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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 prémélanges

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Foreword

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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 P_2O_5 prior to use.

3.2.2 Threonine crystals, purity superior to 99 % mass fraction dried under vacuum in a desiccator for 2 days over P_2O_5 prior to use.

3.2.3 Methionine crystals, purity superior to 99 % mass fraction dried under vacuum in a desiccator for 2 days over P_2O_5 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 P_2O_5 prior to use.

3.4 Sodium hydroxide solution, c(NaOH) = 7,5 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, γ(HCl) = 370 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(HCl) = 0,1mol/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(Nle) = 2,5 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 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 2,5ml 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, $m_{tp\ premix}$, in grams, of test portion based on the amino acid with lowest expected values using Formula (1):

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

where

$m_{tp\ pure\ aa}$ 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 to1,9 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 norleucinestock 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 ($m_{\text{tp premix}}$) 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) nfor 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 aliqout 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.