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Animal feeding stuffs — Determination of soluble nitrogen content after treatment with pepsin in dilute hydrochloric acid

Aliments des animaux — Détermination de la teneur en azote soluble après traitement avec de la pepsine dans l'acide chlorhydrique dilué

Reference number
ISO 6655:1997(E)

Foreword

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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.

International Standard ISO 6655 was prepared by Technical Committee ISO/TC 34, *Agricultural food products*, Subcommittee SC 10, *Animal feeding stuffs*.

Annex A forms an integral part of this International Standard.

Annexes B and C are for information only.

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Animal feeding stuffs — Determination of soluble nitrogen content after treatment with pepsin in dilute hydrochloric acid

1 Scope

This International Standard specifies a method for the determination of the soluble nitrogen content of animal feeding stuffs after treatment with pepsin in dilute hydrochloric acid.

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

NOTES
1 The values obtained on using this method have no direct connection with digestibility *in vivo*.
2 If non-protein nitrogen is to be excluded from the test result, its content should be determined using an appropriate method.

2 Normative references

The following standards contain provisions which, through reference in this text, constitute provisions of this International Standard. At the time of publication, the editions indicated were valid. All standards are subject to revision, and parties to agreements based on this International Standard are encouraged to investigate the possibility of applying the most recent editions of the standards indicated below. Members of IEC and ISO maintain registers of currently valid International Standards.

ISO 5983: 1997[1], *Animal feeding stuffs - Determination of nitrogen content and calculation of crude protein content — Kjeldahl method.*

ISO 6498:1983, *Animal feeding stuffs - Preparation of test samples.*

3 Principle

Incubation of the sample for 48 h at 40 °C in a solution of pepsin in dilute hydrochloric acid.

Filtration of the suspension and determination of the nitrogen content of either the filtrate or the residue remaining in the filter in accordance with the Kjeldahl method specified in ISO 5983. In the latter case, determination of the nitrogen content of the sample as well, in accordance with ISO 5983.

4 Reagents

Use only reagents of recognized analytical grade, and distilled or demineralized water or water of at least equivalent purity.

The reagents (except the standard materials) shall be practically free from nitrogenous compounds.

[1] To be published. (Revision of ISO 5983:1979)

4.1 Dilute hydrochloric acid, $c(HCl)$ = 0,075 mol/l.

4.2 Pepsin, having an activity of 2,0 units per milligram in accordance with the definition given in annex A. Check the pepsin activity in accordance with the method specified in annex A.

4.3 Pepsin solution in hydrochloric acid, with a pepsin activity of about 400 units per litre.

Dissolve 0,2 g ± 0,001 g of pepsin (4.2) in 1 l of dilute hydrochloric acid (4.1). Prepare this solution immediately before use.

If the activity of the pepsin deviates from 2,0 units per milligram, adjust the mass of pepsin to obtain a solution with a pepsin activity of 400 units per litre.

4.4 Hydrochloric acid, $c(HCl)$ = 7,5 mol/l (ρ_{20} = 1,125 g/ml).

5 Apparatus

Usual laboratory apparatus and, in particular, the following.

5.1 Water bath or **incubator,** capable of being maintained at 40 °C ± 1 °C.

5.2 Kjeldahl digestion flasks, of suitable capacity.

5.3 Filter paper, fast filtration rate, acid-resistant.

5.4 Distillation and titration equipment

6 Sampling

Sampling is not part of the method specified in this International Standard. A recommended sampling method is given in ISO 6497 [3].

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

7 Preparation of test sample

Prepare the test sample in accordance with ISO 6498.

If the fat content of the test sample exceeds 10 % (*m*/*m*), extract the fat in accordance with ISO 6498, and take the content of extracted fat into account in the calculation (clause 9).

8 Procedure

8.1 Test portion

Weigh, to the nearest 0,001 g, about 2 g of the prepared test sample.

8.2 Incubation

Place the test portion in a 500 ml volumetric flask and add 450 ml of the pepsin solution (4.3) previously heated to 40 °C. Shake so as to avoid agglomeration. Check that the pH of the suspension is lower than 1,7. Place the flask in the water bath or incubator (5.1) set at 40 °C and leave it there for 48 h. Shake after 8 h, 24 h and 32 h.

After 48 h, add 15 ml of hydrochloric acid (4.4) and cool to 20 °C. Dilute to the mark with water, shake and filter through filter paper (5.3). Proceed in accordance with 8.3 or 8.4.

8.3 Determination of the nitrogen content of the filtrate

8.3.1 Digestion of organic matter

Take 250 ml of the filtrate (8.2) and transfer it into a Kjeldahl digestion flask (5.2). Add the reagents necessary for digestion as specified in ISO 5983:1997, 8.2.1. Mix and boil.

NOTE — It may be advisable to add an antifoaming agent.

Keep the solution boiling vigorously until the water has almost completely evaporated. Eliminate the last traces of water with care, reducing the rate of heating. After the liquid has become clear, continue heating for 1 h, then leave to cool.

8.3.2 Distillation and titration

Proceed as specified in ISO 5983:1997, 8.2.2 and 8.2.3.

8.3.3 Blank test

Carry out a blank test using the same procedure but omitting the test portion. Proceed in accordance with clause 9.

8.4 Determination of the nitrogen content of the residue and of the test sample

8.4.1 Digestion of organic matter in the residue

Wash the filter paper and residue (8.2) with warm water until they are free from acid. Transfer the filter paper with residue to a Kjeldahl digestion flask (5.2). Add the reagents necessary for digestion as specified in ISO 5983:1997, 8.2.1. Mix and boil.

NOTE — It may be advisable to add an antifoaming agent.

Remove the water by boiling vigorously initially and next by reducing the rate of heating to eliminate the last traces of water. After the liquid has become clear, continue heating for 1 h, then leave to cool.

8.4.2 Distillation and titration

Proceed as specified in ISO 5983:1997, 8.2.2 and 8.2.3.

8.4.3 Determination of the nitrogen content of the test sample

Determine the nitrogen content of the prepared test sample (see clause 7) in accordance with ISO 5983.

8.4.4 Blank test

Carry out a blank test using the same procedure but omitting the test portion.

9 Expression of results

9.1 Calculation of the soluble nitrogen content obtained using the procedure specified in 8.3

Provided that the quantities of sulfuric acid used to collect the ammonia for the determination and for the blank test are equal (see ISO 5983), calculate the soluble nitrogen content of the test sample by the equation

$$w_1 = \frac{2(V_0 - V_1) \times c \times M}{m}$$

where

w_1 is the soluble nitrogen content, in grams per kilogram, of the test sample obtained by the procedure specified in 8.3;

c is the concentration, in moles per litre, of the sodium hydroxide solution (see ISO 5983:1997, 4.9.1) used for the titrations;

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

M is the molar mass, in grams per mole, of nitrogen (M = 14 g/mol);

V_0 is the volume, in millilitres, of the sodium hydroxide solution (see ISO 5983:1997, 4.9.1) used for the blank test;

V_1 is the volume, in millilitres, of the sodium hydroxide solution (see ISO 5983:1997, 4.9.1) used for the determination (8.3.2).

Report the result to the nearest 0,1 g/kg.

9.2 Calculation of the soluble nitrogen content obtained using the procedure specified in 8.4

Provided that the quantities of sulfuric acid used to collect the ammonia for the determination and for the blank test are equal (see ISO 5983), calculate the soluble nitrogen content of the test sample by the equation

$$w_2 = w_N - \frac{(V_0 - V_1) \times c \times M}{m}$$

where

w_2 is the soluble nitrogen content, in grams per kilogram, of the test sample obtained by the procedure specified in 8.4;

c is the concentration, in moles per litre, of the sodium hydroxide solution (see ISO 5983:1997, 4.9.1) used for the titrations;

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

M is the molar mass, in grams per mole, of nitrogen (M = 14 g/mol);

V_0 is the volume, in millilitres, of the sodium hydroxide solution (see ISO 5983:1997, 4.9.1) used for the blank test;

V_1 is the volume, in millilitres, of the sodium hydroxide solution (see ISO 5983:1997, 4.9.1) used for the determination (8.4.2);

w_N is the nitrogen content, in grams per kilogram, of the test sample determined in 8.4.3.

Report the result to the nearest 0,1 g/kg.

9.3 Calculation of soluble crude protein content

If it is desired to express the result as soluble crude protein, multiply the soluble nitrogen content determined by the factor 6,25.

10 Precision

10.1 Interlaboratory test

Details of an interlaboratory test on the precision of the method are given in annex B. The values derived from this interlaboratory test may not be applicable to concentration ranges and matrices other than those given.

10.2 Repeatability

The absolute difference between two independent single test results, obtained using the same method on identical test material in the same laboratory by the same operator using the same equipment within a short interval of time, will in not more than 5 % of cases exceed the value of r shown in table 1.

10.3 Reproducibility

The absolute difference between two single test results, obtained using the same method on identical test material in different laboratories by different operators using different equipment, will in not more than 5 % of cases exceed the value of *R* shown in table 1.

Table 1 - Repeatability limit (*r*) and reproducibility limit (*R*)

Sample	Mean soluble crude protein content g/kg[1)]	*r* g/kg	*R* g/kg
Alfalfa meal	136,6	7,2	26,5
Maize gluten feed	141,5	8,9	28,9
Coconut meal	149,5	7,4	31,6
Grass silage	192,9	7,9	36,3
Bone meal	512,0	8,9	63,1
Feather meal	574,3	22,1	166,3

1) Based on dry matter.

11 Test report

The test report shall specify:

- all information necessary for the complete identification of the sample;
- the method with which sampling was carried out, if known;
- the method used;
- the test result(s) obtained, expressed as soluble nitrogen or soluble crude protein and, in the latter case the conversion factor used (i.e. 6,25);
- if the repeatability has been checked, the final quoted result obtained;
- all operating details not specified in this International Standard, or regarded as optional, together with details of any incidents occurred when performing the method, which may have influenced the test result(s).

Annex A
(normative)

Determination of pepsin activity

A.1 Scope

This annex specifies a method for the determination of the activity of the pepsin used in the determination of soluble nitrogen content after treatment with pepsin in dilute hydrochloric acid.

2 Definition

For the purposes of this annex, the following definition applies.

A.2.1 unit of pepsin activity: That quantity of pepsin which liberates per minute, under the conditions specified, a quantity of hydroxyaryl groups whose coloration by Folin-Ciocalteu's reagent has an absorbance equivalent to that of 1 µmol of tyrosine under the same conditions.

A.3 Principle

Treatment of haemoglobin with pepsin in dilute hydrochloric acid. Precipitation of the non-hydrolysed protein fraction by trichloroacetic acid.

Filtration and addition of sodium hydroxide and Folin-Ciocalteu's reagent. Measurement of the absorbance of this solution at a wavelength of 750 nm and determination of the corresponding quantity of tyrosine from a calibration curve.

A.4 Reagents

Use only reagents of recognized analytical grade, and distilled or demineralized water or water of at least equivalent purity.

A.4.1 Hydrochloric acid, c(HCl) = 0,2 mol/l.

A.4.2 Hydrochloric acid, c(HCl) = 0,06 mol/l.

A.4.3 Hydrochloric acid, c(HCl) = 0,025 mol/l.

A.4.4 Trichloroacetic acid solution, $\rho(CCl_3CO_2H)$ = 50 g/l.

A.4.5 Sodium hydroxide solution, c(NaOH) = 0,5 mol/l.

A.4.6 Folin-Ciocalteu's reagent.

Place 100 g of sodium tungstate dihydrate ($Na_2WO_4.2H_2O$), 25 g of sodium molybdate dihydrate ($Na_2MoO_4.2H_2O$) and 700 ml of water in a 2 l round-bottomed flask with a ground glass neck. Add 50 ml of phosphoric acid [$\rho(H_3PO_4)$ = 1,71 g/ml] and 100 ml of concentrated hydrochloric acid [ρ(HCl) = 1,19 g/ml]. Fit a reflux condenser to the flask, bring the solution to the boil and keep gently boiling for 10 h. Allow to cool, detach the reflux condenser, add 175 g of lithium sulfate dihydrate ($Li_2SO_4.2H_2O$), 50 ml of water and 1 ml of bromine. Boil for 15 min to eliminate the excess bromine.

Allow to cool then decant the solution into a 1 l volumetric flask. Dilute to the mark with water, mix and filter. No greenish colouring shall remain.

Before use, dilute 1 volume of this reagent with 2 volumes of water.

A.4.7 Haemoglobin solution.

Weigh a quantity of haemoglobin-protein substrate according to Anson's method (about 2 g) corresponding to 354 mg of nitrogen and place it in a 200 ml flask with a ground glass neck and a reference line at 100 ml.

NOTE — If necessary, determine the nitrogen content of the substrate using a semi-micro Kjeldahl method. The theoretical nitrogen content is 17,7 % (*m*/*m*).

Add a few millilitres of dilute hydrochloric acid (A.4.2), connect the flask to a vacuum pump and shake until the haemoglobin has completely dissolved. Release the vacuum and add, while shaking continuously, dilute hydrochloric acid (A.4.2) to make up to approximately 100 ml.

Prepare this solution immediately before use.

A.4.8 Tyrosine standard solution, $c(C_9H_{11}NO_3)$ = 0,2 mmol/l.

To prepare a stock solution, dissolve in a 1 l volumetric flask 181,2 mg of tyrosine in dilute hydrochloric acid (A.4.1) and dilute to the mark with the same.

Using a pipette, transfer 20 ml of the stock solution to a 100 ml volumetric flask. Add a quantity of dilute hydrochloric acid (A.4.1) and dilute to the mark with the same.

The tyrosine concentration of this standard solution is 0,2 µmol/ml (= 0,2 mmol/l).

A.5 Apparatus

Usual laboratory apparatus and, in particular, the following.

A.5.1 Water bath, capable of being maintained at 25 °C ± 1 °C.

A.5.2 Spectrometer, capable of carrying out measurements at a wavelength of 750 nm.

A.5.3 Precision chronometer, capable of being read to the nearest 1 s.

A.5.4 pH-meter, capable of being read to the nearest 0,1 pH unit.

A.5.5 Glass rod, thicker at one end than at the other.

A.6 Procedure

A.6.1 Preparation of the solution

Dissolve 150 mg of pepsin (or the quantity necessary to obtain an absorbance value of 0,35 ± 0,035) in 100 ml of dilute hydrochloric acid (A.4.2).

Using a pipette, transfer 2 ml of this solution to a 50 ml volumetric flask and dilute to the mark with dilute hydrochloric acid (A.4.3). The pH of this solution shall be 1,6 ± 0,1.

Immerse the flask in the water bath (A.5.1) set at 25 °C .

A.6.2 Hydrolysis

Using a pipette, transfer 5,0 ml of the haemoglobin solution (A.4.7) to a test tube and heat to a temperature of 25 °C ± 1 °C in the water bath (A.5.1). Add 1,0 ml of the pepsin solution obtained in A.6.1 and mix using a glass rod (A.5.5) with about 10 backwards and forwards movements. Keep the test tube in the water bath at 25 °C ± 1 °C for 10 min ± 1 s, timed from the addition of the pepsin solution. The duration and temperature