine within 3 days.

Apparatus: high resolution FT-NMR spectrometer operating at minimum 300 MHz.

Acquisition of ¹³C NMR spectra. The following parameters may be used:

- **sweep width:** 200 ppm (–5 ppm to 195 ppm);
- **irradiation frequency offset:** 95 ppm;
- **time domain:** 64 K;
- **pulse delay:** 2 s;
- **pulse program:** zgig 30 (inverse gated, 30° excitation pulse);
- **dummy scans:** 4;
- **number of scans:** 4096.

Processing and plotting. The following parameters may be used:

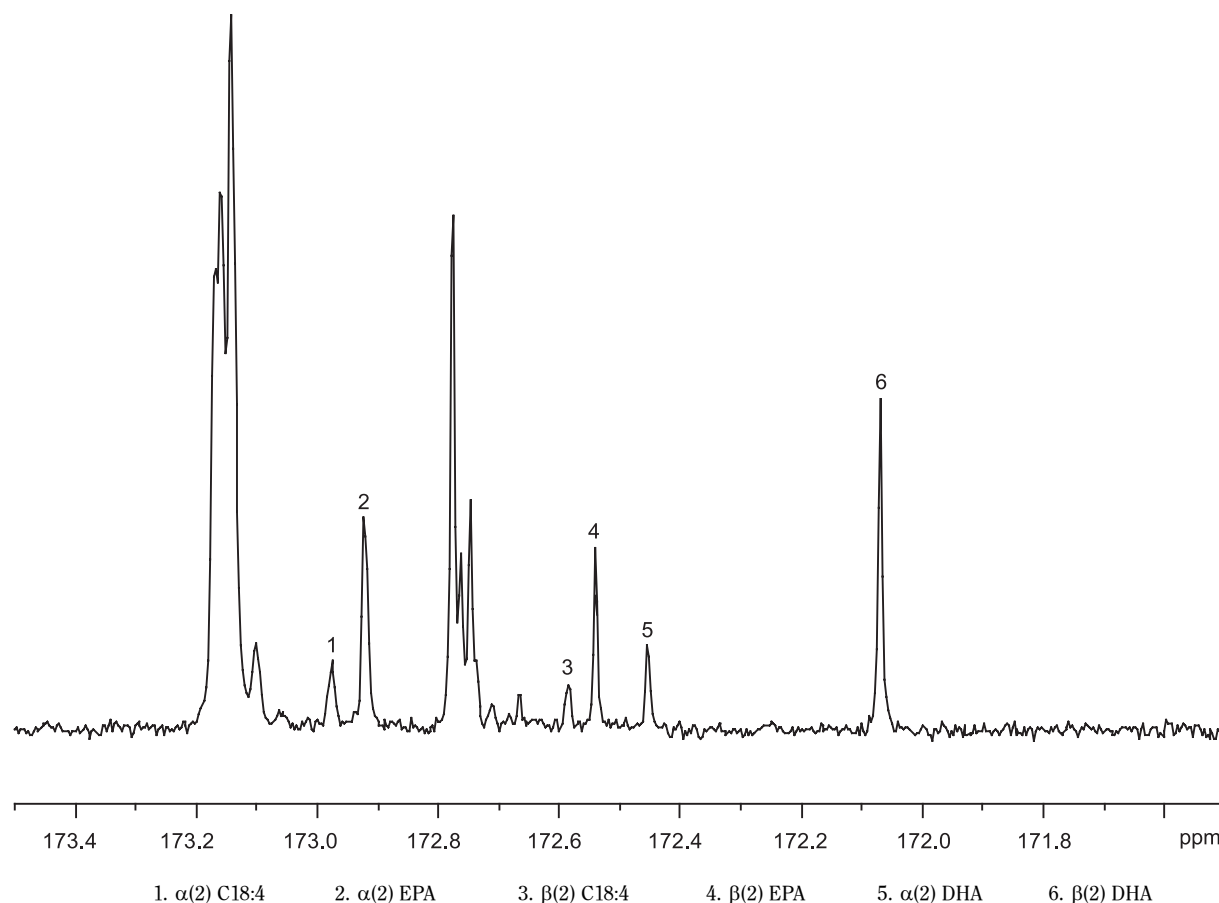
- **size:** 64 K (zero-filling);
- **window multiplication:** exponential;
- **Lorentzian broadening factor:** 0.2 Hz.

Use the CDCl₃ signal for shift referencing. The shift of the central peak of the 1:1:1 triplet is set to 77.16 ppm.

Plot the spectral region δ 171.5-173.5 ppm. Compare the spectrum with the spectrum shown in Figure 2398-1. The shift values lie within the ranges given in Table 2398-1.

Table 2398-1. – Shift values

Signal	Shift range (ppm)
β(2) DHA	172.05 - 172.09
α(2) DHA	172.43 - 172.47
β(2) EPA	172.52 - 172.56
α(2) EPA	172.90 - 172.94
β(2) C18:4	172.56 - 172.60
α(2) C18:4	172.95 - 172.99

Figure 2398.-1. – ^{13}C NMR spectrum carbonyl region of farmed cod-liver oil**System suitability:**

- **signal-to-noise ratio:** minimum 5 for the smallest relevant peak corresponding to $\alpha(2)$ C18:4 signal (in the range δ 172.95-172.99 ppm);
- **peak width at half-height:** maximum 0.02 ppm for the central CDCl_3 signal (at δ 77.16 ppm).

Calculation of positional distribution ($\beta(2)$ -acyl): use the following expression:

$$\frac{100 \times \beta(2)}{\alpha(2) + \beta(2)}$$

- $\alpha(2)$ = peak area of the corresponding $\alpha(2)$ -carbonyl peak;
- $\beta(2)$ = peak area of $\beta(2)$ -carbonyl peak from C22:6 n-3, C20:5 n-3 or C18:4 n-3, respectively.

Limits:

The positional distribution ($\beta(2)$ -acyl) is 71 per cent to 81 per cent for cervonic (docosahexaenoic) acid (C22:6 n-3; DHA), 32 per cent to 40 per cent for timnodonic (eicosapentaenoic) acid (C20:5 n-3; EPA) and 28 per cent to 38 per cent for moroctic acid (C18:4 n-3).

Composition of fatty acids (2.4.29). For identification of the peaks, see the chromatogram shown in Figure 2398.-2.

The 24 largest peaks of the methyl esters account for more than 90 per cent of the total area (these correspond to, in common elution order: 14:0, 15:0, 16:0, 16:1 n-7, 16:4 n-1, 18:0, 18:1 n-9, 18:1 n-7, 18:2 n-6, 18:3 n-3, 18:4 n-3, 20:1 n-11, 20:1 n-9, 20:1 n-7, 20:2 n-6, 20:4 n-6, 20:3 n-3, 20:4 n-3, 20:5 n-3, 22:1 n-11, 22:1 n-9, 21:5 n-3, 22:5 n-3, 22:6 n-3).

Linoleic acid (2.4.29): 3.0 per cent to 11.0 per cent.

ASSAY

EPA and DHA (2.4.29). See the chromatogram shown in Figure 2398.-2.

Vitamin A. Carry out the test as rapidly as possible, avoiding exposure to actinic light and air, oxidising agents, oxidation catalysts (for example, copper and iron) and acids.

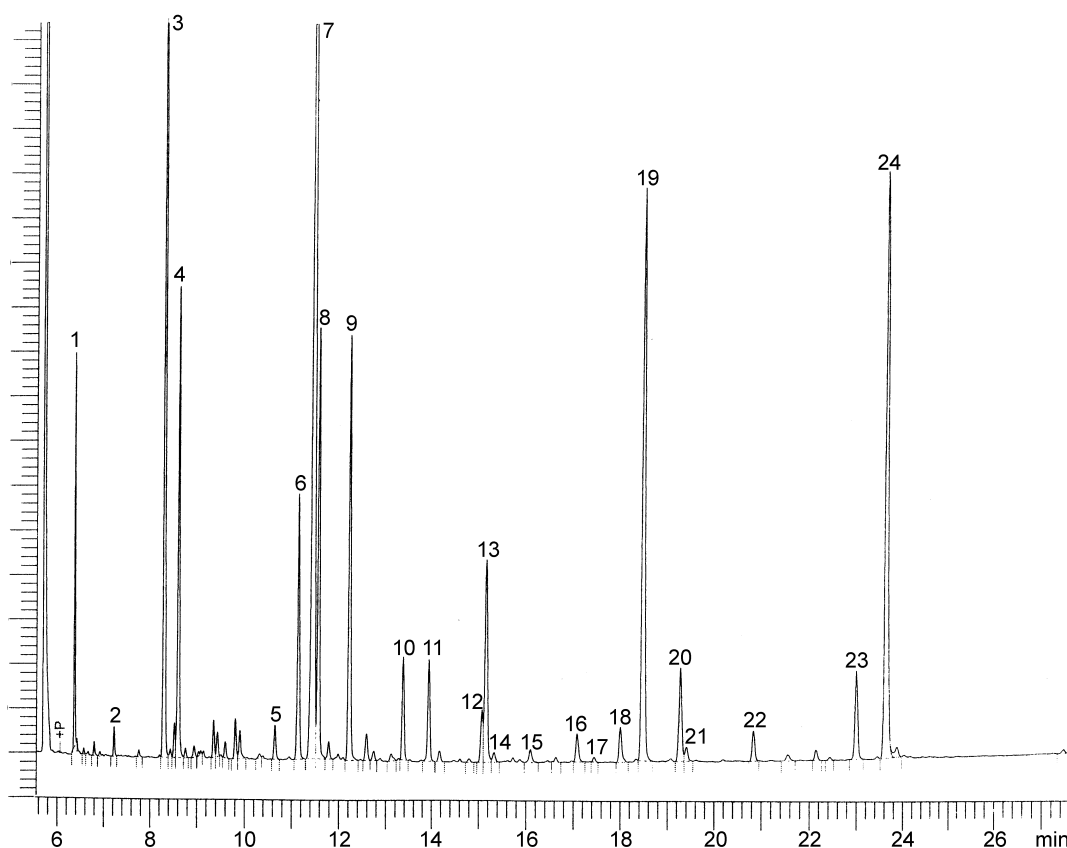
Use method A. If method A is found not to be valid, use method B.

METHOD A

Ultraviolet absorption spectrophotometry (2.2.25).

Test solution. To 1.00 g in a round-bottomed flask, add 3 ml of a freshly prepared 50 per cent *m/m* solution of potassium hydroxide *R* and 30 ml of anhydrous ethanol *R*. Boil under reflux in a current of nitrogen *R* for 30 min. Cool rapidly and add 30 ml of water *R*. Extract with 50 ml of ether *R*. Repeat the extraction 3 times and discard the lower layer after complete separation. Wash the combined upper layers with 4 quantities, each of 50 ml, of water *R*, and evaporate to dryness under a gentle current of nitrogen *R* at a temperature not exceeding 30 °C or in a rotary evaporator at a temperature not exceeding 30 °C under reduced pressure (water ejector). Dissolve the residue in sufficient 2-propanol *RI* to give an expected concentration of vitamin A equivalent to 10-15 IU/ml.

Measure the absorbances of the solution at 300 nm, 310 nm, 325 nm and 334 nm and at the wavelength of maximum absorption with a suitable spectrophotometer in 1 cm specially matched cells, using 2-propanol *RI* as the compensation liquid.



1. C14:0	5. C16:4 n-1	9. C18:2 n-6	13. C20:1 n-9	17. C20:3 n-3	21. C22:1 n-9
2. C15:0	6. C18:0	10. C18:3 n-3	14. C20:1 n-7	18. C20:4 n-3	22. C21:5 n-3
3. C16:0	7. C18:1 n-9	11. C18:4 n-3	15. C20:2 n-6	19. C20:5 n-3	23. C22:5 n-3
4. C16:1 n-7	8. C18:1 n-7	12. C20:1 n-11	16. C20:4 n-6	20. C22:1 n-11	24. C22:6 n-3

Figure 2398-2. – Chromatogram for the test for composition of fatty acids of farmed cod-liver oil

Calculate the content of vitamin A, as all-*trans*-retinol, in International Units per gram, using the following expression:

$$A_{325} \times \frac{1821}{100m} \times V$$

- A_{325} = absorbance at 325 nm;
- m = mass of the substance to be examined, in grams;
- V = total volume of solution containing 10-15 IU of vitamin A per millilitre;
- 1821 = conversion factor for the specific absorbance of all-*trans*-retinol, in International Units.

The above expression can be used only if A_{325} has a value of not greater than $A_{325,corr}/0.970$, where $A_{325,corr}$ is the corrected absorbance at 325 nm and is given by the following equation:

$$A_{325,corr} = 6.815A_{325} - 2.555A_{310} - 4.260A_{334}$$

A designates the absorbance at the wavelength indicated by the subscript.

If A_{325} has a value greater than $A_{325,corr}/0.970$, calculate the content of vitamin A using the following expression:

$$A_{325,corr} \times \frac{1821}{100m} \times V$$

The assay is not valid unless:

- the wavelength of maximum absorption lies between 323 nm and 327 nm;

- the absorbance at 300 nm relative to that at 325 nm is at most 0.73.

METHOD B

Liquid chromatography (2.2.29).

Test solution. Prepare duplicates. To 2.00 g in a round-bottomed flask, add 5 ml of a freshly prepared 100 g/l solution of *ascorbic acid R*, 10 ml of a freshly prepared 800 g/l solution of *potassium hydroxide R* and 100 ml of *anhydrous ethanol R*. Boil under a reflux condenser on a water-bath for 15 min. Add 100 ml of a 10 g/l solution of *sodium chloride R* and cool. Transfer the solution to a 500 ml separating funnel, rinsing the round-bottomed flask with about 75 ml of a 10 g/l solution of *sodium chloride R* and then with 150 ml of a mixture of equal volumes of *light petroleum R1* and *ether R*. Shake for 1 min. When the layers have separated completely, discard the lower layer and wash the upper layer, first with 50 ml of a 30 g/l solution of *potassium hydroxide R* in a 10 per cent *V/V* solution of *anhydrous ethanol R* and then with 3 quantities, each of 50 ml, of a 10 g/l solution of *sodium chloride R*. Filter the upper layer through 5 g of *anhydrous sodium sulphate R* on a fast filter paper into a 250 ml flask suitable for a rotary evaporator. Wash the funnel with 10 ml of fresh extraction mixture, filter and combine the upper layers. Distil them at a temperature not exceeding 30 °C under reduced pressure (water ejector) and fill with *nitrogen R* when evaporation is completed. Alternatively, evaporate the solvent under a gentle current of *nitrogen R* at a temperature not exceeding 30 °C. Dissolve the residue in *2-propanol R*, transfer to a 25 ml volumetric flask and dilute to 25 ml with *2-propanol R*.

Monographs A-C

Gentle heating in an ultrasonic bath may be required. *A large fraction of the white residue is cholesterol, constituting approximately 50 per cent m/m of the unsaponifiable matter of cod-liver oil.*

Reference solution (a). Prepare a solution of *retinol acetate CRS* in *2-propanol R1* so that 1 ml contains about 1000 IU of all-*trans*-retinol.

The exact concentration of reference solution (a) is assessed by ultraviolet absorption spectrophotometry (2.2.25). Dilute reference solution (a) with *2-propanol R1* to a presumed concentration of 10-15 IU/ml and measure the absorbance at 326 nm in matched 1 cm cells using *2-propanol R1* as the compensation liquid.

Calculate the content of vitamin A in International Units per millilitre of reference solution (a) using the following expression, taking into account the assigned content of *retinol acetate CRS*:

$$A_{326} \times \frac{1900 \times V_2}{100 \times V_1}$$

A_{326} = absorbance at 326 nm;

V_1 = volume of reference solution (a) used;

V_2 = volume of the diluted solution;

1900 = conversion factor for the specific absorbance of *retinol acetate CRS*, in International Units.

Reference solution (b). Proceed as described for the test solution but using 2.00 ml of reference solution (a) in place of the substance to be examined.

The exact concentration of reference solution (b) is assessed by ultraviolet absorption spectrophotometry (2.2.25). Dilute reference solution (b) with *2-propanol R1* to a presumed all-*trans*-retinol concentration of 10-15 IU/ml and measure the absorbance at 325 nm in matched 1 cm cells using *2-propanol R1* as the compensation liquid.

Calculate the content of all-*trans*-retinol in International Units per millilitre of reference solution (b), using the following expression:

$$A_{325} \times \frac{1821 \times V_3}{100 \times V_4}$$

A_{325} = absorbance at 325 nm;

V_3 = volume of the diluted solution;

V_4 = volume of reference solution (b) used;

1821 = conversion factor for the specific absorbance of all-*trans*-retinol, in International Units.

Column:

– size: $l = 0.25$ m, $\emptyset = 4.6$ mm;

– stationary phase: *octadecylsilyl silica gel for chromatography R* (5-10 μ m).

Mobile phase: *water R*, *methanol R* (3:97 V/V).

Flow rate: 1 ml/min.

Detection: spectrophotometer at 325 nm.

Injection: 10 μ l; inject in triplicate the test solution and reference solution (b).

Retention time: all-*trans*-retinol = 5 ± 1 min.

System suitability:

– the chromatogram obtained with the test solution shows a peak that corresponds to the peak due to all-*trans*-retinol in the chromatogram obtained with reference solution (b);

– the results obtained with the duplicate test solutions do not differ by more than 5 per cent;

– the recovery of all-*trans*-retinol in reference solution (b) as assessed by direct absorption spectrophotometry is greater than 95 per cent.

Calculate the content of vitamin A using the following expression:

$$A_1 \times \frac{C \times V}{A_2} \times \frac{1}{m}$$

A_1 = area of the peak due to all-*trans*-retinol in the chromatogram obtained with the test solution;

A_2 = area of the peak due to all-*trans*-retinol in the chromatogram obtained with reference solution (b);

C = concentration of *retinol acetate CRS* in reference solution (a) as assessed prior to the saponification, in International Units per millilitre (= 1000 IU/ml);

V = volume of reference solution (a) treated (2.00 ml);

m = mass of the substance to be examined in the test solution (2.00 g).

Vitamin D₃. Liquid chromatography (2.2.29). *Carry out the assay as rapidly as possible, avoiding exposure to actinic light and air.*

Internal standard solution. Dissolve 0.50 mg of *ergocalciferol CRS* in 100 ml of *anhydrous ethanol R*.

Test solution (a). To 4.00 g in a round-bottomed flask, add 5 ml of a freshly prepared 100 g/l solution of *ascorbic acid R*, 10 ml of a freshly prepared 800 g/l solution of *potassium hydroxide R* and 100 ml of *anhydrous ethanol R*. Boil under a reflux condenser on a water-bath for 30 min. Add 100 ml of a 10 g/l solution of *sodium chloride R* and cool the solution to room temperature. Transfer the solution to a 500 ml separating funnel, rinsing the round-bottomed flask with about 75 ml of a 10 g/l solution of *sodium chloride R* and then with 150 ml of a mixture of equal volumes of *light petroleum R1* and *ether R*. Shake for 1 min. When the layers have separated completely, discard the lower layer and wash the upper layer, first with 50 ml of a 30 g/l solution of *potassium hydroxide R* in a 10 per cent V/V solution of *anhydrous ethanol R*, and then with 3 quantities, each of 50 ml, of a 10 g/l solution of *sodium chloride R*. Filter the upper layer through 5 g of *anhydrous sodium sulphate R* on a fast filter paper into a 250 ml flask suitable for a rotary evaporator. Wash the funnel with 10 ml of fresh extraction mixture, filter and combine the upper layers. Distil them at a temperature not exceeding 30 °C under reduced pressure (water ejector) and fill with *nitrogen R* when evaporation is completed. Alternatively, evaporate the solvent under a gentle current of *nitrogen R* at a temperature not exceeding 30 °C. Dissolve the residue in 1.5 ml of the mobile phase described under Purification. Gentle heating in an ultrasonic bath may be required. *A large fraction of the white residue is cholesterol, constituting approximately 50 per cent m/m of the unsaponifiable matter of cod-liver oil.*

Test solution (b). Prepare duplicates. To 4.00 g add 2.0 ml of the internal standard solution and proceed as described for test solution (a).

Reference solution (a). Dissolve 0.50 mg of *cholecalciferol CRS* in 100.0 ml of *anhydrous ethanol R*.

Reference solution (b). In a round-bottomed flask, add 2.0 ml of reference solution (a) and 2.0 ml of the internal standard solution and proceed as described for test solution (a).

PURIFICATION**Column:**

- size: $l = 0.25$ m, $\varnothing = 4.6$ mm;
- stationary phase: nitrile silica gel for chromatography R (10 μ m).

Mobile phase: isoamyl alcohol R, hexane R (1.6:98.4 V/V).

Flow rate: 1.1 ml/min.

Detection: spectrophotometer at 265 nm.

Inject 350 μ l of reference solution (b). Collect the eluate from 2 min before until 2 min after the retention time of cholecalciferol, in a ground-glass-stoppered tube containing 1 ml of a 1 g/l solution of butylhydroxytoluene R in hexane R. Repeat the procedure with test solutions (a) and (b). Evaporate the eluates obtained from reference solution (b) and from test solutions (a) and (b), separately, to dryness at a temperature not exceeding 30 °C under a gentle current of nitrogen R. Dissolve each residue in 1.5 ml of acetonitrile R.

DETERMINATION**Column:**

- size: $l = 0.15$ m, $\varnothing = 4.6$ mm;
- stationary phase: octadecylsilyl silica gel for chromatography R (5 μ m).

Mobile phase: phosphoric acid R, 96 per cent V/V solution of acetonitrile R (0.2:99.8 V/V).

Flow rate: 1.0 ml/min.

Detection: spectrophotometer at 265 nm.

Injection: 2 quantities not exceeding 200 μ l of each of the 3 solutions obtained under Purification.

System suitability:

- resolution: minimum 1.4 between the peaks due to ergocalciferol and cholecalciferol in the chromatogram obtained with reference solution (b);
- the results obtained with the test solution (b) duplicates do not differ by more than 5 per cent.

Calculate the content of vitamin D₃ in International Units per gram using the following expression, taking into account the assigned content of cholecalciferol CRS:

$$\frac{A_2}{A_6} \times \frac{A_3}{A_4 - \left[\frac{A_5}{A_1} \right] \times A_2} \times \frac{m_2}{m_1} \times \frac{V_2}{V_1} \times 40$$

- m_1 = mass of the sample in test solution (b), in grams;
- m_2 = total mass of cholecalciferol CRS used for the preparation of reference solution (a), in micrograms (500 μ g);
- A_1 = area (or height) of the peak due to cholecalciferol in the chromatogram obtained with test solution (a);
- A_2 = area (or height) of the peak due to cholecalciferol in the chromatogram obtained with test solution (b);
- A_3 = area (or height) of the peak due to ergocalciferol in the chromatogram obtained with reference solution (b);
- A_4 = area (or height) of the peak due to ergocalciferol in the chromatogram obtained with test solution (b);
- A_5 = area (or height) of a possible peak in the chromatogram obtained with test solution (a) with the same retention time as the peak co-eluting with ergocalciferol in test solution (b);

- A_6 = area (or height) of the peak due to cholecalciferol in the chromatogram obtained with reference solution (b);
- V_1 = total volume of reference solution (a) (100 ml);
- V_2 = volume of reference solution (a) used for preparing reference solution (b) (2.0 ml).

STORAGE

In an airtight and well-filled container, protected from light. If no antioxidant is added, store under an inert gas.

Once the container has been opened, its contents are used as soon as possible and any part of the contents not used at once is protected by an atmosphere of inert gas.

LABELLING

The label states:

- the concentration of EPA and DHA as a sum;
- the number of International Units of vitamin A per gram;
- the number of International Units of vitamin D₃ per gram.

01/2009:1192

COD-LIVER OIL (TYPE A)**Iecoris aselli oleum A****DEFINITION**

Purified fatty oil obtained from the fresh livers of wild cod, *Gadus morhua* L. and other species of *Gadidae*, solid substances being removed by cooling and filtering. A suitable antioxidant may be added.

Content: 600 IU (180 μ g) to 2500 IU (750 μ g) of vitamin A per gram and 60 IU (1.5 μ g) to 250 IU (6.25 μ g) of vitamin D₃ per gram.

CHARACTERS

Appearance: clear, yellowish, liquid.

Solubility: practically insoluble in water, slightly soluble in ethanol (96 per cent), miscible with light petroleum.

IDENTIFICATION

First identification: A, B, C.

Second identification: C, D.

- In the assay for vitamin A using method A, the test solution shows an absorption maximum (2.2.25) at 325 ± 2 nm. In the assay for vitamin A using method B, the chromatogram obtained with the test solution shows a peak corresponding to the peak of all-*trans*-retinol in the chromatogram obtained with the reference solution.
- In the assay for vitamin D₃, the chromatogram obtained with test solution (a) shows a peak corresponding to the peak of cholecalciferol in the chromatogram obtained with reference solution (b).
- Composition of fatty acids (see Tests).
- To 0.1 g add 0.5 ml of methylene chloride R and 1 ml of antimony trichloride solution R. Mix. A deep blue colour develops in about 10 s.

TESTS

Colour: not more intensely coloured than a reference solution prepared as follows: to 3.0 ml of red primary solution add 25.0 ml of yellow primary solution and dilute to 50.0 ml with a 10 g/l solution of hydrochloric acid R (2.2.2, Method II).

Relative density (2.2.5): 0.917 to 0.930.

Refractive index (2.2.6): 1.477 to 1.484.