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Animal feeding stuffs — Determination of tryptophan content

Aliments des animaux — Dosage du tryptophane

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Foreword

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The committee responsible for this document is ISO/TC 34, *Food products*, Subcommittee SC 10, *Animal feeding stuffs*.

This second edition cancels and replaces the first edition (ISO 13904:2005), which has been technically revised.

Animal feeding stuffs — Determination of tryptophan content

1 Scope

This International Standard specifies a method for determination of the total and free tryptophan (Trp) content in feeding stuffs (e.g. complete and complementary feeds, supplementary feeds, raw materials, ingredients, and concentrates) and determination of free tryptophan in commercial pure substances and premixtures containing more than 2 % of tryptophan.

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. For commercial pure substances and premixtures containing more than 2 % of tryptophan, it is possible to add the internal standard after the extraction.

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 and control substance: tryptophan (purity ≥99 %) dried under vacuum over phosphorus pentoxide.

The two products are considered as 100 % pure. Control substance shall come from another manufacturer than the standard substance (see 3.17.2).

NOTE The control of the purity of the standard substance can be performed by measuring the absorbance of a solution of tryptophan at 280 nm. Prepare a solution of about 5 mg/l in HCl 10^{-3} N from a stock solution and measure the Optical Density (OD) at 280 nm versus HCl 10^{-3} N. Then, the concentration of tryptophan is:

$C = OD/5\,630 * 10^{+06}$

where

5 630 is the molar extinction coefficient of tryptophan in water at 280 nm;

C is expressed in μmole/l.

The standard substance purity is then $(C/C_0)*100$ where C_0 is the theoretical concentration of the diluted solution, expressed in μmole/l (about 25 μmole/l).

The control of the purity is performed every 6 months of use; it shall be ≥99 %.

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

3.4 Barium hydroxide octahydrate.

Care should be taken not to expose the $Ba(OH)_2\ 8H_2O$ 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 standard solution and control solution (3.2), c = 0,50 g/l.

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 approximately -18 °C for a maximum of four weeks.

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

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 approximately -18 °C for a maximum of four weeks.

3.17 Calibration standard solutions of tryptophan and internal standard.

3.17.1 Calibration standard solution for the analysis of tryptophan in feeding stuffs, (c = 0,010 g/l).

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.

3.17.2 Calibration standard solution of tryptophan for the analysis of tryptophan in commercial pure substances and premixtures containing more than 2 % of tryptophan, (c = 0,010 g/l).

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 it with hydrochloric acid 0,1 mol/l (3.13) in a 100 ml volumetric flask. Fill up to the mark.

This solution shall be prepared freshly before use.

Protect from direct sunlight during preparation.

For verification of the calibration standard solution, it is possible to use a control solution of tryptophan. This control solution is prepared and analyzed as it is described for the calibration standard solution, but shall come from another manufacturer than the standard substance (3.2). The recovery of tryptophan in the control solution sample shall be between 99 % and 101 %.

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).

NOTE 1,1,1-trichloro-2-methyl-2-propanol solution (3.19) could be considered critical for environmental reasons and may require special disposal.

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).

Shelf life of the mobile phase (especially stability of the mixture of acetic acid and ethanolamine) has to be checked by the retention times.

See also Annex B for an alternative mobile phase: The mixture of phosphate buffer and methanol is cheaper and harmless; pH adjustment is not necessary. The mixture is very stable.

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 consisting of cellulose acetate (0,45 μm or 0,22 μm pore size).

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 can be used.

4.7 Mechanical shaker or **magnetic stirrer**.

4.8 Vortex mixer.

4.9 Glassware – filters.

4.10 Graduated Erlen meyers flasks: 200 ml, 250 ml, 600 ml.

4.11 Volumetric: 100 ml, 500 ml, 1 000 ml (all class A).

5 Procedure

5.1 Preparation of samples

5.1.1 Feeding stuffs

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.

5.1.2 Commercial pure substances and premixtures containing more than 2 % of tryptophan

Grind the sample to pass through to a 0,25 mm sieve and homogenize it well.

5.2 Determination of free tryptophan (extract)

5.2.1 Feeding stuffs

Weigh, to the nearest 1 mg, an appropriate amount (1 g to 5 g) of the prepared sample (5.1.1) 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 (3.17.1)].

Filter a few millilitres of the solution through a 0,45 μm or 0,22 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 analyze the hydrolysates the same day, they may be stored at 5 °C for a maximum of three days.

5.2.2 Commercial pure substances and premixtures containing more than 2 % of tryptophan

Weigh to the nearest of 0,1 mg, 0,5 g to 5 g of well homogenized sample (5.1.2), depending on the expected concentration of tryptophan in the sample (for example, see Annex C) into a 1 000 ml volumetric flask.

Fill up to volume with 0,1 mol/l hydrochloric acid (3.13).

The mixture is stirred during 30 min on a mechanical shaker or a magnetic stirrer (4.7). Allow the particles to settle.

Transfer an aliquot of 2 ml of clear solution into a 100 ml volumetric flask. Add 2 ml of the concentrated internal standard (3.16).

Fill up to the mark with 0,1 mol/l hydrochloric acid (3.13).

This diluted test solution should have a tryptophan concentration as close as possible as the tryptophan concentration in the calibration standard solution (3.17.2) and the internal standard concentration shall be similar (c = 0,010 8 g/l) to the one of the calibration standard solution (c = 0,010 g/l, 3.17.2). Refer to Annex A for example of samples preparation.

Filter a few millilitres of the solution through a 0,45 μm or 0,22 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 do the analyses the same day, then the extracts shall be stored below 5 °C for not more than three days.

5.3 Determination of total tryptophan (hydrolysates)

Weigh, to the nearest 0,2 mg, from 0,1 g to 1 g of the prepared sample (5.1.1) 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 $Ba(OH)_2$ $8H_2O$, 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 or 0,22 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 analyze 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

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

6.1 Feeding stuffs

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

$$w = \frac{A_{\text{is,cal}} \times A_{\text{trp,sam}} \times V_{\text{trp}} \times c_{\text{trp}} \times V_{\text{is,sam}} \times 100}{A_{\text{is,sam}} \times A_{\text{trp,cal}} \times V_{\text{is,cal}} \times m} \quad (1)$$

where

$A_{\text{is,cal}}$ is the peak area of the internal standard in the calibration standard solution (3.17.1);

$A_{\text{trp,sam}}$ is the peak area of tryptophan in the extract (5.2.1) or hydrolysate (5.3);

V_{trp} is the volume, in millilitres (2 ml), of concentrated tryptophan solution (3.15) added to the calibration solution (3.17.1);

c_{trp} is the concentration, in grams per litre (=0,50), of concentrated tryptophan solution (3.15) added to the calibration standard solution (3.17.1);

$V_{\text{is,sam}}$ is the volume, in millilitres, of concentrated internal standard solution (3.16) added at the extraction (5.2.1) (=5,00 ml) or to the hydrolysate (5.3) (=2,00 ml);

$V_{\text{is,cal}}$ is the volume, in millilitres (=2,00 ml), of concentrated internal standard solution (3.16) added at the calibration solution (3.17.1);

$A_{\text{is,sam}}$ is the peak area of the internal standard in the extract (5.2.1) or hydrolysate (5.3);

$A_{\text{trp,cal}}$ is the peak area of the tryptophan calibration standard solution (3.17.1);

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

6.2 Commercial pure products and premixtures containing more than 2 % of tryptophan

6.2.1 Control of the calibration

It is necessary to verify the quality of this solution. If it does not conform, a new solution must be prepared (3.17.2).

Analyze a calibration solution after every four test solutions.

6.2.2 Calculation

The content of tryptophan, w, in grams per 100 g of sample, is calculated by Formula (2):

$$w = \frac{A_{\text{is,cal}} \times A_{\text{trp,sam}} \times C_{\text{trp}} \times d \times 100}{A_{\text{is,sam}} \times A_{\text{trp,cal}} \times m} \quad (2)$$

where

$A_{\text{is,cal}}$ is the peak area of the internal standard in the calibration standard solution (3.17.2);

$A_{is,sam}$ is the peak area of the internal standard in the diluted test solution (5.2.2);

$A_{trp,sam}$ is the peak area of tryptophan in the diluted test solution (5.2.2);

$A_{trp,cal}$ is the peak area of tryptophan in the calibration standard solution (3.17.2);

C_{trp} concentration of the standard calibration solution (3.17.2) in g/l;

m is the sample mass in g;

d dilution factor (see Annex C).

7 Precision

7.1 Interlaboratory test

7.1.1 Feeding stuffs

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.

7.1.2 Commercial pure substances and premixtures containing more than 2 % of tryptophan

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

7.2 Repeatability

7.2.1 Feeding stuffs

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.

7.2.2 Commercial pure substances and premixtures containing more than 2 % of tryptophan

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 the cases be greater than the repeatability limit, *r*, given in Annex D.

7.3 Reproducibility

7.3.1 Feeding stuffs

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.

7.3.2 Pure products and premixtures containing more than 2 % of tryptophan

The absolute difference between two independent single test results, obtained using the same method on identical test material in different laboratories by different operators using different equipment, will not in more than 5 % of the cases be greater than the reproducibility limit, *R*, given in Annex D.

8 Test report

The test report shall specify the following:

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, i.e ISO 13904:2015;

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.

Annex A
(informative)

Results of an interlaboratory test

An interlaboratory test for feeding stuffs was arranged within the European Union in which three samples were analyzed by up to 12 laboratories to certify the method for hydrolysis. Five replicate analyses were performed on each sample. The results are given in Table A.1.

Table A.1

	Sample 1 Pig feed	**Sample 2** Pig feed supplemented with L-tryptophan	**Sample 3** Feed concentrate for pigs
Number of laboratories submitting results	12	12	12
Number of test results from remaining laboratories	50	55	50
Mean value $\bar{x}$, g/kg	2,42	3,40	4,22
Repeatability standard deviation, s_r, g/kg	0,05	0,05	0,08
Repeatability relative standard deviation, %	1,9	1,6	1,9
Repeatability limit, r (=2,8 s_r), g/kg	0,14	0,14	0,22
Reproducibility standard deviation, s_R, g/kg	0,15	0,20	0,09
Reproducibility relative standard deviation, %	6,3	6,0	2,2
Reproducibility limit, R (=2,8 s_R), g/kg	0,42	0,56	0,25

Another collaborative study was arranged in which two samples were analyzed by up to 13 laboratories to certify the method for extraction of free tryptophan. Five replicate analyses were performed on each sample. The results are given in Table A.2.

Table A.2

	Sample 4 Wheat and soya mixture	**Sample 5** Wheat and soya mixture (=sample 4) with added tryptophan (0,457 g/kg)
Number of laboratories submitting results	12	12
Number of test results from remaining laboratories	55	60
Mean value $\bar{x}$, g/kg	0,391	0,931
Repeatability standard deviation, s_r, g/kg	0,005	0,012
Repeatability relative standard deviation, %	1,34	1,34
Repeatability limit, r (=2,8 s_r), g/kg	0,014	0,034
Reproducibility standard deviation, s_R, g/kg	0,018	0,048
Reproducibility relative standard deviation, %	4,71	5,11
Reproducibility limit, R (=2,8 s_R), g/kg	0,05	0,134

Another interlaboratory test was arranged in which four samples were analyzed by up to seven laboratories with the aim of a tryptophan certification for hydrolysis. The results are given in Table A.3. Five replicate analyses were performed on each sample.

Table A.3

	Sample 1 Mixed pig feed (CRM 117)	**Sample 2** Low-fat fish meal (CRM 118)	**Sample 3** Soybean meal (CRM 119)	**Sample 4** Skimmed milk powder (CRM 120)
Number of laboratories submitting results	7	7	7	7
Number of test results from remaining laboratories	25	30	30	30
Mean value $\bar{x}$, g/kg	2,064	8,801	6,882	5,236
Repeatability standard deviation, s_r, g/kg	0,021	0,101	0,089	0,040
Repeatability relative standard deviation, %	1,04	1,15	1,30	0,76
Repeatability limit, r (=2,8 s_r), g/kg	0,059	0,283	0,249	0,112
Reproducibility standard deviation, s_R, g/kg	0,031	0,413	0,283	0,221
Reproducibility relative standard deviation, %	1,48	4,69	4,11	4,22
Reproducibility limit, R (=2,8 s_R), g/kg	0,087	1,156	0,792	0,619

Annex B
(informative)

Observations on the method

B.1 The following special chromatographic conditions may give better separation between tryptophan and α-methyltryptophan.

Isocratic elution followed by gradient column cleaning:

Liquid chromatographic column:	125 mm × 4 mm, with C_{18}, 5 μm packing or equivalent
Column temperature:	32 °C
Mobile phase:	A: 0,01 mol/l KH_2PO_4/methanol, 95 + 5 (volume fraction)
	B: Methanol
Gradient programme:	0 min 100 % A 0 % B
	15 min 100 % A 0 % B
	17 min 60 % A 40 % B
	19 min 60 % A 40 % B
	21 min 100 % A 0 % B
	33 min 100 % A 0 % B
Flow rate:	1,2 ml/min
Total run time:	Approximately 33 min

B.2 The chromatography will vary according to the type of HPLC and column packing material used. The system chosen should be capable of giving baseline separation between the tryptophan and the internal standard. Moreover, it is important that degradation products are well separated from the tryptophan and the internal standard. Hydrolysates, without the internal standard, should be run in order to check the baseline under the internal standard for impurities. It is important that the run time is sufficiently long for the elution of all the degradation products, otherwise late eluting peaks may interfere with subsequent chromatographic runs.

The chromatographic system should give linear response over the range of operation. The linear response should be measured with a constant (normal) concentration of the internal standard and varying concentrations of tryptophan. It is important that the size of both the tryptophan and internal standard peaks are within the linear range of the HPLC/fluorescence system. If either the tryptophan and/or the internal standard peak(s) is (are) too small or too high, the analysis should be repeated with another sample size and/or a changed final volume.

B.3 With age, barium hydroxide becomes more difficult to dissolve. This results in an unclear solution for the HPLC determination, which may produce low results for tryptophan.

Annex C
(informative)

Sample preparation: Example for analyses in pure products and premixtures

Table C.1

Expected free Trp %	**Sample weight** (g)/1 000 ml	**Dilution**	**Concentrated internal standard solution (3.16)** ml	**Dilution factor *d***	**Expected concentration of free Trp in the solution** (g/l)
2 – 10	5,00 g	2 ml in 100 ml	2	50	0,002 0 to 0,010 0
10 – 20	2,50 g	2 ml in 100 ml	2	50	0,005 0 to 0,010 0
20 – 40	1,25 g	2 ml in 100 ml	2	50	0,005 to 0,010 0
40 – 60	0,80 g	2 ml in 100 ml	2	50	0,006 4 to 0,009 6
60 – 80	0,60 g	2 ml in 100 ml	2	50	0,007 2 to 0,009 6
80 – 100	0,50 g	2 ml in 100 ml	2	50	0,008 0 to 0,010 0

Annex D
(informative)

Results of an interlaboratory test for tryptophan in pure products and premixtures containing more than 2 % of tryptophan

In 2011, an interlaboratory comparison was carried out to assess the method performance characteristics of an analytical method for the determination of free tryptophan in commercial pure substances and premixtures containing more than 2 % of tryptophan.

In total, sixteen samples corresponding to eight double blind determinations were sent to eighteen laboratories.

Seventeen laboratories reported results for the sample set. Five laboratories were excluded because they did not follow the protocol.

The tryptophan content obtained in test materials ranged between 1,26 g/100 g and 49,99 g/100 g for pre mixtures and 90,30 g/100 g and 99,78 g/100 g for commercial pure substances. The measured values were in line with the expected values given by the manufacturer of the premixes, except for the lowest value (wrong expected value for that premix 1 sample).

Table D.1

	Premix 1 (2,5 % free Trp)	**Premix 2 (10,0 % free Trp)**	**Premix 3 (15,0 % free Trp)**	**Premix 4 (50,0 % free Trp)**	**Pure product 1 (90,0 % free Trp)**	**Pure product 2 (94,0 % free Trp)**	**Pure product 3 (96,0 % free Trp)**	**Pure product 4 (98,0 % free Trp)**
Number of laboratories after outlier elimination, p	12	11	12	11	12	11	12	11
Mean value X, g/100 g	**1,26**	**9,94**	**19,33**	**49,99**	**90,30**	**93,84**	**95,77**	**99,78**
Repeatability standard deviation, s_r, g/100 g	0,07	0,14	0,42	0,46	0,63	0,71	0,86	0,51
Relative standard deviation, CV_r (s_r/X %)	5,26 %	1,38 %	2,18 %	0,93 %	0,69 %	0,76 %	0,90 %	0,51 %
Limit of repeatability, r	**0,19**	**0,38**	**1,18**	**1,30**	**1,75**	**1,99**	**2,42**	**1,42**
Reproductibility standard deviation, s_R, g/100 g	0,12	0,30	0,64	1,05	1,18	1,13	1,29	0,96
Relative standard deviation, CV_R (s_R/X, %)	9,50 %	3,04 %	3,31 %	2,11 %	1,30 %	1,20 %	1,34 %	0,96 %
Limit of reproductibility, R, g/100 g	**0,34**	**0,85**	**1,79**	**2,95**	**3,29**	**3,16**	**3,60**	**2,67**

Bibliography

[1] Commission Directive 2000/45/EC of 6 July 2000, establishing Community methods of analysis for the determination of vitamin A, vitamin E and tryptophan in feedingstuffs

[2] Interlaboratory comparisons report from BIPEA: RCIL n° 2011-2012 – 0334: Precision test "Determination of free tryptophan in premixes and pure products"

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