INTERNATIONAL STANDARD

ISO 8968-1

IDF 20-1

First edition
2001-12-15

Milk — Determination of nitrogen content —

Part 1: Kjeldahl method

Lait — Détermination de la teneur en azote

Partie 1: Méthode Kjeldahl

Reference numbers
ISO 8968-1:2001(E)
IDF 20-1:2001(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-idf.org

Printed in Switzerland

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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.

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 8968 | IDF 20 may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

International Standard ISO 8968-1 | IDF 20-1 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.

ISO 8968 | IDF 20 consists of the following parts, under the general title *Milk — Determination of nitrogen content*:

— *Part 1: Kjeldahl method*

— *Part 2: Block-digestion method (Macro method)*

— *Part 3: Block-digestion method (Semi-micro rapid routine method)*

— *Part 4: Determination of the non-protein-nitrogen content*

— *Part 5: Determination of the protein-nitrogen content*

Annex A of this part of ISO 8968 | IDF 20 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 8968-1 | IDF 20-1 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, *Nitrogen compounds*, under the aegis of its project leader, Mr D.M. Barbano (US).

Milk — Determination of nitrogen content —

Part 1:
Kjeldahl method

WARNING — The use of this part of ISO 8968|IDF 20 may involve the use of hazardous materials, operations, and equipment. This standard does not purport to address all the safety risks associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and healthy practices and determine the applicability of local regulatory limitations prior to use.

1 Scope

This part of ISO 8968|IDF 20 specifies a method for the determination of the nitrogen content of liquid milk, whole or skimmed, by the Kjeldahl principle.

2 Normative reference

The following normative document contains provisions which, through reference in this text, constitute provisions of this part of ISO 8968|IDF 20. For dated references, subsequent amendments to, or revisions of, any of these publications do not apply. However, parties to agreements based on this part of ISO 8968|IDF 20 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 385-1, *Laboratory glassware — Burettes — Part 1: General requirements*

3 Term and definition

For the purposes of this part of ISO 8968|IDF 20, the following term and definition apply.

3.1
nitrogen content
mass fraction of nitrogen determined by the procedure specified in this part of ISO 8968|IDF 20

NOTE The nitrogen content is expressed as a percentage by mass.

4 Principle

A test portion is digested with a mixture of concentrated sulfuric acid and potassium sulfate, using copper(II) sulfate as a catalyst to thereby convert organic nitrogen present to ammonium sulfate. The function of the potassium sulfate is to elevate the boiling point of the sulfuric acid and to provide a stronger oxidizing mixture for digestion. Excess sodium hydroxide is added to the cooled digest to liberate ammonia.The liberated ammonia is distilled into excess boric acid solution then titrated with hydrochloric acid. The nitrogen content is calculated from the amount of ammonia produced.

5 Reagents

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

5.1 Potassium sulfate (K_2SO_4), nitrogen free.

5.2 Copper(II) sulfate solution, $c(CuSO_4)$ = 5,0 g per 100 ml.

Dissolve 5,0 g of copper(II) sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$) in water in a 100 ml one-mark volumetric flask. Dilute to the mark with water and mix.

5.3 Sulfuric acid (H_2SO_4), with a mass fraction of at least 95 % to 98 %, nitrogen free (ρ_{20} = 1,84 g/ml approximately).

5.4 Sodium hydroxide solution (NaOH), nitrogen free, containing 50 g of sodium hydroxide per 100 g of solution.

5.5 Indicator solution

Dissolve 0,1 g of methyl red in 95 % (volume fraction) ethanol. Dilute to 50 ml with the ethanol. Dissolve 0,5 g of bromocresol green in 95 % (volume fraction) ethanol. Dilute to 250 ml with the ethanol. Mix amounts of one part of the methyl red solution with five parts of the bromocresol green solution or combine and mix all of both solutions.

5.6 Boric acid solution, $c(H_3BO_3)$ = 40,0 g/l.

Dissolve 40,0 g of boric acid in 1 litre of hot water in a 1 000 ml one-mark volumetric flask. Allow the flask and its contents to cool to 20 °C. Dilute to the mark with water, add 3 ml of the indicator solution (5.5) and mix. Store the solution, which will be light orange in colour, in a borosilicate glass bottle. Protect the solution from light and sources of ammonia fumes during storage.

If using the electronic pH endpoint titration, the addition of the indicator solution to the boric acid solution may be omitted. On the other hand, the change in colour may also be used as a check of proper titration procedures.

5.7 Hydrochloric acid standard volumetric solution, c(HCl) = (0,1 ± 0,000 5) mol/l.

It is recommended that this material be purchased prestandardized by the manufacturer to meet or exceed the above specification.

NOTE Often systematic errors (which can be avoided) introduced by an analyst diluting a concentrated stock acid and then determining the molarity of the acid, can reduce the reproducibility of the method. The analyst should not use a solution for titration that has a higher concentration than 0,1 mol/l, because this will reduce the total titration volume per sample and the uncertainty in readability of the burette will become a larger percentage of the value. This will have a negative impact on the repeatability and reproducibility of the method. The same issues and additional sources of error arise when another acid (e.g. sulfuric acid) is substituted for hydrochloric acid. Thus, these substitutions are not recommended.

5.8 Ammonium sulfate [$(NH_4)_2SO_4$], minimum assay 99,9 % (mass fraction) on dried material.

Immediately before use, dry the ammonium sulfate at 102 °C ± 2 °C for not less than 2 h. Cool to room temperature in a desiccator.

5.9 Tryptophan ($C_{11}H_{12}N_2O_2$) or **lysine hydrochloride** ($C_6H_{15}ClN_2O_2$), minimum assay 99 % (mass fraction).

Do not dry these reagents in an oven before use.

5.10 Sucrose, with a nitrogen content of not more than 0,002 % (mass fraction).

Do not dry the sucrose in an oven before use.

6 Apparatus

Usual laboratory apparatus and, in particular, the following.

6.1 Water bath, capable of being maintained at 38 °C ± 2 °C

6.2 Kjeldahl flasks, of capacity 500 ml or 800 ml.

6.3 Analytical balance, capable of weighing to the nearest 0,1 mg.

6.4 Boiling aids, e.g. glowed pumice, zinc dust, hard pieces of porcelain or high-purity amphoteric alundum (i.e. carbarundum) granules, plain, mesh size 10.

Do not reuse the aids.

NOTE Glass beads of approximately 5 mm diameter are sometimes used, but they might not promote as efficient boiling as the alundum granules and more foaming problems can be encountered during digestion with glass beads.

6.5 Burette or **automatic pipette,** capable of delivering 1,0 ml portions of the copper sulfate solution (5.2).

6.6 Graduated measuring cylinders, of capacity 50 ml, 100 ml and 500 ml.

6.7 Digestion apparatus, to hold the Kjeldahl flasks (6.2) in an inclined position (at approximately 45°), with electric heaters or gas burners that do not heat the flasks above the level of their contents, and with a fume extraction system.

The heater source should be adjustable to control the maximum heater setting to be used during digestion. Preheat the heat source at the heater setting for evaluation. In the case of a gas heater, the preheated period shall be 10 min, and for an electric heater it shall be 30 min. For each of the heaters, determine the heater setting that brings 250 ml of water including 5 to 10 boiling aids with an initial temperature of 25 °C to its boiling point in 5 min to 6 min. This is the maximum heater setting to be used during digestion.

6.8 Distillation apparatus, made of borosilicate glass or other suitable material to which can be fitted a Kjeldahl flask (6.2) consisting of an efficient splash-head connected to an efficient condenser with straight inner tube and an outlet tube attached to its lower end.

The connecting tubing and stopper(s) shall be close fitting and preferably made of neoprene.

6.9 Conical flasks, of capacity 500 ml, graduated at every 200 ml.

6.10 Burette, of capacity 50 ml, graduated at least at every 0,01 ml, complying with the requirements of ISO 385-1, class A.

Alternatively, an automatic burette may be used if it fulfils the same requirements.

6.11 Automatic titrator provided with a pH-meter

The pH-meter should be correctly calibrated in the range of pH 4 to 7 following normal laboratory pH-calibration procedures.

7 Sampling

Sampling is not part of the method specified in this part of ISO 8968 | IDF 20. 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

Warm the test sample in the water bath (6.1) set at 38 °C. Gently mix the test sample thoroughly by repeatedly inverting the sample bottle without causing frothing or churning. Cool the sample to room temperature immediately prior to weighing the test portion (9.1).

NOTE For advice on sample size to apply this method to dairy products other than milk, see annex A.

9 Procedure

9.1 Test portion and pretreatment

Add to a clean and dry Kjeldahl flask (6.2), 5 to 10 boiling aids (6.4), 15,0 g of the potassium sulfate (5.1), 1,0 ml of the copper(II) sulfate solution (5.2), approximately 5 ml ± 0,1 ml of the prepared test sample (clause 8), weighed to the nearest 0,1 mg, and 25 ml of the sulfuric acid (5.3). Use the sulfuric acid to wash down any copper(II) sulfate solution, potassium sulfate or test portion left on the neck of the flask. If any charred digest still is left on the neck, rinse it with a small amount of water. Gently, mix the contents of the Kjeldahl flask.

NOTE For advice on test portion size to apply this method to dairy products other than milk, see annex A of this part.

9.2 Determination

9.2.1 Digestion

Turn on the fume extraction system of the digestion apparatus (6.7) prior to beginning the digestion. Heat the Kjeldahl flask and its contents (9.1) on the digestion apparatus using a heater setting low enough such that charred digest does not foam up the neck of the Kjeldahl flask. Digest at this heat setting until white fumes appear in the flask after approximately 20 min. Increase the heater setting to half-way to the maximum setting determined in 6.7 and continue the heating for 15 min. At the end of the 15 min period increase the heat to the maximum setting determined in 6.7. After the digest clears (clear with light blue-green colour), continue boiling for 1 h to 1,5 h at maximum setting. If the liquid does not boil, the final burner setting may be too low. The total digestion time will be between 1,8 h and 2,25 h.

To determine the specific boiling time required for analysis conditions in a particular laboratory using a particular set of apparatus, select for milk analysis a high-protein, high-fat milk sample and determine its protein content using different boiling times (1 h to 1,5 h) after clearing. The mean protein result increases with increasing boiling time, becomes consistent and then decreases when the boiling time is too long. Select the boiling time that yields the maximum protein result.

At the end of digestion, the digest shall be clear and free of undigested material. Allow the digest to cool to room temperature in an open flask in a separate hood over a period of approximately 25 min. If the flask is left on the hot burners to cool, it will take longer to reach room temperature. The cooled digest should be liquid or liquid with a few small crystals at the bottom of the flask at the end of the 25 min cooling period. Do not leave the undiluted digest in the flasks overnight. The undiluted digest may crystallize during this period and it will be very difficult to get the crystallized digest back into solution.

NOTE Excessive crystallization after 25 min is the result of undue acid loss during digestion and can result in low test values. Undue acid loss is caused by excessive fume aspiration or by an excessively long digestion time caused by an incorrect maximum burner setting.

Add 300 ml of water to the 500 ml Kjeldahl flasks or 400 ml of water when using the 800 ml Kjeldahl flasks. Use the water to wash down the neck of the flask too. Mix the contents thoroughly, ensuring that any crystals that separate out are dissolved. Add 5 to 10 boiling aids (6.4). Allow the mixture to cool again to room temperature prior to the distillation. Diluted digests may be stoppered and held for distillation at a later moment.

9.2.2 Distillation

Turn on the condenser water for the distillation apparatus (6.8). Add 75 ml of sodium hydroxide solution (5.4) to the diluted digest (9.2.1) by carefully pouring the solution down the inclined neck of the Kjeldahl flask to form a layer at the bottom of the bulb of the flask. There should be a clean interface between the two solutions. To reduce the possibility of ammonia loss, immediately after the addition of the sodium hydroxide solution to the Kjeldahl flask, quickly connect it to the distillation apparatus (6.8). The tip of the condenser outlet tube is immersed in 50 ml of the boric acid solution (5.6) contained in a conical flask (6.9). Vigorously swirl the Kjeldahl flask to mix its contents thoroughly until no separate layers of solution are visible in the flask. Set the flask down on the burner. Turn on the burner to a setting high enough to boil the mixture. Continue distillation until irregular boiling (bumping) starts and then immediately disconnect the Kjeldahl flask and turn off the burner. Turn off the condenser water. Rinse the inside and outside of the tip of the outlet tube with water, collecting the rinsing in the conical flask, and mix.

The distillation rate shall be such that approximately 150 ml of distillate are collected before irregular boiling (bumping) starts. The total volume of the contents of the conical flask will be approximately 200 ml. If the volume of distillate collected is less than 150 ml, then it is likely that less than 300 ml of water was added to dilute the digest. The efficiency of the condenser shall be such that the temperature of the contents of the conical flask does not exceed 35 °C during the distillation when using a colorimetric endpoint.

9.2.3 Titration

Titrate the contents of the conical flask (9.2.2) with the hydrochloric acid (5.7) using a burette (6.5). The endpoint is reached at the first trace of pink colour in the contents. Estimate the burette reading at least to its nearest 0,05 ml. An illuminated magnetic stirrer plate may aid visualization of the endpoint.

Alternatively, titrate the contents of the conical flask (9.2.2) with the hydrochloric acid (5.7) using a properly calibrated automatic titrator provided with a pH-meter (6.11). The pH endpoint of the titration is reached at pH 4,6, being the steepest point in the titration curve (inflection point). Read on the automatic titrator the amount of titrant used.

NOTE 1 The first trace of pink is observed between pH 4,6 and 4,3 for the indicator system and 4 % boric acid solution specified in this method. In practice the rate of change of pH as a function of added 0,1 mol/l HCl is very fast within this range of pH. It takes about 0,05 ml of 0,1 mol/l HCl to change the pH by 0,3 units in the range of pH from 4,6 to 4,3 in this system.

NOTE 2 The within- and between-laboratory performance statistics for this method were determined using a colour endpoint titration. Comparing the final test results, including those for their blank tests, obtained with a pH 4,6 endpoint with those of a colour endpoint titration showed that, statistically, no significant difference was demonstrable between them.

9.3 Blank test

Always titrate blanks with the same hydrochloric acid (5.7) and burette (6.5) or automatic titrator provided with a pH-meter (6.11) as used for the test portions. Carry out a blank test following the procedure described in 9.1 to 9.2.3. Replace the test portion with 5 ml of water and about 0,85 g of sucrose (5.10).

Keep a record of the blank values. If the blank values change, identify the cause.

NOTE 1 The purpose of the sucrose in a blank or a recovery standard is to act as organic material to consume an amount of sulfuric acid during digestion that is roughly equivalent to a test portion. If the amount of residual free sulfuric acid at the end of digestion is too low, the recovery of nitrogen by both recovery tests in 9.4.2 and 9.4.3 will be low. If, however, the amount of residual acid present at the end of digestion is sufficient to retain all the nitrogen, but the temperature and time conditions during digestion were not sufficient to release all the nitrogen from a sample, the nitrogen recovery in 9.4.2 will be acceptable and the nitrogen recovery in 9.4.3 will be low.

The amount of titrant used in the blank should always be greater than zero. Blanks within the same laboratory should be consistent across time. Typical blank values are equal to or below 0,2 ml.

NOTE 2 If the blank is already pink before the beginning of titration, something is wrong. Usually in such cases, the conical flasks are not clean or water from the humid air that may condense on the outside of the condenser apparatus has dripped down into the collection flask to cause the contamination.

9.4 Recovery tests

9.4.1 The accuracy of the procedure should be checked regularly by means of the following recovery tests, carried out in accordance with 9.1 to 9.2.3.

9.4.2 Check that no loss of nitrogen occurs by using a test portion of 0,12 g of ammonium sulfate (5.8) together with 0,85 g of sucrose (5.10).

NOTE The ammonium sulfate recovery check does not give information about the capability of the digestion conditions to release nitrogen that is bound in the protein structures.

The percentage of nitrogen recovered shall be between 99,0 % and 100,0 % for all positions on the apparatus. For recoveries less than 99 %, the concentration of the titrant is higher than the stated value, or nitrogen loss may have occurred in the digestion or distillation. It is possible to use a mixture of ammonium sulfate and small amount of sulfuric acid (the amount of residual remaining at the end of a digestion) in a Kjeldahl flask. Dilute it with the normal volume of water, add the normal amount of sodium hydroxide and distil. If the nitrogen recovery is still low by the same amount, the loss of nitrogen is in the distillation apparatus and not in that of the digestion. The probable cause might be leaky tubing in a traditional system or the tips of the condensers not submerged under the surface of the boric acid early in the distillation. The apparatus should pass this test before the recoveries are checked by the procedure in 9.4.3.

In the case where recoveries of nitrogen exceed 100 %, no loss of nitrogen can be seen. In this case, some possible causes are as follows:

a) the ammonium sulfate is contaminated;

b) the actual concentration of the titrant is lower than its stated value;

c) the calibration of the burette for the titrant is wrong;

d) the temperature of the titrant is too far above the temperature of burette calibration; or

e) the flow of titrant out of the burette exceeds the maximum speed at which the burette calibration is valid.

9.4.3 Check the efficiency of the digestion procedure by using 0,16 g of lysine hydrochloride or 0,18 g of tryptophan (5.9) together with 0,67 g of sucrose (5.10).

At least a mass fraction of 98 % of the nitrogen shall be recovered. If the recovery is lower than 98 %, after having a mass fraction of 99 % to 100 % recovery on ammonium sulfate, then the temperature or time of digestion is insufficient (follow procedure in 9.2.1, paragraph 1 and note) or there is undigested sample material (i.e. char) on the inside of the Kjeldahl flask. The final evaluation of performance is best done by participation in a proficiency testing programme, where within- and between-laboratory statistical parameters are computed based on analysis of milk test samples.

9.4.4 Lower results in either of the recovery tests (or higher than 100,0 % in 9.4.2) indicate failures in the procedure and/or inaccurate concentration of the hydrochloric acid solution (5.7).

10 Calculation and expression of results

10.1 Calculation of nitrogen content

10.1.1 Calculate the nitrogen content of the test sample, w_N, using the following equation:

$$w_N = \frac{1{,}400\ 7\ (V_s - V_b) M_r}{m}$$

where

w_N is the nitrogen content of the sample, expressed as a percentage by mass;

V_s is the numerical value of the volume, in millilitres, of the hydrochloric acid (5.7) used in the determination (9.2.3), expressed to at least the nearest 0,05 ml;

V_b is the numerical value of the volume, in millilitres, of the hydrochloric acid (5.7) used in the blank test (9.3), expressed to at least the nearest 0,05 ml;

M_r is the numerical value of the exact molarity of the hydrochloric acid (5.7), expressed to four decimal places;

m is the numerical value, in grams, of the mass of the test portion (9.1), expressed to the nearest 0,1 mg.

10.1.2 Express the obtained results to four decimal places, if needed for further calculations. In the case of end results, express those obtained for the nitrogen content to three decimal places and for the protein content to two decimal places. The obtained results should not be rounded further until the final use of the test value is made.

NOTE This is particularly true when the values are to be used for further calculations. One example is when the individual test values obtained from the analysis of many sample materials are used to calculate method performance statistics for within- and between-laboratory variation. Another example is when the values are used as a reference for instrument calibration (e.g. infrared milk analyser) where the values from many samples will be used in a simple or multiple regression calculation. In such cases the obtained results should not be rounded before they are used for further calculations.

10.2 Calculation of crude protein content

10.2.1 Calculate the crude protein content of the test sample, w_p, using the following equation:

$$w_p = w_N \times 6{,}38$$

where

w_p is the crude protein content of the sample, expressed as a percentage by mass;

w_N is the nitrogen content of the sample, expressed as a percentage by mass to four decimal places (10.1);

6,38 is the generally accepted multiplying factor to express the nitrogen content as crude protein content.

10.2.2 Express the obtained results for the crude protein content to three decimal places, if needed for further calculations. In the case of end results (see 10.1), express these to two decimal places.

11 Precision

11.1 Interlaboratory test

The values for the repeatability and reproducibility limits were derived from the result of an interlaboratory test carried out in accordance with ISO 5725[1)]. Details of the interlaboratory test of the method are summarized in references [5], [6]. The values derived from this test may not be applicable to concentration ranges and matrices other than those given.

1) ISO 5725:1986 (now withdrawn), was used to obtain the precision data.

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 0,006 % for nitrogen content (0,038 % for crude protein content).

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 0,007 7 % for nitrogen content (0,049 % for crude protein content).

12 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, with reference to this part of ISO 8968 | IDF 20;

— all operating details not specified in this part of ISO 8968 | IDF 20, or regarded as optional, together with details of any incident which may have influenced the result(s);

— the test result(s) obtained;

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

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