INTERNATIONAL STANDARD

ISO 12080-2

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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 number
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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 3.

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 12080 may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

International Standard ISO 12080-2 was prepared by Technical Committee ISO/TC 34, *Agricultural food products*, Subcommittee SC 5, *Milk and milk products,* in collaboration with the International Dairy Federation (IDF) and AOAC International, and will also be published by these organizations.

ISO 12080 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*

Annex A of this part of ISO 12080 is for information only.

Introduction

The methods specified in ISO 12080 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 0,000 344 mg 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. This standard does not purport to address all the safety problems associated with its use. It is the responsibility of the user of this standard to establish safety and health practices and determine the applicability of regulatory limitations prior to use.

1 Scope

This part of ISO 12080 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 Term and definition

For the purposes of this part of ISO 12080, the following term and definition 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

NOTE It 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 % (by volume), free from aldehyde.

4.2 Sodium ascorbate solution, 200 g/l.

If not available ready-made, prepare this 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, 50 % (by mass).

Dissolve 50 g of potassium hydroxide (KOH) in 50 ml of water. Mix and cool the obtained solution. Prepare the solution freshly 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. Dilute with water to the 100 ml mark and mix. Prepare the solution freshly before use.

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

4.6 Methanol (CH_3OH), HPLC grade.

4.7 Mobile phase: mixture of methanol and water, ratio 90:10 (by volume), for example (see note in 8.5).

4.8 Vitamin A standard solution

Use US Pharmacopeia standard[1)] 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 solution (4.3).

Saponify and extract as described 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 100 atm);

— 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) The reference vitamin A solution from United States Pharmacopeia Convention, Inc., 12601 Twinbrook Parkway, Rockville, Maryland 20852, USA, is an example of a suitable product available commercially. This information is given for the convenience of users of this part of ISO 12080 and does not constitute an endorsement by ISO of this product.

5.2 Chromatogaphic column, made of stainless steel, 250 mm × 4,6 mm, packed 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.

5.6 One-mark pipettes, of capacities 10 ml, 25 ml and 50 ml.

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 polytetrafluorethylene (PTFE) stopper.

5.10 Ultrasonic bath

5.11 Filter paper, of diameter 9 cm.

6 Sampling

Sampling is not part of the method specified in this International Standard. A recommended sampling method is given in ISO 707 [1].

It is important that the laboratory receive a sample which is truly representative and has not 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 for this, transfer the complete test sample to an airtight container of sufficient capacity.

8 Procedure

8.1 General

NOTE If it is required to check 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 down 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). Dilute with water to the 100 ml mark.

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) using two 30 ml portions of water, two 10 ml portions of ethanol (4.1) and two 40 ml portions 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 with two 10 ml portions 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 with three 40 ml portions of freshly prepared potassium hydroxide alcoholic solution (4.4), 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 (5.11), cut into strips, to the separating funnel and shake.

8.3.6 Transfer the light petroleum extract, dried as described above, 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 and add 10 mg to 20 mg of BHT (4.9). Dilute with light petroleum to the 200 ml mark.

8.4 Preparation of test and reference solutions

Pipette aliquot parts 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) onto 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:

$$w = \frac{c \cdot A_S \cdot V_1 \cdot V_3 \cdot V_4}{A_r \cdot V_2 \cdot V_5 \cdot m}$$

where

c 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 of the test portion, in grams.

10 Precision

10.1 Interlaboratory test

Details of an interlaboratory test carried out in accordance with ISO 5725 on the precision of the method have been published [4]. 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, will 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, will 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 specify:

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

— the sampling method used, if known;

— the test method used, together with reference to this part of ISO 12080;

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

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