BRITISH STANDARD

BS EN 12821:2009

# Foodstuffs — Determination of vitamin D by high performance liquid chromatography — Measurement of cholecalciferol (D3) or ergocalciferol (D2)

ICS 67.050



BSI British Standards

# National foreword

This British Standard is the UK implementation of EN 12821:2009. It supersedes BS EN 12821:2000 which is withdrawn.

The UK participation in its preparation was entrusted to Technical Committee AW/-/3, Food analysis - Horizontal methods.

A list of organizations represented on this committee can be obtained on request to its secretary.

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EUROPEAN STANDARD

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**EN 12821**

April 2009

ICS 67.050

Supersedes EN 12821:2000

English Version

# Foodstuffs - Determination of vitamin D by high performance liquid chromatography - Measurement of cholecalciferol (D3) or ergocalciferol (D2)

Produits alimentaires - Dosage de la vitamine D par chromatographie liquide haute performance - Dosage du cholécalciférol (D3) et de l' ergocalciférol (D2)

Lebensmittel - Bestimmung von Vitamin D mit Hochleistungs-Flüssigchromatographie - Bestimmung von Cholecalciferol (D3) oder Ergocalciferol (D2)

This European Standard was approved by CEN on 21 February 2009.

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## Foreword

This document (EN 12821:2009) has been prepared by Technical Committee CEN/TC 275 “Food analysis - Horizontal methods”, the secretariat of which is held by DIN.

This European Standard shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by October 2009, and conflicting national standards shall be withdrawn at the latest by October 2009.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. CEN [and/or CENELEC] shall not be held responsible for identifying any or all such patent rights.

This document supersedes EN 12821:2000.

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# 1 Scope

This European Standard specifies a method for the determination of vitamin $D_3$ (cholecalciferol) or vitamin $D_2$ (ergocalciferol) in foodstuffs by high performance liquid chromatography (HPLC).

Vitamin $D_3$ is primary in foodstuffs of animal origin, while vitamin $D_2$ is primary in wild mushrooms. Both vitamin $D_3$ and vitamin $D_2$ can be present in fortified foodstuffs. This European Standard is not applicable for samples with a content of vitamin $D_3$ and vitamin $D_2$.

Apart from the vitamin D activity from the parent forms, vitamin $D_3$ and vitamin $D_2$, the corresponding metabolites 25-hydroxy vitamin D and 1,25-dihydroxy vitamin D also contribute to the vitamin D activity. This European Standard does only include measurement of vitamin $D_3$ or vitamin $D_2$.

This European Standard provides the base for the analytical methods. It is intended to serve as a frame in which the analyst can define his own analytical work in accordance to the standard procedure.

This method has been validated in inter-laboratory tests on fortified and non-fortified samples such as margarine, milk, milk powder, liquid infant formula, infant formula, cooking oil, and fish oil at levels from 0,4 µg/100 g to 14 µg/100 g. Further information on the validation data is given in Annex D.

# 2 Normative references

The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

EN ISO 3696*, Water for analytical laboratory use - Specification and test methods (ISO 3696:1987).*

# 3 Principle

Vitamin $D_3$ and vitamin $D_2$ are saponified in the foodstuffs using alcoholic potassium hydroxide solution and extracted by an appropriate solvent. The determination of vitamin $D_3$ or vitamin $D_2$ in an appropriate sample extract solution is carried out by semi-preparative normal phase HPLC followed by reverse-phase analytical HPLC.

If vitamin $D_3$ is to be determined, then vitamin $D_2$ is used as an internal standard. If vitamin $D_2$ is to be determined, then vitamin $D_3$ is used as an internal standard.

Vitamin D is detected by ultraviolet (UV) spectrometry and peaks are identified on the basis of retention times and additionally by UV spectral profile if diode-array detection is used. The determination is carried out by the internal standard procedure using peak areas or peak heights, see [1] to [8].

# 4 Reagents

## 4.1 General

During the analysis, unless otherwise stated, use only reagents of recognized analytical grade and water of at least grade 1 according to EN ISO 3696.

**4.2 Methanol**

**4.3 Ethanol,** volume fraction $\varphi(C_2H_5OH)$ = 100 %.

**4.4 Ethanol,** $\varphi(C_2H_5OH)$ = 96 %.

**4.5 Sodium sulfate,** anhydrous.

**4.6 KOH solutions for saponification,** in suitable concentrations, e.g. mass concentration $\rho$(KOH) = 50 g/100 ml or $\rho$(KOH) = 60 g/100 ml, or alcoholic solutions, e.g. 28 g of KOH in 100 ml of an ethanol and water mixture with a volume fraction of ethanol of 90 %.

**4.7 Antioxidants,** such as ascorbic acid (AA), sodium ascorbate, pyrogallol, sodium sulfide ($Na_2S$) or butylated hydroxytoluene (BHT).

**4.8 Solvents and extraction solvents,** such as diethyl ether (peroxide-free), dichloromethane, light petroleum, *n*-hexane, ethylacetate or appropriate mixtures thereof.

**4.9 HPLC Mobile phases**

**4.9.1 Examples of solvent mixtures for normal phase semi-preparative HPLC**

Examples of appropriate solvent mixtures (given as volume fractions) for normal phase semi-preparative HPLC include:

— *n*-hexane and 2-propanol (98 + 2), (99 + 1) or (95 + 5);

— *n*-hexane and isoamyl alcohol (99 + 1);

— *n*-hexane, 2-propanol and tetrahydrofuran (98 + 1 + 1);

— *iso*-octane and iso-butanol (99 + 1);

— *n*-heptane and 2-propanol (97 + 3).

**4.9.2 Examples of solvent and solvent mixtures for reverse-phase analytical HPLC**

Examples of appropriate solvent and solvent mixtures (given as volume fractions) for reverse-phase analytical HPLC include:

— methanol;

— methanol and water (95 + 5) or (93 + 7);

— acetonitrile and methanol (80 + 20), (90 + 10) or (70 + 30);

— acetonitrile, chloroform and methanol (93 + 4 + 3).

## 4.10 Standard substances

### 4.10.1 Ergocalciferol standard substance (vitamin $D_2$), $M(C_{28}H_{44}O)$ = 396,7 g/mol

Vitamin $D_2$ standard substance shall be of the highest purity obtainable (having a mass fraction of greater than 98 %) and shall be stored according to the supplier's instructions (in the absence of light, typically less than 4 °C).

### 4.10.2 Cholecalciferol standard substance (vitamin $D_3$), $M(C_{27}H_{44}O)$ = 384,6 g/mol

Vitamin $D_3$ standard substance shall be of the highest purity obtainable (having a mass fraction of greater than 98 %) and shall be stored according to the supplier's instructions (in the absence of light, typically less than 4 °C).

## 4.11 Stock solutions

### 4.11.1 Vitamin $D_2$ stock solution

Weigh about 100 mg of vitamin $D_2$ (4.10.1) to the nearest milligram into a one mark 100 ml volumetric flask, dissolve in ethanol (4.4) and dilute to the mark with ethanol. This solution contains approximately 1 mg/ml of vitamin $D_2$. Store below 4 °C and protect from light.

Calculate the mass concentration of the stock solution and the mass fraction of the vitamin $D_2$ standard by the procedure described in 4.12.1.

This solution is stable for 6 months at - 18 °C.

### 4.11.2 Vitamin $D_3$ stock solution

Weigh about 100 mg of vitamin $D_3$ (4.10.2) to the nearest milligram into a one mark 100 ml volumetric flask, dissolve in ethanol (4.4) and dilute to the mark with ethanol. This solution contains approximately 1 mg/ml of vitamin $D_3$. Store below 4 °C and protect from light.

Calculate the mass concentration of the stock solution and the mass fraction of the vitamin $D_3$ standard by the procedure described in 4.12.2.

This solution is stable for 6 months at - 18 °C.

## 4.12 Standard solutions

### 4.12.1 Vitamin $D_2$ standard solution

Pipette 1 ml of the vitamin $D_2$ stock solution (4.11.1) into a one mark 100 ml volumetric flask and dilute to the mark with ethanol (4.4). This solution contains approximately 10 µg/ml of vitamin $D_2$. Prepare this solution on the day of use.

NOTE The mass concentration of the standard solution can be adjusted if necessary to suit the analytical requirements.

Measure the absorption of the vitamin $D_2$ standard solution in a 1 cm quartz cell at a wavelength of 265 nm using ethanol in the reference path. Calculate the mass concentration of vitamin $D_2$, $\rho_{D2}$, in microgram per millilitre of the standard solution using Equation (1):

$$\rho_{D2} = \frac{A_{265} \times M_{D2} \times 1000}{\varepsilon \times b} \quad (1)$$

where:

$A_{265}$ is the absorption of the vitamin $D_2$ standard solution at 265 nm;

$M_{D2}$ is the molar mass of vitamin $D_2$ ($M_{D2}$ = 396,7 g/mol);

$\varepsilon$ is the molar absorption coefficient of vitamin $D_2$ (here: $\varepsilon$ = 18 843 $m^2$/mol, calculated from the $E_{1cm}^{1\%}$ value, see [9]);

$b$ is the optical path length of the quartz cell in centimetres.

#### 4.12.2 Vitamin $D_3$ standard solution

Pipette 1 ml of the vitamin $D_3$ stock solution (4.11.2) into a one mark 100 ml volumetric flask and dilute to the mark with ethanol (4.4). This solution contains approximately 10 µg/ml of vitamin $D_3$. Prepare this solution on the day of use.

NOTE The mass concentration of the standard solution can be adjusted if necessary to suit the analytical requirements.

Measure the absorption of the vitamin $D_3$ standard solution in a 1 cm quartz cell at a wavelength of 265 nm using ethanol (4.4) in the reference path. Calculate the mass concentration of vitamin $D_3$, $\rho_{D3}$, in microgram per millilitre of the standard solution using Equation (2):

$$\rho_{D3} = \frac{A_{265} \times M_{D3} \times 1000}{\varepsilon \times b} \quad (2)$$

where:

$A_{265}$ is the absorption of the vitamin $D_3$ standard solution at 265 nm;

$M_{D3}$ is the molar mass of vitamin $D_3$ ($M_{D3}$ = 384,6 g/mol);

$\varepsilon$ is the molar absorption coefficient of vitamin $D_3$ (here: $\varepsilon$ = 18 461 $m^2$/mol, calculated from the $E_{1cm}^{1\%}$ value, see [9]);

$b$ is the optical path length of the quartz cell in centimetres.

### 4.13 Internal standard solutions

#### 4.13.1 Vitamin $D_2$ internal standard solution

Pipette 10 ml of the vitamin $D_2$ standard solution (4.12.1) into a one mark 100 ml volumetric flask and dilute to the mark with ethanol (4.4). Prepare this solution on the day of use.

#### 4.13.2 Vitamin $D_3$ internal standard solution

Pipette 10 ml of the vitamin $D_3$ standard solution (4.12.2) into a one mark 100 ml volumetric flask and dilute to the mark with ethanol (4.4). Prepare this solution on the day of use.

NOTE If vitamin $D_3$ is to be determined, then vitamin $D_2$ is used as an internal standard. If vitamin $D_2$ is to be determined, then vitamin $D_3$ is used as an internal standard.

### 4.14 Vitamin $D_2$ and vitamin $D_3$ semi-preparative standard solution

Pipette 5 ml of the vitamin $D_2$ standard solution (4.12.1) and 5 ml of the vitamin $D_3$ standard solution (4.12.2) into a rotary evaporator flask and carefully remove the solvent (at not more than 40 °C). Re-dissolve the residue in 100 ml of the semi-preparative HPLC mobile phase (4.9.1).

The concentration of the semi-preparative standard may be adjusted if necessary to suit the HPLC system in use (5.4 or 5.5).

### 4.15 Vitamin $D_2$ and vitamin $D_3$ analytical standard solution

Pipette 5 ml of the vitamin $D_2$ standard solution (4.12.1) and 5 ml of the vitamin $D_3$ standard solution (4.12.2) into a rotary evaporator flask and carefully remove the solvent (at not more than 40 °C). Re-dissolve the residue in 50 ml of the analytical HPLC mobile phase (4.9.2).

## 5 Apparatus

### 5.1 General

Usual laboratory apparatus and, in particular, the following.

**5.2 UV spectrometer,** capable of measuring at a wavelength of 265 nm.

### 5.3 Rotary evaporator, with water bath and vacuum unit

NOTE The use of nitrogen is recommended for releasing the vacuum.

**5.4 Semi-preparative HPLC system,** consisting of a pump, sample injection device, UV detector, a means of collecting a defined aliquot portion of column eluent, and a recorder or integrator.

**5.5 Analytical HPLC system,** consisting of a pump, sample injection device, UV detector, recorder/integrator or similar data capture device.

### 5.6 HPLC columns

**5.6.1 Semi-preparative normal phase column,** e.g. silica or bonded cyano-amino, particle size 5 µm, diameter 4,0 mm to 8,0 mm, length 250 mm to 300 mm. See Annex A for more information.

**5.6.2 Analytical reverse phase column,** e.g. $C_{18}$ reverse phase, particle size 5 µm, diameter 4,0 mm to 4,6 mm, length 250 mm. See Annex A for more information.

#### 5.6.3 Packing materials

Particle sizes and column dimensions other than those specified in this European Standard may be used, but the analyst has to ensure that they provide adequate separation of the vitamins D from matrix interferences if equivalent results are to be obtained.

### 5.7 Filter device

Large and small scale filter devices to filter HPLC mobile phases and sample solutions respectively, e.g. of 0,45 µm pore size or similar is appropriate.

NOTE Filtering of the mobile phase as well as of the sample test solution through a membrane filter prior to use or injection usually increases longevity of the columns.

# 6 Procedure

## 6.1 General

Vitamin $D_2$ and vitamin $D_3$ are sensitive to UV radiation and to oxidizing agents (e.g. atmospheric oxygen). It is therefore necessary to exclude UV light by using amber glassware, aluminium foil or UV absorbing materials. Antioxidants need to be added to solutions containing extracted vitamin, and nitrogen flushing should be used. The solvents shall be evaporated under reduced pressure using a rotary evaporator at not more than 40 ºC.

## 6.2 Preparation of the test sample

Homogenize the test sample. Comminute coarse material thoroughly and homogenize in a food blender or liquidiser. Precautions such as pre-cooling the sample shall be taken to avoid exposure to high temperatures. After this preparation the test sample shall be analysed without delay. Protect samples from light.

## 6.3 Preparation of the sample test solution

### 6.3.1 Saponification

Saponify 10 g to 30 g of the test sample by refluxing, preferably under nitrogen, using suitable amounts of ethanol (4.4), water, an antioxidant (4.7) such as ascorbic acid, sodium ascorbate or pyrogallol and one of the potassium hydroxide solutions (4.6). Add the antioxidants to the sample prior to the addition of potassium hydroxide. Sodium sulfide (4.7) may also be added to obviate the oxidative catalytic effects of traces of metals.

If vitamin $D_3$ is to be determined, pipette an appropriate amount of vitamin $D_2$ internal standard solution (4.13.1) into the saponification flask. The amount of vitamin $D_2$ internal standard solution added shall be similar to the amount of vitamin $D_3$ expected in the sample. If vitamin $D_2$ is to be determined then vitamin $D_3$ standard solution (4.13.2) shall be added as the internal standard.

A sample that does not contain the internal standard should be taken through the analytical procedure to ensure that there is no sample matrix interference at the internal standard retention time.

Examples of suitable ratios of reagents are given in Table 1.

**Table 1 — Examples of suitable ratios of reagents**

| Sample | Ethanol | Pyrogallol | Ascorbic acid / Na ascorbate | Potassium hydroxide |
|---|---|---|---|---|
| 10 g to 30 g | 100 ml | 0,5 g to 1 g | 1,0 g to 2,5 g | 50 ml of a 50 g/100 ml solution |

The usual time of saponification ranges from 20 min to 45 min with temperatures of 70 °C to 100 °C. Saponification may also be carried out at room temperature overnight (approximately 16 h) under otherwise same conditions.

If after saponification and cooling, fat or oil is present on the surface of the saponification mixture, additional ethanolic potassium hydroxide has to be added and saponification time extended.

NOTE Conditions found suitable for saponification of a margarine and a milk powder are shown in Annex B.

#### 6.3.2 Extraction

In order to avoid emulsions, an amount of water has to be added to the saponified sample solution so that the ratio of alcohol to water in the resulting solution is 1:1.

Extract the vitamins $D_2$ and $D_3$ from the cooled saponification mixture using a suitable solvent, or mixture of solvents (4.8), and repeat the procedure two to four times with volumes ranging from 100 ml to 200 ml. Wash the combined solvent extracts to neutral pH with water (typically 5 times with 50 ml to 100 ml).

NOTE Some methods prescribe washing to neutrality with 3 % or 5 % potassium hydroxide in 0,9 % sodium chloride solution buffered in 2,6 mol/l sodium acetate (pH = 7), or similar mixtures. Annex B shows extraction conditions found suitable for a margarine and a milk powder.

#### 6.3.3 Concentration

Evaporate sample extracts using a rotary evaporator (5.3) under reduced pressure, and at a temperature not exceeding 40 °C. Prior to evaporation it is good practice to add an antioxidant (e.g. 2 ml of 1 mg/ml BHT in *n*-hexane) to the sample extract.

Absolute ethanol (4.3) or anhydrous sodium sulfate (4.5) should be added to the concentrated sample extract to assist in the removal of traces of water (azeotropic distillation).

At this stage in the analytical procedure, additional cleanup of the sample extract may be employed to remove potential interferences. If additional cleanup is employed, the procedure shall be fully validated for use.

NOTE Annex E outlines three different additional cleanup steps. The cleanup step with use of column chromatography (E.2) and with use of SPE (E.3) should always be combined, and have shown to be useful for foods, for example margarine and oil. The cleanup step with use of preparative TLC (E.1) is preferable for feed and supplements like tablets or capsules. For supplements it may be combined with E.3 if necessary.

#### 6.3.4 Dilution

Re-dissolve the residue in a small, known volume of solvent which is compatible with the semi-preparative HPLC system. Addition of a small amount of anhydrous sodium sulfate will remove residual traces of water.

### 6.4 Calibration

Use standard solutions of vitamin $D_2$ (4.12.1) and vitamin $D_3$ (4.12.2) to calibrate the semi-preparative (5.6.1) and analytical HPLC (5.6.2) systems and assess system suitability.

### 6.5 HPLC system suitability

Chromatograph a mixed vitamin $D_2$ and $D_3$ semi-preparative standard (4.14) on the semi-preparative HPLC system (5.6.1) until a single vitamin D peak is eluted with a reproducible retention time. Once achieved, this will allow precise band-cut collection of the vitamin D fraction from sample extracts.

The chromatographic conditions of the semi-preparative HPLC have to be adjusted to achieve optimal separation of vitamin D from tocopherols and other food matrix interferences. See Annex C for example chromatograms.

Chromatograph a mixed vitamin $D_2$ and $D_3$ analytical standard solution (4.15) on the analytical HPLC system and adjust the chromatographic conditions until the resolution of vitamin $D_2$ from vitamin $D_3$ is at least 98 % complete (i.e. the resolution factor shall be greater than 1,0), and the vitamins are resolved from all food matrix interferences.

## 6.6 Determination

### 6.6.1 Semi-preparative HPLC

Inject an aliquot portion of the concentrated sample extract onto the semi-preparative HPLC system (5.6.1) and collect the vitamin D fraction via a band-cut. The time window for band-cut collection shall have been previously determined using a vitamin D standard (6.5). The band-cut shall be sufficiently wide to collect all of the vitamin D band but sufficiently narrow to reduce the possibility of collecting tocopherols or other interfering compounds.

A typical semi-preparative chromatogram is shown in Annex C.

### 6.6.2 Analytical HPLC

Evaporate the band-cut from the semi-preparative HPLC to dryness and re-dissolve in solvent compatible with the analytical HPLC mobile phase.

Inject aliquot portions of the sample extract onto the analytical HPLC system and identify the vitamin $D_2$ and $D_3$ peaks (6.6.3). The vitamin $D_2$ and $D_3$ peaks shall be resolved from sample matrix interferences.

A typical analytical HPLC chromatogram is shown in Annex C.

### 6.6.3 Identification

Identify vitamins $D_2$ and $D_3$ by comparing retention times from sample chromatograms with those obtained from standards under the same chromatographic conditions (6.5). The use of diode array detection allows the UV profile of the vitamin D peaks to be scrutinised and peak purity assessed. Re-chromatographing sample extracts using different UV detector wavelengths may also be used to assess vitamin D peak purity and confirm peak identity.

### 6.6.4 Number of determinations

Perform at least two independent determinations.

## 6.7 Internal standard procedure and response factor

Calculate the response factor of vitamin $D_3$ to $D_2$, $R_f$, by internal standard procedure using standards of known concentration (4.13) using Equation (3):

$$R_f = \frac{A_{STD3} \times \rho_{STD2}}{A_{STD2} \times \rho_{STD3}} \quad (3)$$

where:

$A_{STD3}$ is the peak area or height for the vitamin $D_3$ standard solution;
$A_{STD2}$ is the peak area or height for the vitamin $D_2$ standard solution;
$\rho_{STD2}$ is the mass concentration of vitamin $D_2$ in the standard solution, in microgram per millilitre;
$\rho_{STD3}$ is the mass concentration of vitamin $D_3$ in the standard solution in microgram per millilitre.

# 7 Calculation

Calculate the mass fraction, $w_{D3}$, of vitamin $D_3$ in µg/100 g, using Equation (4):

$$w_{D3} = \frac{A_{SD3} \times I_S \times 100}{A_{SD2} \times R_f \times m} \quad (4)$$

where:

$I_S$ is the mass of the internal standard of vitamin $D_2$, in the test portion, in microgram;
$m$ is the mass of the sample taken for the saponification, in grams;
$R_f$ see Equation (3);
$A_{SD3}$ is the peak area or height for vitamin $D_3$ in the sample solution;
$A_{SD2}$ is the peak area or height for vitamin $D_2$ in the sample solution.

# 8 Precision

## 8.1 Statistical summary

The precision data of different HPLC methods for the determination of vitamin $D_3$ were established in 1994 by an international comparison study organized on behalf of the European Commission's Standards Measurement and Testing Programme on a sample of margarine (Certified Reference Material (CRM 122)) and milk powder (CRM 421) and provided the statistical information shown in Annex D.

The precision data on porridge and milk powder were established in an interlaboratory test on a method using a calculation based on external standard, in accordance with ISO 5725:1986. See Annex D.

The precision data on milk, liquid infant formula, cooking oil, margarine, infant formula and fish oil were established in an interlaboratory test in accordance with the AOAC Guidelines for collaborative study procedures to validate characteristics of a method of analysis, see Annex D.

The data derived from these comparison studies may not be applicable to analyte concentration ranges and sample matrices other than those given in Annex D.

## 8.2 Repeatability

The absolute difference between two single test results found on identical test material by one operator using the same apparatus within the shortest feasible time interval will exceed the repeatability limit $r$ in not more than 5 % of the cases.

The values are:

| | | |
|---|---|---|
| Margarine: | $\bar{x}$ = 12,3 µg/100 g | $r$ = 2,32 µg/100 g |
| Milk powder: | $\bar{x}$ = 14,3 µg/100 g | $r$ = 2,09 µg/100 g |

| | | |
|---|---|---|
| Milk: | $\bar{x}$ = 0,418 µg/100 g | $r$ = 0,054 µg/100 g |
| Liquid infant formula: | $\bar{x}$ = 1,38 µg/100 g | $r$ = 0,23 µg/100 g |
| Cooking oil: | $\bar{x}$ = 4,61 µg/100 g | $r$ = 0,96 µg/100 g |
| Margarine: | $\bar{x}$ = 8,39 µg/100 g | $r$ = 1,52 µg/100 g |
| Infant formula: | $\bar{x}$ = 10,1 µg/100 g | $r$ = 0,7 µg/100 g |
| Fish oil: | $\bar{x}$ = 11,6 µg/100 g | $r$ = 0,7 µg/100 g |

## 8.3 Reproducibility

The absolute difference between two single test results found on identical test material reported by two laboratories will exceed the reproducibility limit $R$ in not more than 5 % of the cases.

The values are:

| | | |
|---|---|---|
| Margarine: | $\bar{x}$ = 12,3 µg/100 g | $R$ = 2,66 µg/100 g |
| Milk powder: | $\bar{x}$ = 14,3 µg/100 g | $R$ = 2,21 µg/100 g |
| | | |
| Milk: | $\bar{x}$ = 0,418 µg/100 g | $R$ = 0,106 µg/100 g |
| Liquid infant formula: | $\bar{x}$ = 1,38 µg/100 g | $R$ = 0,47 µg/100 g |
| Cooking oil: | $\bar{x}$ = 4,61 µg/100 g | $R$ = 3,11 µg/100 g |
| Margarine: | $\bar{x}$ = 8,39 µg/100 g | $R$ = 1,60 µg/100 g |
| Infant formula: | $\bar{x}$ = 10,1 µg/100 g | $R$ = 2,0 µg/100 g |
| Fish oil: | $\bar{x}$ = 11,6 µg/100 g | $R$ = 5,8 µg/100 g |

## 9 Test report

The test report shall contain at least the following data:

a) all information necessary for the identification of the sample;

b) a reference to this European Standard or to the method used;

c) the results and the units in which the results have been expressed;

d) the date and type of sampling procedure (if known);

e) the date of receipt;

f) the date of test;

g) any particular points observed in the course of the test;

h) any observations not specified in the method or regarded as optional which might have affected the results.

# Annex A
# (informative)

# Examples of suitable HPLC systems

**Table A.1 — Examples of semi-preparative HPLC systems used for sample test solution cleanup by participants in the EU MAT certification study for vitamin D [8]**

| Column | Dimensions, mm | Mobile phase, (V + V) | Detector, λ |
|---|---|---|---|
| Polygosil® 60, 5 µm | 250 x 8 | *iso*-octane + *iso*-butanol (99 + 1) | 265 nm |
| LiChrospher® Si 60, 5 µm | 250 x 4 | *n*-hexane + *2*-propanol (99 + 1) | 265 nm |
| LiChrospher® Si 100, 5 µm | 250 x 8 | *n*-hexane + *2*-propanol (98 + 2) | 265 nm |
| µ Porasil® silica | 300 x 3,9 | *n*-hexane + THF + *2*-propanol (98 + 1 + 1) | 265 nm |
| Partisil® PAC, 5 µm | 250 x 4,6 | *n*-hexane + isoamylalcohol (99 + 1) | 265 nm |
| LiChrosorb® Si 60 | 250 x 4 | *n*-hexane + *2*-propanol + THF (98 + 1 + 1) | 265 nm |
| LiChrosorb® Si 60 | 250 x 4 | *n*-hexane + *2*-propanol (95 + 5) | 265 nm |
| LiChrosorb® Si 60 | 250 x 4 | *n*-hexane + *2*-propanol (97 + 3) | 265 nm |
| All trade names are given for the convenience of users of this European Standard and do not constitute an endorsement of these products by CEN. | | | |

**Table A.2 — Examples of analytical HPLC systems used to determine vitamin D in sample test solutions by participants in the EU MAT certification study, see [8]**

| Column | Dimensions, mm | Mobile phase, (V + V) | Detector, λ |
|---|---|---|---|
| Hypersil® ODS, 5 µm | 250 x 4,6 | methanol | 265 nm |
| LiChrospher® 100 RP 18, 5 µm | 250 x 4 | methanol + water (95 + 5) | 264 nm |
| Vydac® 201TP54 | 250 x 4,6 | methanol + water (93 + 7) | 265 nm |
| Vydac® 201TP54 | 250 x 4,6 | acetontrile + methanol (80 + 20) | 265 nm |
| Spherisorb® ODS 2, 5 µm | 250 x 4,6 | acetonitrile + dichloromethane + methanol (93 + 4 + 3) | diode array |
| Nucleosil® $C_{18}$, 5 µm | 250 x 4 | acetonitrile + methanol (70 + 30) | 265 nm |
| Zorbax® ODS | 250 x 4,6 | acetonitrile + methanol (95 + 5) | 265 nm |
| All trade names are given for the convenience of users of this European Standard and do not constitute an endorsement of these products by CEN. | | | |

# Annex B
# (informative)

# Examples of suitable extraction and saponification conditions

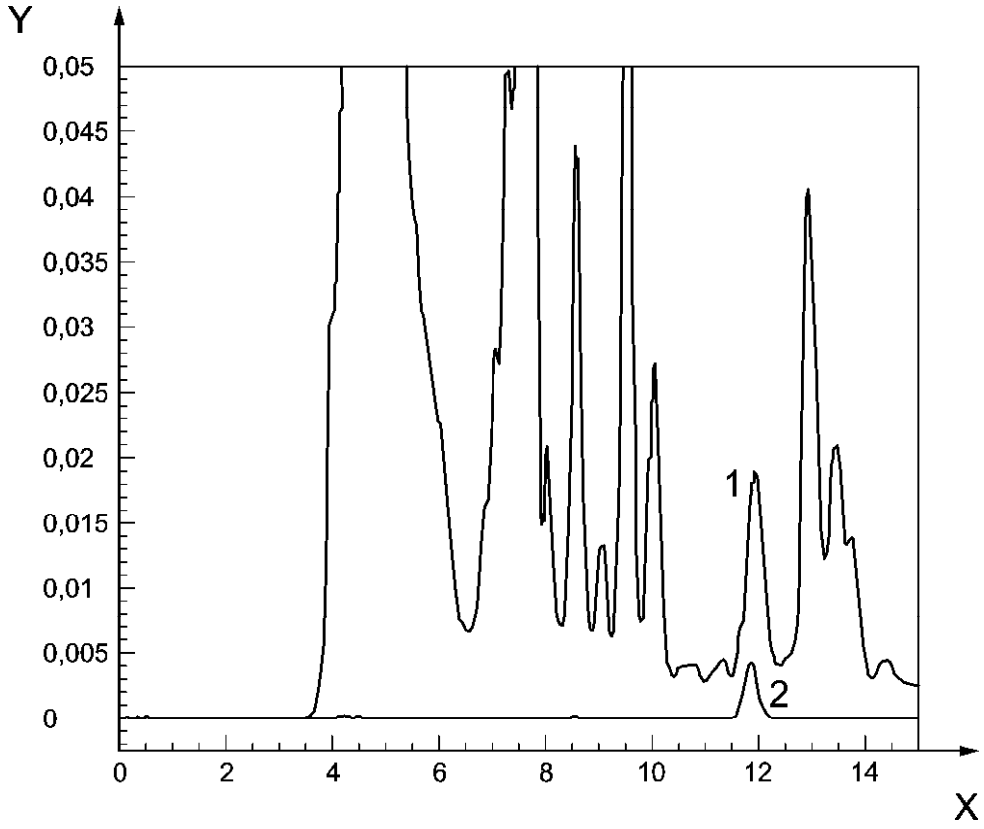
The examples of extraction and saponification conditions were used for the determination of vitamin D by participants in the EU MAT certification study [8]. Sample masses are expressed as grams of fat (margarine 82 % fat, milk powder 27 % fat).

**Table B.1 — Examples of analytical HPLC systems used to determine vitamin D in sample test solutions by participants in the EU MAT certification study**

| **Saponification** | **Extraction** |
|---|---|
| 16 g fat + 150 ml ethanol + 1g pyrogallol + [75 ml water + 30 g KOH[a)]] + nitrogen flushing; 70 °C for 30 min | PE [b)] + DEE[c)] (9 + 1) 2 x 100 ml; water wash 5 x 100 ml |
| 8 g fat + 100 ml ethanol + 1 g sodium ascorbate + 0,04 g sodium sulfide + 12 g KOH + 50 ml water + nitrogen flushing; 80 °C for 30 min | *n*-hexane, 3 x 100 ml and 3 x 50 ml; water wash 4 x 100 ml |
| 8 g fat + 35 ml ethanol + 2 g sodium ascorbate + 10 g KOH + 50 ml water; 100 °C for 45 min | *n*-hexane, 1 x 100 ml; 5 % KOH wash, 1 x 100 ml; 30 % ethanol in 9 % sodium chloride wash 1 x 100 ml; 0,9 % sodium chloride wash; 1 x 100 ml |
| 12 g fat + 30 ml ethanol + 30 ml methanol + 0,1 g ascorbic acid + 30 ml 50 % KOH + nitrogen flushing; 100 °C for 30 min | DEE, 2 x 100 ml; water wash, 4 x 50 ml |
| 6 g to 8 g fat + 100 ml ethanol + 1 g ascorbic acid + 50 ml 50 % KOH + nitrogen flushing; 20 °C for 20 h | PE + DEE (1 + 1), 2 x 200 ml; water wash, 6 x 50 ml |
| 8 g fat + 1 g pyrogallol + 150 ml 28 % KOH in ethanol and water (9 + 1) + nitrogen flushing; 100 °C reflux for 30 min | DEE + PE (1 + 1), 2 x 500 ml; water wash, 5 x 150 ml |
| 24 g fat + 90 ml ethanol + 0,5 g sodiumascorbate + 30 ml 60 % KOH + nitrogen flushing; 100 °C for 45 min, reflux | DEE, 1 x 150 ml, 3 x 75 ml; wash, 5 x 200 ml |
| a) KOH = potassium hydroxide<br>b) PE = light petroleum<br>c) DEE = diethyl ether | |

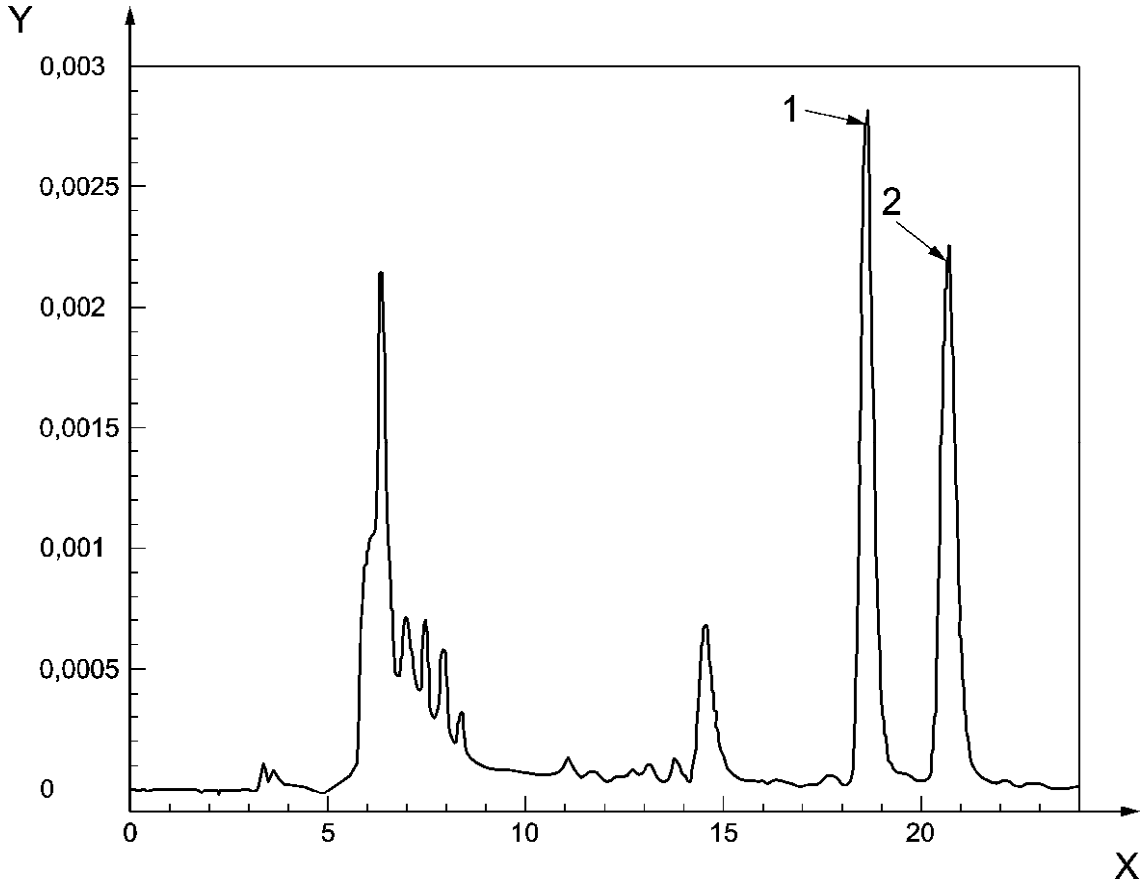
# Annex C
(normative)

## Examples of suitable semi-preparative and analytical HPLC chromatograms




**Key**
x time (min)
y signal (arbitrary units)
1 vitamin D in milk powder
2 vitamin D in standard

**Figure C.1 — Typical chromatogram of a normal phase semi-preparative HPLC of a saponified and liquid/liquid treated milk powder (CRM 421) and a standard of vitamin D (vitamin $D_2$ and vitamin $D_3$)**




**Key**
x time (min)
y signal (arbitrary units)
1 vitamin $D_2$
2 vitamin $D_3$

**Figure C.2 — Typical chromatogram of extract milk powder (CRM 421) reverse phase HPLC of the vitamin D fraction between 11,5 min and 12,5 min in semi-preparative step (see Figure C.1)**

# Annex D
(informative)

# Precision data

The parameters in Table D.1 on margarine (CRM 122) and milk powder (CRM 421) have been defined in an interlaboratory test [8], in accordance with the EU MAT Certification Study Guidelines. The study was organized by the Laboratory of the Government Chemist, UK.

The parameters in Table D.2 on milk, liquid infant formula, cooking oil, margarine, infant formula and fish oil have been defined in an interlaboratory test according to AOAC Guidelines for collaborative study procedures to validate characteristics of a method of analysis [13]. The study was organized by NMKL (Nordic Committee on Food Analysis) [11]. The method used included saponification, extraction, concentration, preparative HPLC and analytical HPLC.

**Table D.1 — Precision data for margarine and milk powder**

| **Sample** | **1** | **2** |
|---|---|---|
| Year of interlaboratory test | 1994 | 1994 |
| Number of laboratories | 11 | 11 |
| Number of samples | 5 | 5 |
| Number of laboratories retained after eliminating outliers | 11 | 11 |
| Number of outliers (laboratories) | 0 | 0 |
| Number of accepted results | 48 | 47 |
| Mean value $\bar{x}$ , µg/100 g | 12,30 | 14,30 |
| Repeatability standard deviation $s_r$, µg/100 g | 0,82 | 0,74 |
| Repeatability relative standard deviation $RSD_r$, % | 6,7 | 5,2 |
| Repeatability limit $r$ [$r = 2{,}8 \times s_r$], µg/100 g | 2,32 | 2,09 |
| Reproducibility standard deviation $s_R$, µg/100 g | 0,94 | 0,78 |
| Reproducibility relative standard deviation $RSD_R$, % | 7,6 | 5,5 |
| Reproducibility limit $R$ [$R = 2{,}8 \times s_R$], µg/100 g | 2,66 | 2,21 |
| Horrat $R$ index | 0,3 | 0,3 |
| 1 Margarine fortified with vitamin $D_3$ (CRM 122) | | |
| 2 Milk powder, spray-dried, vitamin $D_3$ fortified whole milk (CRM 421) | | |

**Table D.2 — Precision data for milk, liquid infant formula, cooking oil, margarine, infant formula and fish oil**

| **Sample** | **1** | **2** | **3** | **4** | **5** | **6** |
|---|---|---|---|---|---|---|
| Year of interlaboratory test | 1997 | 1997 | 1997 | 1997 | 1997 | 1997 |
| Number of laboratories | 8 | 8 | 8 | 8 | 8 | 8 |
| Number of samples | 2 | 2 | 2 | 2 | 2 | 2 |
| Number of laboratories retained after eliminating outliers | 7 | 8 | 8 | 7 | 7 | 8 |
| Number of outliers (laboratories) | 1 | 0 | 0 | 1 | 1 | 0 |
| Number of accepted results | 14 | 16 | 16 | 14 | 14 | 16 |
| Mean value $\bar{x}$, µg/100 g | 0,418 | 1,38 | 4,61 | 8,39 | 10,1 | 11,6 |
| Repeatability standard deviation $s_r$, µg/100 g | 0,019 | 0,08 | 0,34 | 0,54 | 0,2 | 0,3 |
| Repeatability relative standard deviation $RSD_r$, % | 4,6 | 5,9 | 7,4 | 6,5 | 2,4 | 2,2 |
| Repeatability limit $r$ [$r = 2{,}8 \times s_r$], µg/100 g | 0,054 | 0,23 | 0,96 | 1,52 | 0,7 | 0,7 |
| Reproducibility standard deviation $s_R$, µg/100 g | 0,038 | 0,17 | 1,11 | 0,57 | 0,7 | 2,1 |
| Reproducibility relative standard deviation $RSD_R$, % | 9,1 | 12,1 | 24,1 | 6,8 | 7,1 | 17,7 |
| Reproducibility limit $R$ [$R = 2{,}8 \times s_R$], µg/100 g | 0,106 | 0,47 | 3,11 | 1,60 | 2,0 | 5,8 |
| Recovery, % | - | - | 102 | - | 93,9 | 92,9 |
| Horrat $R$ index [a] | 0,4 | 0,6 | 1,1 | 0,3 | 0,3 | 0,8 |

1 low-lactose ultra-high temperature (UHT) milk, non-fortified

2 ready-to-feed, fortified, liquid infant formula (gruel), vitamin $D_3$ label claim of 1,3 µg/100 g

3 cooking oil with added vitamin $D_3$ for the collaborative trial

4 margarine with added vitamin $D_3$ for the collaborative trial

5 powdered infant formula with added vitamin $D_3$ for the collaborative trial

6 fish oil with added vitamin $D_3$ for the collaborative trial

a) These values differ from the ones in the reference [10] as Horwitz value is set to 22 % for mean values less than 120 ppb according to [12].

# Annex E
# (informative)

# Additional cleanup step for the determination of vitamin D with use of preparative TLC, column chromatography and or SPE

## E.1 Additional cleanup step with use of preparative TLC

### E.1.1 General

The outlined procedure for TLC has been widely used for cleanup of vitamin D extracts before the introduction of preparative HPLC. It has been validated for feedstuffs like piglet feed with 50 µg vitamin D/kg.

The advantages are:

— the vitamin D containing zone can be easily visualized under UV-light. Depending on the contributing matrix it will be decided from sample to sample how small the scraped $D_2/D_3$ zone should be. So matrix interferences could be minimized;

— using multiple step development of the TLC-plates the discrimination of $D_2/D_3$ zone and matrix-zones can be additionally improved.

A drawback of this method is that it can not be performed automatically. Turn-over times are restricted. More information on this procedure is given in [11].

### E.1.2 Principle

An aliquot of the raw extract after saponification is cleaned up by preparative TLC. The vitamin $D_2/D_3$ containing zone is scraped of the plate, transferred to a small column and the vitamins $D_2/D_3$ are extracted. The volume of this extract is reduced before application to analytical HPLC.

### E.1.3 Reagents

**E.1.3.1 TLC plates,** e.g. silica gel 60 F 254, Merck 5715 [1] , 0,25 mm, 20 cm x 20 cm

**E.1.3.2 Butylated hydroxytoluene (BHT)**

**E.1.3.3 *n*-hexane**

**E.1.3.4 Methanol**

**E.1.3.5 Acetone**

**E.1.3.6 Diethyl-ether**

**E.1.3.7 Solvent for developing the TLC-plate,** mixture of 88 ml of *n*-hexane, 2 ml of methanol, 10 ml of acetone and 10 mg of BHT

**E.1.3.8 Solvent for elution of the isolated vitamin $D_2$/$D_3$ zone,** mixture of 60 ml of diethylether and 40 ml of *n*-hexane

**E.1.3.9 Vitamin $D_3$ standard solution,** 250 µg/ml in methanol

### E.1.4 Apparatus

**E.1.4.1 TLC chamber for 20 cm x 20 cm TLC plates**

**E.1.4.2 Syringe,** 1 ml gastight or suitable spotting system for TLC

**E.1.4.3 UV lamp,** 254 nm

**E.1.4.4 Open tubular glas column,** diameter of 1 cm, length of approximately 15 cm, polytetrafluoroethylene (PTFE) stop cock

**E.1.4.5 Small, sharp-edged spatula**

### E.1.5 Procedure

Fill the TLC developing chamber with solvent (E.1.3.8) and wait approximately 2 h for saturation.

Evaporate a suitable aliquot of the raw extract after saponification to dryness under reduced pressure and immediately redissolve it in 2 ml of *n*-hexane. Spot 1 ml from this solution in multiple portions on the TLC-plate (E.1.3.1) to give a 10 cm long and a not broader than 1 cm zone. For localisation and identification, spot 5 µl to 10 µl of the vitamin $D_3$ (E.1.3.9) standard on both sides of this zone.

Develop the plate twice up to nearly the upper end. To improve separation it could be suitable to develop the plate multi-step wise. Before starting a new separation after the last run the solvent is evaporation under a stream of nitrogen. Visualize the separated vitamin $D_2$/$D_3$–zone under UV-light (E.1.4.3) and mark with a pencil (0,5 cm to 1,5 cm). Scrape the corresponding zone off with the spatula (E.1.4.5) onto a piece of paper and transfer it to the open tubular column (E.1.4.4), which has been filled with 10 ml of solvent (E.1.3.8).

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[1] Silica gel 60 F 254, Merck 5715 is an example of a suitable product available commercially. This information is given for the convenience of users of this European Standard and does not constitute an endorsement by CEN of this product.

Vitamin $D_2/D_3$ is eluted 5 times with 10 ml portions of solvent (E.1.3.8). Evaporate the combined eluates to dryness under reduced pressure and immediately redissolve in 1 ml of HPLC solvent (sample solution).

## E.2 Additional cleanup step with use of column chromatography

### E.2.1 Principle

The cleanup method is performed on a polyethylenglycol-celite column. A drawback of this cleanup method is that it cannot be performed automatically. Turn-over times are restricted.

### E.2.2 Reagents

**E.2.2.1 Petroleum ether,** boiling range 40 °C to 60 °C, distilled.

**E.2.2.2 Petroleum ether,** boiling range 60 °C to 70 °C, distilled.

**E.2.2.3 Diatomaceous earth,** soda ash flux calcined CAS No.: 68855-54-9.

**E.2.2.4 Sodium sulfate**

**E.2.2.5 Polyethylenglycol 600**

### E.2.3 Apparatus

**E.2.3.1 Rotation evaporator,** with water bath at 40 °C.

**E.2.3.2 Explosion proof high speed blender**

**E.2.3.3 Chromatography tubes,** 30 cm long, 2,5 cm internal diameter, equipped with a tap at the bottom for a polytetrafluoroethylene (PTFE) plug. 10 cm long, 1,4 cm internal diameter, narrowed at the bottom and equipped with a 100 ml reservoir at the top.

**E.2.3.4 Ultraviolet lamp,** hand-held model for long-wave light (366 nm).

### E.2.4 Procedure

#### E.2.4.1 Preparation of the column

Create a suspension of 25 g of diatomaceous earth (E.2.2.3) and 200 ml of petroleum ether (E.2.2.2) in a blender (E.2.3.2). While mixing, rapidly add 15 ml of polyethylenglycol (E.2.2.5) and continue mixing for approximately 20 s.

Alternatively, create the suspension using a method that produces an equivalent trituration of polyethylenglycol on the carrier substance. Pour a small quantity of petroleum ether (E.2.2.2) into the chromatography tube (E.2.3.3), put an air-free cotton wool plug in the bottom and cover with a 1 cm layer of sodium sulfate (E.2.2.4). Pour the suspension into the bottle and compress the column using a plunger on a stick until the length of the finished column measures approximately 15 cm. Any surplus petroleum ether will run off at the same time. Cover the top with a 1 cm layer of sodium sulfate.

#### E.2.4.2 Mounting of sample solution, elution and collection of eluate

Transfer the extract from the round-bottomed flask and rinse the flask using 3 batches of petroleum ether (E.2.2.2) of approximately 3 ml each. These should also be transferred to the column. Elute using petroleum

ether (E.2.2.2). Discard the first 30 ml of the eluate. Collect the following 50 ml eluate or until the retinol tape reaches the lower end of the diatomaceous earth column. This can be seen using a long-wave ultraviolet lamp (E.2.3.4). Evaporate the eluate until dryness in a rotation evaporator (E.2.3.1) and add 2 ml to 3 ml petroleum ether (E.2.2.1) or (E.2.2.2). Continue with cleanup step SPE (see E.3).

## E.3 Additional cleanup step with use of SPE

### E.3.1 Principle

This clause describes a cleanup method with use of SPE and silica. A drawback of this cleanup method is that it cannot be performed automatically. Turn-over times are restricted.

### E.3.2 Reagents

#### E.3.2.1 *n*-heptane p.a.

#### E.3.2.2 Diethylether

Remove peroxides by distilling over KOH pellets every day before use.

**E.3.2.3 Petroleum ether,** boiling range 40 °C to 60 °C, distilled.

### E.3.3 Apparatus

**E.3.3.1 Solid phase silica cartridge,** approximately 700 mg.

**E.3.3.2 Tapered 50 ml flasks with ground joints B 29**

### E.3.4 Procedure

Evaporate the eluate and dissolve it in 10 ml of heptane (E.3.2.1). Activate the cartridge (E.3.3.1) with 10 ml of heptane. Mount and eluate 10 ml of sample extract. Rinse using 10 ml of 6 % diethylether (E.3.2.2) in heptane and elute using 20 ml of 20 % diethylether (E.3.2.2) in heptane. Collect the eluate in a tapered flask (E.3.3.2), evaporate and dissolve it in 1 ml to 2 ml of petroleum ether (E.3.2.3). Continue with cleanup step TLC or semi-preparative HPLC.

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