International Standard 5510

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Animal feeding stuffs — Determination of available lysine

Aliments des animaux — Dosage de la lysine disponible

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Foreword

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Animal feeding stuffs — Determination of available lysine

0 Introduction

Lysine in food products is generally considered to be assimilable when its terminal amino group (amino ϵ) is not combined. This fraction of lysine can be determined because these groups combine with 2,4-dinitrofluorobenzene.

1 Scope and field of application

This International Standard specifies a method for the determination of the available lysine in animal feeding stuffs containing animal or vegetable proteins.

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Compared with biological determination, the method does, however, overestimate the amount of available lysine and care is necessary in interpreting the results.

2 References

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ISO 5983, *Animal feeding stuffs — Determination of nitrogen content and calculation of crude protein content.*

ISO 6497, *Animal feeding stuffs — Sampling.*[1]

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

3 Definition

available lysine: The quantity of lysine corresponding to the difference between the total lysine and the non-available lysine determined under the conditions specified in this International Standard.

It is expressed as a percentage by mass of the raw product.

4 Principle

Hydrolysis with hydrochloric acid of a ground test portion, followed by separation of the total lysine by column chromatography and determination by spectrometry at 570 nm.

Reaction of a second ground test portion with an ethanolic solution of 2,4-dinitrofluorobenzene in an alkaline medium, purification, then hydrolysis with hydrochloric acid, separation of the non-available lysine by column chromatography, and determination by spectrometry at 570 nm.

5 Reagents

All the reagents shall be of recognized analytical grade. The water used shall be distilled water or water of at least equivalent purity.

5.1 Diethyl ether, free from peroxides.

5.2 Sodium bicarbonate, 80 g/l solution.

5.3 2,4-Dinitrofluorobenzene (DNFB), ethanolic solution.

Dissolve 0,15 ml of DNFB in 12 ml of 95 % (*V*/*V*) ethanol.

Prepare this solution at the time of use.

5.4 Hydrochloric acid, approximately 6 mol/l .

Mix 1 volume of hydrochloric acid (ϱ_{20} = 1,19 g/ml) with 1 volume of water.

5.5 Sodium citrate, buffer solution, pH 2,2 approximately.

Successively dissolve in water

21 g of citric acid monohydrate;

8 g of sodium hydroxide;

16 ml of hydrochloric acid (ϱ_{20} = 1,19 g/ml);

0,1 ml of octanoic (caprylic) acid;

20 ml of thiodiglycol;

3 ml of a 30 % (*V*/*V*) aqueous solution of polyoxyethylene dodecyl ether with approximately 23 molecules of oxyethylene.[2]

Dilute to 1 000 ml with water.

1) At present at the stage of draft.

2) A suitable commercially available product is BRIJ 35. This information is given for the convenience of the user of this International Standard and does not constitute an endorsement of this product by ISO.

5.6 Sodium citrate, buffer solution, pH 5,28.

Successively dissolve in water

24,5 g of citric acid monohydrate;

14 g of sodium hydroxide;

6,8 ml of hydrochloric acid (ϱ_{20} = 1,19 g/ml);

0,1 ml of octanoic (caprylic) acid;

3 ml of a 30 % (*V*/*V*) aqueous solution of polyethoxyl dodecyl ether with approximately 23 molecules of oxyethylene.[1)]

Dilute to 1 000 ml with water. Adjust the pH to 5,28 ± 0,02 using hydrochloric acid (ϱ_{20} = 1,19 g/ml) or 50 % (*m*/*m*) sodium hydroxide solution.

5.7 Ninhydrin reagent.

5.7.1 Sodium propionate, buffer solution, pH 5,5 ± 0,1.

Dissolve 168 g of sodium hydroxide in about 400 ml of water. Cool, then add while shaking, 498 ml of propionic acid. Dilute to 1 000 ml with water.

5.7.2 Preparation of reagent.

Prepare the ninhydrin reagent in an atmosphere of nitrogen and in the dark. Into a 2 l flask, place 1 litre of peroxide-free ethylene glycol monomethyl ether, 1 litre of the sodium propionate buffer solution (5.7.1) and 40 g of ninhydrin. Shake until completely dissolved, then add 1,33 g of tin(II) chloride dihydrate ($SnCl_2.2H_2O$). Shake until completely dissolved. (See the note.)

This reagent is stable for 1 month if kept at 4 °C under light pressure in an atmosphere of nitrogen and in the dark.

NOTE — If precipitation of $SnCl_2$ occurs, replace the tin(II) chloride with 7,5 ml of 150 g/l titanium (III) chloride solution or by 1,5 g of hydrindantin per litre of reagent.

5.8 Lysine, standard solution, corresponding to 1 mmol of lysine base per litre.

Dissolve 182,5 mg of lysine monohydrochloride in 100 ml of 0,1 mol/l hydrochloric acid. Take exactly 10 ml of this solution and dilute it to 100 ml with sodium citrate buffer solution of pH 2,2 (5.5).

1 ml of this solution contains 1 µmol of lysine base.

6 Apparatus

Usual laboratory equipment, and in particular:

6.1 Grinder, having the following characteristics:

a) constructed of a material which does not absorb moisture;

b) easy to clean and having minimum dead space;

c) permitting rapid and uniform grinding, without causing undue heating and preventing, as much as possible, contact with the outside air;

d) capable of being regulated to give particles of the sizes specified in 7.1.

6.2 Flasks, of capacities 250 ml and 1 000 ml, flat-bottomed, short-necked with ground conical joints and **reflux condensers** to fit the flasks.

6.3 Crucible, with a sintered glass plate of grade P 16 (pore size index 10 to 16 µm).

6.4 Oil-bath, capable of being maintained at a temperature which will ensure refluxing (120 to 130 °C).

6.5 Rotary evaporator.

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6.6 Apparatus for automatic analysis of amino acids, or if this is not available, **manual chromatographic system** comprising:

a) a chromatography column, of length about 250 mm and of internal diameter 6 mm, thermostatically maintained at 55 °C by means of a water jacket and circulation bath, and filled up to 200 mm with an 8 % cross-linked strongly acidic, cation exchange resin with sulfonic functional groups, of particle size 13 ± 2 µm;[2)]

b) small piston pump, producing a flow of 50 ml/h;

c) fraction collector;

d) boiling water-bath;

e) spectrometer, set at 570 nm, with cells of thickness 10 mm.

6.7 Graduated pipettes, of capacities 1; 5; and 10 ml.

6.8 Volumetric flasks, of capacities 10; 20; and 100 ml.

6.9 Analytical balance.

7 Sampling

See ISO 6497.

1) A suitable commercially available product is BRIJ 35. This information is given for the convenience of the user of this International Standard and does not constitute an endorsement of this product by ISO.

2) A suitable commercially available product is Aminex A5. This information is given for the convenience of the user of this International Standard and does not constitute an endorsement of this product by ISO.

8 Procedure

8.1 Preparation of the test sample

Grind 5 to 10 g of the laboratory sample to obtain particles which will pass completely through a sieve of aperture size 315 µm.

NOTE — The aperture size specified is smaller than that recommended in ISO 6498 in order to ensure maximum contact with the DNFB.

8.2 Test portions

Weigh, to the nearest 1 mg, and place respectively in the two flasks (6.2), two test portions each of which corresponds to approximately 100 mg of crude protein. (See ISO 5983 for the determination of the protein content.)

8.3 Total lysine

8.3.1 Hydrolysis with hydrochloric acid

Into the 1 000 ml flask (see 8.2), place 500 ml of the 6 mol/l hydrochloric acid (5.4). Fit a reflux condenser and place the flask in the oil-bath (6.4), previously heated to 120 to 130 °C.

After boiling gently for 24 h, cool the flask and filter the contents through the crucible (6.3). Evaporate the filtrate at a temperature not exceeding 40 °C, using the rotary evaporator (6.5). Add water to the residue thus obtained and evaporate. Repeat this operation until most of the hydrochloric acid has been removed; in general, four rinses with 30 ml of water are sufficient.

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Dissolve the residue in sodium citrate buffer solution of pH 2,2 (5.5) and transfer quantitatively to a 100 ml one-mark volumetric flask (6.8). Make up to the mark with the sodium citrate buffer solution of pH 2,2 (5.5). Filter through a folded filter paper.

8.3.2 Final preparation of the column and adsorption of the hydrolysate

Connect the pump of the apparatus (6.6) to a reservoir containing sodium citrate buffer solution of pH 5,28 (5.6); then adjust it to obtain a flow of 50 ml/h. Connect the pump to the resin column, previously heated to 55 °C, and allow the buffer solution (5.6) to pass through the column for 20 min in order to establish equilibrium. Disconnect the pump. Remove most of the liquid above the resin, taking care that the surface of the resin does not become dry.

By means of a pipette (6.7), take 0,5 ml of hydrolysate (or 1 ml if using the manual system), place it on the column, then allow it to pass through the resin with the aid of a slight nitrogen pressure. Rinse the walls of the column with 0,5 ml of sodium citrate buffer solution of pH 5,28 (5.6) and pass through the resin. Fill the column to the top with sodium citrate buffer solution (5.6) and connect it to the pump.

8.3.3 Determination

8.3.3.1 Automatic system

Set the analyser in operation. Calibrate the apparatus, proceeding as described in 8.3.2 using 0,25 µmol of lysine [0,25 ml of the lysine standard solution (5.8)] or the amount specified in the manufacturer's instructions.

8.3.3.2 Manual system

8.3.3.2.1 Localization of lysine

The fraction of eluate containing the lysine shall be checked by using a lysine solution of known concentration, for example the 1 µmol/ml solution (5.8). For this purpose, discard the first 25 to 30 ml of eluate and then collect fractions of 1 ml in test-tubes until the 50th fraction is obtained, reckoned from the beginning of the elution. Add to each fraction, between the 30th and the 50th fractions, 1 ml of the ninhydrin reagent (5.7). Mix, transfer to the boiling water-bath and leave for 15 min. Cool. Dilute by adding 10 ml of sodium citrate buffer solution of pH 5,28 (5.6). Mix and measure the absorbance at 570 nm, by means of the spectrometer, using sodium citrate buffer solution of pH 5,28 (5.6) as the reference liquid.

NOTE — For information only, under the conditions described, lysine generally elutes between the 39th and 45th fractions.

8.3.3.2.2 Determination

If the lysine is eluted between the 39th and 45th fractions discard the first 38 ml of eluate, collect and combine the fractions (Nos 39 to 45) corresponding to the lysine peak and evaporate using the rotary evaporator (6.5).

NOTE — After recovering the fractions containing the lysine, allow about 200 ml of buffer solution to pass through the column in order to remove any undesirable components which may remain.

Dissolve the residue in 5 ml of the sodium citrate buffer solution of pH 5,28 (5.6) and add 5 ml of the ninhydrin reagent (5.7). Mix, transfer to the boiling water-bath and leave for 15 min. Cool. Dilute the mixture with the sodium citrate buffer solution of pH 5,28 (5.6) so that the lysine concentration of the test solution is approximately 0,02 µmol/ml. (Let V_1 be the volume of test solution thus obtained.)

Measure the absorbance at 570 nm by means of the spectrometer, using as the reference liquid a mixture of one part by volume sodium citrate buffer solution (5.6) and one part by volume ninhydrin reagent (5.7), which has been placed for 15 min in the boiling water-bath and diluted to the volume V_1 after cooling.

8.3.3.2.3 Calibration of the spectrometer

Take exactly 5 ml of the standard lysine solution (5.8). Add 5 ml of the ninhydrin reagent (5.7). Mix, transfer to the boiling water-bath and leave for 15 min. Cool and dilute to 100 ml with sodium citrate buffer solution of pH 5,28 (5.6).

Measure the absorbance, by means of the spectrometer, under the same conditions as in 8.3.3.2.2.

8.4 Non-available lysine

8.4.1 Dinitrophenylation reaction

By means of a pipette (6.7), transfer 8 ml of the sodium bicarbonate solution (5.2) to the 250 ml flask (see 8.2). Leave for

about 10 min, stirring from time to time. Then add the DNFB solution (5.3), stopper the flasks, agitate, and ensuring that there are no particles adhering to the walls of the flask, leave the mixture overnight at ambient temperature and in darkness.

8.4.2 Purification

Evaporate to dryness at a temperature below 40 °C, using the rotary evaporator (6.5). Add 75 ml of the diethyl ether (5.1) to the flask, stir and allow to separate. Pour off most of the diethyl ether, taking care to avoid entraining solid particles with it. Repeat these operations twice more, using each time 50 ml of the diethyl ether. Evaporate the last traces of diethyl ether by heating on the rotary evaporator.

8.4.3 Hydrolysis with hydrochloric acid

Transfer to the flask 150 ml of the hydrochloric acid (5.4) and proceed as described in 8.3.1 but transfer quantitatively the residue, dissolved in sodium citrate buffer solution of pH 2,2 (5.5) to a 20 ml volumetric flask (6.8) (or 10 ml if a manual system is being used).

8.4.4 Deposition of the hydrolysate and determination

Proceed as described in 8.3.2 and 8.3.3.

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In the case of the manual system, the final reading on the spectrometer may be made by diluting the volume of the lysine plus reagent mixture to 20 ml with sodium citrate buffer solution of pH 5,28 (5.6) (Let V_2 be the volume of test solution thus obtained.)

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8.5 Number of determinations

Carry out two determinations on the same test sample using two different pairs of test portions.

9 Expression of results

9.1 Method of calculation and formulae

9.1.1 Total lysine

9.1.1.1 Determination using an automatic analyser

The total lysine, w_1, expressed as a percentage by mass, is equal to

$$\frac{0{,}365}{m_1 \times V_0} \times \frac{S_1}{S_0}$$

where

S_0 is the area of the peak corresponding to 0,25 µmol of lysine;

S_1 is the area of the peak corresponding to the total lysine determined in 8.3.3.1;

m_1 is the mass, in grams, of the test portion placed in the first flask;

V_0 is the volume, in millilitres, of hydrolysate transferred to the column (generally, V_0 = 0,5 ml).

9.1.1.2 Determination using a manual system

The total lysine, w_1, expressed as a percentage by mass, is equal to

$$\frac{V_1 \times 0{,}073}{m_1 \times V_0} \times \frac{A_1}{A_0}$$

where

A_0 is the absorbance of the standard lysine solution determined in 8.3.3.2.3;

A_1 is the absorbance determined in 8.3.3.2.2;

V_0 is the volume, in millilitres, of hydrolysate transferred to the column (generally, V_0 = 1 ml);

V_1 is the volume, in millilitres, of the test solution;

m_1 is the mass, in grams, of the test portion placed in the first flask.

9.1.2 Non-available lysine

9.1.2.1 Determination using an automatic analyser

The non-available lysine, w_2, expressed as a percentage by mass, is equal to

$$\frac{0{,}073}{m_2 \times V_0} \times \frac{S_2}{S_0}$$

where

S_0 is the area of the peak corresponding to 0,25 µmol of lysine;

S_2 is the area of the peak corresponding to the non-available lysine, determined in 8.4.4;

m_2 is the mass, in grams, of the test portion placed in the second flask;

V_0 is the volume, in millilitres, of hydrolysate transferred to the column (generally, V_0 = 0,5 ml).

9.1.2.2 Determination using a manual system

The non-available lysine, w_2, expressed as a percentage by mass, is equal to

$$\frac{V_2 \times 0{,}007\,3}{m_2 \times V_0} \times \frac{A_2}{A_0}$$

where

A_0 is the absorbance of the standard lysine solution determined in 8.3.3.2.3;

A_2 is the absorbance determined in 8.4.4;

V_0 is the volume, in millilitres, of hydrolysate transferred to the column (generally, V_0 = 1 ml);

V_2 is the volume, in millilitres, of the test solution (generally, V_2 = 20 ml);

m_2 is the mass, in grams, of the test portion placed in the second flask.

9.1.3 Available lysine

The available lysine, w_3, expressed as a percentage by mass, is equal to

$$w_1 - w_2$$

where

w_1 is the total lysine (see 9.1.1);

w_2 is the non-available lysine (see 9.1.2).

NOTE — The available lysine, expressed as a percentage by mass of total lysine is given by the formula

$$\frac{(w_1 - w_2)}{w_1} \times 100$$

9.2 Repeatability

The difference between the results of two determinations carried out simultaneously or in rapid succession by the same analyst shall not exceed 10 % of the mean value of the results obtained.

10 Test report

The test report shall show the method used and the results obtained. It shall also mention all operating details not specified in this International Standard, or regarded as optional, together with details of any incidents likely to have influenced the results.

The test report shall include all the information necessary for the complete identification of the sample.

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