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Milk and milk products — Determination of nitrogen content —

Part 1: Kjeldahl principle and crude protein calculation

Lait et produits laitiers — Détermination de la teneur en azote —
Partie 1: Méthode Kjeldahl et calcul de la teneur en protéines brutes

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Forewords

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The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2. www.iso.org/directives

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received. www.iso.org/patents

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

For an explanation on the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the WTO principles in the Technical Barriers to Trade (TBT) see the following URL: Foreword - Supplementary information

The committee responsible for this document is ISO/TC 34, *Food and food products*, Subcommittee SC 5, *Milk and milk products* and the International Dairy Federation (IDF) and is being published jointly by ISO and IDF.

This second edition of ISO 8968-1|IDF 20-1 cancels and replaces the first edition of ISO 8968-1|IDF 20-1:2001, ISO 8968-2|IDF 20-2:2001, ISO 5549:1978/IDF 92:1979 and ISO/TS 17837|IDF/RM 25:2008 which have been technically revised.

The International Dairy Federation (IDF) 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 in the development of standard methods of analysis and sampling for milk and milk products.

Draft International Standards adopted by the Standing Committees are circulated to the National Committees for voting. Publication as an International Standard requires approval by at least 50 % of IDF National Committees casting a vote.

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ISO 8968-1|IDF 20-1 was prepared by the International Dairy Federation and Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products*. It is being published jointly by ISO and IDF.

The work was carried out by the IDF-ISO Project Group on Nitrogen, of the Standing Committee on *Analytical Methods for Composition (SCAMC)*, under the aegis of its project leaders: Mr. R. Johnson (NZ), Mr. J. Romero (US), Dr. Barbano (US), Dr. Orlandini (IT), and Mr. Psathas (CY).

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Milk and milk products — Determination of nitrogen content —

Part 1:
Kjeldahl principle and crude protein calculation

WARNING — The use of this International Standard might involve the use of hazardous materials, operations, and equipment. This International Standard does not purport to address all the safety risks associated with its use. It is the responsibility of the user of this International Standard to establish appropriate safety and health practices and determine the applicability of local regulatory limitations prior to use.

1 Scope

This International Standard specifies a method for the determination of the nitrogen content and crude protein calculation of milk and milk products by the Kjeldahl principle, using traditional and block digestion methods.

The methods are applicable to:

— liquid cow's (whole, partially skimmed or skimmed milk), goat's and sheep's whole milk;

— hard, semi-hard and processed cheese;

— dried milk and dried milk products (including milk-based infant formulae, milk protein concentrate, whey protein concentrate, casein and caseinate).

The methods are not applicable to samples containing ammonium caseinate.

NOTE Inaccurate crude protein results will be obtained if non-milk sources of nitrogen are present in the products specified in this International Standard.

2 Normative references

The following documents, in whole or in part, are normatively referenced in this document and are indispensable to its application. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 385, *Laboratory glassware — Burettes*

ISO 8655-3, *Piston-operated volumetric apparatus — Part 3: Piston burettes*

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

3.1
nitrogen content
mass fraction of nitrogen determined by the specified procedure

Note 1 to entry: It is expressed as a percentage.

3.2
crude protein content
mass fraction of crude protein calculated as specified

Note 1 to entry: It is expressed as a percentage.

4 Principle

A test portion is digested with a mixture of concentrated sulfuric acid and potassium sulfate. Using copper sulfate (II) as a catalyst to thereby convert any 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 steam distilled into the excess boric acid solution and titration with hydrochloric acid standard volumetric solution is carried out. 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.

NOTE The solutions specified in this procedure might be different than those required for the operation of automated titrators. An effort is made to address those, but it is the responsibility of the operator to follow the directions of the equipment manufacturer.

5.1 Potassium sulfate (K_2SO_4), nitrogen free.

5.2 Copper (II) sulfate pentahydrate solution, $c(CuSO_4.5H_2O)$ = 5,0 g/100 ml.

Dissolve 5,0 g of copper(II) sulfate pentahydrate 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 between 95 % and 98 %, nitrogen-free (approximately ρ_{20} = 1,84 g/ml).

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

With automated distillation systems, other mass fractions of sodium hydroxide may be used, provided an excess of sodium hydroxide is dispensed to the distillation mixture; for example, a mass fraction of 40 % sodium hydroxide solution may be used instead of a mass fraction of 50 %, where plugging of the automated flow system is a problem. The total volume of such sodium hydroxide solution should be considered in order to maintain the suitable distillation volumes.

5.5 Indicator solution

Dissolve 0,1 g of methyl red in 95 % (volume fraction) ethanol in a 50 ml one-mark volumetric flask (6.16). Dilute to 50 ml with ethanol and mix. Dissolve 0,5 g of bromocresol green in 95 % (volume fraction) ethanol in a 250 ml one-mark volumetric flask (6.16). Dilute to 250 ml with ethanol and mix. Mix 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 (H_3BO_3) in 1 l of hot water in a 1 000 ml one-mark volumetric flask (6.16). Allow the flask and its contents to cool to 20 °C. Adjust 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 fume during storage.

With automated distillation systems, other boric acid concentrations may be used after validating accordingly.

If using the electronic pH end point 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 on proper titration procedures.

5.7 Hydrochloric acid standard volumetric solution, c(HCl) = (0,1 ± 0,0005) mol/l.

It is recommended to purchase this material prestandardized by the manufacturer, which meets, or exceeds, these specifications. Often, the systematic errors (which can be avoided) introduced by an analyst diluting a concentrated stock acid and then determining the molarity of the acid, cause poor reproducibility performance of the method in this part. 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 method repeatability and reproducibility performance.

If sulfuric acid is substituted for hydrochloric acid, the solution should have a concentration of 0,05 ± 0,000 3 mol/l.

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 mass fraction of nitrogen of not more than 0,002 %.

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 maintaining a water temperature between 38 °C and 40 °C.

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

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

6.4 Graduated measuring cylinders, of capacity 25 ml, 50 ml, 100 ml and 500 ml.

6.5 Conical flasks, of capacity 500 ml.

6.6 Automatic burette, of suitable capacity e.g. 20 ml, with resolution of at least 0,004 ml, complying with the requirements of ISO 8655-3. Alternatively, a burette, of capacity 50 ml, graduated at least at every 0,1 ml, complying with the requirements of ISO 385, class A may be used for the analysis of milk.

NOTE The manual burette does not have sufficient resolution to achieve the required number of significant figures for all other products.

6.7 Grinding device

6.8 Digestion flasks (Kjeldahl), of 500 ml or 800 ml capacities. Suitable to the digestion system to be used and to the specifications of the manufacturer of the digestion apparatus (6.10 or 6.11).

6.9 Boiling aids, e.g. hard pieces of porcelain or high-purity amphoteric alundum (i.e. carbarundum) granules, plain, mesh size 10. Do not reuse the boiling aids.

NOTE Glass beads of approximately 5 mm diameter can also be 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.

6.10 Digestion apparatus, to hold the digestion flasks (6.8) in an inclined position (approximately 45 °), with electric heaters or gas burners, which 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 preheating 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.

6.11 Distillation apparatus (traditional method), made of borosilicate glass or other suitable material to which can be fitted a digestion flask (6.8) 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 polychloroprene.

NOTE The distillation apparatus mentioned above can be replaced by the complete Parnas-Wagner[1)] distillation configuration or other suitable equipment.

6.12 Digestion block (block digesting method), aluminium alloy block or equivalent block, fitted with an adjustable temperature control and device for measuring block temperature.

6.13 Digestion tubes (block digesting method), of 250 ml in capacity, suitable for use with the digestion block (6.12).

6.14 Exhaust manifold (block digesting method), suitable for use with the digestion tubes (6.13).

6.15 Centrifugal scrubber apparatus or filter pump or aspirator (block digesting method), constructed of acid-resistant material, for use with mains water supply.

6.16 Volumetric flasks, one mark of 50 ml, 250 ml and 1 000 ml capacities.

6.17 Distillation unit (block digesting method), capable of steam distilling, manual or semi–automatic, suited to accept the 250 ml digestion tubes (6.13) and the 500 ml conical flasks (6.5).

1) Parnas-Wagner is an example of glassware configuration utilized for Kjeldahl distillation available commercially. This information is given for the convenience of users and does not constitute an endorsement by either ISO or IDF of this product.

6.18 Automatic titrator provided with a pH-meter.

The pH-meter should be calibrated properly in the range of pH 4 to pH 7 following normal laboratory pH-calibration procedures. The automatic titrator burette shall comply with the requirements of 6.6.

6.19 Spatula or suitable transfer device.

6.20 Filter paper, nitrogen–free, of dimensions and porosity suitable to hold the cheese test portion.

6.21 Illuminated magnetic stirrer plate.

7 Sampling

Sampling is not part of the method specified in this International Standard. A recommended sampling method is given in ISO 707|IDF 50.

It is important that the laboratory receive a sample which is representative and has not been damaged or changed during transport or storage.

8 Preparation of test sample

8.1 Whole, partially skimmed or skimmed liquid milk

Place the test sample in the water bath (6.1) set at 38 °C to 40 °C. Mix gently by inversion without causing frothing or churning. Once the sample is mixed thoroughly, cool to room temperature.

Proceed as indicated in 9.1 or 9.2.

8.2 Hard, semi-hard and processed cheese

Remove the rind, smear or mouldy surface layer of the cheese, in such a way as to provide a test sample representative of the cheese as it is usually consumed.

Grind the test sample by means of an appropriate device (6.7). Quickly mix the whole mass and, preferably, grind the mass again quickly. Analyse the test sample as soon as possible after grinding.

Using the spatula (6.19), weigh the required amount (Table A.1) of either prepared ground cheese onto a pre-folded, tared filter paper (6.20). Enclose cheese in filter paper and drop the filter paper containing the cheese into the bottom of a digestion flask (6.8) or digestion tube (6.13) as indicated in 9.1.1 or 9.2.1.

NOTE Use of a filter paper can promote foam formation in block digestion systems. To avoid this, when using the block digestion method (9.2), the filter paper can be omitted by weighing the sample into a suitable vessel, weighing the cheese and vessel, transferring the cheese to the digestion vessel, reweighing the empty vessel and determining the sample mass by subtracting the mass of the empty vessel from the mass of the cheese and vessel.

8.3 Dried milk and dried milk products

Let the test sample reach a temperature of between 20 °C and 25 °C before transferring to a container of internal volume approximately twice the volume of the test sample. Close the container immediately to avoid changing the moisture content of the sample. Thoroughly mix the sample by repeatedly rotating and inverting the container.

Proceed as indicated in 9.1 or 9.2.

9 Procedures

9.1 Traditional method

9.1.1 Test portion and pre-treatment

Add to a clean and dry digestion flask (6.8), 5 to 10 boiling aids (6.9), 15,0 g of potassium sulfate (5.1), 1,0 ml of copper (II) sulfate solution (5.2), the amount of prepared test sample (8.1, 8.2 or 8.3) as indicated in Table A.1, weighed to the nearest 0,1 mg, and 25 ml of sulfuric acid (5.3) while using sulfuric acid to wash down any copper (II) sulfate solution, potassium sulfate or test portion left on the neck of the flask. Gently mix the contents of the digestion flask.

As an alternative to 5.1 and 5.2, commercially available tablets containing, for example 15 g of potassium sulfate and 0,05 g of copper (II) sulfate pentahydrate, may be used, provided

a) the tablets contain a quantity of potassium sulfate such that the required amount can be dispensed using an integer number of whole tablets to maintain a similar salt to acid (5.3) ratio. For instance, three tablets each containing 5 g of potassium sulfate, may be used with 20 ml of sulfuric acid (5.3), and

b) the tablets do not contain salts of toxic metals, such as selenium or mercury.

9.1.2 Determination

9.1.2.1 Digestion

Turn on the fume extraction system of the digestion apparatus (6.10) prior to beginning the digestion. Heat the digestion flask and its contents (9.1.1) on the digestion apparatus using a heater setting low enough such that charred digest does not foam up the neck of the digestion 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 6.10 and continue the heating period for 15 min. At the end of the 15-min period increase the heat to maximum setting determined in 6.10. After the digest clears (clear with light blue-green colour), continue boiling for 1 h to 2,5 h at maximum setting. If visible boiling of the clear liquid is not apparent as bubbles forming at the surface of the hot liquid, the temperature of the heating device might be too low. The total digestion time will be between 1,8 h and 3,25 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 2,5 h) after clearing. Other milk products require samples of compositions similar to those being tested. The mean protein result increases with increasing boil time, becomes constant and then decreases when boil time is too long. Select the boil time that yields the maximum protein result for the product tested.

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 heating device 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 might 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 digestion flasks or 400 ml of water when using the 800 ml digestion flasks. Wash down the neck of the flask during the addition of water. Mix the contents thoroughly ensuring that any crystals that separate out are dissolved. Allow the mixture to cool again to room

temperature prior to distillation. Diluted digests may be stoppered and held for distillation at a later stage.

9.1.2.2 Distillation

Turn on the condenser water for the distillation apparatus (6.11). Add 75 ml of sodium hydroxide solution (5.4) to the diluted digest (9.1.2.1) by carefully pouring the solution down the inclined neck of the digestion 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 (6.11), the tip of whose condenser outlet tube is immersed in 50 ml of the boric acid solution (5.6) contained in a conical flask (6.5). Vigorously swirl the digestion flask to mix its contents thoroughly until no separate layers of solution are visible in the flask anymore. Turn on the burner of the steam generator to a setting high enough to boil the contents of the digestion flask. Continue distillation until irregular boiling (bumping) starts and then immediately disconnect the digestion 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 is 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, 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 colourimetric end point.

9.1.2.3 Titration

Titrate the contents of the conical flask (9.1.2.2) with the hydrochloric acid standard volumetric solution (5.7) using a burette (6.6).The end point 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 (6.21) may aid visualization of the end point.

Alternatively, titrate the contents of the conical flask (9.1.2.2) with the hydrochloric acid standard volumetric solution (5.7) using a properly calibrated automatic titrator provided with a pH meter (6.18). The pH end point of the titration is reached at pH 4,6, being the steepest point in the titration curve (inflection point). Read the amount of the used titrant on the automatic titrator.

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 N HCl is very fast within this range of pH. It takes about 0,05 ml of 0,1 N HCl to change 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 lab method performance statistics for this method were determined using a colour end point titration. Comparing the final test results, including those for their blank tests, obtained with a pH 4,6 end point with those of a colour end point titration showed that, statistically, no significant difference was demonstrable between them.

9.2 Block digestion method

9.2.1 Test portion and pre-treatment

Add to a clean and dry digestion tube (6.13), 12,0 g of potassium sulfate (5.1), 1,0 ml of copper (II) sulfate solution (5.2), the amount of prepared test sample (8.1, 8.2 or 8.3) as indicated in Table A.1, weighed to the nearest 0,1 mg, and 20 ml of sulfuric acid (5.3) while using sulfuric acid to wash down any copper

(II) sulfate solution, potassium sulfate or test portion left on the upper walls of the digestion tube. Gently mix the contents of the tube.

NOTE Volumes of acid greater than 20 ml in the block digestion systems give excessive foaming problems during digestion and variable results. Users of block digestors are expected to note that maintaining sufficient residual sulfuric acid at the end of digestion needs more attention by the analyst in block digestors than in traditional systems. Excessive acid loss due to over aspiration of fumes is of greater concern in block digestors than in traditional systems.

As an alternative to 5.1 and 5.2, commercially available tablets containing, for example 3,5 g of potassium sulfate, 0,105 g of copper (II) sulfate pentahydrate and 0,105 g of titanium dioxide may be used, provided

a) the tablets contain a quantity of potassium sulfate such that the required amount can be dispensed using an integer number of whole tablets maintain a similar salt to acid (5.3) ratio. For instance, two tablets each containing 3,5 g potassium sulfate, may be used with 12 ml of sulfuric acid (5.3), and

b) the tablets do not contain salts of toxic metals, such as selenium or mercury.

9.2.2 Determination

9.2.2.1 Digestion

Set the digestion block (6.12) at a low initial temperature to control foaming (approximately at between 180 °C and 230 °C). Transfer the tube to the digestion block and place the exhaust manifold (6.14), which itself is connected to a centrifugal scrubber of similar device (6.15), 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.

In instances where foaming is not an issue, the digestion tubes (6.13) with the test portion (9.2.1) may be transferred to the digestion block (6.12) initially set at a temperature between 410 °C and 430 °C without further temperature adjustments.

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 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 period, 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, the temperature of the block might be too low.

The total digestion time might be between 1,75 h and 3 h. Longer digestion times will be required for those products where the foaming was controlled by lowering the initial digestion temperature below 410 °C.

To determine the specific boiling time required for analysis conditions in a particular laboratory using a particular set of apparatus, select for fluid milk analysis a high-protein, high-fat milk sample and determine its protein content using different boil times (1 h to 2,5 h) after clearing. Other milk products will require samples of compositions similar to those being tested. The mean protein result increases with increasing boil time, becomes constant and then decreases when boil time is too long. Select the boil time that yields the maximum protein result for the product tested.

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 temperature 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 approximately 25 min, remove the exhaust manifold and carefully add 85 ml of water to each tube. Swirl to mix while ensuring that any crystals that have separated out are dissolved. Allow the contents of the tube to cool to room temperature.

9.2.2.2 Distillation

Turn on the condenser water for the distillation apparatus. Attach the digestion tube containing the diluted digest to the distillation unit (6.18). Place a conical flask (6.5) 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 (6.17) to dispense 55 ml of sodium hydroxide solution (5.4).

In cases where a mass fraction of 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, large variability in duplicate results will occur.

Having due regard to 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 is collected. Remove the conical flask from the distillation unit and completely drain the distillation tip. Rinse the in- and outside of the tip with water collecting the rinsing in the conical flask. Rinse the tip always 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 colourimetric end point.

9.2.2.3 Titration

Titrate the contents of the conical flask (9.2.2.2) with the hydrochloric acid standard volumetric solution (5.7) using a burette (6.6). The end-point 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 (6.21) may aid visualization of the end-point.

Alternatively, titrate the contents of the conical flask (9.2.2.2) with the hydrochloric acid standard volumetric solution (5.7) using a properly calibrated automatic titrator provided with a pH meter (6.18). The pH end point of the titration is reached at pH 4,6, being the steepest point in the titration curve (inflection point). Read the amount of the used titrant on the automatic titrator.

NOTE 1 The first trace of pink is observed between 4,6 pH and 4,3 pH 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 N HCl is very fast within this range of pH. It takes about 0,05 ml of 0,1 N HCl to change pH by 0,3 units in the range of pH from 4,6 pH to 4,3 pH in this system.

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

9.3 Blank test

Always titrate blanks with the same hydrochloric acid standard volumetric solution (5.7) and burette (6.6) or automatic titrator provided with a pH meter (6.18) as used for the test portions. Carry out a

blank test following the procedure described in 9.1 or 9.2. Replace the test portion with 5 ml of water and about 0,85 g of sucrose (5.10).

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

Sucrose (5.10) in the amount of 0,85 g may be used without water addition for this purpose.

NOTE 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 0,00 ml. Blanks within the same laboratory should be consistent across 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 humid air that can condense on the outside of the condenser apparatus has dripped down into the collection flask. Typical blank values are equal to or below 0,2 ml.

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 or 9.2.

9.4.2 Check that no loss of nitrogen occurs by using a test portion of 0,12 g of ammonium sulfate (5.8) along 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, which is bound in the protein structures.

The percentage of nitrogen recovered shall be greater than 99 % for all positions on the apparatus. For recoveries less than 99 %, the normality of the titrant may be 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 distill. 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 being submerged under the surface of the boric acid early in the distillation. The apparatus should pass this test before going on to check recoveries by the procedure in 9.4.3.

In the case where recoveries of nitrogen exceed 100 % and no loss of nitrogen can be seen, some possible causes are

a) the ammonium sulfate is contaminated,

b) the actual normality 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.

Although the maximum theoretical recovery should not exceed 100 %, recoveries higher than the maximum can be obtained in practice due to uncertainty of measurement, i.e. 99 % to 101 % might be obtained. If the mean recovery of a number of tests is greater than 100 %; the cause should be investigated.

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) along with 0,67 g of sucrose (5.10).

NOTE While tryptophan (5.9) is an adequate indicator of digestion efficiency for liquid milks, it is not an adequate indicator for dried milk and dried milk products; lysine hydrochloride (5.9) is intended to be used to provide a more representative indication of digestion efficiencies for these dried products.

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, the temperature or time of digestion is insufficient or there is undigested sample material (i.e. char) on the inside of the Kjeldahl flask.

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 standard volumetric hydrochloric acid solution (5.7).

Laboratories performing this test are encouraged to participate in an international proficiency testing programme.

10 Calculation and expression of results

10.1 Calculation

10.1.1 Nitrogen content

Calculate the nitrogen content of the test sample, w_n, using Formula (1):

$$w_n = \frac{1,400\,7 \times (V_s - V_b) \times M_t}{m} \quad (1)$$

where

w_n is the nitrogen content of the sample, expressed as a percentage mass fraction;

V_s is the numerical value of the volume of the hydrochloric acid standard volumetric solution (5.7) used in the determination (9.1.2.3 or 9.2.2.3), in millilitres, expressed to at least the nearest 0,05 ml;

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

M_t is the numerical value of the exact molarity of the hydrochloric acid standard volumetric solution (5.7), expressed to four decimal places. If sulfuric acid is substituted for hydrochloric acid, M_t is the exact molarity of the sulfuric acid multiplied by a factor of 2, expressed to four decimal places;

m is the numerical value of the mass of the test portion (9.1.1 or 9.2.1), in grams, expressed to the nearest 0,1 mg.

10.1.2 Crude protein content

Calculate the crude protein content, w_p, using Formula (2):

$$w_p = w_n \times 6{,}38 \quad (2)$$

where

w_p is the crude protein content, expressed as a percentage mass fraction;

w_n is the nitrogen content of the sample, expressed as a percentage mass fraction, to four decimal places (10.1.1);

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

10.1.3 Recovery

Calculate the nitrogen recovery, R_n, using Formula (3):

$$R_n = \frac{w_n \times 100}{T_n} \quad (3)$$

where

R_n is recovery of nitrogen, expressed as a percentage mass fraction;

w_n is the nitrogen content of the sample, expressed as a percentage mass fraction;

T_n is the theoretical nitrogen content of the substance, expressed as a percentage mass fraction.

The theoretical nitrogen content for ammonium sulfate is 21,20 %, tryptophan is 13,72 % and lysine hydrochloride is 15,34 %.

The lower limits of recovery, in 9.4, are based on the minimum assay specified in 5.7 and 5.8. No additional allowance for reagent purity should be made in the calculation of recovery.

10.2 Expression of results

10.2.1 General

The obtained results should not be rounded further until the final use of the test value is made.

This is particularly true when the values are going to be used for further calculation. 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 lab 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.

10.2.2 Nitrogen content

Express the obtained results to four decimal places, if needed for further calculations. In cases where the results are end results, express the results to three decimal places.

10.2.3 Crude protein content

Express the obtained results to three decimal places, if needed for further calculations. In cases where the results are end results, express the results to two decimal places.

11 Precision

11.1 Interlaboratory tests

Interlaboratory trials were conducted according to ISO 5725-1 and ISO 5725-2. The values for repeatability and reproducibility for milk and milk products described in this International Standard have been published.[5][6][7][8][9][10] The values derived from these trials might not be applicable to concentration ranges and matrices other than those given.

11.2 Liquid milk, whole milk and skimmed milk

11.2.1 Repeatability

11.2.1.1 Cow's milk

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

11.2.1.2 Goat's milk

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 0,008 4 % nitrogen content (0,052 % for crude protein).

11.2.1.3 Sheep's milk

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 0,007 8 % nitrogen content (0,050 % for crude protein).

11.2.2 Reproducibility

11.2.2.1 Cow's milk

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

11.2.2.2 Goat's milk

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 0,013 1 % nitrogen content (0,084 % for crude protein).

11.2.2.3 Sheep's milk

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 0,011 4 % nitrogen content (0,073 % for crude protein).

11.3 Hard, semi-hard and processed cheese

11.3.1 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 0,048 9 % nitrogen content (0,312 % for crude protein).

11.3.2 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 0,067 0 % nitrogen content (0,428 % for crude protein).

11.4 Dried milk and dried milk products

11.4.1 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 not in more than 5 % of cases be greater than 0,007 M, where M is the arithmetic mean of the two results.

11.4.2 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 0,013 M, where M is the arithmetic mean of the two results.

12 Test report

The test report shall specify:

a) all the information required for the complete identification of the sample;

b) the sampling method used, if known;

c) the method used, with reference to this International Standard, i.e. ISO 8968-1|IDF 20-1;

d) all operating details not specified in this International Standard, or regarded as optional, together with details of any incident which might have influenced the result(s);

e) the test result(s) obtained and, if the repeatability or the recovery has been checked, the final quoted result obtained.

Annex A
(informative)

Test portion

Table A.1 — Test portion sizes for the determination of nitrogen and calculation of crude protein in milk and milk products using the Kjeldahl principle

Material	Test portion size
Fluid cow's milk, whole and skimmed	5 g ± 0,10 g
Fluid goat's milk, whole	5 g ± 0,10 g
Fluid sheep's milk, whole	2,5 g ± 0,10 g
Hard, semi-hard and processed cheese	1 g ± 0,05 g
Dried milk and milk-based infant formulae	0,5 g ± 0,05 g
Milk protein concentrate, whey protein concentrates, casein and caseinates	0,25 g ± 0,05 g

Annex B
(informative)

Collaborative trials

Table B.1 — Summary of collaborative trial statistical parameters for percentage crude protein (N × 6,38) in milk and milk products determined by the Kjeldahl principle, with outliers excluded[5][6][7][8][9]

Material	Statistics						
	Mean (%)	s_r	s_R	$C_{V,r}$ (%)	$C_{V,R}$ (%)	r (2,8 s_r)	R (2,8 s_R)
Fluid cow's milk, whole and skimmed	3,395	0,014	0,017	0,39	0,50	0,038	0,049
Fluid goat's milk, whole	4,807	0,018	0,030	0,37	0,62	0,052	0,084
Fluid sheep's milk, whole	5,398	0,018	0,026	0,35	0,49	0,050	0,073
Cheese	26,461	0,111	0,153	0,42	0,58	0,312	0,428

Table B.2 — Summary of collaborative trial statistical parameters for percentage crude protein (N × 6,38) in milk and milk products determined by the Kjeldahl principle, with outliers excluded[10]

Material	Statistics						
	Mean (%)	s_r	s_R	$C_{V,r}$ (%)	$C_{V,R}$ (%)	r (2,8 s_r)	R (2,8 s_R)
Whole milk powder (2000)	23,47	0,067	0,077	0,28	0,33	0,19	0,22
Whole milk powder (1995)	25,37	0,042	0,100	0,16	0,39	0,12	0,28
Skim milk powder (2004)	33,85	0,066	0,111	0,20	0,33	0,18	0,31
Skim milk powder (2009)	32,32	0,083	0,142	0,26	0,44	0,23	0,40
Infant formula (2011)	11,71	0,039	0,064	0,34	0,54	0,11	0,18
Infant formula (2010)	11,90	0,022	0,049	0,19	0,41	0,06	0,14
Milk protein concentrate (1971)	42,98	0,181	0,275	0,42	0,64	0,51	0,77
Milk protein concentrate (1992)	84,67	0,273	0,422	0,32	0,50	0,76	1,18
Whey protein concentrate (2005)	78,86	0,122	0,262	0,15	0,33	0,34	0,73
Whey protein concentrate (2002)	92,36	0,292	0,355	0,32	0,38	0,82	0,99
Casein (2006)	86,45	0,251	0,464	0,29	0,54	0,70	1,30
Casein (2001)	87,20	0,150	0,583	0,17	0,67	0,42	1,63
Caseinate (1988)	92,08	0,157	0,477	0,17	0,52	0,44	1,34
Caseinate (1984)	92,27	0,170	0,363	0,18	0,39	0,48	1,02

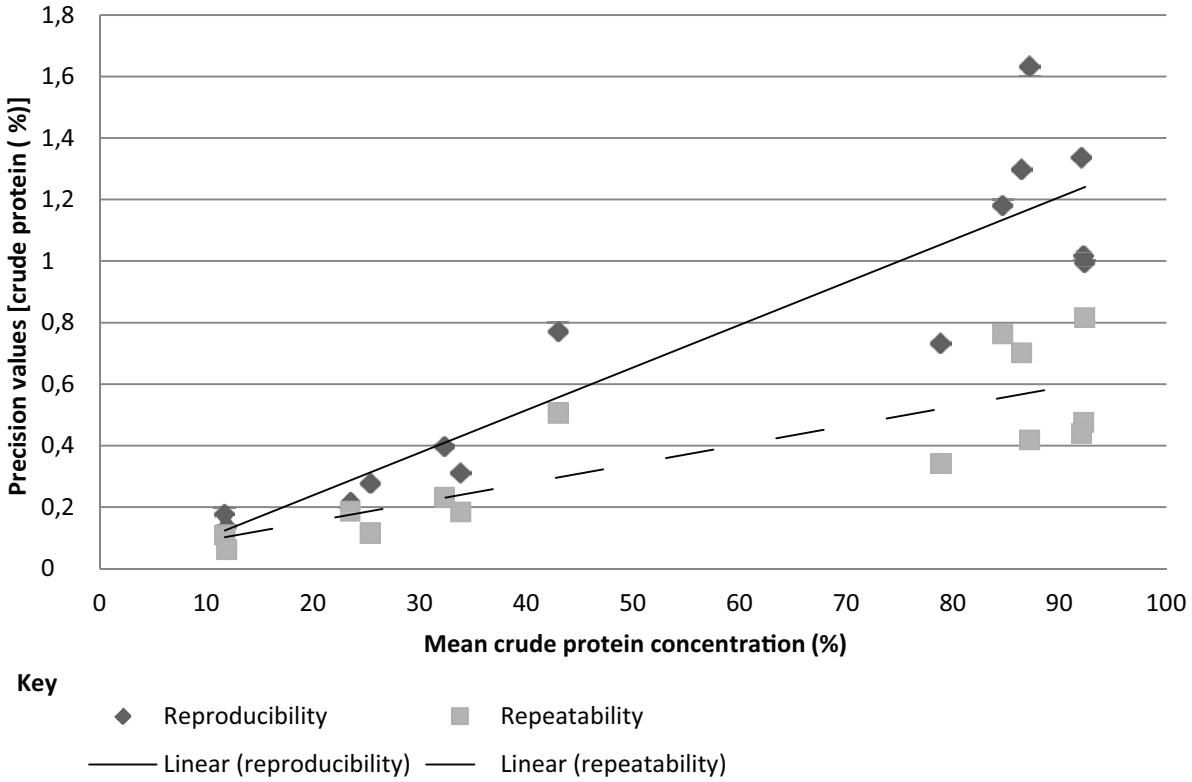

Figure B.1 — Relationship of *r* and *R* with concentration of crude protein (%)[10]

As the precision values are strongly correlated with protein concentration, the use of relative repeatability and reproducibility values is appropriate.

The mean relative repeatability, *r*, is 0,69 % and the mean relative reproducibility, *R*, is 1,28 %.

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