International Standard

ISO 5738

Milk and milk products — Determination of copper content — Photometric reference method

Lait et produits laitiers — Détermination de la teneur en cuivre — Méthode photométrique de référence

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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards institutes (ISO member bodies). The work of developing International Standards is carried out through ISO technical committees. Every member body interested in a subject for which a technical committee has been set up 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.

Draft International Standards adopted by the technical committees are circulated to the member bodies for approval before their acceptance as International Standards by the ISO Council.

International Standard ISO 5738 was developed by Technical Committee ISO/TC 34, *Agricultural food products*, and was circulated to the member bodies in June 1977.

It has been approved by the member bodies of the following countries :

Australia	Hungary	Portugal
Austria	India	Romania
Belgium	Israel	South Africa, Rep. of
Bulgaria	Kenya	Spain
Canada	Korea, Rep. of	Thailand
Czechoslovakia	Mexico	Turkey
Egypt, Arab Rep. of	Netherlands	United Kingdom
Ethiopia	New Zealand	USSR
France	Philippines	Yugoslavia
Germany, F.R.	Poland	

No member body expressed disapproval of the document.

NOTE — The method specified in this International Standard has been developed jointly with the International Dairy Federation (IDF) and the Association of Official Analytical Chemists (AOAC) and will also be published by these organizations.

Milk and milk products — Determination of copper content — Photometric reference method

1 Scope and field of application

This International Standard specifies a photometric reference method for the determination of the copper content of milk and milk products.

The method is applicable to

— milk, skimmed milk and buttermilk;

— evaporated milk and sweetened condensed milk;

— whole and skimmed milk powder;

— cream and butter;

— butterfat;[1]

— ice cream;

— hard, semi-hard and soft cheese of various ages, and processed cheese;

— caseins, caseinates and co-precipitates.

2 Reference

ISO/R 707, *Milk and milk products — Sampling.*

3 Definition

copper content of milk or a milk product : The content of substances determined by the procedure described in this International Standard and expressed as milligrams of copper per kilogram.

4 Principle

Digestion of the organic material with a mixture of nitric and sulphuric acids, preceded in the case of cream, butter and butterfat by removal of the fat.

Neutralization with ammonium hydroxide solution, followed by complexing the copper as a salt of diethyldithiocarbamic acid. Extraction of the copper(II) salt with amyl acetate. Photometric measurement of the absorbance of the yellow solution.

NOTE — The presence of bismuth and/or tellurium interferes with the determination of copper. See the check for absence and method of removal specified in 8.6.

5 Reagents

All reagents shall be of recognized analytical grade and, with the exception of the standard copper(II) sulphate solution (5.11), free from copper. The water used shall be double-distilled, the final distillation being carried out in a copper-free distillation unit.

5.1 Ethanol, about 96 % (V/V).

Distil, if necessary, in a copper-free distillation unit.

5.2 Diethyl ether.

Distil, if necessary, in a copper-free distillation unit.

5.3 Petroleum ether (light petroleum), boiling range 40 to 60 °C.

Distil, if necessary, in a copper-free distallation unit.

5.4 Nitric acid, concentrated, ϱ_{20} 1,42 g/ml.

Distil in a copper-free distillation unit, discarding the first 50 ml.

5.5 Sulphuric acid, concentrated, ϱ_{20} 1,84 g/ml.

5.6 Hydrogen peroxide solution, ϱ_{20} 1,099 to 1,103 g/ml.

5.7 Ammonium hydroxide solution, ϱ_{20} 0,91 g/ml.

Purify, if necessary, by vacuum distillation in a copper-free distillation unit.

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1) The designation "butterfat" covers all the products described in IDF Standard 68 : 1971.

5.8 Citrate EDTA, solution.

Dissolve in water, in a 1 000 ml volumetric flask, 400 g of ammonium citrate $[(NH_4)_3C_6H_5O_7]$ and 100 g of EDTA disodium salt dihydrate [ethylenedinitrilotetraacetic acid, disodium salt, dihydrate] $(Na_2C_{10}H_{14}N_2O_8.2H_2O)$. Make up to the mark.

If necessary, purify the solution as follows :

Extract the citrate EDTA solution with 25 ml of a 1 g/l solution of diethylammonium diethyldithiocarbamate $[(C_2H_5)_2NCSSNH_2(C_2H_5)_2]$ in carbon tetrachloride (CCl_4). Repeat the extraction with 25 ml portions of the diethylammonium diethyldithiocarbamate solution until the carbon tetrachloride remains nearly colourless.

5.9 Sodium diethyldithiocarbamate solution.

Dissolve, in a 100 ml volumetric flask, 400 mg of sodium diethyldithiocarbamate $[(C_2H_5)_2NCSSNa]$ (or 500 mg of the trihydrate) in 90 ml of water. Make up to the mark with the ammonium hydroxide solution (5.7).

Store the solution in the dark in a refrigerator (0 to 8 °C). Renew the solution every week.

5.10 Amyl acetate.

Dry 1 litre of amyl acetate for 24 h on 15 g of anhydrous sodium sulphate. Distil in a copper-free distillation unit, collecting the fraction which distils between 136 and 140 °C.

NOTE — Instead of amyl acetate, xylene distilled in a copper-free distillation unit may be used.

5.11 Copper(II) sulphate, standard solution.

5.11.1 Stock solution

Dissolve in water 196,5 mg of copper(II) sulphate pentahydrate $(CuSO_4.5H_2O)$. Add 5 ml of 0,5 M sulphuric acid solution and dilute to 1 000 ml with water.

5.11.2 Working solution

Prepare this solution on the day of use.

Add 10 ml of the stock solution (5.11.1) to 5 ml of 0,5 M sulphuric acid solution and dilute to 500 ml with water.

1 ml of this working solution contains 1 µg of Cu.

5.12 Phenolphthalein solution.

Dissolve 1 g of phenolphthalein in 100 ml of 90 % (V/V) ethanol.

6 Apparatus

Keep the clean glassware, including the glass beads (6.7), in

10 % (m/m) nitric acid solution. Rinse before use three times with distilled water and then three times with double-distilled water. Dry, if necessary, by successively rinsing with ethanol and diethyl ether.

6.1 Analytical balance.

6.2 Device for grinding or grating cheese, capable of being easily cleaned.

6.3 Sieve, of nominal aperture size 0,5 mm, made of copper-free material.

6.4 Water baths, maintaining temperatures of 30 to 40 °C, 40 to 60 °C 60 to 65 °C and 80 to 90 °C.

6.5 Micro gas burners.

6.6 Digestion flasks (Kjeldahl), with ground glass stoppers, calibrated on the lower part of the neck at 50 ml.

6.7 Glass beads.

6.8 Graduated cylinders, of capacity 5 and 25 ml.

6.9 Graduated pipettes, of capacity 5 and 25 ml, graduated in 0,1 ml, complying with the requirements of ISO/R 835, class A.

6.10 One-mark pipettes, delivering 1, 2, 5 and 20 ml, complying with the requirements of ISO/R 648, class A.

6.11 Spectrophotometer, operating at 436 nm equipped with cells of optical path length 10 mm.

7 Sampling

7.1 See ISO/R 707.

Avoid copper contamination.

Store glass sampling jars in 10 % (m/m) nitric acid solution.

7.2 Store the sample in such a way that deterioration and change in composition are prevented.

8 Procedure

Avoid copper contamination.

8.1 Preparation of the test sample

8.1.1 Milk and skimmed milk

Bring the sample to 20 ± 2 °C and mix carefully.

If, in the case of milk, the fat is not evenly dispersed, heat the sample slowly to 35 to 40 °C, mix gently by tilting and cool quickly to 20 ± 2 °C.

8.1.2 Buttermilk

If necessary, remove butter granules. Bring the sample to 20 ± 2 °C and mix carefully, immediately before weighing (8.2.1).

8.1.3 Cream

Bring the sample to 20 ± 2 °C. Mix or stir thoroughly but not so vigorously as to cause frothing or churning.

If the cream is very thick, or if the fat is not evenly dispersed, warm slowly to 35 to 40 °C to facilitate mixing.

Cool the sample quickly to 20 ± 2 °C.

NOTE — Correct results cannot be expected if adequate mixing of the sample is not achieved or if the sample shows any evidence of churning or any other signs of abnormality.

8.1.4 Evaporated milk

Shake and invert the container. Open the container and pour the milk slowly into a second container provided with an air-tight lid. Mix by repeated transfer between the two containers, taking care to incorporate in the sample any fat or other constituents adhering to the wall and ends of the first container.

Finally, transfer the milk as completely as possible to the second container, and close the latter.

Heat the closed container in a water bath at 40 to 60 °C. Remove and shake the container vigorously every 15 min. After 2 h, remove the container and cool to 20 ± 2 °C. Remove the lid and mix thoroughly by stirring the milk with a spoon or spatula.

NOTE — If the fat separates, correct results cannot be expected.

8.1.5 Sweetened condensed milk

Open the container and thoroughly mix the milk with a spoon or spatula. Use an up and down rotary movement in such a way that the top layers and the contents of the lower corners of the container are moved and mixed. Take care to incorporate in the sample any milk adhering to the wall and ends of the container. Transfer the milk as completely as possible to a second container provided with an airtight lid, and close this container.

NOTE — In the case of a collapsible tube, open it and transfer the contents to a sample container. Cut open the tube and transfer all material adhering to the interior as completely as possible to the container.

Heat the closed container in a water bath at 30 to 40 °C. Open the container and transfer the milk as completely as possible to a sample container. Take care to incorporate any milk adhering to the interior of the second container. Cool to 20 ± 2 °C. Stir the milk in the container thoroughly. Mix until the whole mass is homogeneous. Close the container.

8.1.6 Whole and skimmed milk powder

Transfer the milk powder into a container, provided with an air-tight lid, of a capacity about twice the volume of the powder. Close the container immediately. Mix the milk powder thoroughly by repeatedly shaking and inverting the container.

8.1.7 Butter

8.1.7.1 If the sample is not visibly inhomogeneous, cool to 4 ± 2 °C before weighing to facilitate transfer to the digestion flask.

8.1.7.2 If the sample is visibly inhomogeneous, bring the sample to a temperature at which it will be soft enough to facilitate thorough mixing to a homogeneous state without any rupture of emulsion. The temperature of mixing shall normally not exceed 35 °C, While mixing, cool the sample to about 20 °C (or until rather firm). As soon as possible after cooling, open the sample container and stir briefly (not exceeding 10 s) with a spoon or spatula. Proceed as in 8.1.7.1.

8.1.8 Butterfat

Before weighing, bring the sample to 40 °C, keep it at this temperature for 5 min and mix gently. Cool to about 20 °C.

8.1.9 Ice cream

For samples taken in small packages, remove the packaging and place the sample in a container provided with an airtight lid.

For samples taken from bulk or from large packages, keep them in their sample containers.

In either case, melt the sample by standing it in a water bath at 45 ± 1 °C for just enough time to allow the sample to become fluid. Mix the sample by shaking. Cool to ambient temperature, continuing to mix until cooling is completed.

8.1.10 Cheese and processed cheese

Remove the rind or mouldy surface layer of the cheese, in such a way as to provide a sample representative of the cheese as it is usually consumed. Grind or grate the sample by means of an appropriate device (6.2); mix the ground or grated mass quickly, and if possible grind or grate a second time and again mix thoroughly. If the sample cannot be ground or grated, mix it thoroughly by intensive stirring and kneading.

Transfer the test sample to an air-tight container to await analysis, which should be carried out as soon as possible after grinding. If delay is unavoidable, take all precautions to ensure proper preservation of the sample and to prevent condensation of moisture on the inside surface of the container. The storage temperature should be 10 to 12 °C.

Clean the device after grinding or grating each sample.

8.1.11 Casein, caseinates and co-precipitates

8.1.11.1 Thoroughly mix the laboratory sample by repeatedly shaking and inverting the container (if necessary, after having transferred all of the laboratory sample to an air-tight container of sufficient capacity to allow this operation to be carried out).

8.1.11.2 Transfer about 50 g of the thoroughly mixed laboratory sample to the test sieve (6.3).

8.1.11.3 If the 50 g portion passes the sieve completely or almost completely, use for the determination the sample as prepared in 8.1.11.1.

8.1.11.4 Otherwise, grind the 50 g portion, using the grinding device (6.2), until it passes the sieve. Immediately transfer all the sieved sample to an air-tight container of sufficient capacity and mix thoroughly by repeatedly shaking and inverting. During these operations, take precautions to avoid any change in the water content of the product.

8.1.11.5 After the test sample has been prepared, the determination should be proceeded with as soon as possible.

8.2 Weighing and pre-treatment of the test portion

8.2.1 Milk, skimmed milk and buttermilk

Weigh into a digestion flask (6.6) 20 g of the test sample (8.1), to the nearest 10 mg. Add 3 ml of the nitric acid (5.4) and 2,5 ml of the sulphuric acid (5.5).

Continue as in 8.3.

8.2.2 Evaporated milk

Weigh into a digestion flask (6.6) 8 g of the test sample (8.1), to the nearest 10 mg. Add 3 ml of the nitric acid (5.4) and 2,5 ml of the sulphuric acid (5.5).

Continue as in 8.3.

8.2.3 Sweetened condensed milk

Weigh into a digestion flask (6.6) 5 g of the test sample (8.1), to the nearest 10 mg. Add 10 ml of water and 2,5 ml of the sulphuric acid (5.5).

NOTE — No nitric acid should be added at this stage because of excessive foaming.

Continue as in 8.3.

8.2.4 Whole and skimmed milk powder

Weigh into a digestion flask (6.6) 2 g of the test sample (8.1), to the nearest 1 mg. Add 5 ml of water and mix well. Then add 3 ml of the nitric acid (5.4) and 2,5 ml of the sulphuric acid (5.5).

Continue as in 8.3.

8.2.5 Cream, butter and butterfat

Weigh into a digestion flask (6.6) 20 g of the test sample (8.1), to the nearest 10 mg. Add in the case of cream or butter 8 ml of the nitric acid (5.4) and in the case of butterfat 4 ml of water and 8 ml of the nitric acid (5.4).

Heat the flask in a water bath at 80 to 90 °C for 1 h. Shake thoroughly every 3 min in order to wash the fat with the nitric acid. Cool to 40 °C and as far as possible remove the fat layer by means of a pipette.

Add 15 ml of the petroleum ether (5.3), swirl carefully, and as far as possible remove the solvent by means of a pipette.

Repeat twice with fresh 15 ml portions of the petroleum ether. Remove residual petroleum ether by warming in a water bath at 80 to 90 °C.

Cool to room temperature. Add 2,5 ml of the sulphuric acid (5.5).

Continue as in 8.3.

8.2.6 Ice cream, cheese and processed cheese

Weigh into a digestion flask (6.6) 3 g of the test sample (8.1), to the nearest 1 mg. Add 3 ml of the nitric acid (5.4) and 2,5 ml of the sulphuric acid (5.5).

Continue as in 8.3.

8.2.7 Caseins, caseinates and co-precipitates

Weigh into a digestion flask (6.6) 1 g of the test sample (8.1), to the nearest 1 mg. Add 5 ml of water, 3 ml of the nitric acid (5.4) and 2,5 ml of the sulphuric acid (5.5).

Continue as in 8.3.

8.3 Digestion

8.3.1 Add three glass beads (6.7). Operating under a well ventilated fume hood, place the flask in an inclined position and heat with a micro-burner (6.5). The height of the flame shall be controlled so as to limit the production of foam in the flask. Foaming into the neck of the flask is allowed but the foam shall not escape. Keep the mixture gently boiling.

Avoid local overheating.

NOTE — A blank test (8.5) shall be carried out simultaneously.

8.3.2 When the solution turns brown, carefully add 3 to 5 drops of the nitric acid (5.4). *Heat vigorously as soon as possible*. Continue heating and adding the nitric acid 5 to 20 drops at a time, swirling the flask occasionally to remove any material adhering to the wall, until the mixture remains colourless.

Cool to room temperature.

8.3.3 Carefully add 2 ml of water and 1 ml of the hydrogen peroxide solution (5.6). Swirl and heat again until white fumes are emitted. If the solution becomes yellow, add a further 0,5 ml of the hydrogen peroxide solution. Continue heating for 45 min after the beginning of the emission of white fumes. Cool to room temperature and add water to give a total volume of approximately 25 ml.

8.3.4 Add 5 ml of the citrate EDTA solution (5.8) and 0,1 ml of the phenolphthalein solution (5.12.) Add ammonium hydroxide solution (5.7), while swirling occasionally, until the mixture remains light red.

8.4 Colour development

During the colour development and the photometric determination, avoid unnecessary exposure to light stronger than an illuminance of 150 lx (subdued daylight).

Add 5 ml of the sodium diethyldithiocarbamate solution (5.9), while swirling.

Make up to the 50 ml mark with water. Pipette 4 ml of the amyl acetate (5.10) into the solution and close the flask with a stopper. Heat the closed flask for 10 min in a water bath at 60 to 65 °C. Then shake the flask vigorously for 1 min, with repeated inversion, ensuring that the stopper remains in position.

NOTE — If xylene is used instead of amyl acetate, the time of shaking shall be 2 min.

Cool the closed flask immediately under running tap water for at least 10 min.

Eliminate the turbidity appearing in the amyl acetate layer by tilting the flask cautiously from a vertical to a horizontal position a number of times.

Continue cautiously tilting the flask if turbidity persists. Keep the flask for 1 h in the dark.

8.5 Blank test

Simultaneously with the analysis of the test portion, carry out a reagents blank test using all the reagents and 20 ml of water instead of the test portion.

During the digestion period, use the same amount of the nitric acid (5.4) and the hydrogen peroxide solution (5.6) as for the digestion of the test portion.

8.6 Photometric measurements

Transfer the amyl acetate layer, by means of a pipette, into a 10 mm cell of the spectrophotometer (6.11). Measure the absorbances of the amyl acetate layers of the test solution (8.4) and the reagents blank solution (8.5) against that of water at a wavelength of 436 nm.

Subtract the value for the reagents blank solution from that the test solution.

NOTES

1 The absorbance value of the reagents blank should correspond to less than 0,4 µg Cu/50 ml. If the blank value corresponds to more than 0,4 µg Cu/50 ml, all reagents should be checked.

2 Bismuth and tellurium can be assumed to be absent if the amyl acetate layer turns colourless after shaking with 5 % (*m/m*) potassium cyanide solution. Otherwise they can be removed by washing the amyl acetate layer with 1 M sodium hydroxide solution.

8.7 Number of determinations

All determinations, that of the reagents blank (8.5) included, shall be carried out in duplicate.

8.8 Calibration curve

8.8.1 Pipette 0 (blank), 0,5, 1, 2, 3, 4 and 6 ml of the copper(II) sulphate standard working solution (5.11.2) into a series of seven digestion flasks (6.6). Dilute with water to about 25 ml.

Add 2,5 ml of the sulphuric acid (5.5). Mix well.

Add 5 ml of the citrate EDTA solution (5.8) and 0,1 ml of the phenolphthalein solution (5.12). Add ammonium hydroxide solution (5.7), while swirling occasionally, until the mixture remains light red.

8.8.2 Carry out the procedure described in 8.4.

8.8.3 Transfer each amyl acetate layer, by means of a pipette, into a cell of the spectrophotometer (6.11). Measure the absorbances of the amyl acetate layers against that of water at a wavelength of 436 nm.

Subtract the blank value from the values found for the other solutions.

8.8.4 Plot these absorbances against the amounts of copper added.

8.8.5 Check the calibration curve monthly.

9 Expression of results

9.1 Method of calculation and formula

Calculate the copper content of the sample, expressed as milligrams per kilogram, using the formula

$$\frac{m_1}{m_0}$$

where

m_1 is the mass, in grams, of the test portion;

m_0 is the mass, in micrograms, of copper, read from the calibration curve, that corresponds with the absorbance of the test solution.

Provided that the requirement for repeatability (see 9.2), is satisfied, take as the result the arithmetic mean of the values obtained, expressed to the number of decimal places shown in the table.

9.2 Repeatability

The difference between the results of a determination in duplicate (results obtained almost simultaneously or in rapid succession by the same analyst) shall not be greater than the repeatability value for the product analysed, as detailed in the table.

10 Test report

The test report shall show the method used, by reference to this International Standard, and the result obtained.

It shall also mention all operational conditions not specified in the International Standard, or regarded as optional, as well as any cirumstances that may have influenced the result.

The report shall include all details necessary for complete identification of the sample.

Table — Expression of results and repeatability

Product	Expression of results to the nearest	Repeatability
	mg/kg	mg/kg
Milk	0,001	0,004
Skimmed milk	0,001	0,004
Buttermilk	0,001	0,004
Evaporated milk	0,001	0,008
Sweetened condensed milk	0,001	0,008
Whole milk powder	0,01	0,05
Skimmed milk powder	0,01	0,05
Cream	0,001	0,004
Butter	0,001	0,004
Butterfat	0,001	0,004
Ice cream	0,001	0,03
Cheese and processed cheese	0,001	0,03
Caseins, caseinates and co-precipitates	0,01	0,15

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