
**Animal feeding stuffs —
Determination of lysine, methionine
and threonine in commercial amino
acid products and premixtures**

*Aliments des animaux — Détermination de la teneur en lysine,
méthionine et thréonine dans les acides aminés industriels et les pré-
mélanges*



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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.

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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.

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Animal feeding stuffs — Determination of lysine, methionine and threonine in commercial amino acid products and premixtures

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1 Scope

This International Standard specifies a method for the quantitative determination of free (non-protein-bound) lysine, methionine, and threonine in commercial products and premixtures containing more than about 10 % mass fraction of the respective amino acid. It does not distinguish between D- and L-forms.

NOTE For the purposes of this International Standard, the term “amino acids” used in [Clause 2](#) onwards refers to lysine, methionine, and threonine.

2 Principle

The samples are treated in dilute hydrochloric acid and then diluted with sodium citrate buffer. Norleucine internal standard is added and the amino acids are separated by an amino acid analyser or high performance liquid chromatography (HPLC), using a cation exchange resin and sodium citrate buffer eluent solutions. The amino acids are measured colourimetrically following post-column reaction with ninhydrin reagent or by fluorescence detection after post-column reaction with *ortho*-phthaldialdehyde (OPA).

3 Reagents and materials

Use only reagents of recognized analytical grade, unless otherwise specified.

3.1 Water, double distilled water or equivalent purity (conductivity <10 µS/cm).

3.2 Standard substances.

3.2.1 Lysine·HCl crystals, purity superior to 99 % mass fraction dried under vacuum in a desiccator for 2 days over P₂O₅ prior to use.

3.2.2 Threonine crystals, purity superior to 99 % mass fraction dried under vacuum in a desiccator for 2 days over P₂O₅ prior to use.

3.2.3 Methionine crystals, purity superior to 99 % mass fraction dried under vacuum in a desiccator for 2 days over P₂O₅ prior to use.

3.3 Norleucine crystals, for use as internal standard, purity superior to 99 % dried under vacuum in a desiccator for 2 days over P₂O₅ prior to use.

3.4 Sodium hydroxide solution, $c(\text{NaOH}) = 7,5 \text{ mol/l}$, for pH adjustment of sodium citrate buffer.

Carefully dissolve 300 g sodium hydroxide in water ([3.1](#)) and make up to 1 l.

3.5 Hydrochloric acid, $\gamma(\text{HCl}) = 370 \text{ g/kg}$.

3.6 Sodium citrate dihydrate.

3.7 2,2'-Thiodiethanol (thiodiglycol).

3.8 Phenol crystals, purity superior to 98,5 % mass fraction.

3.9 Sodium citrate buffer, pH 2,20.

Dissolve 19,61 g of sodium citrate dihydrate (3.6), 5 ml of thiodiglycol (3.7), 1 g of phenol (3.8) and 16,5 ml of HCl (3.5) in 800 ml of water. Adjust the pH to 2,2 with a few drops of HCl (3.5) or NaOH solution (3.4). The phenol acts to preserve the buffer solution.

Sodium citrate buffer can be also prepared from citric acid and sodium chloride.

3.10 Hydrochloric acid solution, $c(\text{HCl}) = 0,1 \text{ mol/l}$.

Take 8,2 ml of HCl (3.5), dilute with approximately 900 ml of water (3.1). Mix thoroughly and make up to 1 l with water.

3.11 Standard solutions of amino acids.

3.11.1 Internal standard substance norleucine stock solution, $c(\text{Nle}) = 2,5 \text{ mmol/l}$.

Dissolve 0,328 g norleucine (3.3) and transfer quantitatively with 0,1 mol/l HCl (3.10) into a 1 l volumetric flask.

Store below 5 °C for no more than 6 months.

3.11.2 Amino acids standard stock solutions, $c = 2,5 \text{ mmol/l}$.

Prepare a standard stock solution for each molecule.

Standard solutions shall contain only the amino acid being analysed, i.e. lysine, threonine or methionine. Commercially available mixed standard solutions, e.g. containing 18 amino acids, do not give optimal results.

Transfer 0,456 g lysine·HCl (3.2.1), 0,297 g threonine (3.2.2), and 0,373 g methionine (3.2.3) into a 1 l volumetric flask and make up to the mark with 0,1 mol/l HCl (3.10).

Store below 5 °C for no more than 6 months.

3.11.3 Amino acids calibration solution.

3.11.3.1 Weight dilution. Weigh 2,5 ml of each amino acid stock solution m_{aa} (3.11.2) and 2,5 ml of norleucine stock solution m_{Nle} (3.11.1) into a 50 ml volumetric flask. Make up to volume with sodium citrate buffer (3.9).

Store the solution below 5 °C for not more than 1 week.

3.11.3.2 Volume dilution. Using a pipette (4.4) or dilutor (4.7), dilute equal volumes of amino acid stock solution (3.11.2) and norleucine solution (3.11.1) with sodium citrate buffer (3.9), e.g. 50 µl norleucine solution and 50 µl amino acid stock solution are diluted by the dilutor to 1 000 µl with sodium citrate buffer (3.9).

3.12 Elution buffers for cation exchanger column. Use commercially available buffers or prepare them according to the requirements specified by the analyser manufacturer. Typically three to five

buffer solutions are used containing sodium citrate or carbonate and small quantities of additives and preservatives.

3.13 Ninhydrin or OPA reagent. Use commercially available reagents or prepare them according to the requirements specified by the analyser manufacturer.

4 Apparatus

Usual laboratory apparatus and in particular the following.

4.1 Glass beakers, of capacity 1 000 ml.

4.2 Volumetric flask, of volume 50 ml, 100 ml, 500 ml, 1 000 ml.

4.3 Graduated measuring cylinders, of volume 100 ml, 1 000 ml.

4.4 Graduated pipettes, of volume 5 ml and 10 ml.

4.5 Magnetic stirring plates or mechanical shaker.

4.6 Membrane filters 0,2 µm consisting of cellulose acetate or PVDF.

4.7 Dilutor, optional for volumetric dilution.

If derivatization is done with ninhydrin, use a volume dilution ratio of 1→20. Use a higher ratio for OPA derivatization. Check the coefficient of variation (CV) of the dilution regularly with a balance; the CV shall be less than 1 %.

4.8 pH meter.

4.9 Analytical balance, readability 0,1 mg.

4.10 Amino acid analyser or equivalent **high performance liquid chromatography (HPLC)** equipment.

4.10.1 Cation exchange resin column placed in an oven.

4.10.2 Guard column.

4.10.3 Automatic or manual injection system, able to inject volumes from 10 µl to 100 µl.

4.10.4 HPLC pumps for buffers.

4.10.5 Ninhydrin or OPA post- column derivatization pump.

4.10.6 Channel UV detector set at 440 nm and 570 nm for ninhydrin post-column reaction or **fluorescence detector** set at excitation wavelength 330 nm and emission wavelength 460 nm for OPA post-column reaction.

4.10.7 Data acquisition and handling system for integration of peaks.

5 Procedure

5.1 Preparation of samples

Grind the sample (mill or mortar) until it passes through a sieve with 0,25 mm openings. Homogenize it thoroughly.

5.2 Number of prepared extracts

Prepare two different extracts of each sample.

5.3 Extraction using dilution by weighing

5.3.1 Pure amino acids trade products

Weigh 0,45 g to 0,47 g lysine·HCl (3.2.1), 0,29 g to 0,31 g threonine (3.2.2), 0,36 g to 0,38 g methionine (3.2.3) into weighed 500 ml bottles. Add approximately 400 ml 0,1 mol/l HCl (3.10). Dissolve the amino acid while stirring with a magnetic stirrer (4.5) for 30 min. Determine the total mass of extraction volume (m_{ex}) and weigh an aliquot of 1 ml using a graduated pipette (4.4) into a 50 ml volumetric flask (m_{ali}). Weigh additionally 2,5 ml norleucine solution (3.11.1) (m_{Nle}) into that flask, then fill to the mark with citrate buffer (3.9) and mix well.

If the sample solutions are not to be examined the same day, they shall be stored below 5 °C for not more than 1 week.

5.3.2 For premixes or impure amino acid trade products

Calculate the approximate mass, $m_{tp\text{ premix}}$, in grams, of test portion based on the amino acid with lowest expected values using Formula (1):

$$m_{tp\text{ premix}} = \frac{m_{tp\text{ pure aa}}}{w_{exp}} \times 100 \quad (1)$$

where

- $m_{tp\text{ pure aa}}$ is the mass, in grams, of the test portion used for pure amino acid;
- w_{exp} is the expected mass fraction, expressed in grams per 100 g, of the amino acid of lowest content.

For example, a premix with a 20 % mass fraction of DL-methionine requires test portion amount of 1,8 g to 1,9 g.

Weigh in the calculated test portion amount into weighed 500 ml bottles. Add approximately 400 ml 0,1 mol/l HCl. Dissolve the premix while stirring with a magnetic stirrer for 30 min. Determine the total mass of extraction volume (m_{ex}) and weigh an aliquot of 1 ml using a graduated pipette (4.4) into a 50 ml volumetric flask (m_{ali}). Weigh additionally 2,5 ml norleucine stock solution (3.11.1) (m_{Nle}) into that flask. Fill to the mark with citrate buffer (3.9) and mix well.

If the premix contains amino acids with high expected contents, dilutions using an aliquot of less than 1 ml of extract plus 2,5 ml norleucine (3.11.1) (m_{Nle}) may be prepared.

If the sample solutions are not to be examined the same day, they shall be stored below 5 °C for not more than 3 days.

5.4 Extraction using volumetric dilution

5.4.1 Pure amino acids trade products

Weigh 0,45 g to 0,47 g lysine·HCl, 0,29 g to 0,31 g threonine, 0,36 g to 0,38 g methionine. Transfer quantitatively with 0,1 mol/l HCl (3.10) into a 1 l volumetric flask. Dissolve in about 900 ml of 0,1 mol/l HCl, while stirring with a magnetic stirrer (4.5) for 30 min. Fill to volume with 0,1 mol/l HCl and mix well.

Dilute and add norleucine solution (3.11.1), following the procedure specified in 3.11.3.2 for the preparation of the calibration solution.

If the sample solutions are not to be examined the same day, they shall be stored below 5 °C for not more than 3 days.

5.4.2 For premixes or impure amino acid trade products

Calculate the approximate mass of test portion ($m_{tp\text{ premix}}$) in grams based on the amino acid with lowest expected values (w_{exp}) as described in 5.3.2. Use for extraction a test portion amount to ensure that all amino acids peak areas are in the linear range of the calibration. Weigh in this amount and transfer quantitatively with 0,1 mol/l HCl (3.10) into a 1 l volumetric flask. Dissolve in about 900 ml of 0,1 mol/l HCl, while stirring with a magnetic stirrer (4.5) for 30 min. Fill to volume with 0,1 mol/l HCl and mix well. Dilute and add norleucine solution (3.11.1), following the procedure specified in 3.11.3.2 for the preparation of the calibration solution.

If the premix contains other amino acids with higher expected contents, dilutions using an aliquot of less than 1 ml of extract plus 2,5 ml norleucine may be prepared.

If the sample solutions are not to be examined the same day, they shall be stored below 5 °C for not more than 3 days.

5.5 Chromatography

Filter a suitable amount of the test solution through membrane filter unit (4.6) into an auto sampler vial and inject into the analyser or HPLC system (4.10). The injection volume normally is 20 µl to 50 µl.

The chromatographic system is required to separate the amino acids from each other and from any other components (e.g. ammonia, amines, peptides or amino sugars).

The analyte amino acids are required to be 100 % baseline separated from all other peaks that are eluted to avoid erroneous results caused by peak overlap. The chromatographic system is required to provide a linear response over the concentration range of the standard curve.

6 Calculation of results

6.1 Principle of the method and control of the calibration

Contents in samples are calculated using the result of the initial calibration solution (3.11.3). In order to check there is no drift, inject the calibration solution as quality control after every four test solutions. If the recovery of these control solutions is outside the range 99 % to 101 %, repeat the analysis.

Determine the peak area of the amino acid peaks in the calibration solutions and test extracts by integration and calculate the content of the amino acid as a percentage mass fraction of the test portion as specified in 6.2 or 6.3.

6.2 Calculation if dilution by weighing is applied

Calculate the response factor ($F_{R\ aa}$) for each amino acid using Formula (2):

$$F_{R\ aa} = \frac{A_{Nle\ c} m_{aa}}{A_{aa\ c} m_{Nle}} \quad (2)$$

where

- $A_{Nle\ c}$ is peak area of norleucine in calibration solution;
- m_{aa} is mass of 2,5 ml amino acid stock solution expressed in grams (g);
- $A_{aa\ c}$ is peak area of amino acid in calibration solution;
- m_{Nle} is mass of 2,5 ml norleucine stock solution expressed in grams (g).

Calculate the amino acid content of the test samples, w_{aa} , expressed as percentage mass fraction, using Formula (3):

$$w_{aa} = \frac{A_{aa\ ts} F_{R\ aa} \gamma_{aa} m_{Nle\ ts} m_{ex}}{A_{Nle\ ts} m_{tp} m_{ali} \times 10} \quad (3)$$

where

- $A_{aa\ ts}$ is peak area of amino acid in test solution;
- $A_{Nle\ ts}$ is peak area of norleucine in test solution;
- $F_{R\ aa}$ is response factor of amino acids;
- γ_{aa} is concentration of amino acid in stock solution (g/1 000 g) assuming 1 l solution weighs 1 000 g;
- $m_{Nle\ ts}$ is mass of 2,5 ml norleucine stock solution in test solution; expressed in grams (g)
- m_{ex} is mass of total extract solution expressed in grams (g);
- m_{ali} is mass of extract aliquot used expressed in grams (g);
- m_{tp} is mass of test portion expressed in grams (g).

6.3 Calculation if volumetric dilution is applied

Calculate the response factor ($F_{R\ aa}$) for each amino acid using Formula (4)

$$F_{R\ aa} = \frac{A_{Nle\ c}}{A_{aa\ c}} \quad (4)$$

where

- $A_{Nle\ c}$ is peak area of norleucine in calibration solution;
- $A_{aa\ c}$ is peak area of amino acid in calibration solution.

Calculate the amino acid content of the test samples, w_{aa} , expressed as a percentage mass fraction, using Formula (5)

$$w_{aa} = \frac{A_{aa\ ts} F_{R\ aa} \gamma_{aa}}{A_{Nle\ ts} m_{tp}} \times 100 \quad (5)$$

where

- $A_{aa\ ts}$ is peak area of amino acid in test solution;
- $A_{Nle\ ts}$ is peak area of norleucine in test solution;
- $F_{R\ aa}$ is response factor of amino acids;
- γ_{aa} concentration of amino acid in stock solution (g/l);
- m_{tp} mass of test portion expressed in grams (g).

NOTE Results of the calculation are expressed on the product "as is". For the calculation of purity according to a specification, i.e. amino acid content in dry product, determine the dry matter on the product. The purity is directly obtained, if the product is dried prior the analysis.

The result for lysine·HCl can be converted into lysine by the following equation:

$$w_{Lys} = \frac{w_{Lys\cdot HCl}}{1,25}$$

where

- w_{Lys} is the lysine mass fraction;
- $w_{Lys\cdot HCl}$ is the lysine·HCl mass fraction.

7 Precision

7.1 Interlaboratory test

Details of interlaboratory tests on the precision of the method are summarized in [Annex A](#).

7.2 Repeatability

Perform the analyses in duplicate.

Calculate the relative spread, expressed as a percentage

$$D = \frac{|w_{aa1} - w_{aa2}|}{w_{aa1} + w_{aa2}} \times 200 \quad (6)$$

where

- w_{aa1} is the first value;
- w_{aa2} is the second value;
- $|w_{aa1} - w_{aa2}|$ is the absolute difference between the two values.

Report the mean value if D is lower than 1,5 %.

If the spread is greater, perform the analysis on a third extraction and report the mean of the three determinations.

If the CV is less than or equal to 3 %, record the mean value of the three determinations.

If the CV is above 3 %, reanalyse the test solutions after homogenization.

If the results from the two new extractions again are not confirmed ($D > 1,5$ %), the error is due to the chromatography. Check the analyser and repeat all affected measurements.

7.3 Reproducibility

The absolute difference between two test results (averages of two determinations) obtained using the same method on identical test material in different laboratories with different operators using different equipment will be, in 95 % of cases, less than the reproducibility limit R given in [Annex A](#).

8 Test report

The test report shall contain at least the following information:

- a) all information necessary for the complete identification of the sample;
- b) the sampling method used, if known;
- c) the test method used, with reference to this International Standard (ISO 17180:2013);
- d) all operating details not specified in this International Standard, or regarded as optional, together with details of any incidents which may have influenced the test result(s);
- e) the test result(s) obtained;
- f) if the repeatability has been checked, the final quoted result obtained.