INTERNATIONAL STANDARD

ISO 12080-1

First edition
2000-06-01

Dried skimmed milk — Determination of vitamin A content —

Part 1: Colorimetric method

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

Partie 1: Méthode colorimétrique

Reference number
ISO 12080-1:2000(E)

ISO copyright office
Case postale 56 • CH-1211 Geneva 20
Tel. + 41 22 749 01 11
Fax + 41 22 734 10 79
E-mail copyright@iso.ch
Web www.iso.ch

Printed in Switzerland

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 12080-1 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 1:
Colorimetric method

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 colorimetric method 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

This part of ISO 12080 specifies a colorimetric method for the determination of vitamin A in dried skimmed milk containing at least 10 IU (International Units) of vitamin A per gram.

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. The unsaponifiable matter is reacted with trifluoroacetic acid. The absorbance at 620 nm is measured.

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 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 % (by mass)

Dissolve 50 g of potassium hydroxide in 50 ml of water. Mix and cool the solution. Prepare this 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 this 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 Chloroform ($CHCl_3$)

4.7 Trifluoroacetic acid (CF_3COOH)

WARNING — Chloroform and trifluoroacetic acid are carcinogenic. Take all necessary precautions.

4.8 Colour reagent

Mix by volume 1 part of pure trifluoroacetic acid (4.7) and 2 parts of chloroform (4.6).

4.9 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.

A secondary standard solution may be used if standardized against this primary standard reference solution or by UV measurement.

Cut the tip from the capsule containing the vitamin A standard reference solution and express the oil into a (tared) 100 ml amber-coloured one-mark volumetric flask. Weigh the contents to the nearest 0,1 mg. Dilute with chloroform (4.6) to the 100 ml mark. Use the vitamin A standard solution as soon as possible. Discard the solution after 8 h.

4.10 Butylated hydroxytoluene (BHT)

5 Apparatus

Usual laboratory apparatus and, in particular, the following.

5.1 Photoelectric colorimeter or spectrometer, with an optical mechanism or filter for 620 nm wavelength (vitamin A derivative).

Use matched absorption cells. An instrument providing linearity between absorbance and concentration is to be preferred.

5.2 Beaker or conical flask, of capacity 250 ml.

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

5.4 One-mark volumetric flasks, of capacities 100 ml and 200 ml.

5.5 One-mark pipettes, of capacities 2 ml, 10 ml, 25 ml and 50 ml.

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.

5.6 Automatic pipettes, suitable for organic solvents, or a pipette, to deliver 10 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 polytetrafluoroethylene (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 part of ISO 12080. 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 the test sample into a beaker or conical flask (5.2) and dissolve in 50 ml of water at a temperature of least 80 °C. Break down any lumps with a spatula or by using a ultrasonic bath (5.10). Cool to room temperature. Transfer quantitatively to a 100 ml one-mark volumetric flask (5.4). Dilute with water to the 100 ml mark.

8.3 Saponification and extraction

8.3.1 Transfer, by means of a pipette (5.5), 25 ml of the prepared test solution (8.2) to a saponification flask (5.3). Add 20 ml of potassium hydroxide solution (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), swirling 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 (5.4). 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 (4.10). Dilute with light petroleum to the 200 ml mark and mix well.

8.4 Preparation of test colorimetric solution

Pipette an aliquot part of the diluted extract (8.3.6) into a round-bottom flask. 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 chloroform (4.6).

NOTE A typical concentration in the test solution is 10 IU of vitamin A per millilitre of chloroform. The amount of the aliquot may be adjusted depending on the size and sensitivity of the colorimetric cell.

8.5 Determination

Designate two suitable matched colorimetric cells as 1 and 2. Pipette 2 ml of the test colorimetric solution (8.4) into cell 1. Pipette 2 ml of the diluted vitamin A standard solution (8.6) into cell 2. Rapidly add to each cell, preferably using an automatic pipette (5.6), 10,0 ml of the colour reagent (4.8) and mix. Monitor the absorbance of the solutions at 620 nm with the spectrometer or photoelectric colorimeter (5.1) measured against a blank of 2 ml of chloroform (4.6) and 10 ml of colour reagent (4.8) until the absorbance reaches its maximum. Plot a graph of the obtained absorbance against the vitamin A content (see 8.6).

The volumes and proportions used may be adjusted proportionally according to the capacity of the colorimeter cells.

8.6 Preparation of calibration graph

Make five-fold dilutions (or greater) of the vitamin A standard solution (4.9) with chloroform (4.6) so that 2 ml aliquot portions treated in the colorimetric determination give absorbances in the range of 0,07 to 0,7 at 620 nm. Plot absorbance against vitamin A content in micrograms. If the graph is a straight line, a factor for the content of vitamin A in the samples may be calculated.

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:

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

where

c is the concentration, in micrograms of retinol per millilitre (or vitamin A activity in IU per millilitre) in the test colorimetric solution (8.4), calculated from the calibration graph (8.6);

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 chloroform 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 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 International Standard;

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

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