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Milk — Determination of nitrogen content —

Part 2: Block-digestion method (Macro method)

Lait — Détermination de la teneur en azote —

Partie 2: Méthode de minéralisation en bloc (Méthode macro)

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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 8968-2 | IDF 20-2 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*

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 8968-2 | IDF 20-2 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 2:
Block digestion method (Macro 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 block-digestion 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 substances 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 by using a block-digestion apparatus 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 environment. Excess sodium hydroxide is added to the cooled digest to liberate ammonia. The liberated ammonia is steam distilled, using either a manual or semi-automatic steam distillation unit, into an excess of 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 faction of between 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.

A 40 % sodium hydroxide solution may be used instead of a 50 %, if plugging of the flow system in an automatic distillation unit is a problem.

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

6.3 Digestion block, aluminium alloy block or equivalent block, fitted with an adjustable temperature control and device for measuring the temperature of the block.

6.4 Digestion tubes, of capacity 250 ml, suitable for use with the digestion block (6.3).

6.5 Exhaust manifold, suitable for use with the digestion tubes (6.4).

6.6 Centrifugal scrubber apparatus or **filter pump** or **aspirator**, constructed of acid-resistant material, for use with mains water supply.

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

6.8 Graduated measuring cylinders, of capacity 25 ml, 50 ml and 100 ml.

6.9 Distillation unit, capable of steam distilling, manual or semi-automatic, suited to accept the 250 ml digestion tubes (6.4) and the 500 ml conical flasks (6.10).

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

6.11 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.

6.12 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 of ISO 8968-1 | IDF 20-1:2001.

9 Procedure

9.1 Test portion and pretreatment

Add to a clean and dry digestion tube (6.4), 12,0 g of 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, weighed to the nearest 0,1 mg, and 20 ml of the sulfuric acid (5.3). Use the sulfuric acid to wash down any copper sulfate solution, potassium sulfate or test portion left on the upper walls of the digestion tube. Gently mix the contents of the tube.

CAUTION — Users of block digestors should note that maintaining sufficient residual sulfuric acid at the end of digestion needs more attention by the analyst using block digestors than with traditional systems. Excessive acid loss due to over aspiration of fumes is more of concern in block digestors than traditional systems.

NOTE 1 The amount of acid used in block digestors is less than that used in the traditional system described in ISO 8968-1 | IDF 20-1. It was determined in the collaborative study (see ref. [6]) that volumes of acid greater than 20 ml in the block-digestion systems gave excessive foaming problems during digestion and variable results.

NOTE 2 For advice on test portion size to apply this method to dairy products other than milk, see annex A of ISO 8968-1 | IDF 20-1:2001.

9.2 Determination

9.2.1 Digestion

Set the digestion block (6.3) at a low initial temperature to control foaming (at approximately between 180 °C and 230 °C). Transfer the tube to the digestion block and place the exhaust manifold (6.5), which itself is connected to a centrifugal scrubber of similar device (6.6), in the top of the tube. The aspiration rate of the centrifugal scrubber or similar device shall be just sufficient to remove fumes. The complete digestion apparatus may need to be kept inside a fume hood.

Digest the test portion for 30 min or until white fumes develop. Then increase the temperature of the digestion block to between 410 °C and 430 °C. Continue digestion of the test portion until the digest is clear.

It may be necessary to increase the temperature gradually over a period of approximately 20 min to control foaming. In any event, do not let the foam rise higher than 4 cm to 5 cm below the surface of the exhaust manifold inserted into the top of the digestion tube.

After the digest clears (clear with light blue-green colour), continue digestion at between 410 °C and 430 °C for at least 1 h. During this time the sulfuric acid shall be boiling. If visible boiling of the clear liquid is not apparent as bubbles forming at the surface of the hot liquid around the perimeter of the tube, then the temperature of the block may be too low. The total digestion time will be between 1,75 h and 2,5 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. Remove the tube from the block with the exhaust manifold in place.

Allow the digest to cool to room temperature over a period of approximately 25 min. The cooled digest should be liquid or liquid with a few small crystals at the bottom of the tube. Do not leave the undiluted digest in the tubes 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 an excessively long digestion time caused by digestions for

too long a period at temperatures below the maximum temperature of the analysis. To reduce acid loss, reduce the rate of fume aspiration.

After the digest has cooled to room temperature in approx. 25 min, remove the exhaust manifold and carefully add 85 ml of water to each tube. Swirl to mix while ensuring that any separated out crystals are dissolved. Allow the contents of the tube to cool to room temperature again.

9.2.2 Distillation

Turn on the condenser water for the distillation apparatus. Attach the digestion tube containing the diluted digest to the distillation unit (6.9). Place a conical flask (6.10) containing 50 ml of the boric acid solution (5.6) under the outlet of the condenser, in such a way that the outlet is below the surface of the boric acid solution. Adjust the distillation unit to dispense 55 ml of sodium hydroxide solution (5.4).

In cases where a 40 % sodium hydroxide solution is used, the dispensed volume should be adjusted to 65 ml. If the automatic delivery of sodium hydroxide solution is extremely variable due to partial plugging of the delivery tubing for the sodium hydroxide, then a large variability in duplicate results will occur.

Following the manufacturer's instructions, operate the distillation unit in such a way as to steam distil the ammonia liberated by addition of the sodium hydroxide solution, collecting the distillate in the boric acid solution. Continue with the distillation process until at least 150 ml of distillate are collected. Remove the conical flask from the distillation unit and completely drain the distillation tip. Rinse the inside and outside of the tip with water, collecting the rinsing in the conical flask. Always rinse the tip with water between samples. 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 (6.11). 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 (6.12). 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 the 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 (6.11) or automatic titrator provided with a pH-meter (6.12) 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 by 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 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 test should always be greater than zero. Typical blank values are equal to or below 0,2 ml

NOTE 2 Blanks within the same laboratory should be consistent over time. 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 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 1 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 digestion 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 a small amount of sulfuric acid (the amount of residual remaining at the end of a digestion) in a 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 the digestion. The probable cause is leaky tubing in a traditional system or the tips of the condensers not being submerged under the surface of the boric acid early in the distillation. The apparatus should pass this test the recoveries are checked by the procedure in 9.4.3.

NOTE 2 In older steam distillation systems that have had heavy use, it has been found that erosion of the glass parts by the hot sodium hydroxide inside the distillation system of the apparatus can result in small pin-hole leaks or cracks in the glass and loss of ammonia through these leaks. This is very difficult to detect, but this has been documented as the cause of low recoveries in some laboratories with older steam distillation equipment.

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 98,0 % 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 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 samples.

9.4.4 Lower results in either of the recovery tests (9.4.2 and 9.4.3) indicate failures in the procedure and/or inaccurate concentration of the hydrochloric acid solution (5.7).