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Animal feeding stuffs — Determination of tryptophan content

Aliments des animaux — Détermination de la teneur en tryptophane

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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 tryptophan content

1 Scope

This International Standard describes determination of the total and free tryptophan content in feeding stuffs (e.g. complete and complementary feeds, supplementary feeds, raw materials, ingredients, premixtures and concentrates). It does not distinguish between D- and L-forms.

2 Principle

For the determination of the total tryptophan, the sample is hydrolysed under alkaline conditions with saturated barium hydroxide solution and heated to 110 °C for 20 h. After hydrolysis, an internal standard is added.

For the determination of free tryptophan, the sample is extracted under mild acidic conditions in the presence of an internal standard.

The tryptophan and the internal standard in the hydrolysate or in the extract are determined by reversed phase C_{18} HPLC with fluorescence detection.

3 Reagents and materials

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

3.1 Double-distilled water, or water of equivalent purity (conductivity < 10 µS/cm).

3.2 Standard substance: tryptophan (purity/content ⩾ 99 %) dried under vacuum over phosphorus pentoxide.

3.3 Internal standard substance: α-methyltryptophan (purity/content ⩾ 99 %), dried under vacuum over phosphorus pentoxide.

3.4 Barium hydroxide octahydrate.

Care should be taken not to expose the $Ba(OH)_2 \cdot 8H_2O$ excessively to air in order to avoid formation of $BaCO_3$, which could disturb the determination (see observation in B.3).

3.5 Sodium hydroxide.

3.6 Orthophosphoric acid, w = 85 %.

3.7 Concentrated hydrochloric acid, ρ_{20} = 1,19 g/ml.

3.8 Methanol, HPLC grade.

3.9 Light petroleum, boiling range 40 °C to 60 °C.

3.10 Sodium hydroxide solution, c = 1 mol/l.

Dissolve 40,0 g of NaOH (3.5) in water (3.1) and make up to 1 l with water (3.1).

3.11 Hydrochloric acid, c = 6 mol/l.

Take 492 ml of HCl (3.7) and make up to 1 l with water (3.1).

3.12 Hydrochloric acid, c = 1 mol/l.

Take 82 ml of HCl (3.7) and make up to 1 l with water (3.1).

3.13 Hydrochloric acid, c = 0,1 mol/l.

Take 8,2 ml of HCl (3.7) and make up to 1 l with water (3.1).

3.14 Orthophosphoric acid, c = 0,5 mol/l.

Take 34 ml of orthophosphoric acid (3.6) and make up to 1 l with water (3.1).

3.15 Concentrated tryptophan solution (3.2), c = 0,000 5000 g/ml.

In a 500 ml volumetric flask, dissolve 0,25 g of tryptophan (3.2) (weighed to the nearest 0,1 mg) in hydrochloric acid (3.13) and make up to the mark with hydrochloric acid (3.13). Store at –18 °C for a maximum of four weeks.

3.16 Concentrated internal standard solution, c = 0,000 54 g/ml.

In a 500 ml volumetric flask, dissolve 0,27 g of α-methyltryptophan (3.3) (weighed to the nearest 0,1 mg) in hydrochloric acid (3.13) and make up to the mark with hydrochloric acid (3.13). Store at –18 °C for a maximum of four weeks.

3.17 Calibration standard solution of tryptophan and internal standard.

Take 2,00 ml of the concentrated tryptophan solution (3.15) and 2,00 ml of concentrated internal standard solution (α-methyltryptophan) (3.16). Dilute with water (3.1) and methanol (3.8) to approximately the same volume and to approximately the same concentration of methanol (10 % to 30 %) as the finished hydrolysate.

This solution shall be prepared freshly before use.

Protect from direct sunlight during preparation.

3.18 Ethanolamine > 98 %.

3.19 1,1,1-Trichloro-2-methyl-2-propanol solution.

Add 1 g of 1,1,1-trichloro-2-methyl-2-propanol to 100 ml of methanol (3.8).

3.20 Mobile phase for HPLC.

Dissolve 3,00 g of acetic acid in 900 ml of water (3.1) and add 50,0 ml of 1,1,1-trichloro-2-methyl-2-propanol solution (3.19). Adjust the pH to 5,00 using ethanolamine (3.18). Make up to 1 000 ml with water (3.1).

4 Apparatus

Usual laboratory apparatus and, in particular, the following.

4.1 HPLC equipment with a spectrofluorimetric detector.

4.2 Liquid chromatographic column, 125 mm × 4 mm, with C_{18}, 3 µm packing, or equivalent.

4.3 pH-meter.

4.4 Polypropylene flask, of capacity 125 ml, with wide neck and screw cap.

4.5 Membrane filter, 0,45 µm.

4.6 Autoclave, capable of being maintained at (110 ± 2) °C, [(140 ± 10) kPa (1,4 ± 0,1) bar].

A pressure-tight covered dish that may be put into a drying oven adjustable to (110 ± 2) °C may be used.

4.7 Mechanical shaker or **magnetic stirrer**.

4.8 Vortex mixer.

5 Procedure

5.1 Preparation of samples

Grind the sample to pass through a 0,5 mm sieve. Samples high in moisture shall be either air-dried at a temperature not exceeding 50 °C or freeze-dried prior to grinding. Samples with high fat content shall be extracted with light petroleum (3.9) prior to grinding.

5.2 Determination of free tryptophan (extract)

Weigh, to the nearest 1 mg, an appropriate amount (1 g to 5 g) of the prepared sample (5.1) into a conical flask. Add 100,0 ml of hydrochloric acid, (3.13) and 5,00 ml of concentrated internal standard solution (3.16). Shake or mix for 60 min using a mechanical shaker or a magnetic stirrer (4.7). Allow the sediment to settle and pipette 10,0 ml of the supernatant solution into a beaker. Add 5 ml of orthophosphoric acid (3.14). Adjust the pH to 3,0 using sodium hydroxide (3.10). Add sufficient methanol (3.8) to give a concentration of between 10 % and 30 % of methanol in the final volume. Transfer to a volumetric flask of appropriate volume and dilute with water (3.1) to a volume necessary for the chromatography [approximately the same volume as the calibration standard solution (3.17)].

Filter a few millilitres of the solution through a 0,45 µm membrane filter (4.5) before injection on the HPLC column. Proceed to the chromatography step according to 5.4.

Protect the standard solution and extracts against direct sunlight. If it is not possible to analyse the extracts the same day, the extracts may be stored at 5 °C for a maximum of three days.

5.3 Determination of total tryptophan (hydrolysate)

Weigh, to the nearest 0,2 mg, from 0,1 g to 1 g of the prepared sample (5.1) into the polypropylene flask (4.4). The weighed test portion should have a nitrogen content of about 10 mg. Add 8,4 g of barium hydroxide octahydrate (3.4) and 10 ml of water (3.1). Mix on a vortex mixer (4.8) or magnetic stirrer (4.7). Leave the Teflon-coated magnet in the mixture. Wash down the walls of the vessel with 4 ml of water (3.1). Put on the screw cap and close the flask loosely. Transfer to an autoclave (4.6) which contains boiling water, and steam for 30 min to 60 min. Close the autoclave and autoclave at (110 ± 2) °C for 20 h.

Before opening the autoclave, reduce the temperature to just under 100 °C. In order to avoid crystallization of $Ba(OH)_2 \cdot 8H_2O$, add to the warm mixture 30 ml of water (3.1) which is at room temperature. Shake or stir gently. Add 2,00 ml of concentrated internal standard solution (α-methyltryptophan) (3.16). Cool the vessel in a water/ice bath for 15 min.

Then, add 5 ml of orthophosphoric acid (3.14). Keep the vessel in the cooling bath and neutralize with 6 mol/l HCl (3.11) whilst stirring and adjust the pH to 3,0 using 1 mol/l HCl (3.12). Add sufficient methanol to give a concentration of between 10 % and 30 % of methanol in the final volume. Transfer to a volumetric flask of appropriate volume and dilute with water (3.1) to the defined volume necessary for the chromatography (for example 100 ml). The addition of methanol should not cause precipitation.

Filter a few millilitres of the solution through a 0,45 µm membrane filter (4.5) before injection on the HPLC column. Proceed to the chromatography step according to 5.4.

Protect the standard solution and hydrolysates against direct sunlight. If it is not possible to analyse the hydrolysates the same day, they may be stored at 5 °C for a maximum of three days.

5.4 HPLC determination

The following conditions for isocratic elution are offered for guidance; other conditions may be used, provided they yield equivalent results (see also observations B.1 and B.2):

Liquid chromatographic column (4.2):	125 mm × 4 mm, with C_{18}, 3 µm packing or equivalent
Column temperature:	Room temperature
Mobile phase (3.20):	Dissolve 3,00 g of acetic acid in 900 ml of water (3.1) and add 50,0 ml of 1,1,1-trichloro-2-methyl-2-propanol solution (3.19). Adjust the pH to 5,00 using ethanolamine (3.18). Make up to 1 000 ml with water.
Flow rate:	1 ml/min
Total run time:	approximately 34 min
Detection wavelength:	excitation: 280 nm; emission: 356 nm
Injection volume:	20 µl

6 Calculation of results

The content of tryptophan, w, in grams per 100 g of sample, is calculated by

$$w = \frac{A_{\text{is,cal}} \times A_{\text{try,sam}} \times V_{\text{try}} \times c_{\text{try}} \times V_{\text{is,sam}} \times 100}{A_{\text{is,sam}} \times A_{\text{try,cal}} \times V_{\text{is,cal}} \times m}$$

where

$A_{\text{is,cal}}$ is the peak area of the internal standard in the calibration standard solution (3.17);

$A_{\text{try,sam}}$ is the peak area of tryptophan in the extract (5.2) or hydrolysate (5.3);

V_{try} is the volume, in millilitres (2 ml), of concentrated tryptophan solution (3.15) added to the calibration solution (3.17);

c_{try} is the concentration, in grams per millilitre (= 2,50), of concentrated tryptophan solution (3.15) added to the calibration solution (3.17);

$V_{\text{is,sam}}$ is the volume, in millilitres, of concentrated internal standard solution (3.16) added at the extraction (5.2) (= 5,00 ml) or to the hydrolysate (5.3) (= 2,00 ml);

$A_{\text{is,sam}}$ is the peak area of the internal standard in the extract (5.2) or hydrolysate (5.3);

$A_{\text{try,cal}}$ is the peak area of the tryptophan calibration standard solution (3.17);

$V_{\text{is,cal}}$ is the volume, in millilitres (= 2,00 ml), of the concentrated internal standard solution (3.16) added to the calibration standard solution (3.17);

m is the sample mass, in grams (corrected to the original mass if dried and/or defatted).

7 Precision

7.1 Interlaboratory test

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

7.2 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 not in more than 5 % of cases be greater than the repeatability limit r given in Tables A.1 to A.3.

7.3 Reproducibility

The absolute difference between two single test results, obtained using the same method on identical test material in different laboratories with different operators using different equipment, will not in more than 5 % of cases be greater than the reproducibility limit R given in Tables A.1 to A.3.

8 Test report

The test report shall specify:

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;

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, or, if the repeatability has been checked, the final quoted result obtained.