DRAFT TANZANIA STANDARD

Foodstuffs – Determination of vitamin D by high performance liquid chromatography – Measurement of cholecalciferol (D3) or ergocalciferol (D2)
0. Foreword

Vitamin D is a group of fat-soluble secosteroids responsible for increasing intestinal absorption of calcium, magnesium, and phosphate, and multiple other biological effects. In humans, the most important compounds in this group are vitamin D₃ (also known as cholecalciferol) and vitamin D₂ (ergocalciferol). A diet with insufficient vitamin D causes rickets, a softening and weakening of bones in children and osteoporosis in older adults.

This Tanzania standard is applicable to foodstuffs and it deals with the measurement of cholecalciferol (D₃) or ergocalciferol (D₂) in food.

In the preparation of this Tanzania Standard assistance was drawn from the following publications:

BS: EN 12821:2009 Foodstuffs Determination of vitamin D by high performance liquid chromatography – Measurement of cholecalciferol (D₃) or ergocalciferol (D₂)

In reporting the results of a test or analysis made in accordance with this Tanzania Standard, if the final value, observed or calculated is to be rounded off, it shall be done in accordance with TZS 4.

1. Scope

This Tanzania Standard specifies a method for the determination of vitamin D₃ (cholecalciferol) or vitamin D₂ (ergocalciferol) in foodstuffs by high performance liquid chromatography (HPLC).

Vitamin D₃ is primary in foodstuffs of animal origin, while vitamin D₂ is primary in wild mushrooms. Both vitamin D₃ and vitamin D₂ can be present in fortified foodstuffs. This Tanzania Standard is not applicable for samples with a content of vitamin D₃ and vitamin D₂.

Apart from the vitamin D activity from the parent forms, vitamin D₃ and vitamin D₂, the corresponding metabolites 25-hydroxy vitamin D and 1,25-dihydroxy vitamin D also contribute to the vitamin D activity. This Tanzania Standard does only include measurement of vitamin D₃ or vitamin D₂.

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.

TZS 4, Rounding off numerical values

TZS 59, Water for analytical laboratory use - Specification and test methods

3. Principle

Vitamin D₃ and vitamin D₂ are saponified in the foodstuffs using alcoholic potassium hydroxide solution and extracted by an appropriate solvent. The determination of vitamin D₃ or vitamin D₂ in an appropriate sample extract solution is carried out by semi-preparative normal phase HPLC followed by reverse-phase analytical HPLC.

If vitamin D₃ is to be determined, then vitamin D₂ is used as an internal standard. If vitamin D₂ is to be determined, then vitamin D₃ 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.
4. Reagents

4.1 General

During the analysis, unless otherwise stated, use only reagents of analytical grade and water of at least grade 1 according to TZS 59.

4.2 Methanol

4.3 Ethanol (Absolute), volume fraction $\varphi(C_2H_5OH) = 99.9\%$.

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$_2$S) 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):

$$
\rho_{D2} = \frac{A_{265} \times M_{D2} \times 1000}{\epsilon \times b}
$$

(1)

Where:

$A_{265}$ is the absorption of the vitamin D$_2$ standard solution at 265nm;

$M_{D2}$ is the molar mass of vitamin D$_2$ ($M_{D2}$=396.7 g/mol);

$\epsilon$ is the molar absorption coefficient of vitamin D$_2$(here; $\epsilon$ =18 843 m$^2$/mol, calculated from the $E_{1cm}^{\%}$ 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 D₃ 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 D₃, ρ₃, in microgram per millilitre of the standard solution using Equation (2):

$$\rho_3 = \frac{A_{265} \times M_{D3} \times 1000}{\varepsilon \times b}$$  (2)

Where;

- $A_{265}$ is the absorption of the vitamin D₃ standard solution at 265 nm;
- $M_{D3}$ is the molar mass of vitamin D₃ ($M_{D3}$=384.6 g/mol);
- $\varepsilon$ is the molar absorption coefficient of vitamin D₃ (here; $\varepsilon$=18 461 m²/mol, calculated from the $E_{1%}^{1\text{cm}}$ value)
- $b$ is the optical path length of the quartz cell in centimetres.

4.13 Internal standard solutions

4.13.1 Vitamin D₂ internal standard solution

Pipette 10 ml of the vitamin D₂ 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 D₃ internal standard solution

Pipette 10 ml of the vitamin D₃ 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 D₃ is to be determined, then vitamin D₂ is used as an internal standard. If vitamin D₂ is to be determined, then vitamin D₃ is used as an internal standard.

4.14 Vitamin D₂ and vitamin D₃ semi-preparative standard solution

Pipette 5 ml of the vitamin D₂ standard solution (4.12.1) and 5 ml of the vitamin D₃ standard solution (4.12.2) into a rotary evaporator flask and carefully remove the solvent (at not more than 40 °C). Redissolve 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 D₂ and vitamin D₃ analytical standard solution

Pipette 5 ml of the vitamin D₂ standard solution (4.12.1) and 5 ml of the vitamin D₃ standard solution (4.12.2) into a rotary evaporator flask and carefully remove the solvent (at not more than 40 °C). Redissolve the residue in 50 ml of the analytical HPLC mobile phase (4.9.2).

5 Equipment and Apparatus

5.1 General

Usual laboratory equipment and apparatus 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-arnino, 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. C18 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 Tanzania 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 D₂ and vitamin D₃ 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 °C.

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 D₃ is to be determined, pipette an appropriate amount of vitamin D₂ internal standard solution (4.13.1) into the saponification flask. The amount of vitamin D₂ 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>
<thead>
<tr>
<th>Sample</th>
<th>Ethanol</th>
<th>Pyrogallop</th>
<th>Ascorbic acid / Na ascorbate</th>
<th>Potassium hydroxide</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 g to 30 g</td>
<td>100 ml</td>
<td>0.5 g to 1.9</td>
<td>1.0 g to 2.5 g</td>
<td>50 ml of a 50 g/100 ml solution</td>
</tr>
</tbody>
</table>

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 clean-up of the sample extract may be employed to remove potential interferences. If additional clean-up is employed, the procedure shall be fully validated for use.

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$_2$, $R_i$, by internal standard procedure using standards of known concentration (4.13) using Equation (3):

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

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
$7$ Calculation

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

$$w_{D_3} = \frac{A_{SD_3} \times I_s \times 100}{A_{SD_2} \times R_f \times m}$$

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)
$A_{SD_3}$ is the peak area or height for the vitamin $D_3$ in the sample solution;
$A_{SD_2}$ is the peak area or height for the vitamin $D_2$ in the sample solution;

$8$ Precision

$8.2$ 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.

$8.3$ 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.

$9$. Test report

The test report shall contain at least the following data:

- All information necessary for the identification of the sample;
- A reference to this Tanzanian standard, or to the method used;
- The results and the units in which the results have been expressed;
- The date and type of sampling procedure (if known);
- The date of receipt;
- The date of test;
- Any particular points observed in the course of the test;
- Any operations 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

<table>
<thead>
<tr>
<th>Column</th>
<th>Dimensions, mm</th>
<th>Mobile phase, (V + V)</th>
<th>Detector, A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polygosil® 60, 5 µm</td>
<td>250 x 8</td>
<td>iso-octane + iso-butanol (99 + 1)</td>
<td>265 nm</td>
</tr>
<tr>
<td>LiChrospher® Si 60, 5 µm</td>
<td>250 x 4</td>
<td>n-hexane + 2-propanol (99 + 1)</td>
<td>265 nm</td>
</tr>
<tr>
<td>LiChrospher® Si 100, 5 µm</td>
<td>250 x 8</td>
<td>n-hexane + 2-propanol (98 + 2)</td>
<td>265 nm</td>
</tr>
<tr>
<td>µ Porasil® silica</td>
<td>300 x 3,9</td>
<td>n-hexane + THF + 2-propanol (98 + 1 + 1)</td>
<td>265 nm</td>
</tr>
<tr>
<td>Partisil® PAC, 5 µm</td>
<td>250 x 4,6</td>
<td>n-hexane + isoamylalcohol (99 + 1)</td>
<td>265 nm</td>
</tr>
<tr>
<td>LiChrosorb® Si 60</td>
<td>250 x 4</td>
<td>n-hexane + 2-propanol + THF (98 + 1 + 1)</td>
<td>265 nm</td>
</tr>
<tr>
<td>LiChrosorb® Si 60</td>
<td>250 x 4</td>
<td>n-hexane + 2-propanol (95 + 5)</td>
<td>265 nm</td>
</tr>
<tr>
<td>LiChrosorb® Si 60</td>
<td>250 x 4</td>
<td>n-hexane + 2-propanol (97 + 3)</td>
<td>265 nm</td>
</tr>
</tbody>
</table>

*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.

<table>
<thead>
<tr>
<th>Column</th>
<th>Dimensions, mm</th>
<th>Mobile phase, (V + V)</th>
<th>Detector, A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypersil® ODS, 5 µm</td>
<td>250 x 4,6</td>
<td>methanol</td>
<td>265 nm</td>
</tr>
<tr>
<td>LiChrospher® 100 RP 18, 5 µm</td>
<td>250 x 4</td>
<td>methanol + water (95 + 5)</td>
<td>264 nm</td>
</tr>
<tr>
<td>Vydac® 201TP54</td>
<td>250 x 4,6</td>
<td>methanol + water (93 + 7)</td>
<td>265 nm</td>
</tr>
<tr>
<td>Vydac® 201TP54</td>
<td>250 x 4,6</td>
<td>acetonitrile + methanol (80 + 20)</td>
<td>265 nm</td>
</tr>
<tr>
<td>Spherisorb® ODS 2, 5 µm</td>
<td>250 x 4,6</td>
<td>acetonitrile + dichloromethane + methanol</td>
<td>diode array</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(93 + 4 + 3)</td>
<td></td>
</tr>
<tr>
<td>Nucleosil® C18, 5 µm</td>
<td>250 x 4</td>
<td>acetonitrile + methanol (70 + 30)</td>
<td>265 nm</td>
</tr>
<tr>
<td>Zorbax® ODS</td>
<td>250 x 4,6</td>
<td>acetonitrile + methanol (95 + 5)</td>
<td>265 nm</td>
</tr>
</tbody>
</table>

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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. 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

<table>
<thead>
<tr>
<th>Saponification</th>
<th>Extraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>16 g fat + 150 ml ethanol + 1 g pyrogallol + [75 ml water + 30 g KOH(^\text{[9]})] + nitrogen flushing; 70 °C for 30 min</td>
<td>PE(^\text{[1]}) + DEE(^\text{[1]}) (9 + 1) 2 x 100 ml; water wash 5 x 100 ml</td>
</tr>
<tr>
<td>8 g fat + 100 ml ethanol + 1 g sodium ascorbate + 0.84 g sodium sulfide + 12 g KOH + 50 ml water + nitrogen flushing; 80 °C for 30 min</td>
<td>n-hexane, 3 x 100 ml and 3 x 50 ml; water wash 4 x 100 ml</td>
</tr>
<tr>
<td>8 g fat + 35 ml ethanol + 2 g sodium ascorbate + 10 g KOH + 50 ml water; 100 °C for 45 min</td>
<td>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</td>
</tr>
<tr>
<td>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</td>
<td>DEE, 2 x 100 ml; water wash, 4 x 50 ml</td>
</tr>
<tr>
<td>6 g to 8 g fat + 100 ml ethanol + 1 g ascorbic acid + 50 ml 50 % KOH + nitrogen flushing; 20 °C for 20 h</td>
<td>PE + DEE (1 + 1), 2 x 200 ml; water wash, 6 x 50 ml</td>
</tr>
<tr>
<td>6 g fat + 1 g pyrogallol + 150 ml 28 % KOH in ethanol and water (9 + 1) + nitrogen flushing; 100 °C reflux for 30 min</td>
<td>DEE + PE (1 + 1), 2 x 500 ml; water wash, 5 x 150 ml</td>
</tr>
<tr>
<td>24 g fat + 90 ml ethanol + 0.5 g sodium ascorbate + 30 ml 60 % KOH + nitrogen flushing; 100 °C for 45 min, reflux</td>
<td>DEE, 1 x 150 ml, 3 x 75 ml; wash, 5 x 200 ml</td>
</tr>
</tbody>
</table>

a) KOH = potassium hydroxide
b) PE = light petroleum
c) DEE = diethyl ether
Examples of suitable semi-preparative and analytical HPLC chromatograms

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
y  signal (arbitrary units)
1  vitamin D in milk powder
2  vitamin D in standard
Figure C.2 - Typical chromatogram of extract milk powder (CRM 421) reverse phase HPLC of the vitamin 0 fraction between 11.5 min and 12.5 min in semi-preparative step (see Figure C.1)