INTERNATIONAL STANDARD

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Dried skimmed milk — Determination of vitamin A content —

Part 2: Method using high-performance liquid chromatography

Lait écrémé en poudre — Détermination de la teneur en vitamine A —

Partie 2: Méthode par chromatographie en phase liquide à haute performance

Reference numbers
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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

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 document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

ISO 12080-2 | IDF 142-2 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products*, and the International Dairy Federation (IDF). It is being published jointly by ISO and IDF.

ISO 12080 | IDF 142 consists of the following parts, under the general title *Dried skimmed milk — Determination of vitamin A content*:

— *Part 1: Colorimetric method*

— *Part 2: Method using high-performance liquid chromatography*

This second edition of ISO 12080-2 | IDF 142-2 cancels and replaces the first edition (ISO 12080-2:2000), of which it constitutes a minor revision.

Foreword

IDF (the International Dairy Federation) is a non-profit organization representing the dairy sector worldwide. IDF membership comprises National Committees in every member country as well as regional dairy associations having signed a formal agreement on cooperation with IDF. All members of IDF have the right to be represented on the IDF Standing Committees carrying out the technical work. IDF collaborates with ISO in the development of standard methods of analysis and sampling for milk and milk products.

The main task of Standing Committees is to prepare International Standards. 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 the IDF National Committees casting a vote.

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All work was carried out by the former Joint ISO-IDF Group of Experts (E46 — *Vitamins A and D in dried milk*) which is now part of the Joint ISO-IDF Action Team on *Organic contaminants and veterinary residues* of the Standing Committee on *Analytical methods for additives and contaminants*.

ISO 12080|IDF 142 consists of the following parts, under the general title *Dried skimmed milk — Determination of vitamin A content*:

— *Part 1: Colorimetric method*

— *Part 2: Method using high-performance liquid chromatography*

This edition of ISO 12080-2|IDF 142-2, together with ISO 12080-1|IDF 142-1, cancels and replaces IDF 142:1990, of which it constitutes a minor revision.

Introduction

The methods specified in ISO 12080 | IDF 142 (all parts) have been selected after consideration and laboratory testing of a variety of alternative procedures. Their advantages include the absence of highly dangerous reagents as in, for example, the Carr-Price method, and the avoidance of reagents that are not universally available.

The decision to provide two separate methods was taken to meet the needs both of laboratories with sophisticated equipment (HPLC) and those without such apparatus.

Although the International Standard for vitamin A was discontinued in 1954, the International Unit for this substance has continued to be widely used and its use has been maintained in this International Standard. The International Unit for vitamin A was redefined in 1960 as the activity of 0,344 µg of pure all-*trans*-vitamin A acetate (see Annex A).

Dried skimmed milk — Determination of vitamin A content —

Part 2:
Method using high-performance liquid chromatography

WARNING — The use of this International Standard may involve hazardous materials, operations and equipment. Persons using this International Standard should be familiar with normal laboratory practice. This standard does not purport to address all of the safety problems, if any, associated with its use. It is the responsibility of the user to establish health and safety practices and determine the applicability of regulatory limitations prior to use.

1 Scope

This part of ISO 12080 | IDF 142 specifies a method using high-performance liquid chromatography (HPLC) for the determination of vitamin A in dried skimmed milk containing at least 10 IU (International Units) of vitamin A per gram.

2 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

2.1
vitamin A content of dried skimmed milk
mass fraction of substances determined by the procedure specified in this part of ISO 12080 | IDF 142

NOTE Vitamin A content is expressed either in micrograms of retinol per gram or in International Units of vitamin A activity per gram.

3 Principle

The test sample is saponified and extracted. Vitamin A is separated from impurities by HPLC. The content is determined using an ultraviolet detector or a fluorescence detector.

4 Reagents

Use only reagents of recognized analytical grade, unless otherwise specified, and distilled or demineralized water or water of equivalent purity.

4.1 Ethanol (CH_3CH_2OH), 95 % volume fraction, free from aldehyde.

4.2 Sodium ascorbate solution, 200 g/l. If not available ready-made, prepare by dissolving 3,5 g of ascorbic acid ($C_6H_8O_6$) in 20 ml of 1 mol/l sodium hydroxide (NaOH) solution and mix. Prepare this solution fresh daily.

4.3 Potassium hydroxide aqueous solution (KOH), 50 % mass fraction. Dissolve 50 g of potassium hydroxide in 50 ml of water. Mix and cool the solution. Prepare the solution just before use.

4.4 Potassium hydroxide aqueous alcoholic solution, 30 g/l. Dissolve 3 g of potassium hydroxide (KOH) in water and add 10 ml of ethanol (4.1) in a 100 ml one-mark volumetric flask. Make up to the mark with water and mix. Prepare the solution just before use.

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

4.6 Methanol (CH_3OH), HPLC grade.

4.7 Mobile phase: mixture of methanol (4.6) and water, 90 + 10 (parts by volume), for example (see Caution in 8.5).

4.8 Vitamin A standard solution. Use US Pharmacopeia[1)] standard reference solution of vitamin A made from crystalline all-*trans*-retinyl acetate in cottonseed oil, equivalent to 30 mg of retinol (vitamin A alcohol, $C_{20}H_{30}O$) per gram of oil, or as stated when purchased.

Cut the tip from the capsule containing the vitamin A standard solution and express the oil into a saponification flask. Weigh, to the nearest 0,1 mg, approximately 20 mg of the standard solution. Add 40 ml of ethanol (4.1), 10 ml of sodium ascorbate solution (4.2), and 10 ml of potassium hydroxide aqueous solution (4.3).

Saponify and extract as specified in 8.3.2 to 8.3.6. Prepare a standard reference solution by proceeding as in 8.4.

4.9 Butylated hydroxytoluene (BHT).

5 Apparatus

Usual laboratory apparatus and, in particular, the following.

5.1 Liquid chromatograph, fitted with an ultraviolet detector.

Typical operating conditions are:

— variable UV detector that monitors absorption at 325 nm, or a fixed wavelength detector that monitors at a wavelength of between 300 nm and 360 nm with a detector sensivity of 0,128 AUFS (absorption units, full scale);

— eluent flow rate of 2 ml/min (at approximately 10 MPa);

— ambient temperature;

— injection volume of 20 µl;

— chart speed of 10 mm/min.

When a fluorescence detector is used, set it at 325 nm for excitation and at 450 nm for emission.

1) Example of a suitable product available commercially. This information is given for the convenience of users of this part of ISO 12080 | IDF 142 and does not constitute an endorsement by ISO or IDF of this product.

5.2 Chromatogaphic column, of stainless steel, 250 mm × 4,6 mm, with 10 µm particle size packing of C8 or C18, chemically bonded to totally porous microsilica particles or a column of equivalent performance.

5.3 Beaker or **conical flask**, of capacity 250 ml.

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

5.5 One-mark volumetric flasks, of capacities 100 ml and 200 ml, ISO 1042[3] class A.

5.6 One-mark pipettes, of capacities 10 ml, 25 ml and 50 ml, ISO 648[1] class A.

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

5.8 Water bath, capable of operating at a temperature of up to 40 °C.

5.9 Separating funnel, of capacity 500 ml, preferably with a polytetrafluoroethylene (PTFE) stopper.

5.10 Ultrasonic bath.

5.11 Filter paper, of diameter 90 mm.

6 Sampling

Sampling is not part of the method specified in this part of ISO 12080 | IDF 142. A recommended sampling method is given in ISO 707 | IDF 50[2].

A representative sample should have been sent to the laboratory. It should not have been damaged or changed during transport or storage.

7 Preparation of test sample

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

8 Procedure

8.1 General

If a check is required of whether the repeatability limit (10.2) is met, carry out two single determinations in accordance with 8.2 to 8.5.

For all operations, work in subdued light or use low-actinic glassware.

8.2 Test solution

Weigh, to the nearest 0,001 g, about 20 g of dried milk into a beaker or conical flask (5.3) and dissolve in 50 ml of hot water at a temperature of at least 80 °C. Break up any lumps with a spatula or by using an ultrasonic bath (5.10). Cool to room temperature. Transfer quantitatively to a 100 ml one-mark volumetric flask (5.5). Make up to the mark with water.

8.3 Saponification and extraction

8.3.1 Transfer, by means of a pipette (5.6), 25 ml of the prepared test portion (8.2) to a saponification flask (5.4). Add 20 ml of potassium hydroxide (4.3) and 10 ml of sodium ascorbate solution (4.2). Add 50 ml of ethanol (4.1) and mix well.

8.3.2 Reflux for 30 min on a steam bath (5.7) and swirl from time to time. Cool immediately under running water.

8.3.3 Transfer the liquid to a separating funnel (5.9) and wash twice, each time using 30 ml of water, 10 ml of ethanol (4.1), and 40 ml of light petroleum (4.5). Shake vigorously for 30 s and allow to stand until the two layers are clear.

Transfer the aqueous (lower) phase to a second separating funnel and shake with a mixture of 10 ml of ethanol (4.1) and 40 ml of light petroleum (4.5). Leave to separate.

8.3.4 Transfer the aqueous phase to a third separating funnel and the light petroleum phase to the first separating funnel. Wash the second separating funnel twice with 10 ml of light petroleum (4.5). Add the washings to the first separating funnel.

8.3.5 Shake the aqueous phase with 40 ml of light petroleum (4.5) and 10 ml of ethanol (4.1). Add the light petroleum phase to the first separating funnel. Wash the combined light petroleum extracts three times with 40 ml freshly prepared potassium hydroxide aqueous alcoholic solution (4.4), shaking vigorously. Then wash with 40 ml volumes of water until the last washing is neutral to phenolphthalein. Drain the last few drops of water, add two sheets of filter paper (5.11), cut into strips, to the separating funnel and shake.

8.3.6 Transfer the light petroleum extract from which water has been removed (8.3.5) to a 200 ml one-mark volumetric flask (5.5). Rinse the separating funnel and paper with light petroleum (4.5), add the rinsings to the volumetric flask, then add 10 mg to 20 mg of BHT (4.9). Make up to the mark with light petroleum.

8.4 Preparation of test and reference solutions

Pipette aliquots of the diluted extracts (8.3.6) obtained from both the test solution (8.2) and the vitamin A standard solution (4.8) into separate round-bottom flasks. Evaporate to dryness under vacuum by swirling in a water bath (5.8) at a temperature not exceeding 40 °C. Cool under running water and restore atmospheric pressure, preferably with nitrogen. Dissolve the residue immediately in 10,0 ml of methanol (4.6).

8.5 Determination

Inject 20 µl of the test solution and the reference solution (8.4) on to the column and adjust the operation conditions of the detector to give the largest possible on-scale peaks of vitamin A. Measure the peak areas of vitamin A.

CAUTION — The details of the chromatographic procedure depend, among others, on the equipment, the type, age, and supplier of the column, the means of introduction of the test and reference solution, the sample size and the detector. The ratio of methanol to water will vary according to these factors; increasing the water content of the mobile phase causes an increase in retention time.

9 Calculation and expression of results

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

$$w = \frac{\rho\, A_s\, V_1\, V_3\, V_4}{A_r\, V_2\, V_5\, m}$$

where

ρ is the concentration, in micrograms of retinol per millilitre (or vitamin A activity in IU per millilitre), in the reference solution (8.4);

A_s is the numerical value of the peak area of vitamin A in the test solution (8.5);

A_r is the numerical value of the peak area of vitamin A in the reference solution (8.5);

V_1 is the total volume, in millilitres, of light petroleum extract (V_1 = 200 ml);

V_2 is the volume, in millilitres, of the aliquot taken from V_1 (8.4);

V_3 is the volume, in millilitres, of methanol in which the residue is dissolved (V_3 = 10 ml);

V_4 is the total volume, in millilitres, of the test solution (8.2) (V_4 = 100 ml);

V_5 is the volume, in millilitres, of the aliquot part of the test solution (8.3.1) (V_5 = 25 ml);

m is the mass, in grams, of the test portion (8.2).

10 Precision

10.1 Interlaboratory test

Details of an interlaboratory test carried out in accordance with ISO 5725-1[4] and ISO 5725-2[5] on the precision of the method have been published (Reference [6]). The values derived from this interlaboratory test may not be applicable to concentration ranges and matrices other than those given.

10.2 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, shall in not more than 5 % of cases be greater than 14 % of the arithmetic mean of the two results.

10.3 Reproducibility

The absolute difference between two independent single test results, obtained using the same method on identical test material in different laboratories with different operators using different equipment, shall in not more than 5 % of cases be greater than 42 % of the arithmetic mean of the results.

11 Test report

The test report shall contain at least the following information:

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

b) the sampling method used, if known;

c) the test method used, together with reference to this part of ISO 12080|IDF 142;

d) all operating details not specified in this part of ISO 12080|IDF 142, or regarded as optional, together with details of any incidents which may have influenced the test result(s);

e) the test result(s) obtained, and, if the repeatability has been checked, the final result obtained.

Annex A
(informative)

Activity expressed in International Units (IU)

A.1 Activity of vitamin A

The activity of vitamin A is expressed in International Units. It has been defined (see Reference [7]) that 1 IU of vitamin A corresponds to the activity of 0,344 µg of all-*trans*-retinyl acetate.

The activity of the other vitamin A compounds is calculated stoichiometrically so that 1 IU corresponds to the activity of 0,300 µg of all-*trans*-retinol, 0,359 µg of all-*trans*-retinyl propionate or 0,500 µg of all-*trans*-retinyl palmitate, respectively.

That means that the activity of 1 g of pure all-*trans*-vitamin A alcohol and ester, expressed in International Units, is equal to:

— vitamin A alcohol (retinol) 3 333 000 IU;

— vitamin A acetate 2 907 000 IU;

— vitamin A propionate 2 785 000 IU;

— vitamin A palmitate 1 818 000 IU.

A.2 Assay of vitamin A standard (vitamin A ester, pure or dissolved in oil)

See Reference [7].

Weigh, to an accuracy of 0,1 %, 25 mg to 100 mg of vitamin A ester in a flask. Dissolve this in 5 ml of pentane and dilute, depending on the weighed amount, with 2-propanol to a presumed concentration of 10 IU/ml to 15 IU/ml.

Verify that the absorption maximum, A_m, of the solution lies between 325 nm and 327 nm using 2-propanol as compensation liquid (blank). Measure the absorbance, A_n, at 300 nm, 326 nm and 370 nm.

Calculate the ratio A_n/A_m for each of the wavelengths mentioned. If the ratios do not exceed 0,593 at 300 nm, 0,537 at 350 nm or 0,142 at 370 nm, respectively, then calculate the vitamin A content, w, in International Units per gram, using the equation:

$$w = \frac{A_m V f}{100 \times m}$$

where

A_m is the numerical value of the maximum absorbance obtained at 326 nm;

V is the total volume, in millilitres, to which the vitamin A ester has been diluted to give 10 IU/ml to 15 IU/ml;