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**Animal feeding stuffs — Determination  
of amino acids content**

*Aliments des animaux — Détermination de la teneur en acides aminés*

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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.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

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.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

ISO 13903 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 10, *Animal feeding stuffs*.

ISO 13903 is based on Commission Directive 98/64/EC of September 1998 [1].

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# Animal feeding stuffs — Determination of amino acids content

## 1 Scope

This International Standard describes the determination of free (synthetic and natural) and total (peptide-bound and free) amino acids in feeding stuffs, using an amino acid analyser or HPLC equipment. It is applicable to the following amino acids:

- sum of cystine and cysteine;
- methionine;
- lysine;
- threonine;
- alanine;
- arginine;
- aspartic acid;
- glutamic acid;
- glycine;
- histidine;
- isoleucine;
- leucine;
- phenylalanine;
- proline;
- serine;
- tyrosine;
- valine.

The method does not distinguish between the salts of amino acids, nor does it differentiate between D and L forms of amino acids. It is not valid for the determination of tryptophan or hydroxy analogues of amino acids.

Limits of quantification depend on the chromatographic equipment, but levels as low as: 0,3 g/kg total lysine; 0,25 g/kg total methionine; 0,35 g/kg total cystine plus cysteine; 0,2 g/kg total threonine; 0,035 g/kg free lysine; 0,035 g/kg free methionine; and 0,03 g/kg free threonine can typically be analysed.

NOTE A lower limit of quantification or detection might be achievable but this is to be validated by the users.

## 2 Principle

### 2.1 Free amino acids

The free amino acids are extracted with dilute hydrochloric acid. Co-extracted nitrogenous macromolecules are precipitated with sulfosalicylic acid and removed by filtration. The filtered solution is adjusted to pH 2,20. The amino acids are separated by ion exchange chromatography and determined by reaction with ninhydrin with photometric detection at 570 nm.

## 2.2 Total amino acids

The procedure chosen depends on the amino acids under investigation. Cyst(e)ine and methionine shall be oxidized to cysteic acid and methionine sulphone, respectively, prior to hydrolysis. Tyrosine shall be determined in hydrolysates of unoxidized samples. All the other amino acids listed in Clause 1 may be determined in either the oxidized or unoxidized sample.

Oxidation is performed at 0 °C with a performic acid/phenol mixture. Excess oxidation reagent is decomposed with sodium disulfite. The oxidized or unoxidized sample is hydrolysed with hydrochloric acid ( $c = 6 \text{ mol/l}$ ) for 23 h. The hydrolysate is adjusted to pH 2,20. The amino acids are separated by ion exchange chromatography and determined by reaction with ninhydrin, using photometric detection at 570 nm (440 nm for proline).

## 3 Reagents and materials

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

**3.1 Water**, double distilled water or water of equivalent quality shall be used (conductivity  $< 10 \mu\text{S}$ ).

**3.2 Hydrogen peroxide**,  $w = 30 \%$ .

**3.3 Formic acid**,  $w = 98 \%$  to  $100 \%$ .

**3.4 Hydrochloric acid**, density approximately  $1,19 \text{ g/ml}$ .

**3.5 2,2'-Thiodiethanol** (thiodiglycol)

**3.6 Light petroleum**, boiling rate  $40 \text{ }^\circ\text{C}$  to  $60 \text{ }^\circ\text{C}$

**3.7 Norleucine**, or any other compound suitable for use as internal standard.

**3.8 Nitrogen gas** ( $< 10$  parts per million oxygen).

**3.9 Amino acids.**

**3.9.1 Standard substances** listed under Clause 1.

Use pure compounds containing no water of crystallization. Dry under vacuum over  $\text{P}_2\text{O}_5$  or  $\text{H}_2\text{SO}_4$  for 1 week prior to use.

**3.9.2 Cysteic acid**

**3.9.3 Methionine sulfone.**

**3.10 Sodium hydroxide solution I**,  $c = 7,5 \text{ mol/l}$ .

Dissolve 300 g of NaOH (3.6) in water and make up to 1 l.

**3.11 Sodium hydroxide solution II**,  $c = 1 \text{ mol/l}$ .

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

**3.12 Formic acid-phenol solution.**

Mix 889 g of formic acid (3.3) with 111 g of water (3.1) and add 4,73 g of phenol.

**3.13 Hydrolysis mixture**,  $c = 6$  mol/l HCl containing 1 g of phenol per litre.

Add 1 g of phenol to 492 ml of HCl (3.4) and make up to 1 l with water (3.1).

**3.14 Extraction mixture**,  $c = 0,1$  mol/l HCl containing 2 % thiodiglycol.

Take 8,2 ml of HCl (3.4), dilute with approximately 900 ml of water (3.1). Add 20 ml of thiodiglycol (3.5) and make up to 1 l with water. Do not mix 3.4 and 3.5 directly.

**3.15 5-Sulfosalicylic acid**,  $\beta = 6$  %.

Dissolve 60 g of 5-sulfosalicylic acid dihydrate in water (3.1) and make up to 1 l with water.

**3.16 Oxidation mixture** (performic acid-phenol).

Mix 0,5 ml of hydrogen peroxide (3.2) with 4,5 ml of formic acid-phenol solution (3.12) in a small beaker. Incubate at between 20 °C and 30 °C for 1 h in order to form performic acid, then cool in an ice-water bath (15 min) before adding to the sample.

Avoid contact with skin and wear protective clothing.

**3.17 Citrate buffer**,  $c = 0,2$  mol/l  $\text{Na}^+$ , pH 2,20.

Dissolve 19,61 g of sodium citrate dihydrate, 5 ml of thiodiglycol (3.5), 1 g of phenol and 16,50 ml of HCl (3.4) in approximately 800 ml of water (3.1). Adjust the pH to 2,20. Make up to 1 l with water.

**3.18 Elution buffers**, prepared according to conditions for the analyser used (4.9).

**3.19 Ninhydrin reagent**, prepared according to conditions for the analyser used (4.9).

**3.20 Standard solutions of amino acids.**

These solutions shall be stored below 5 °C.

**3.20.1 Stock standard solution of amino acids** (3.9.1),  $c = 2,5$   $\mu\text{mol/ml}$  of each in hydrochloric acid.

These may be obtained commercially.

**3.20.2 Stock standard solution of cysteic acid and methionine sulfone**,  $c = 1,25$   $\mu\text{mol/ml}$ .

Dissolve 0,211 5 g of cysteic acid (3.9.2) and 0,226 5 g of methionine sulphone (3.9.3) in citrate buffer (3.17) in a 1 l graduated flask and make up to mark with citrate buffer. Store below 5 °C for not more than 12 months. This solution shall not be used if the stock standard solution (3.20.1) contains cysteic acid and methionine sulfone.

**3.20.3 Stock standard solution of the internal standard** e.g. norleucine,  $c = 20$   $\mu\text{mol/ml}$ .

Dissolve 0,656 0 g of norleucine (3.7) in citrate buffer (3.17) in a graduated flask and make up to 250 ml with citrate buffer. Store below 5 °C for no more than 6 months.

**3.20.4 Calibration solution of standard amino acids**, for use with hydrolysates,  $c = 0,1$   $\mu\text{mol/ml}$  of cysteic acid and methionine sulfone and  $c = 0,2$   $\mu\text{mol/ml}$  of the other amino acids.

Dissolve 2,2 g of sodium chloride in 100 ml beaker with 30 ml of citrate buffer (3.17). Add 4,00 ml of stock standard solution of amino acids (3.20.1), 4,00 ml of stock standard solution of cysteic acid and methionine sulfone (3.20.2), and 0,50 ml of stock standard solution of internal standard (3.20.3) if used. Adjust the pH to 2,20 with sodium hydroxide (3.11). Transfer quantitatively to a 50 ml graduated flask and make up to the mark with citrate buffer (3.17) and mix. Store below 5 °C for not more than 3 months. See also 9.1.

**3.20.5 Calibration solution of standard amino acids**, for use with hydrolysates prepared according to 5.3.3.2 and for use with extracts (5.2).

Prepare the calibration solution according to 3.20.4 but omitting sodium chloride. Store below 5 °C for not more than 3 months.

## 4 Apparatus

Usual laboratory apparatus and, in particular, the following.

- 4.1 **Round bottomed flask**, of capacity 100 ml or 250 ml, fitted with a reflux condenser.
- 4.2 **Borosilicate glass bottle**, of capacity 100 ml, with screw cap with rubber/teflon liner (e.g. Duran, Schott) for use in the oven.
- 4.3 **Oven**, with forced ventilation and a temperature regulator, with an accuracy better than  $\pm 2$  °C.
- 4.4 **pH-meter**, reading to three decimal places.
- 4.5 **Membrane filter**, 0,2  $\mu\text{m}$ .
- 4.6 **Centrifuge**.
- 4.7 **Rotary vacuum evaporator**.
- 4.8 **Mechanical shaker or magnetic stirrer**.
- 4.9 **Amino acid analyser or HPLC equipment** with ion exchange column, device for ninhydrin, post-column derivatization and photometric detector

The column is filled with sulfonated polystyrene resins capable of separating the amino acids from each other and from other ninhydrin-positive materials. The flow in the buffer and ninhydrin lines is provided by pumps having a flow stability of  $\pm 0,5$  % in the period covering both the standard calibration run and the analysis of the sample.

With some amino acid analysers, hydrolysis procedures may be used in which the hydrolysate has a sodium concentration of  $c = 0,8$  mol/l and contains all the residual formic acid from the oxidation step. Others do not give a satisfactory separation of certain amino acids if the hydrolysate contains excess formic acid and/or high sodium ion concentrations. In this case, reduce the volume of acid by evaporation to approx. 5 ml after the hydrolysis and prior to pH adjustment. The evaporation shall be performed under vacuum at 40 °C maximum.

## 5 Procedure

### 5.1 Preparation of test sample

Grind the sample until it passes through a 0,5 mm sieve. Samples high in moisture shall either be air-dried at a temperature not exceeding 50 °C or freeze-dried prior to grinding. Samples with a high fat content shall be extracted with light petroleum (3.6) prior to grinding.

### 5.2 Determination of free amino acids in feeding stuffs and premixtures

Weigh, to the nearest 0,2 mg, an appropriate amount (1 g to 5 g) of the prepared test sample (5.1) into a conical flask and add 100,0 ml of extraction mixture (3.14). Shake the mixture for 60 min using a mechanical shaker or a magnetic stirrer (4.8). Allow the sediment to settle and pipette 10,0 ml of the supernatant solution into a 100 ml beaker.

Add 5,0 ml of sulfosalicylic acid solution (3.15), with stirring, and continue to stir with the aid of magnetic stirrer for 5 min. Filter or centrifuge the supernatant in order to remove any precipitate. Place 10,0 ml of the resulting solution into a 100 ml beaker and adjust the pH to 2,20 using sodium hydroxide solution (3.11). Transfer to a volumetric flask of appropriate volume using citrate buffer (3.17), and make up to the mark with the buffer solution.

If an internal standard is being used, add 1,00 ml of internal standard (3.20.3) for each 100 ml of final solution and make up to the mark with the buffer solution (3.17).

Proceed to the chromatography step according to 5.4.

If the extracts are not being examined the same day, they shall be stored below 5 °C.

### 5.3 Determination of total amino acids

#### 5.3.1 Oxidation

Weigh, to the nearest 0,2 mg, from 0,1 g to 1 g of the prepared test sample (5.1) into

- a 100 ml round-bottomed flask (4.1) for open hydrolysis (5.3.2.3), or
- a 250 ml round-bottomed flask (4.1) if a low sodium concentration is required (5.3.3.2), or
- a 100 ml bottle, fitted with a screw cap (4.2), for closed hydrolysis (5.3.2.4).

The weighed test portion shall have a nitrogen content of about 10 mg and a moisture content not exceeding 100 mg.

Place the flask/bottle in an ice-water bath and cool to 0 °C. Add 5 ml of oxidation mixture (3.16) and mix using a glass spatula with a bent tip. Seal the flask/bottle containing the spatula with an airtight film, place the ice-water bath containing the sealed container in a refrigerator at 0 °C and leave for 16 h. After 16 h, remove from the refrigerator and decompose the excess oxidation reagent by the addition of 0,84 g of sodium disulfite.

Proceed to 5.3.2.1.

#### 5.3.2 Hydrolysis

##### 5.3.2.1 Hydrolysis of oxidized samples

To the oxidized sample prepared according to 5.3.1, add 25 ml of hydrolysis mixture (3.13), taking care to wash down any sample residue adhering to the sides of the vessel and the spatula. Depending on the hydrolysis procedure being used, proceed according to 5.3.2.3 or 5.3.2.4.

##### 5.3.2.2 Hydrolysis of unoxidized samples

Weigh, to the nearest 0,2 mg, into a 100 ml or 250 ml round-bottom flask (4.1) or into a 100 ml bottle fitted with a screw cap (4.2) from 0,1 g to 1 g of the prepared sample (5.1). The weighed sample portion shall have a nitrogen content of about 10 mg. Carefully add 25 ml of hydrolysis mixture (3.13) and mix with the sample. Proceed according to either 5.3.2.3 or 5.3.2.4.

##### 5.3.2.3 Open hydrolysis

Add three glass beads to the mixture in the flask (prepared in accordance with 5.3.2.1 or 5.3.2.2) and boil with continuous bubbling under reflux for 23 h. On completion of hydrolysis, wash the condenser down with 5 ml of citrate buffer (3.17). Disconnect the flask and cool it in an ice bath. Proceed according to 5.3.3.

#### 5.3.2.4 Closed hydrolysis

Place the bottle containing the mixture prepared in accordance with 5.3.2.1 or 5.3.2.2 in an oven (4.3) set at 110 °C. During the first hour, in order to prevent a build up of pressure (due to the evolution of gaseous substances) and to avoid explosion, place the screw cap over the top of the vessel. Do not close the vessel with the cap. After 1 h, close the vessel with the cap and leave in the oven (4.3) for 23 h. On completion of hydrolysis, remove the bottle from the oven, carefully open the cap of the bottle and place the bottle in an ice-water bath. Leave to cool.

Depending on the procedure for pH adjustment (5.3.3), quantitatively transfer the contents of the bottle to a 250 ml beaker or a 250 ml round-bottom flask, using citrate buffer (3.17).

Proceed according to 5.3.3.

#### 5.3.3 Adjustment of pH

**5.3.3.1** Depending on the sodium tolerance of the amino acid analyser (4.9), proceed according to 5.3.3.2 or 5.3.3.3 for the pH adjustment.

**5.3.3.2** When amino acid analysers requiring a low sodium concentration are used (when the acid volume has to be reduced) and for chromatographic systems (4.9) requiring a low sodium concentration, it is advisable to use an internal stock standard solution (3.20.3).

In this case, add 2,00 ml of the internal stock standard solution (3.20.3) to the hydrolysate before the evaporation.

Add two drops of 1-octanol to the hydrolysate obtained in accordance with 5.3.2.3 or 5.3.2.4.

Using a rotary evaporator (4.7), reduce the volume to 5 ml to 10 ml under vacuum at 40 °C. If the volume is accidentally reduced to less than 5 ml, the hydrolysate shall be discarded and the analysis recommenced.

Adjust the pH to 2,20 with sodium hydroxide solution II (3.11) and proceed to 5.3.4.

**5.3.3.3** For chromatographic systems (4.9) not requiring a low sodium concentration, take the hydrolysates obtained in accordance with 5.3.2.3 or 5.3.2.4 and partly neutralize them by carefully adding, with stirring, 17 ml of sodium hydroxide solution I (3.10), ensuring that the temperature is kept below 40 °C.

Adjust the pH to 2,20 at room temperature using sodium hydroxide solution I (3.10) and finally sodium hydroxide solution II (3.11). Proceed to 5.3.4.

#### 5.3.4 Sample solution for chromatography

Quantitatively transfer the pH-adjusted hydrolysate (5.3.3.2 or 5.3.3.3) with citrate buffer (3.17) to a 200 ml graduated flask, and make up to the mark with the buffer.

If an internal standard has not already been used, add 2,00 ml of internal standard (3.20.3) and make up to the mark with citrate buffer (3.17). Mix thoroughly.

Proceed to the chromatography step (5.4).

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

### 5.4 Chromatography

Before conducting the chromatography, bring the extract (5.2) or hydrolysate (5.3.4) to room temperature. Shake the mixture and filter a suitable amount through a 0,2 µm membrane filter (4.5). Subject the resulting clear solution to ion exchange chromatography, using an amino acid analyser or HPLC equipment (4.9).

The injection may be performed manually or automatically. It is important that the same quantity of  $\pm 0,5\%$  solution is added to the column for the analysis of standards and samples except when an internal standard is used, and that the sodium:amino acid ratios in the standard and sample solutions are as similar as is practicable.

In general, the frequency of the calibration runs depends on the stability of the ninhydrin reagent and the analytical system. Dilute the standard or sample with citrate buffer (3.17) to give a peak area of the standard of 30 % to 200 % of the sample amino acid peak area.

The chromatogram of amino acids will vary slightly according to the type of analyser employed and resin used. The chosen system shall be capable of separating the amino acids from each other and from the ninhydrin-positive materials. In the range of operation the chromatographic system shall give a linear response to changes in the amounts of amino acids added to the column.

During the chromatography step, the valley:peak height ratios mentioned below apply, when an equimolar solution (of the amino acids being determined) is analysed. This equimolar solution shall contain at least 30 % of the maximum load of each amino acid that can be accurately measured with the amino acid analyser system (4.9).

For separation of threonine-serine, the valley:peak height ratio of the lower of the two overlapping amino acids on the chromatogram shall not exceed 2:10 (if only cyst(e)ine, methionine, threonine and lysine are determined, insufficient separation from adjoining peaks will adversely influence the determination). For all other amino acids, the separation shall be better than 1:10.

The system shall ensure that lysine is separated from 'lysine artifacts' and ornithine.

## 6 Calculation of results

The area of the sample and standard peaks is measured for each individual amino acid and the amount,  $w$ , in grams of amino acid per kilogram of sample, is calculated:

$$w = \frac{A_e \times c \times M \times V_e}{A_c \times m \times 1000}$$

If an internal standard is used, multiply by  $\frac{A_{ic}}{A_{ie}}$

where

$A_e$  is the peak area of the hydrolysate or extract;

$A_c$  is the peak area of the calibration standard solution;

$A_{ie}$  is the peak area of the internal standard in the hydrolysate or extract;

$A_{ic}$  is the peak area, of the internal standard in the calibration standard solution;

$M$  is the molecular mass of the amino acid being determined;

$c$  is the concentration of standard, in  $\mu\text{mol/ml}$ ;

$m$  is the sample mass, in grams (corrected to original mass if dried or defatted);

$V_e$  is the volume of total hydrolysate (5.3.4), in millilitres, the calculated total dilution volume of extract (6.1), in millilitres.

Cystine and cysteine are both determined as cysteic acid in hydrolysates of the oxidized sample, but calculated as the sum of cystine and cysteine by using  $M = 120,15$  ( $= 0,5 \times 240,30$ ) ( $C_6H_{12}N_2O_4S_2$ ,  $M = 240,30$ ).

Methionine is determined as methionine sulfone in hydrolysates of the oxidized sample, but calculated as methionine by using  $M$  of methionine = 149,21.

Added free methionine is determined after extraction as methionine; for the calculation, the same value of  $M$  is used.

The total dilution volume of extracts ( $V_e$ ) for determination of free amino acids (5.2) is calculated as follows:

$$V_e = 100 \times \frac{(10 + 5)}{10} \times \frac{V_{ef}}{10}$$

where  $V_{ef}$  is the volume of final extract, in millilitres.

The results may be expressed as a percentage: result in percent =  $0,1 \times$  result in grams per kilogram.

## 7 Precision

### 7.1 Interlaboratory tests

Details of interlaboratory tests on the precision of the method are summarized in Annex A. The values derived from these interlaboratory tests 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 in not more than 5 % of cases be greater than the repeatability limit  $r$  ( $= 2,83 s_r$ ) given in Table A.2 for the first test, and in Tables A.6 to A.10 for the second test

### 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 in not more than 5 % of cases be greater than the reproducibility limit  $R$  ( $= 2,83 s_R$ ) given in Table A.4 for the first test, and in Tables A.6 to A.10 for the second test

## 8 Use of reference materials

The correct application of the method shall be verified by making replicate measurements of certified reference materials when available. Calibration with certified amino acid calibration solution is recommended.

## 9 Observations on the method

**9.1** Because of differences between amino acid analysers, the final concentrations of the calibration solutions of standard amino acids (see 3.20.4 and 3.20.5) and of the hydrolysate (see 5.3.4) shall be taken as a guideline.

The range of linear response of the apparatus shall be checked for all amino acids.

The standard solution is diluted with citrate buffer to give peak areas in the middle of the range.

**9.2** Where high-performance liquid chromatographic equipment is used to analyse the hydrolysates, the experimental conditions shall be optimized in accordance with the manufacturer's recommendations.

**9.3** By applying the method to feeding stuffs containing more than 1 % chloride (concentrate, mineral feeds, supplementary feeds), underestimation of methionine could occur and special treatment shall be carried out in the following way (the Slump method). After addition of 5 ml of the oxidation mixture in 5.3.1, add 12,5 ml of water and stir the sample mixture on a stirrer for 15 min. Afterwards follow the normal procedure. This procedure is not quantitative for determination of the sum of cysteine and cystine.

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## Annex A (informative)

### Results of interlaboratory tests

#### A.1 First test

The method has been tested in a collaborative test made at the international level in 1990 using four different feeding stuffs (mixed pig feed, broiler compound, protein concentrate and premixture). The results, after elimination of outliers, are given in Table A.1. The repeatability expressed as within-laboratory standard deviation of this test is given in Tables A.2 and A.3. The results for the between-laboratory standard deviation for this test are given in Tables A.4 and A.5.

**Table A.1 — Amino acids content**

Reference material	Amino acid			
	threonine	sum of cystine and cysteine	methionine	lysine
	g/kg	g/kg	g/kg	g/kg
Mixed pig feed	6,94 <i>n</i> = 15	3,01 <i>n</i> = 17	3,27 <i>n</i> = 17	9,55 <i>n</i> = 13
Broiler compound	9,31 <i>n</i> = 16	3,92 <i>n</i> = 18	5,08 <i>n</i> = 18	13,93 <i>n</i> = 16
Protein concentrate	22,32 <i>n</i> = 16	5,06 <i>n</i> = 17	12,01 <i>n</i> = 17	47,74 <i>n</i> = 15
Premixture	58,42 <i>n</i> = 16	—	90,21 <i>n</i> = 16	98,03 <i>n</i> = 16

*n* is the number of participating laboratories.

**Table A.2 — Within-laboratory standard deviation ( $s_r$ )**

Reference material	Amino acid			
	threonine	sum of cystine and cysteine	methionine	lysine
	g/kg	g/kg	g/kg	g/kg
Mixed pig feed	0,13 <i>n</i> = 15	0,10 <i>n</i> = 17	0,11 <i>n</i> = 17	0,26 <i>n</i> = 13
Broiler compound	0,20 <i>n</i> = 16	0,11 <i>n</i> = 18	0,16 <i>n</i> = 18	0,28 <i>n</i> = 16
Protein concentrate	0,48 <i>n</i> = 16	0,13 <i>n</i> = 17	0,27 <i>n</i> = 17	0,99 <i>n</i> = 15
Premixture	1,30 <i>n</i> = 16	—	2,19 <i>n</i> = 16	2,06 <i>n</i> = 16

*n* is the number of participating laboratories.

Table A.3 — Coefficient of variation (%) for within-laboratory standard deviation ( $s_r$ )

Reference material	Amino acid			
	threonine	sum of cystine and cysteine	methionine	lysine
	g/kg	g/kg	g/kg	g/kg
Mixed pig feed	1,9 $n = 15$	3,3 $n = 17$	3,4 $n = 17$	2,8 $n = 13$
Broiler compound	2,1 $n = 16$	2,8 $n = 18$	3,1 $n = 18$	2,1 $n = 16$
Protein concentrate	2,7 $n = 16$	2,6 $n = 17$	2,2 $n = 17$	2,4 $n = 15$
Premixture	2,2 $n = 16$	—	2,4 $n = 16$	2,1 $n = 16$

$n$  is the number of participating laboratories.

Table A.4 — Between-laboratory standard deviation ( $s_R$ )

Reference material	Amino acid			
	threonine	sum of cystine and cysteine	methionine	lysine
	g/kg	g/kg	g/kg	g/kg
Mixed pig feed	0,28 $n = 15$	0,30 $n = 17$	0,23 $n = 17$	0,30 $n = 13$
Broiler compound	0,48 $n = 16$	0,34 $n = 18$	0,55 $n = 18$	0,75 $n = 16$
Protein concentrate	0,85 $n = 16$	0,62 $n = 17$	1,57 $n = 17$	1,24 $n = 15$
Premixture	2,49 $n = 16$	—	6,20 $n = 16$	6,62 $n = 16$

$n$  is the number of participating laboratories.

Table A.5 — Coefficient of variation (%) for between-laboratory standard deviation ( $s_R$ )

Reference material	Amino acid			
	threonine	sum of cystine and cysteine	methionine	lysine
	[g/kg]	[g/kg]	[g/kg]	[g/kg]
Mixed pig feed	4,1 $n = 15$	9,9 $n = 17$	7,0 $n = 17$	3,2 $n = 13$
Broiler compound	5,2 $n = 16$	8,8 $n = 18$	10,9 $n = 18$	5,4 $n = 16$
Protein concentrate	3,8 $n = 16$	12,3 $n = 17$	13,0 $n = 17$	3,0 $n = 15$
Premixture	4,3 $n = 16$	—	6,9 $n = 16$	6,7 $n = 16$

$n$  is the number of participating laboratories.

## A.2 Second test

Blind duplicate pairs of broiler finisher feed, broiler starter feed, corn, fishmeal and poultry meal samples were submitted to twenty-three laboratories. The results are given in Tables A.6 to A.10.

**Table A.6 — Method performance for analysis of amino acids in poultry meal**

Amino acid	No. of single determinations	Mean	$s_r$	Standard deviation of repeatability %	$s_R$	Standard deviation of reproducibility %
Alanine	42	4,26	0,087	2,04	0,210	4,93
Arginine	46	4,35	0,144	3,31	0,420	9,66
Aspartic acid	44	4,92	0,132	2,68	0,376	7,64
Cystine plus cysteine	42	0,81	0,037	4,57	0,143	17,65
Glutamic acid	46	7,97	0,216	2,71	0,728	9,13
Glycine	38	6,90	0,085	1,23	0,286	4,14
Histidine	38	1,31	0,036	2,75	0,242	18,47
Isoleucine	46	2,24	0,060	2,68	0,261	11,65
Leucine	46	4,09	0,101	2,47	0,310	7,58
Lysine	46	3,63	0,112	3,09	0,360	9,92
Methionine	40	1,17	0,025	2,14	0,140	11,97
Phenylalanine	44	2,33	0,082	3,52	0,215	9,23
Proline	34	4,53	0,102	2,25	0,283	6,25
Serine	44	2,76	0,116	4,20	0,347	12,57
Threonine	44	2,32	0,073	3,15	0,212	9,14
Valine	44	2,82	0,090	3,19	0,361	12,80

**Table A.7 — Method performance for analysis of amino acids in broiler finisher feed**

Amino acid	No. of single determinations	Mean	$s_r$	Standard deviation of repeatability %	$s_R$	Standard deviation of reproducibility %
Alanine	44	1,17	0,032	2,74	0,099	8,46
Arginine	44	1,28	0,030	2,34	0,110	8,59
Aspartic acid	46	1,68	0,047	2,80	0,121	7,20
Cystine plus cysteine	38	0,32	0,010	3,13	0,036	11,25
Glutamic acid	44	3,25	0,052	1,60	0,226	6,95
Glycine	46	1,27	0,029	2,28	0,085	6,69
Histidine	34	0,50	0,020	4,00	0,099	19,80
Isoleucine	42	0,76	0,024	3,16	0,052	6,84
Leucine	46	1,66	0,044	2,65	0,105	6,33
Lysine	44	1,07	0,037	3,46	0,096	8,97
Methionine	38	0,53	0,006	1,13	0,040	7,55
Phenylalanine	44	0,87	0,038	4,37	0,127	14,60
Proline	36	1,39	0,044	3,17	0,124	8,92
Serine	44	0,94	0,044	4,68	0,127	13,51
Threonine	40	0,73	0,020	2,74	0,060	8,22
Valine	44	0,92	0,035	3,80	0,117	12,72

Table A.8 — Method performance for analysis of amino acids in broiler starter feed

Amino acid	No. of single determinations	Mean	$s_r$	Standard deviation of repeatability %	$s_R$	Standard deviation of reproducibility %
Alanine	46	1,28	0,027	2,11	0,100	7,81
Arginine	46	1,57	0,042	2,68	0,129	8,22
Aspartic acid	40	2,29	0,035	1,53	0,137	5,98
Cystine plus cysteine	38	0,35	0,006	1,71	0,056	16,00
Glutamic acid	46	4,04	0,072	1,78	0,339	8,39
Glycine	46	1,27	0,034	2,68	0,090	7,09
Histidine	40	0,65	0,018	2,77	0,100	15,38
Isoleucine	46	0,95	0,019	2,00	0,098	10,32
Leucine	46	1,97	0,033	1,68	0,124	6,29
Lysine	46	1,35	0,032	2,37	0,122	9,04
Methionine	42	0,62	0,013	2,10	0,063	10,16
Phenylalanine	42	1,12	0,025	2,23	0,101	9,02
Proline	36	1,47	0,060	4,08	0,128	8,71
Serine	44	1,12	0,028	2,50	0,153	13,66
Threonine	44	1,12	0,024	2,73	0,087	9,89
Valine	40	1,11	0,019	1,71	0,098	8,83

Table A.9 — Method performance for analysis of amino acids in corn

Amino acid	No. of single determinations	Mean	$s_r$	Standard deviation of repeatability %	$s_R$	Standard deviation of reproducibility %
Alanine	44	0,61	0,009	1,48	0,049	8,03
Arginine	44	0,40	0,013	3,25	0,038	9,50
Aspartic acid	44	0,54	0,015	2,78	0,045	8,33
Cystine plus cysteine	38	0,18	0,007	3,89	0,025	13,89
Glutamic acid	40	1,51	0,036	2,38	0,094	6,23
Glycine	46	0,33	0,010	3,03	0,030	9,09
Histidine	42	0,27	0,019	7,04	0,063	23,33
Isoleucine	44	0,28	0,015	5,38	0,041	14,62
Leucine	44	0,99	0,019	1,92	0,069	6,97
Lysine	44	0,26	0,008	3,08	0,034	13,08
Methionine	42	0,18	0,010	5,56	0,021	11,67
Phenylalanine	40	0,38	0,009	2,37	0,073	19,21
Proline	34	0,73	0,019	2,60	0,044	6,03
Serine	40	0,39	0,007	1,79	0,040	10,26
Threonine	44	0,29	0,012	4,14	0,034	11,72
Valine	46	0,38	0,009	2,37	0,061	16,05