INTERNATIONAL STANDARD

ISO 14892

IDF 177

First edition
2002-02-01

Dried skimmed milk — Determination of vitamin D content using high-performance liquid chromatography

Lait écrémé sec — Détermination de la teneur en vitamine D par chromatographie liquide à haute performance

Reference numbers
ISO 14892:2002(E)
IDF 177:2002(E)

ISO copyright office
Case postale 56 • CH-1211 Geneva 20
Tel. + 41 22 749 01 11
Fax + 41 22 749 09 47
E-mail copyright@iso.ch
Web www.iso.ch

International Dairy Federation
41 Square Vergote • B-1030 Brussels
Tel. + 32 2 733 98 88
Fax + 32 2 733 04 13
E-mail info@fil-idf.org
Web www.fil-org.com

Printed in Switzerland

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Foreword

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International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 3.

The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

Attention is drawn to the possibility that some of the elements of this part of ISO 14892 | IDF 177 may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

International Standard ISO 14892 | IDF 177 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products*, and the International Dairy Federation (IDF), in collaboration with AOAC International. It is being published jointly by ISO and IDF and separately by AOAC International.

Annex A forms a normative part of this International Standard. Annex B is for information only.

Foreword

IDF (the International Dairy Federation) is a worldwide federation of the dairy sector with a National Committee in every member country. Every National Committee has the right to be represented on the IDF Standing Committees carrying out the technical work. IDF collaborates with ISO and AOAC International in the development of standard methods of analysis and sampling for milk and milk products.

Draft International Standards adopted by the Action Teams and Standing Committees are circulated to the National Committees for voting. Publication as an International Standard requires approval by at least 50 % of National Committees casting a vote.

International Standard ISO 14892 | IDF 177 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products*, and the International Dairy Federation (IDF), in collaboration with AOAC International. It is being published jointly by ISO and IDF and separately by AOAC International.

All work was carried out by the Joint ISO/IDF/AOAC Action Team, *Selected food additives and vitamins*, under the aegis of its project leader, Mr E.J. de Vries (NL).

Introduction

The method described in this International Standard was selected after consideration and laboratory testing of a variety of alternative procedures in an interlaboratory study of methods for the determination of vitamin D in foodstuffs in the Measurements and Testing programme. The study was organized by the Community Bureau of Reference (BCR) of the Commission of the European Communities. The method is based upon the same principles as the method described in the European Pharmacopoeia in the monograph on cod-liver oil for the determination of vitamin D in cod-liver oil.

Although the International Standard for vitamin D was withdrawn in 1984, the International Unit for this substance has continued to be widely used and therefore its use has been maintained in this International Standard. Vitamin D is used here as a generic term for vitamin D_2 (ergocalciferol) or D_3 (cholecalciferol). The International Unit for vitamin D_3 is defined as the activity of 0,025 µg of pure cholecalciferol, and for vitamin D_2 defined as the activity of 0,025 µg of ergocalciferol.

Dried skimmed milk — Determination of vitamin D content using high-performance liquid chromatography

1 Scope

This International Standard specifies a method for the determination of vitamin D in a test sample containing at least 10 µg of vitamin D per 100 g [equal to 400 International Units (IU) of vitamin D per 100 g] by using high-performance liquid chromatography (HPLC).

The results of the determination are only reliable if, in the case of determination of vitamin D_3, the test sample contains only vitamin D_3, and no vitamin D_2 (which will be added as an internal standard) and, in the case of determination of vitamin D_2, the test sample contains only vitamin D_2 and no vitamin D_3 (which will be added as an internal standard). This is to be verified by the procedure carried out without the addition of the internal standard (vitamin D_2).

2 Normative reference

The following normative document contains provisions which, through reference in this text, constitute provisions of this International Standard. For dated references, subsequent amendments to, or revisions of, any of these publications do not apply. However, parties to agreements based on this International Standard are encouraged to investigate the possibility of applying the most recent edition of the normative document indicated below. For undated references, the latest edition of the normative document referred to applies. Members of ISO and IEC maintain registers of currently valid International Standards.

ISO 3696, *Water for analytical laboratory use — Specification and test methods*

3 Term and definition

For the purposes of this International Standard, the following term and definition apply.

3.1
vitamin D content of dried skimmed milk
mass fraction of substances determined by the procedure specified in this International Standard

NOTE It is expressed either in micrograms per gram or in International Units (IU) of vitamin D activity per gram.

4 Principle

The test sample is saponified and extracted. Vitamin D is separated from impurities by a semi-preparative clean-up using normal-phase HPLC. The vitamin D from the clean-up column is collected. The content is determined using reverse-phase HPLC with UV detection. Vitamin D_2 is used as an internal standard in the determination of vitamin D_3 and *vice versa*. The internal standard is added to each test portion prior to saponification.

5 Reagents and materials

Use only reagents of recognized analytical grade, unless otherwise specified.

5.1 Water, complying with at least grade 1 in accordance with ISO 3696.

5.2 Ethanol (C_2H_5OH), 95 % (by volume), free from aldehydes.

5.3 Sodium ascorbate solution, c($NaC_6H_5O_6 \cdot H_2O$) = 200 g/l.

If not available ready-made, prepare this solution by dissolving 3,5 g of ascorbic acid ($C_6H_8O_6$) in 20 ml of 1 mol/l sodium hydroxide (NaOH) solution. Prepare this solution fresh daily.

5.4 Potassium hydroxide aqueous solution, c(KOH) = 500 g/l.

Dissolve 50 g of potassium hydroxide (KOH) in 50 ml of water in a 100 ml volumetric flask (6.6). Mix and cool the obtained solution to ambient temperature. Dilute to the mark with water then mix again. Prepare this solution freshly before use.

5.5 Potassium hydroxide aqueous alcoholic solution, c(KOH) = 30 g/l.

Dissolve 3 g of potassium hydroxide (KOH) in water. Add 10 ml of ethanol (5.2) in a 100 ml one-mark volumetric flask (6.6). Dilute with water to the 100 ml mark and mix. Prepare this solution freshly before use.

5.6 Light petroleum, with a boiling range of between 40 °C and 60 °C, or of between 60 °C and 80 °C.

5.7 Methanol (CH_3OH), HPLC grade.

5.8 *n*-Hexane (C_6H_{14}), HPLC grade.

5.9 *n*-Pentanol ($C_5H_{11}OH$), HPLC grade.

5.10 Acetonitrile (CH_3CN), HPLC grade.

5.11 Propan-2-ol (C_3H_7OH), HPLC grade.

5.12 Nitrogen, chemically pure (oxygen free).

5.13 Butylated hydroxytoluene (BHT), chemically pure.

5.14 Antioxidant solution, 10 mg BHT (5.13) per milliltre of *n*-hexane (5.8).

5.15 Filter paper, of diameter 9 cm.

5.16 Vitamin D_3 standard solutions

5.16.1 Vitamin D_3 standard stock solution, e.g. Ph Eur or USP[1)] reference standard cholecalciferol.

Accurately weigh *ca.* 20 mg of cholecalciferol in a 100-ml one-mark volumetric flask (6.6). Dilute to the mark with ethanol (5.2) and mix.

This solution may be stored in a freezer at a temperature of (– 18 ±1) °C for 1 month.

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1) Ph Eur and USP are examples of suitable grades of product available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of these products.

5.16.2 Vitamin D_3 standard working solution I

Use a pipette (6.7) to add 10 ml of the vitamin D_3 standard stock solution (5.16.1) to a 100-ml one-mark volumetric flask (6.6). Dilute to the mark with ethanol (5.2) and mix.

5.16.3 Vitamin D_3 standard working solution II

Use a pipette (6.7) to add 1 ml of the vitamin D_3 standard working solution I (5.16.2) to a second 100-ml one-mark volumetric flask (6.6). Dilute to the mark with ethanol (5.2) and mix. This solution contains 0,2 µg/ml (= 8 IU/ml).

5.17 Vitamin D_2 standard solutions

5.17.1 Vitamin D_2 standard stock solution, e.g. Ph Eur or USP[1)] reference standard ergocalciferol.

Accurately, weigh *ca*. 20 mg of ergocalciferol in a 100-ml one-mark volumetric flask. Dilute to the mark with ethanol (5.2) and mix.

This solution may be stored in a freezer for 1 month.

5.17.2 Vitamin D_2 standard working solution I

Use a pipette (6.7) to add 10 ml of the vitamin D_2 standard stock solution (5.17.1) to a 100-ml one-mark volumetric flask (6.6). Dilute to the mark with ethanol (5.2) and mix.

15.17.3 Vitamin D_2 standard working solution II

Use another pipette (6.7) to add 1 ml of the vitamin D_2 standard working solution I (5.17.2) to a second 100-ml one-mark volumetric flask (6.6). Dilute to the mark with ethanol (5.2) and mix. This solution contains 0,2 µg/ml (8 IU/ml).

5.18 Reference solution

Prepare a reference solution by mixing 1,00 ml of the vitamin D_3 standard working solution I (5.16.2) and 1,00 ml of the vitamin D_2 standard working solution I (5.17.2) in a 100-ml volumetric flask and dilute to the mark with methanol (5.7). This reference solution contains 0,2 µg/ml of cholecalciferol and 0,2 µg/ml of ergocalciferol (8 IU/ml of vitamin D_3 and 8 IU/ml of vitamin D_2).

5.19 Mobile phase for clean-up column

Degas *n*-hexane (5.8) under reduced pressure. Mix 3 ml of *n*-pentanol (5.9) with 997 ml of the just-degassed *n*-hexane (see 6.3 and A.2.1).

5.20 Mobile phase for analytical column

Use a mixture of acetonitrile/propan-2-ol/water (ratio 90:7:3); for example, mix amounts of 900 ml acetonitrile (5.10), 70 ml propan-2-ol (5.11) and 30 ml water (5.1) (see 6.4 and A.3.1).

6 Apparatus

Usual laboratory equipment and, in particular, the following.

6.1 Analytical balance, capable of weighing to the nearest 0,1 mg, with readability to 0,01 mg.

6.2 Liquid chromatograph, fitted with an ultraviolet detector.

Typical operating conditions are the following:

UV detector that monitors absorption at 265 nm or at the nearest available wavelength for a fixed wavelength detector;

two columns, clean-up and analytical;

detector sensitivity, 0,128 AUFS (absorbance units full scale);

chart speed, 10 mm/min;

temperature, ambient.

6.3 Clean-up chromatographic column, made of stainless steel, of length 250 mm and internal diameter 4,6 mm, packed with 5 µm particle size packing of silica (normal-phase material), passing system suitability test (A.2.1); eluent flow rate 3 ml/min; injection volume 600 µl.

6.4 Analytical chromatographic column, made of stainless steel, of length 250 mm and internal diameter 4,6 mm, packed with 5 µm particle size packing of silica [C-18 or ODS (octadecyldimethylsilane) reverse-phase material], passing system suitability test (A.3.1); eluent flow rate 2 ml/min; injection volume 200 µl.

6.5 Beakers or **conical flasks**, of capacity 250 ml.

6.6 One-mark volumetric flasks, of capacity 100 ml and 250 ml.

6.7 One-mark pipettes, of capacity 1 ml, 4 ml, 5 ml, 10 ml, 20 ml and 50 ml.

6.8 Saponification flask, of capacity approximately 200 ml, fitted with a reflux condenser.

6.9 Steam bath, boiling water bath or **electric heating mantle**.

6.10 Separating funnel, of capacity 500 ml, fitted preferably with a polytetrafluoroethylene (PTFE) stopcock.

6.11 Tapered-bottom flasks, of capacity 20 ml and 250 ml.

6.12 Water baths, capable of operating at 40 °C ± 1 °C and at a temperature up to 39 °C.

6.13 Rotary evaporator, fitted with vacuum unit.

6.14 Stirrer, overhead.

7 Sampling

Sampling is not part of the method specified in this International Standard. A recommended sampling method is given in ISO 707.

It is important that the laboratory receive a sample which is truly representative and has not been damaged or changed during transport or storage.

8 Preparation of test sample

Thoroughly mix the test sample by repeatedly rotating and inverting the sample container. If necessary for this, transfer the complete test sample to an airtight container of sufficient capacity.

9 Procedure

9.1 General

For all operations, work in subdued light and use low-actinic glassware or protect ordinary glassware with aluminium foil.

All solvent evaporations shall be carried out under reduced pressure at a temperature of less than 40 °C. Do not evaporate completely to dryness under reduced pressure. Restore atmospheric pressure with nitrogen. Evaporate the last drops using a stream of nitrogen.

See annex A for additional details of the chromatographic procedure.

9.2 Test portion

Weigh, to the nearest 0,01 g, about 50 g (m_1) of the test sample into a beaker or conical flask (6.5). Dissolve the sample in 50 ml of hot water (60 °C to 80 °C). Break down any lumps with a spatula. Add another 50 ml portion of hot water (60 °C to 80 °C) and mix until homogeneous. Cool the obtained mixture to room temperature. Weigh to the nearest 0,01 g and calculate the mass (m_2) of the contents of the beaker (or conical flask). Homogenize and weigh, to the nearest 0,01 g, about 20 g (m_3) of the thus-prepared test portion into a saponification flask (6.8).

9.3 Saponification and extraction

9.3.1 Add to the test portion in the saponification flask (9.2), 20 ml of potassium hydroxide solution (5.4), 10 ml of sodium ascorbate solution (5.3), 50 ml of ethanol (5.2) and, as internal standard, 4,00 ml of vitamin D_2 standard working solution II (5.16.3). Mix the solution.

9.3.2 Reflux the obtained solution (9.3.1) for 30 min on a steam bath (6.9) while swirling from time to time. Cool immediately under running water.

9.3.3 Transfer the cooled solution (9.3.2) to a separating funnel (6.10) using two 30 ml portions of water, two 10 ml portions of ethanol (5.2) and two 40 ml portions of light petroleum (5.6). Shake the solution vigorously for 30 s and allow it to stand until the two layers are clear. Transfer the aqueous (lower) phase to a second separating funnel (6.10) and shake with a mixture of 10 ml of ethanol (5.2) and 40 ml of light petroleum (5.6). Leave it to separate.

9.3.4 Transfer the obtained aqueous phase to a third separating funnel and the obtained light petroleum phase to the first-used separating funnel (9.3.3). Wash the second-used separating funnel with two 10 ml portions of light petroleum (5.6). Add the washings to the first separating funnel.

9.3.5 Shake the aqueous phase with 40 ml of light petroleum (5.6) and 10 ml of ethanol (5.2). Add the light petroleum phase to the first separating funnel. Wash the combined light petroleum extracts with three 40 ml portions of freshly prepared potassium hydroxide alcoholic solution (5.5), shaking vigorously. Then wash with 40 ml portions of water until the last washing is neutral to phenolphthalein. Drain the last few drops of water, add two sheets of filter paper (5.15), cut into strips, to the separating funnel and shake.

9.3.6 Transfer the light petroleum extract, dried as described above, to a 250 ml tapered bottom flask (6.11). Rinse the separating funnel and paper with light petroleum. Add the rinsings to the flask. Add to its contents 10 mg to 20 mg of BHT (5.13).

9.3.7 Evaporate the contents of the tapered bottom flask (9.3.6) to almost dryness under vacuum by swirling in a water bath (6.14) at a maximum temperature of 39 °C. Cool under running water and restore the atmospheric pressure with nitrogen (5.12). Evaporate the last drops of light petroleum by a stream of nitrogen. Dissolve the residue immediately in 2,00 ml of *n*-hexane (5.8).

9.4 Purification

Inject 600 µl of the redissolved test solution (9.3.7) through the sampling valve into a clean-up column. Adjust the operating conditions of the detector to give the largest possible on-scale peaks of vitamin D_2 and D_3. Collect the fraction between 3 min before and 3 min after the vitamin D peak in a 20 ml tapered bottom flask (6.11). Add 1 ml of antioxidant solution (5.14) and evaporate to dryness under a stream of nitrogen. Dissolve the thus-purified test solution immediately in 2,0 ml of methanol (5.7). Use this solution for injection into the analytical column.

9.5 Determination

Inject 200 µl of purified test solution (9.4) through the sampling valve into the analytical column. Adjust the operating conditions of the detector to give the largest possible on-scale peaks of vitamin D_2 and D_3. Measure the peak responses of vitamin D_2 and D_3 in the purified test solution.

Inject 200 µl of the reference solution (5.18). Use the same operating conditions as above for the test solution. Measure the peak responses of vitamin D_2 and of vitamin D_3 in the reference solution.

10 Calculation and expression of results

10.1 Calculation

10.1.1 Relative response factor

Calculate the relative response factor, R_f, using the following equation:

$$R_f = \frac{A_e \cdot m_c}{A_c \cdot m_e}$$

where

A_e is the numerical value representing the peak area (or height) for ergocalciferol in the chromatogram obtained with the reference solution (9.5);

m_e is the mass, in micrograms, of ergocalciferol in 1 ml of reference solution;

A_c is the numerical value representing the peak area (or height) for cholecalciferol in the chromatogram obtained with reference solution (9.5);

m_c is the mass, in micrograms, of cholecalciferol in 1 ml of reference solution.

10.1.2 Vitamin D content

Calculate the vitamin D content, w, expressed in micrograms of cholecalciferol per gram or in International Units (IU) of vitamin D activity per gram, using the following equation:

$$w = \frac{A_{cs} \cdot m_{es} \cdot R_f}{A_{es} \cdot m_s}$$

where

A_{cs} is the numerical value of the peak area (or height) for cholecalciferol in the chromatogram obtained with the test solution (9.5);

m_{es} is the mass, in micrograms, of ergocalciferol (internal standard) added to the test solution (9.3.1);

A_{es} is the numerical value of the peak area (or height) for ergocalciferol in the chromatogram obtained with the test solution (9.5);

m_s is the mass, in grams, of the test portion obtained using the following equation (see 9.1):

$$m_s = \frac{m_1 \cdot m_3}{m_2}$$

10.2 Expression of results

Express the test results to two decimal places.

11 Precision

11.1 Interlaboratory test

Details of an interlaboratory test carried out in accordance with ISO 5725-1 and ISO 5725-2 on the precision of the method have been published (see reference [4]). The values derived from this interlaboratory test may not be applicable to concentration ranges and matrices other than those given.

Note that when vitamins are dry-mixed, the precision is strongly influenced by the homogeneity of the product.

11.2 Repeatability

The absolute difference between two independent single test results, obtained using the same method on identical test material in the same laboratory by the same operator using the same equipment within a short interval of time, will in not more than 5 % of cases be greater than 14 % (relative) of the arithmetic mean of the two results.

11.3 Reproducibility

The absolute difference between two single test results, obtained using the same method on identical test material in different laboratories with different operators using different equipment, will in not more than 5 % of cases be greater than 17 % of the arithmetic mean of the two results.

12 Test report

The test report shall specify:

— all information required for the complete identification of the sample;

— the sampling method used, if known,

— the test method used, with reference to this International Standard;

— all operating details not specified in this International Standard, or regarded as optional, together with details of any incidents that may have influenced the test result(s);

— the test result(s) obtained;

— if the repeatability has been checked, the final quoted result obtained.

Annex A
(normative)

Notes on procedure

A.1 General

The details of the chromatographic procedure depend, among other things, on the equipment, the type, age and supplier of the column, the means of introduction of the test and reference solutions, the sample size and the detector. Different column lengths and brands of packing may be used, and injection volumes may be varied, if the system suitability tests (A.2.1 and A.3.1) are met.

A.2 Normal-phase chromatography

A.2.1 Test of the suitability of the clean-up column

Inject 600 µl of a mixture of vitamin D_2 and D_3. Prepare this mixture by mixing in a 100 ml one-mark volumetric flask (6.6), 1 ml of the vitamin D_3 standard stock solution (5.16.1) and 1 ml of the vitamin D_2 standard stock solution (5.17.1). Dilute to the 100 ml mark with *n*-hexane (5.8). No separation of vitamin D_2 and vitamin D_3 should be shown in the chromatogram.

The ratio of *n*-pentanol to *n*-hexane influences the chromatographic behavior: an increase of the alcohol content of the mobile phase causes a decrease in retention time.

A.2.2 Retention times

The approximate retention time of vitamin D_2 and D_3 in the normal-phase chromatography with *n*-pentan-2-ol/*n*-hexane (3 ml + 997 ml) as mobile phase and Partisil PAC 5 µm column is 8 min.

A.3 Reverse-phase chromatography

A.3.1 Test of the suitability of the analytical column

Inject 200 µl of the reference solution (5.18). The resolution between the vitamin D_2 and D_3 peaks should be at least 1,5.

Calculate the separation or resolution factor, R, by the following equation:

$$R = \frac{2D}{B + C}$$

where

D is the distance between the peak maxima of vitamin D_2 and vitamin D_3, in centimetres;

B is the peak width of vitamin D_2, in centimetres;

C is the peak width of vitamin D_3, in centimetres.

NOTE The water content in the mobile phase influences the chromatographic behaviour: an increase of the water content causes an increase in retention time.

A.3.2 Retention times

The approximate retention times of vitamin D_2 and D_3 in the reverse-phase chromatography with acetonitrile/propanol-2-ol/water with ratio 90:7:3 as mobile phase and a column with Zorbax ODS 10 µm packing are 13,5 min and 15 min respectively.

To ensure the peak purity of the analyte and the internal standard, use a dual wavelength monitoring at 265 nm and 280 nm respectively.

A.4 Limit of detection

The limit of detection is 2,5 µg per 100 g which is equal to 100 IU per 100 g.

Annex B
(informative)

Activity expressed in International Units (IU)

B.1 Activity of vitamin D

The activity of vitamin D is expressed in International Units (IU). It has been defined (see reference [5]) that one IU of vitamin D corresponds to the antirachitic activity of 0,025 µg of cholecalciferol.

That means that the antirachitic activity of 1 g of pure cholecalciferol, expressed in International Units, is equal to 4×10^7 IU.

B.2 Assay of vitamin D standard (cholecalciferol, pure)

See reference [5] for details.

Weigh, to the nearest 0,1 mg, 50 mg of cholecalciferol in a 100 ml one-mark volumetric flask (6.6). Dissolve the cholecalciferol rapidly and without heating in aldehyde-free ethanol. Dilute to the 100 ml mark with the aldehyde-free ethanol and mix the obtained solution (solution A). Pipette 5,0 ml of solution (A) into a 250 ml one-mark volumetric flask (6.6). Dilute to the 250 ml mark with the aldehyde-free ethanol and mix solution (solution B). The presumed concentration of the vitamin D will be about 400 IU/ml.

Measure the absorbance of solution B within 30 min of the start of preparing solution A.

Verify that the absorption maximum of solution B, A_m, lies at (265 ± 1) nm using ethanol as compensation liquid. Measure the absorbance, A_n, at 265 nm. The numerical value of the obtained absorbance should be between 0,46 and 0,50.

NOTE The numerical value of the specific absorbance of cholecalciferol in ethanol is equal to 480 at 265 nm.

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ICS 67.100.10

Price based on 11 pages