INTERNATIONAL STANDARD

ISO 5983-2

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Animal feeding stuffs — Determination of nitrogen content and calculation of crude protein content —

Part 2: Block digestion/steam distillation method

Aliments des animaux — Détermination de la teneur en azote et calcul de la teneur en protéines brutes —
Partie 1: Méthode de digestion en bloc et distillation à la vapeur

Reference number
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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 2.

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 document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

ISO 5983-2 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 10, *Animal feeding stuffs*.

This first edition of ISO 5983-2, together with ISO 5983-1:2005, cancels and replaces ISO 5983:1997, which has been technically revised.

ISO 5983 consists of the following parts, under the general title *Animal feeding stuffs — Determination of nitrogen content and calculation of crude protein content*:

— *Part 1: Kjeldahl method*

— *Part 2: Block digestion/steam distillation*

Animal feeding stuffs — Determination of nitrogen content and calculation of crude protein content —

Part 2:
Block digestion/steam distillation method

WARNING — The use of this method 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 method to establish appropriate health and safety practices and determine the applicability of local regulatory limitations prior to use.

1 Scope

This part of ISO 5983 specifies a method for the determination of nitrogen content of animal feeding stuffs according to the Kjeldahl method, and a method for the calculation of the crude protein content.

It concerns a semi-micro rapid routine method using block-digestion, copper catalyst and steam distillation into boric acid.

The method is applicable to the determination of greater than 0,5 % Kjeldahl nitrogen in animal feeding stuffs, pet foods and their raw materials.

The method does not measure oxidized forms of nitrogen nor heterocyclic nitrogen compounds.

The method does not distinguish between protein nitrogen and non-protein nitrogen.

NOTE If it is of importance to determine the content of non-protein-nitrogen, an appropriate method can be used.

2 Normative references

The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referred document (including any amendments) applies.

ISO 385:2005, *Laboratory glassware — Burettes*

ISO 1871, *Agricultural food products — General directions for the determination of nitrogen by the Kjeldahl method*

ISO 6498:1998, *Animal feeding stuffs — Preparation of test samples*

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 procedure specified in this document

NOTE The nitrogen content is expressed as a percentage by mass or in grams per kilogram.

3.2
crude protein content
amount of nitrogen content (3.1) multiplied by the factor 6,25

NOTE The crude protein content is expressed as a percentage by mass or in grams per kilogram.

4 Principle

The test portion is digested using a block-digestion or equivalent apparatus. Concentrated sulfuric acid is used to convert protein nitrogen to ammonium sulfate at a boiling point elevated by the addition of potassium sulfate. A copper catalyst is used to enhance the reaction rate. An excess of sodium hydroxide is added to the cooled digest to liberate ammonia.

The liberated ammonia is distilled, using a manual, semi-automatic or fully automatic steam distillation unit. In the case of manual or semi-automatic steam distillation, distillation of the ammonia into an excess of boric acid solution is followed by titration with hydrochloric acid solution to a colorimetric endpoint. Where a fully automatic system is employed, automatic titration of the ammonia is carried out simultaneously with the distillation.

The nitrogen content is calculated from the amount of ammonia produced. The crude protein content is obtained by multiplying the result by the conventional conversion factor of 6,25.

NOTE 1 As in ISO 5983-1, the automatic titration of the ammonia can also be carried out with endpoint detection using a potentiometric pH system (see Annex B).

NOTE 2 In principle, sulfuric acid could also be used for the titration.

5 Reagents

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

5.1 Kjeldahl catalyst tablets, comprising of 3,5 g of potassium sulfate and 0,4 g of copper(II) sulfate pentahydrate per tablet.

These tablets may be purchased ready prepared.

Other types of tablet may be used provided that

a) they contain a quantity of potassium sulfate such that 7 g of potassium sulfate and 0,8 g of copper(II) sulfate pentahydrate can be dispensed using an integer number of whole tablets, and

b) they do not contain salts of toxic metals such as selenium or mercury.

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

5.3 Hydrogen peroxide solution, containing approximately 30 g of H_2O_2 per 100 ml.

5.4 Antifoaming agent.

A silicone preparation is recommended, for example with a mass fraction of 30 % aqueous emulsion.

5.5 Sodium hydroxide (NaOH) solution, approximately 40 % (mass fraction), nitrogen-free (< 5 µg of N per gram).

5.6 Indicator solutions.

5.6.1 Methyl red solution.

Dissolve 100 mg of methyl red ($C_{15}H_{15}N_3O_2$) in 100 ml of ethanol or methanol.

5.6.2 Bromocresol green solution.

Dissolve 100 mg of bromocresol green ($C_{21}H_{14}Br_4O_5S$) in 100 ml of ethanol or methanol.

5.7 Concentrated boric acid solution, $c(H_3BO_3) = 40{,}0$ g/l.

Dissolve 400 g of boric acid in about 5 l to 6 l of hot deionized water. Mix and add more hot deionized water to a volume of about 9 l. Allow to cool to room temperature. Add 70 ml of the methyl red solution (5.6.1) and 100 ml of the bromocresol green solution (5.6.2) and mix. Dilute to a final volume of 10 l with water and mix well. Depending on the water used, the pH of the boric acid solution can differ from batch to batch. Often an adjustment with a small volume of alkali is necessary to obtain a positive blank (0,05 ml to 0,15 ml of titrant). The colour shall turn green when 100 ml of distilled water are added to 25 ml of the boric acid solution. If still red, titrate with 0,1 mol/l NaOH until "neutral grey" and calculate the amount of alkali needed for the 10 l batch.

Store the solution, which will be red in colour, at room temperature and protect the solution from light and sources of ammonia fumes during storage.

5.8 Dilute boric acid solution, $c(H_3BO_3) = 10{,}0$ g/l (optional trapping solution for titrators that automatically begin titration when distillation begins).

Dissolve 100 g of boric acid in about 5 l to 6 l of hot deionized water, mix and add more hot deionized water to a volume of about 9 l. Allow to cool to room temperature. Add 70 ml of the methyl red solution (5.6.1) and 100 ml of the bromocresol green solution (5.6.2) and mix. Dilute to a final volume of 10 l. Depending on the water used, the pH of the boric acid solution can differ from batch to batch. Often an adjustment with a small volume of alkali is necessary to obtain a positive blank (0,05 ml to 0,15 ml of titrant). The colour shall turn green when 100 ml of distilled water are added to 25 ml of the boric acid solution. If still red, titrate with 0,1 mol/l NaOH until "neutral grey" and calculate the amount of alkali needed for the 10 l batch.

Store the solution, which will be light green in colour, at room temperature and protect the solution from light and sources of ammonia fumes during storage.

NOTE The addition of about 3 ml to 4 ml of 0,1 M NaOH into 1 l of 1 % boric acid usually gives good adjustments.

5.9 Hydrochloric acid standard volumetric solution, $c(HCl) = 0{,}100\ 0$ mol/l.

Other concentrations of HCl or sulfuric acid may be used if this is corrected for in the calculations. The concentrations should always be expressed to four decimal places.

5.10 Ammonium sulfate [$(NH_4)_2SO_4$], min. 99,5 % (mass fraction), with certified purity. Dry ammonium sulfate at 102 °C ± 2 °C for 4 h and store in a desiccator.

Percent nitrogen in ammonium sulfate (at 99,5 % purity) is 21,09.

5.11 Ammonium iron(II) sulfate [$(NH_4)_2{\cdot}Fe(SO_4)_2{\cdot}6H_2O$], with certified purity.

Percent nitrogen in ammonium iron(II) sulfate (at 100 % purity) is 7,145.

5.12 Standard materials

One of the following (5.12.1 or 5.12.2) standard materials may be used.

In addition to the standard materials listed below, suitable reference materials with certified values for Kjeldahl nitrogen/protein should be used whenever possible.

NOTE The moisture content can be checked on a separate portion.

5.12.1 Tryptophan ($C_{11}H_{12}N_2O_2$), with melting point 282 °C; nitrogen content 137,2 g/kg. Before use, dry the tryptophan.

5.12.2 Acetanilide (C_8H_9NO), minimum assay 99 % (mass fraction). Nitrogen content 103,6 g/kg. Do not dry in an oven before use.

5.13 Sucrose ($C_{12}H_{22}O_{11}$), with a nitrogen content of not more than 0,002 % (mass fraction). Do not dry in an oven before use.

6 Apparatus

Usual laboratory apparatus and, in particular, the following.

6.1 Analytical balance, capable of weighing to the nearest 0,1 mg, with a readability of 0,1 mg.

6.2 Digestion block, aluminium alloy block or equivalent block, fitted with an adjustable temperature control and device for measuring block temperature, warmed up to 420 °C ± 5 °C.

6.3 Digestion tubes, of capacity 250 ml, suitable for use with the digestion block (6.2).

6.4 Exhaust manifold, suitable for use with the digestion tubes (6.3).

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

6.6 Automatic pipettes (dispensers), capable of delivering up to 25 ml portions.

6.7 Graduated measuring cylinders, of capacity 50 ml.

6.8 Distillation unit, capable of steam distilling, manual or semi-automatic, suitable to accept the digestion tubes (6.3) and the conical flasks (6.9), or capable of steam distillation and autotitration.

6.9 Conical flasks, of capacity 250 ml.

6.10 Burette, of capacity 25 ml or suitable capacity, with at least a readability of 0,05 ml, complying with the requirements of ISO 385:2005, class A.

Alternatively, an automatic burette may be used fulfilling the same requirements.

7 Sampling

A representative sample should have been sent to the laboratory. It should not have been damaged or changed during transport or storage.

Sampling is not part of the method specified in this part of ISO 5983. A recommended sampling method is given in ISO 6497.

8 Preparation of test sample

Prepare the test sample in accordance with ISO 6498.

9 Procedure

9.1 General

Usually test samples should be analysed in batches according to the described procedure. For general requirements on the application of the Kjeldahl method, see ISO 1871.

9.2 Test portion

As the test portion, weigh, to the nearest 0,1 mg,

— approximately 1,0 g for materials with 3 % to 30 % protein,

— approximately 0,5 g for materials with 30 % to 80 % protein, or

— approximately 0,3 g for materials with more than 80 % protein.

Do not exceed 1,2 g.

Always perform quality control and standards as well as a reagent blank with each batch.

9.3 Determination

9.3.1 Digestion

Transfer the test portion (9.2) to the digestion tube (6.3) and add two catalyst tablets (5.1) to each tube. Using a pipetting dispenser, add 12 ml of sulfuric acid (5.2) to each tube. Use 15 ml for high fat materials (> 10 % fat). It is possible to stop at this point and continue work the following day.

If foaming is a problem, slowly add 3 ml to 5 ml of hydrogen peroxide (5.3). Swirl gently and let the reaction subside. Alternatively a few drops of antifoaming agent (5.4) may be used.

Attach the heat side shields to the tube rack. Place the exhaust manifold (6.4) tightly on the tubes and turn the water aspirator or scrubber (6.5) on completely. Place the rack of tubes in the pre-heated (420 °C) digestion block.

After 10 min, turn the water aspirator down until the acid fumes are just contained within the exhaust hood. A condensation zone should be maintained within the tube. After the bulk of the sulfur oxide fumes are produced during the initial stages of digestion, the vacuum source shall be reduced to prevent loss of sulfuric acid.

Digest for an additional 50 min. The total digestion time should be approximately 60 min.

Turn the digestor off. Remove the rack of tubes with the exhaust still in place and put it in the stand to cool for 10 min to 20 min. When fuming has stopped, remove the manifold and shut off the aspirator. Remove the side shields.

Allow the tubes to cool. It is recommended to predilute samples manually prior to distilling. Wearing gloves and eye protection, carefully add a few millilitres of deionized water to each tube. If spattering occurs, this

means that the tubes are still too hot. Allow to cool for a few more minutes. Add water to each tube to a total volume of approximately 80 ml.

If the sample solidifies, place the tube containing the diluted digest in the block digester and carefully warm with occasional swirling until salts dissolve, or distil for a further 30 s to 60 s.

NOTE 1 Some instruments perform the addition of water automatically. The predilution before placing the tube in the instrument is only required if very solid cakes form.

NOTE 2 Some distillation instruments start with the addition of steam before the addition of alkali, which leads to a dissolution of salt cakes and a less violent reaction during alkali addition. Crystallization during digestion can cause nitrogen losses.

9.3.2 Distillation

Transfer the digestion tube (see 9.3.1) to the distillation unit (6.8).

Where titration of the ammonia content of the distillate is performed manually, the procedure mentioned below applies. Where the distillation unit is fully automated to include titration of the ammonia content of the distillate, follow the manufacturer's instructions for operation of the distillation unit.

Place a conical flask (6.9) containing 25 ml to 30 ml of the concentrated boric acid solution (5.7) under the outlet of the condenser in such a way that the delivery tube is below the surface of the excess boric acid solution. Adjust the distillation unit to dispense 50 ml of sodium hydroxide solution (5.5). Operate the distillation unit in accordance with the manufacturer's instructions and distil off the ammonia liberated by the addition of the sodium hydroxide solution. Collect the distillate in the boric acid receiving solution. The amount of distillate (time of steam distillation) depends on the amount of nitrogen in the sample. Follow the manufacturer's instructions.

NOTE In a semi-automatic distillation unit, the addition of excess sodium hydroxide and the steam distillation are performed automatically.

9.3.3 Titration

Titrate the contents of the conical flask (9.3.2) with the hydrochloric acid standard volumetric solution (5.9) using a burette (6.10) and read the amount of titrant used. The endpoint is reached at the first trace of pink colour in the contents. Estimate the burette reading to the nearest 0,05 ml. An illuminated magnetic stirrer plate or a photometric detector may aid visualization of the endpoint.

This can be done automatically using a steam distiller with automatic titration.

Follow the manufacturer's instructions for operation of the specific distiller or distiller/titrator.

When an automatic titration system is used, titration begins immediately after distillation starts and the 1 % boric acid solution (5.8) should be used.

Where a fully automatic distillation unit is used, the automatic titration of the ammonia may also be carried out with endpoint detection using a potentiometric pH system (see Annex B).

9.4 Blank test

Carry out a blank test following the procedure described in 9.1 to 9.3.3 taking 2 ml of water and about 0,7 g of sucrose (5.13) instead of the test portion. Keep a record of blank values. If blank values change, identify the cause.

The amount of titrant used in the blank test should always be greater than 0,0 ml. Blanks within the same laboratory should be consistent over time.

9.5 Recovery tests

9.5.1 General

The regularly run recovery tests to check the accuracy of the procedure and equipment are described in 9.5.2 to 9.5.4.

9.5.2 Nitrogen loss

Use 0,12 g of ammonium sulfate (5.10) and 0,67 g of sucrose (5.13) per flask. Add all other reagents as stated in 9.3. Digest and distil under the same conditions as for the sample. Recoveries shall be $\geqslant$ 99 %.

9.5.3 Digestion efficiency

Use a test portion of at least 0,15 g of tryptophan (5.12.1) or acetanilide (5.12.2), weighed to the nearest 0,1 mg, and with addition of about 0,7 g of sucrose (5.13). Determine the nitrogen content according to the procedure described in 9.1 to 9.3.3. The recoveries should be $\geqslant$ 99,5 % for acetanilide (5.12.2) and $\geqslant$ 98,5 % for tryptophan (5.12.1) [5].

9.5.4 Distillation and titration efficiency

Weigh 0,10 g to 0,15 g, to the nearest 0,000 1 g of ammonium sulfate (5.10), or 0,3 g to 0,5 g, to the nearest 0,000 1 g, of ammonium iron(II) sulfate (5.11) into a tube. Add 80 ml of distilled water and proceed according to 9.3.2 and 9.3.3. The recovery shall be $\geqslant$ 99,5 %.

9.5.5 Limits

Recoveries less than specified or more than 101,0 % in any of the above recovery tests indicate failures in the procedures and/or inaccurate concentration of the standard volumetric hydrochloric acid solution (5.9).

10 Calculation and expression of results

10.1 Calculation

10.1.1 Calculation of nitrogen content

Calculate the nitrogen content of the sample, w_N, as a percentage by mass:

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

where

V_s is the numerical value of the volume of the hydrochloric acid standard volumetric solution (5.9) used in the determination (9.3), in millilitres, expressed to the nearest 0,05 ml;

V_b is the numerical value of the volume of the hydrochloric acid standard volumetric solution (5.9) used in the blank test (9.4), in millilitres, expressed to the nearest 0,05 ml;

c_s is the numerical value of the exact concentration, in moles per litre, of the hydrochloric acid standard volumetric solution (5.9), expressed to four decimal places;

m is the numerical value of the mass of the test portion (9.2), in grams.

For reporting the result in grams per kilogram, a factor of 14,007 may be used in the above equation.