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

(Text with EEA relevance)

THE COMMISSION OF THE EUROPEAN COMMUNITIES,

Having regard to the Treaty establishing the European Community,

Having regard to Council Directive 70/373/EEC of 20 July 1970 on the introduction of Community methods of sampling and analysis for the official control of feedingstuffs [1], as last amended by the Act of Accession of Austria, Finland and Sweden [2], and in particular Article 2 thereof,

Whereas:

(1) Directive 70/373/EEC stipulates that official controls of feedingstuffs for the purpose of checking compliance with the requirements arising under the laws, regulations and administrative provisions governing their quality and composition must be carried out using Community methods of sampling and analysis.

(2) Council Directive 70/524/EEC of 23 November 1970 concerning additives in feedingstuffs [3], as last amended by Commission Regulation (EC) No 2439/1999 of 17 November 1999 [4] stipulates that the vitamin A and vitamin E content must be indicated on the labelling where these substances are added to premixtures and feedingstuffs.

(3) Council Directive 79/373/EEC of 2 April 1979 on the marketing of compound feedingstuffs [5] as last amended by Commission Directive 2000/16/EC [6] and Council Directive 93/74/EEC of 13 September 1993 on feedingstuffs intended for particular nutritional purposes [7], as last amended by Directive 96/25/EC [8], stipulate that amino acids are to be set out on the feedingstuffs labelling.

(4) Community methods of analysis must be established for checking these substances.

(5) The measures provided for in this Directive are in accordance with the opinion of the Standing Committee for Feedingstuffs,

HAS ADOPTED THIS DIRECTIVE:

Article 1

The Member States shall provide that analyses conducted with a view to official controls of the vitamin A, vitamin E and tryptophan content of feedingstuffs and premixtures are carried out using the method set out in Annex hereto.

Article 2

The Member States shall bring into force, not later than 31 August 2000, the laws, regulations or administrative provisions necessary to comply with the provisions of this Directive. They shall immediately inform the Commission thereof.

They shall apply the measures from 1 September 2000.

When Member States adopt these measures, they shall contain a reference to this Directive or shall be accompanied by such reference at the time of their official publication. The procedure for such reference shall be adopted by Member States.

Article 3

This Directive shall enter into force on the 20th day following its publication in the *Official Journal of the European Communities*.

Article 4

This Directive is addressed to the Member States.

Done at Brussels, 6 July 2000.

For the Commission

David BYRNE

Member of the Commission

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[1] OJ L 170, 3.8.1970, p. 2.
[2] OJ C 241, 29.8.1994, p. 1.
[3] OJ L 270, 14.12.1970, p. 1.
[4] OJ L 297, 18.11.1999, p. 8.
[5] OJ L 86, 6.4.1979, p. 30.
[6] OJ L 105, 3.5.2000, p. 36.
[7] OJ L 237, 22.9.1993, p. 23.
[8] OJ L 125, 23.5.1996, p. 35.

ANNEX

PART A

DETERMINATION OF VITAMIN A

1. **Purpose and scope**

This method is for the determination of vitamin A (retinol) in feedingstuffs and premixtures. Vitamin A includes all-*trans*-retinyl alcohol and its *cis* isomers which are determined by this method. The content of vitamin A is expressed in International Units (IU) per kg. One IU corresponds to the activity of 0,300 µg all-*trans*-vitamin A alcohol or 0,344 µg all-*trans*-vitamin A acetate or 0,550 µg all-*trans*-vitamin A palmitate.

The limit of determination is 2 000 IU vitamin A/kg.

2. **Principle**

The sample is hydrolysed with ethanolic potassium hydroxide solution and the vitamin A is extracted into light petroleum. The solvent is removed by evaporation and the residue is dissolved in methanol and, if necessary, diluted to the required concentration. The content of vitamin A is determined by reversed phase high performance liquid chromatography (RP-HPLC) using a UV or a fluorescence detector. The chromatographic parameters are chosen so that there is no separation between the all-*trans*-vitamin A alcohol and its *cis* isomers.

3. **Reagents**

3.1. Ethanol, σ = 96 %

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

3.3. Methanol

3.4. Potassium hydroxide solution, β = 50 g/100 ml

3.5. Sodium ascorbate solution, β = 10 g/100 ml (see 7.7 observations)

3.6. Sodium sulfide, $Na_2S \cdot x\ H_2O$ (x = 7-9)

3.6.1. Sodium sulfide solution, c = 0,5 mol/l in glycerol, β = 120 g/l (for x = 9) (see 7.8 observations)

3.7. Phenolphthalein solution, β = 2 g/100 ml in ethanol (3.1)

3.8. 2-Propanol

3.9. Mobile phase for HPLC: mixture of methanol (3.3) and water, e.g. 980 + 20 (v + v). The exact ratio will be determined by the characteristics of the column employed.

3.10. Nitrogen, oxygen free

3.11. All-*trans*-vitamin A acetate, extra pure, of certified activity, e.g. $2{,}80 \times 10^6$ IU/g

3.11.1. Stock solution of all-*trans*-vitamin A acetate: Weigh to the nearest 0,1 mg, 50 mg of vitamin A acetate (3.11) into a 100 ml graduated flask. Dissolve in 2-propanol (3.8) and make up to the mark with the same solvent. The nominal concentration of this solution is 1 400 IU vitamin A per ml. The exact content has to be determined according to 5.6.3.1.

3.12. All-*trans*-vitamin A palmitate, extra pure, of certified activity, e.g. $1{,}80 \times 10^6$ IU/g

3.12.1. Stock solution of all-*trans*-vitamin A palmitate: Weigh to the nearest 0,1 mg, 80 mg of vitamin A palmitate (3.12) into a 100 ml graduated flask. Dissolve in 2-propanol (3.8) and make up to the mark with the same solvent. The nominal concentration of this solution is 1 400 IU vitamin A per ml. The exact content has to be determined according to 5.6.3.2.

3.13. 2,6-Di-*tert*-butyl-4-methylphenol (BHT) (see 7.5 observations)

4. **Apparatus**

4.1. Vacuum rotary evaporator

4.2. Amber glassware

4.2.1. Flat bottom or conical flasks, 500 ml, with ground-glass socket

4.2.2. Graduated flasks with ground-glass stoppers, narrow-necked, 10, 25, 100 and 500 ml

4.2.3. Separating funnels, conical, 1 000 ml, with ground-glass stoppers

4.2.4. Pear shaped flasks, 250 ml, with ground-glass sockets

4.3. Allihn condenser, jacket length 300 mm, with ground-glass joint, with adapter for gas feed pipe

4.4. Pleated filter paper for phase separation, diameter 185 mm (e.g. Schleicher & Schuell 597 HY 1/2)

4.5. HPLC equipment with injection system

4.5.1. Liquid chromatographic column, 250 mm x 4 mm, C_{18}, 5 or 10 µm packing, or equivalent (performance criterion: only a single peak for all retinol isomers under the HPLC-conditions)

4.5.2. UV or fluorescence detector, with variable wavelength adjustment

4.6. Spectrophotometer with 10 mm quartz cells

4.7. Water-bath with magnetic stirrer

4.8. Extraction apparatus (see figure 1) consisting of:

4.8.1. Glass cylinder of 1l capacity fitted with a ground glass neck and stopper

4.8.2. Ground glass insert equipped with a side-arm and an adjustable tube passing through the centre. The adjustable tube should have a U-shaped lower end and a jet at the opposite end so that the upper liquid layer in the cylinder may be transferred into a separating funnel.

5. **Procedure**

Note: Vitamin A is sensitive to (UV) light and to oxidation. All operations should be carried out in the absence of light (using amber glassware, or glassware protected with aluminium foil) and oxygen (flush with nitrogen). During extraction air above the liquid should be replaced by nitrogen (avoid excess pressure by loosening the stopper from time to time).

5.1. Preparation of the sample

Grind the sample so that it passes a 1 mm mesh sieve, taking care to avoid generation of heat. Grinding must be carried out immediately before weighing and saponification otherwise there may be losses of vitamin A.

5.2. Saponification

Depending on the vitamin A content weigh, to the nearest 0,01 g, 2 g to 25 g of the sample into a 500 ml flat bottom or conical flask (4.2.1). Add successively with swirling 130 ml ethanol (3.1), approximately 100 mg BHT (3.13), 2 ml sodium ascorbate solution (3.5) and 2 ml sodium sulfide solution (3.6). Fit a condenser (4.3) to the flask and immerse the flask in a water-bath with magnetic stirrer (4.7). Heat to boiling and allow to reflux for five minutes. Then add 25 ml potassium hydroxide solution (3.4) through the condenser (4.3) and allow to reflux for a further 25 min, with stirring under a slow stream of nitrogen. Then rinse the condenser with approximately 20 ml water and cool the content of the flask to room temperature.

5.3. Extraction

Transfer by decantation the saponification solution quantitatively by rinsing with a total volume of 250 ml water to a 1000 ml separating funnel (4.2.3) or to the extraction apparatus (4.8). Rinse the saponification flask successively with 25 ml ethanol (3.1) and 100 ml light petroleum (3.2) and transfer the rinsings to the separating funnel or to the extraction apparatus. The proportion of water and ethanol in the combined solutions should be about 2:1. Shake vigorously for two minutes and allow to settle for two minutes.

5.3.1. Extraction using a separating funnel (4.2.3)

When the layers have separated (see observation 7.3) transfer the light petroleum layer to another separating funnel (4.2.3). Repeat this extraction twice, with 100 ml light petroleum (3.2) and twice, with 50 ml light petroleum (3.2).

Wash the combined extracts in the separating funnel twice by gently swirling (to avoid formation of emulsions) with 100 ml portions of water and then by repeated shaking with further 100 ml portions of water until the water remains colourless on addition of phenolphthalein solution (3.7) (washing four times is usually sufficient). Filter the washed extract through a dry pleated filter for phase separation (4.4) to remove any suspended water into a 500 ml graduated flask (4.2.2). Rinse the separating funnel and the filter with 50 ml light petroleum (3.2), make up to the mark with light petroleum (3.2) and mix well.

5.3.2. Extraction using an extraction apparatus (4.8)

When the layers have separated (see observation 7.3) replace the stopper of the glass cylinder (4.8.1) by the ground glass insert (4.8.2) and position the U-shaped lower end of the adjustable tube so that it is just above the level of the interface. By application of pressure from a nitrogen line to the side-arm, transfer the upper light petroleum-layer to a 1 000 ml separating funnel (4.2.3). Add 100 ml light petroleum (3.2) to the glass cylinder, stopper and shake well. Allow the layers to separate and transfer the upper layer to the separating funnel as before. Repeat the extraction procedure with further 100 ml of light petroleum (3.2), then twice with 50 ml portions of light petroleum (3.2) and add the light petroleum layers to the separating funnel.

Wash the combined light petroleum extracts as described in 5.3.1 and proceed as described there.

5.4. Preparation of the sample solution for HPLC

Pipette an aliquot portion of the light petroleum solution (from 5.3.1 or 5.3.2) into a 250 ml pear shaped flask (4.2.4). Evaporate the solvent nearly to dryness on the rotary evaporator (4.1) with reduced pressure at a bath temperature not exceeding 40 °C. Restore atmospheric pressure by admitting nitrogen (3.10) and remove the flask from the rotary evaporator. Remove the remaining solvent with a stream of nitrogen (3.10) and dissolve the residue immediately in a known volume (10-100 ml) of methanol (3.3) (the concentration of vitamin A should be in the range of 5 IU/ml to 30 IU/ml).

5.5. Determination by HPLC

Vitamin A is separated on a C_{18} reversed phase column (4.5.1) and the concentration is measured by means of a UV detector (325 nm) or a fluorescence detector (excitation: 325 nm, emission: 475 nm) (4.5.2).

Inject an aliquot portion (e.g. 20 µl) of the methanolic solution obtained in 5.4 and elute with the mobile phase (3.9). Calculate the mean peak height (area) of several injections of the same sample solution and the mean peak heights (areas) of several injections of the calibration solutions (5.6.2).

HPLC conditions

The following conditions are offered for guidance; other conditions may be used provided that they give equivalent results.

Liquid chromatographic column (4.5.1):	250 mm x 4 mm, C_{18}, 5 or 10 µm packing, or equivalent
Mobile phase (3.9):	Mixture of methanol (3.3) and water, e.g. 980 + 20 (v + v)
Flow rate:	1-2 ml/min
Detector (4.5.2):	UV detector (325 nm) or fluorescence detector (excitation: 325 nm/emission: 475 nm)

5.6. Calibration

5.6.1. Preparation of the working standard solutions

Pipette 20 ml of the vitamin A acetate stock solution (3.11.1) or 20 ml of the vitamin A palmitate stock solution (3.12.1) into a 500 ml flat bottom or conical flask (4.2.1) and hydrolyse as described under 5.2, but without addition of BHT. Subsequently extract with light petroleum (3.2) according to 5.3 and make up to 500 ml with light petroleum (3.2). Evaporate 100 ml of this extract on the rotary evaporator (see 5.4) nearly to dryness, remove the remaining solvent with a stream of nitrogen (3.10) and redissolve the residue in 10,0 ml of methanol (3.3). The nominal concentration of this solution is 560 IU vitamin A per ml. The exact content has to be determined according to 5.6.3.3. The working standard solution has to be freshly prepared before use.

Pipette 2,0 ml of this working standard solution into a 20 ml graduated flask, make up to the mark with methanol (3.3) and mix. The nominal concentration of this diluted working standard solution is 56 IU vitamin A per ml.

5.6.2. Preparation of the calibration solutions and calibration graph

Transfer 1,0, 2,0, 5,0 and 10,0 ml of the diluted working standard solution into a series of 20 ml graduated flasks, make up to the mark with methanol (3.3) and mix. The nominal concentrations of these solutions are 2,8, 5,6, 14,0 and 28,0 IU vitamin A per ml.

Inject 20 µl of each calibration solution several times and determine the mean peak heights (areas). Using the mean peak heights (areas) plot a calibration graph considering the results of the UV control (5.6.3.3).

5.6.3. UV standardisation of the standard solutions

5.6.3.1. Vitamin A acetate stock solution

Pipette 2,0 ml of the vitamin A acetate stock solution (3.11.1) into a 50 ml graduated flask (4.2.2) and make up to the mark with 2-propanol (3.8). The nominal concentration of this solution is 56 IU vitamin A per ml. Pipette 3,0 ml of this diluted vitamin A acetate solution into a 25 ml graduated flask and make up to the mark with 2-propanol (3.8). The nominal concentration of this solution is 6,72 IU vitamin A per ml. Measure the UV spectrum of this solution against 2-propanol (3.8) in the spectrophotometer (4.6) between 300 nm and 400 nm. The extinction maximum must be between 325 nm and 327 nm.

Calculation of the vitamin A content:

$$\text{IU vitamin A/ml} = E_{326} \times 19{,}0$$

($E^{1\,\%}_{1\,cm}$ for vitamin A acetate = 1 530 at 326 nm in 2-propanol)

5.6.3.2. Vitamin A palmitate stock solution

Pipette 2,0 ml of the vitamin A palmitate stock solution (3.12.1) into a 50 ml graduated flask (4.2.2) and make up to the mark with 2-propanol (3.8). The nominal concentration of this solution is 56 IU vitamin A per ml. Pipette 3,0 ml of this diluted vitamin A palmitate solution into a 25 ml graduated flask and make up to the mark with 2-propanol (3.8). The nominal concentration of this solution is 6,72 IU vitamin A per ml. Measure the UV spectrum of this solution against 2-propanol (3.8) in the spectrophotometer (4.6) between 300 nm and 400 nm. The extinction maximum must be between 325 nm and 327 nm.

Calculation of the vitamin A content:

$$\text{IU vitamin A/ml} = E_{326} \times 19{,}0$$

($E^{1\,\%}_{1\,cm}$ for vitamin A palmitate = 957 at 326 nm in 2-propanol)

5.6.3.3. Vitamin A working standard solution

Pipette 3,0 ml of the undiluted vitamin A working standard solution, prepared according to 5.6.1 into a 50 ml graduated flask (4.2.2) and make up to the mark with 2-propanol (3.8). Pipette 5,0 ml of this solution into a 25 ml graduated flask and make up to the mark with 2-propanol (3.8). The nominal concentration of this solution is 6,72 IU vitamin A per ml. Measure the UV spectrum of this solution against 2-propanol (3.8) in the spectrophotometer (4.6) between 300 nm and 400 nm. The extinction maximum must be between 325 nm and 327 nm.

Calculation of the vitamin A content:

$$\text{IU vitamin A/ml} = E_{325} \times 18{,}3$$

($E^{1\,\%}_{1\,cm}$ for vitamin A alcohol = 1 821 at 325 nm in 2-propanol)

6. **Calculation of the results**

From the mean height (area) of the vitamin A peaks of the sample solution determine the concentration of the sample solution in IU/ml by reference to the calibration graph (5.6.2).

The vitamin A content w in IU/kg of the sample is given by the following formula:

$$w = \frac{500 \cdot \beta \cdot V_2 \cdot 1\,000}{V_1 \cdot m} \text{ [IU/kg]}$$

in which:

β = vitamin A concentration of the sample solution (5.4) in IU/ml

V_1 = volume of sample solution (5.4) in ml

V_2 = volume of aliquot taken in 5.4 in ml

m = mass of the test portion in g

7. **Observations**

7.1. For samples with low vitamin A concentration it may be useful to combine the light petroleum-extracts of two saponification-charges (amount weighed: 25 g) to one sample solution for HPLC-determination.

7.2. The weight of the sample taken for the analysis should not contain more than 2 g fat.

7.3. If phase separation does not occur add approximately 10 ml ethanol (3.1) to break the emulsion.

7.4. With cod-liver oil and other pure fats the saponification time should be extended to 45-60 minutes.

7.5. Hydroquinone can be used instead of BHT.

7.6. Using a normal phase-column the separation of retinol isomers is possible.

7.7. Approximately 150 mg ascorbic acid can be used instead of sodium ascorbate solution.

7.8. Approximately 50 mg EDTA can be used instead of sodium sulfide solution.

8. **Repeatability**

The difference between the results of two parallel determinations carried out on the same sample must not exceed 15 % relative to the higher result.

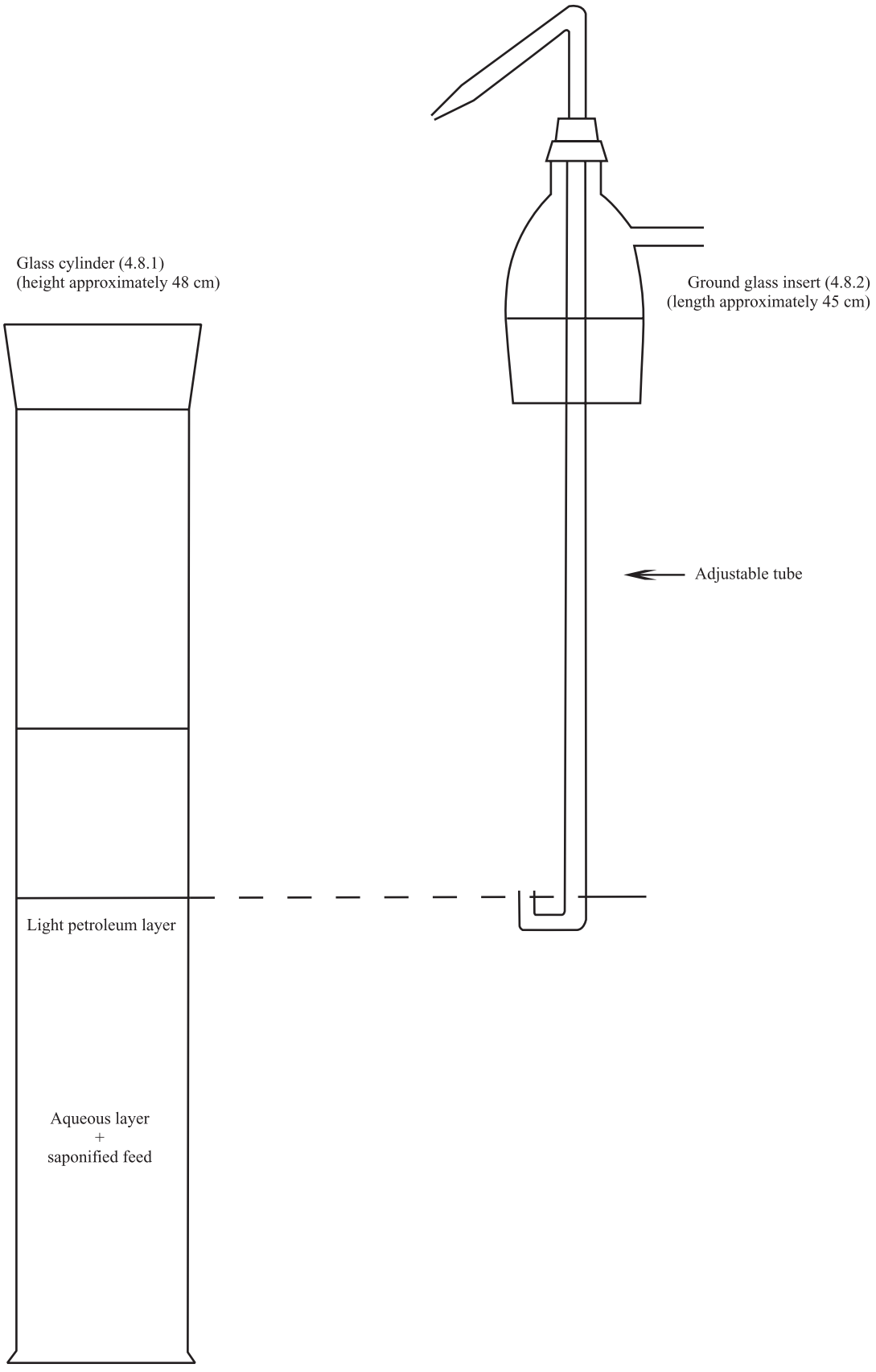
9. **Results of a collaborative study** [1]

	Premix	Premix feed	Mineral concentrate	Protein feed	Piglet
L	13	12	13	12	13
n	48	45	47	46	49
mean [IU/kg]	$17{,}02 \times 10^6$	$1{,}21 \times 10^6$	537 100	151 800	18 070
s_r [IU/kg]	$0{,}51 \times 10^6$	$0{,}039 \times 10^6$	22 080	12 280	682
r [IU/kg]	$1{,}43 \times 10^6$	$0{,}109 \times 10^6$	61 824	34 384	1 910
CV_r [%]	3,0	3,5	4,1	8,1	3,8
s_R [IU/kg]	$1{,}36 \times 10^6$	$0{,}069 \times 10^6$	46 300	23 060	3 614
R [IU/kg]	$3{,}81 \times 10^6$	$0{,}193 \times 10^6$	129 640	64 568	10 119
CV_R [%]	8,0	6,2	8,6	15	20

L: number of laboratories
n: number of single values
s_r: standard deviation of repeatability
s_R: standard deviation reproducibility
r: repeatability
R: reproducibility
CV_r: coefficient of variation of repeatability
CV_R: coefficient of variation of reproducibility

[1] Conducted by the feedingstuffs working group of Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten (VDLUFA).

Figure 1: Extraction apparatus (4.8)



PART B

DETERMINATION OF VITAMIN E

1. **Purpose and scope**

This method is for the determination of vitamin E in feedingstuffs and premixtures. The content of vitamin E is expressed as mg DL-α-tocopherol acetate per kg. 1 mg DL-α-tocopherol acetate corresponds to 0,91 mg DL-α-tocopherol (vitamin E).

The limit of determination is 2 mg vitamin E/kg.

2. **Principle**

The sample is hydrolysed with ethanolic potassium hydroxide solution and the vitamin E is extracted into light petroleum. The solvent is removed by evaporation and the residue is dissolved in methanol and, if necessary, diluted to the required concentration. The content of vitamin E is determined by reversed phase high performance liquid chromatography (RP-HPLC) using a fluorescence or a UV detector.

3. **Reagents**

3.1. Ethanol, σ = 96 %

3.2. Light petroleum, boiling range 40 °C - 60 °C

3.3. Methanol

3.4. Potassium hydroxide solution, β = 50 g/100 ml

3.5. Sodium ascorbate solution, β = 10 g/100 ml (see 7.7 observations)

3.6. Sodium sulfide, $Na_2S \cdot x\,H_2O$ (x = 7-9)

3.6.1. Sodium sulfide solution, c = 0,5 mol/l in glycerol, β = 120 g/l (for x = 9) (see 7.8 observations)

3.7. Phenolphthalein solution, β = 2 g/100 ml in ethanol (3.1)

3.8. Mobile phase for HPLC: mixture of methanol (3.3) and water, e.g. 980 + 20 (v + v). The exact ratio will be determined by the characteristics of the column employed.

3.9. Nitrogen, oxygen free

3.10. DL-α-tocopherol acetate, extra pure, of certified activity

3.10.1. Stock solution of DL-α-tocopherol acetate: Weigh to the nearest 0,1 mg, 100 mg of DL-α-tocopherol acetate (3.10) into a 100 ml graduated flask. Dissolve in ethanol (3.1) and make up to the mark with the same solvent. 1 ml of this solution contains 1 mg DL-α-tocopherol acetate. (UV control see 5.6.1.3; stabilisation see 7.4 observations).

3.11. DL-α-tocopherol, extra pure, of certified activity

3.11.1. Weigh to the nearest 0,1 mg, 100 mg of DL-α-tocopherol (3.10) into a 100 ml graduated flask. Dissolve in ethanol (3.1) and make up to the mark with the same solvent. 1 ml of this solution contains 1 mg DL-α-tocopherol. (UV control see 5.6.2.3; stabilisation see 7.4 observations).

3.12. 2,6-Di-*tert*-butyl-4-methylphenol (BHT) (see 7.5 observations)

4. **Apparatus**

4.1. Vacuum rotary evaporator

4.2. Amber glassware

4.2.1. Flat bottom or conical flasks, 500 ml, with ground-glass socket

4.2.2. Graduated flasks with ground-glass stoppers, narrow-necked, 10, 25, 100 and 500 ml

4.2.3. Separating funnels, conical, 1 000 ml, with ground-glass stoppers

4.2.4. Pear shaped flasks, 250 ml, with ground-glass sockets

4.3. Allihn condenser, jacket length 300 mm, with ground-glass joint, with adapter for gas feed pipe

4.4. Pleated filter paper for phase separation, diameter 185 mm (e.g. Schleicher & Schuell 597 HY 1/2)

4.5. HPLC equipment with injection system

4.5.1. Liquid chromatographic column, 250 mm x 4 mm, C_{18}, 5 or 10 µm packing, or equivalent

4.5.2. Fluorescence or UV detector, with variable wavelength adjustment

4.6. Spectrophotometer with 10 mm quartz cells

4.7. Water-bath with magnetic stirrer

4.8. Extraction apparatus (see figure 1) consisting of:

4.8.1. Glass cylinder of 1 l capacity fitted with a ground glass neck and stopper

4.8.2. Ground glass insert equipped with a side-arm and an adjustable tube passing through the centre. The adjustable tube should have a U-shaped lower end and a jet at the opposite end so that the upper liquid layer in the cylinder may be transferred into a separating funnel.

5. **Procedure**

Note: Vitamin E is sensitive to (UV) light and to oxidation. All operations should be carried out in the absence of light (using amber glassware, or glassware protected with aluminium foil) and oxygen (flush with nitrogen). During extraction air above the liquid should be replaced by nitrogen (avoid excess pressure by loosening the stopper from time to time).

5.1. Preparation of the sample

Grind the sample so that it passes a 1 mm mesh sieve, taking care to avoid generation of heat. Grinding must be carried out immediately before weighing and saponification otherwise there may be losses of vitamin E.

5.2. Saponification

Depending on the vitamin E content weigh, to the nearest 0,01 g, 2 g to 25 g of the sample into a 500 ml flat bottom or conical flask (4.2.1). Add successively with swirling 130 ml ethanol (3.1), approximately 100 mg BHT (3.12), 2 ml sodium ascorbate solution (3.5) and 2 ml sodium sulfide solution (3.6). Fit the condenser (4.3) to the flask and immerse the flask in a water-bath with magnetic stirrer (4.7). Heat to boiling and allow to reflux for five minutes. Then add 25 ml potassium hydroxide solution (3.4) through the condenser (4.3) and allow to reflux for a further 25 min with stirring under a slow stream of nitrogen. Then rinse the condenser with approximately 20 ml water and cool the content of the flask to room temperature.

5.3. Extraction

Transfer by decantation the saponification solution quantitatively by rinsing with a total volume of 250 ml water to a 1000 ml separating funnel (4.2.3) or to the extraction apparatus (4.8). Rinse the saponification flask successively with 25 ml ethanol (3.1) and 100 ml light petroleum (3.2) and transfer the rinsings to the separating funnel or to the extraction apparatus. The proportion of water and ethanol in the combined solutions should be about 2:1. Shake vigorously for two minutes and allow to settle for two minutes.

5.3.1. Extraction using a separating funnel (4.2.3)

When the layers have separated (see observation 7.3) transfer the light petroleum layer to another separating funnel (4.2.3). Repeat this extraction twice, with 100 ml light petroleum (3.2) and twice, with 50 ml light petroleum (3.2).

Wash the combined extracts in the separating funnel twice by gently swirling (to avoid formation of emulsions) with 100 ml portions of water and then by repeated shaking with further 100 ml portions of water until the water remains colourless on addition of phenolphthalein solution (3.7) (washing four times is usually sufficient). Filter the washed extract through a dry pleated filter for phase separation (4.4) to remove any suspended water into a 500 ml graduated flask (4.2.2). Rinse the separating funnel and the filter with 50 ml light petroleum (3.2), make up to the mark with light petroleum (3.2) and mix well.

5.3.2. Extraction using an extraction apparatus (4.8)

When the layers have separated (see observation 7.3) replace the stopper of the glass cylinder (4.8.1) by the ground glass insert (4.8.2) and position the U-shaped lower end of the adjustable tube so that it is just above the level of the interface. By application of pressure from a nitrogen line to the side-arm, transfer the upper light petroleum-layer to a 1 000 ml separating funnel (4.2.3). Add 100 ml light petroleum (3.2) to the glass cylinder, stopper and shake well. Allow the layers to separate and transfer the upper layer to the separating funnel as before. Repeat the extraction procedure with further 100 ml of light petroleum (3.2), then twice with 50 ml portions of light petroleum (3.2) and add the light petroleum layers to the separating funnel.

Wash the combined light petroleum extracts as described in 5.3.1 and proceed as described there.

5.4. Preparation of the sample solution for HPLC

Pipette an aliquot portion of the light petroleum solution (from 5.3.1 or 5.3.2) into a 250 ml pear shaped flask (4.2.4). Evaporate the solvent nearly to dryness on the rotary evaporator (4.1) with reduced pressure at a bath temperature not exceeding 40 °C. Restore atmospheric pressure by admitting nitrogen (3.9) and remove the flask from the rotary evaporator. Remove the remaining solvent with a stream of nitrogen (3.9) and dissolve the residue immediately in a known volume (10-100 ml) of methanol (3.3) (the concentration of DL-α-tocopherol should be in the range 5 µg/ml to 30 µg/ml).

5.5. Determination by HPLC

Vitamin E is separated on a C_{18} reversed phase column (4.5.1) and the concentration is measured using a fluorescence detector (excitation: 295 nm, emission: 330 nm) or a UV detector (292 nm) (4.5.2).

Inject an aliquot portion (e.g. 20 µl) of the methanolic solution obtained in 5.4 and elute with the mobile phase (3.8). Calculate the mean peak heights (areas) of several injections of the same sample solution and the mean peak heights (areas) of several injections of the calibration solutions (5.6.2).

HPLC conditions

The following conditions are offered for guidance; other conditions may be used provided that they give equivalent results.

Liquid chromatographic column (4.5.1):	250 mm x 4 mm, C_{18}, 5 or 10 µm packing, or equivalent
Mobile phase (3.8):	Mixture of methanol (3.3) and water, e.g. 980 + 20 (v + v)
Flow rate:	1-2 ml/min
Detector (4.5.2):	Fluorescence detector (excitation: 295 nm/emission: 330 nm) or UV detector (292 nm)

5.6. Calibration (DL-α-tocopherol acetate or DL-α-tocopherol)

5.6.1. DL-α-tocopherol acetate standard

5.6.1.1. Preparation of the working standard solution

Transfer by pipette 25 ml of the DL-α-tocopherol acetate stock solution (3.10.1) into a 500 ml flat bottom or conical flask (4.2.1) and hydrolyse as described under 5.2. Subsequently extract with light petroleum (3.2) according to 5.3 and make up to 500 ml with light petroleum. Evaporate 25 ml of this extract on the rotary evaporator (see 5.4) nearly to dryness, remove the remaining solvent with a stream of nitrogen (3.9) and redissolve the residue in 25,0 ml of methanol (3.3). The nominal concentration of this solution is 45,5 µg DL-α-tocopherol per ml, equivalent to 50 µg DL-α-tocopherol acetate per ml. The working standard solution has to be freshly prepared before use.

5.6.1.2. Preparation of the calibration solutions and calibration graph

Transfer 1,0, 2,0, 4,0 and 10,0 ml of the working standard solution into a series of 20 ml graduated flasks, make up to the mark with methanol (3.3) and mix. The nominal concentrations of these solutions are 2,5, 5,0, 10,0 and 25,0 µg/ml DL-α-tocopherol acetate, i. e. 2,28, 4,55, 9,10 µg/ml and 22,8 µg/ml DL-α-tocopherol.

Inject 20 µl of each calibration solution several times and determine the mean peak heights (areas). Using the mean peak heights (areas) plot a calibration graph.

5.6.1.3. UV standardisation of the DL-α-tocopherol acetate stock solution (3.10.1)

Dilute 5,0 ml of the DL-α-tocopherol acetate stock solution (3.10.1) to 25,0 ml with ethanol and measure the UV spectrum of this solution against ethanol (3.1) in the spectrophotometer (4.6) between 250 nm and 320 nm.

The absorption maximum should be at 284 nm:

$$E^{1\,\%}_{1\,cm} = 43{,}6 \text{ at } 284 \text{ nm in ethanol}$$

At this dilution an extinction value of 0,84 to 0,88 should be obtained.

5.6.2. DL-α-tocopherol standard

5.6.2.1. Preparation of the working standard solution

Transfer by pipette 2,0 ml of the DL-α-tocopherol stock solution (3.11.1) into a 50 ml graduated flask, dissolve in methanol (3.3) and make up to the mark with methanol. The nominal concentration of this solution is 40 µg DL-α-tocopherol per ml, equivalent to 44,0 µg DL-α-tocopherol acetate per ml. The working standard solution has to be freshly prepared before use.

5.6.2.2. Preparation of the calibration solutions and calibration graph

Transfer 1,0, 2,0, 4,0 and 10,0 ml of the working standard solution into a series of 20 ml graduated flasks, make up to the mark with methanol (3.3) and mix. The nominal concentrations of these solutions are 2,0, 4,0, 8,0 and 20,0 µg/ml DL-α-tocopherol, i. e. 2,20, 4,40, 8,79 µg/ml and 22,0 µg/ml DL-α-tocopherol acetate.

Inject 20 µl of each calibration solution several times and determine the mean peak heights (areas). Using the mean peak heights (areas) plot a calibration graph.

5.6.2.3. UV standardisation of the DL-α-tocopherol stock solution (3.11.1)

Dilute 2,0 ml of the DL-α-tocopherol stock solution (3.11.1) to 25,0 ml with ethanol and measure the UV spectrum of this solution against ethanol (3.1) in the spectrophotometer (4.6) between 250 nm and 320 nm. The absorption maximum should be at 292 nm:

$$E^{1\,\%}_{1\,cm} = 75{,}8 \text{ at } 292 \text{ nm in ethanol}$$

At this dilution an extinction value of 0,6 should be obtained.

6. **Calculation of the results**

From the mean height (area) of the vitamin E peaks of the sample solution determine the concentration of the sample solution in µg/ml (calculated as α-tocopherol acetate) by reference to the calibration graph (5.6.1.2 or 5.6.2.2).

The vitamin E content w in mg/kg of the sample is given by the following formula:

$$w = \frac{500 \cdot \beta \cdot V_2}{V_1 \cdot m} \text{ [mg/kg]}$$

in which:

β = vitamin E concentration of the sample solution (5.4) in µg/ml

V_1 = volume of sample solution (5.4) in ml

V_2 = volume of aliquot taken in 5.4 in ml

m = mass of the test portion in g

7. **Observations**

7.1. For samples with low vitamin E concentration it may be useful to combine the light petroleum-extracts of two saponification-charges (amount weighed: 25 g) to one sample solution for HPLC-determination.

7.2. The weight of the sample taken for the analysis should not contain more than 2 g fat.

7.3. If phase separation does not occur add approximately 10 ml ethanol (3.1) to break the emulsion.

7.4. After the spectrophotometric measurement of the DL-α-tocopherol acetate or DL-α-tocopherol solution according to 5.6.1.3 or 5.6.2.3 respectively add approximately 10 mg BHT (3.12) to the solution (3.10.1 or 3.10.2) and keep the solution in a refrigerator (storage life maximum of four weeks).

7.5. Hydroquinone can be used instead of BHT.

7.6. Using a normal phase-column the separation of α-, β-, χ- and δ-tocopherol is possible.

7.7. Approximately 150 mg ascorbic acid can be used instead of sodium ascorbate solution.

7.8. Approximately 50 mg EDTA can be used instead of sodium sulfide solution.

8. **Repeatability**

The difference between the results of two parallel determinations carried out on the same sample must not exceed 15 % relative to the higher result.

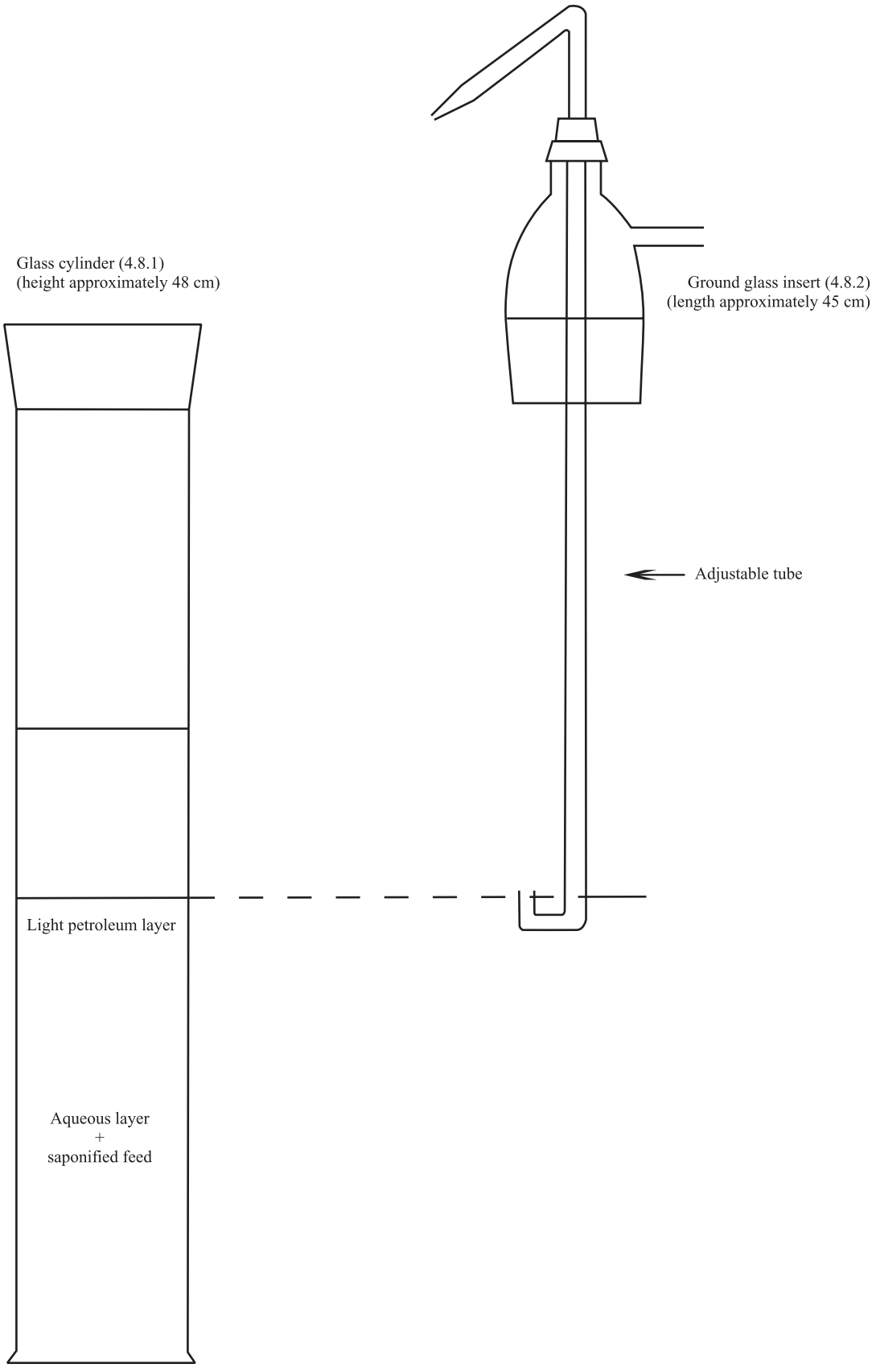
9. **Results of a collaborative study** [1]

	Premix	Premix feed	Mineral concentrate	Protein feed	Piglet
L	12	12	12	12	12
n	48	48	48	48	48
mean [mg/kg]	17 380	1 187	926	315	61,3
s_r [mg/kg]	384	45,3	25,2	13,0	2,3
r [mg/kg]	1 075	126,8	70,6	36,4	6,4
CV_r [%]	2,2	3,8	2,7	4,1	3,8
s_R [mg/kg]	830	65,0	55,5	18,9	7,8
R [mg/kg]	2 324	182,0	155,4	52,9	21,8
CV_R [%]	4,8	5,5	6,0	6,0	12,7

L: number of laboratories
n: number of single values
s_r: standard deviation of repeatability
s_R: standard deviation of reproducibility
r: repeatability
R: reproducibility
CV_r: coefficient of variation of repeatability
CV_R: coefficient of variation of reproducibility

[1] Conducted by the feedingstuffs working group of Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten (VDLUFA).

Figure 1: Extraction apparatus (4.8)



PART C

DETERMINATION OF TRYPTOPHAN

1. **Purpose and scope**

The method is for the determination of the total and free tryptophan in feedingstuffs. It does not distinguish between D- and L- forms.

2. **Principle**

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

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

The tryptophan and the internal standard in the hydrolysate or in the extract are determined by HPLC with fluorescence detection.

3. **Reagents**

3.1. Double distilled water or water of equivalent quality must be used (conductivity < 10 µS/cm)

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

3.3. Internal standard substance: α-methyl-tryptophan (purity/content ≥ 99 %), dried under vacuum over phosphorous pentoxide

3.4. Barium hydroxide octa-hydrate (care should be taken not to expose the $Ba(OH)_2 \cdot 8\ H_2O$ excessively to air in order to avoid formation of $BaCO_3$, which could disturb the determination) (see observation 9.3).

3.5. Sodium hydroxide

3.6. Ortho-phosphoric acid, w = 85 %

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

3.8. Methanol, HPLC grade

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

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

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

3.11. Hydrochloric acid, c = 6 mol/l:

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

3.12. Hydrochloric acid, c = 1 mol/l:

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

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

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

3.14. Ortho-phosphoric acid, c = 0,5 mol/l:

Take 34 ml ortho-phosphoric acid (3.6) and make up to 1 l with water (3.1)

3.15. Concentrated solution of tryptophan (3.2), c = 2,50 µmol/ml:

In a 500 ml volumetric flask dissolve 0,2553 g tryptophan (3.2) 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 = 2,50 µmol/ml:

In a 500 ml volumetric flask dissolve 0,2728 g α-methyl-tryptophan (3.3) 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 concentrated solution of tryptophan (3.15), and 2,00 ml of concentrated internal standard (α-methyl-tryptophan) solution (3.16). Dilute with water (3.1) and methanol (3.8) to approximately the same volume and to approximately the same concentration of methanol (10-30 %) as the finished hydrolysate.

This solution must be prepared freshly before use.

Protect from direct sunlight during preparation.

3.18. Acetic acid

3.19. 1,1,1-trichloro-2-methyl-2-propanol

3.20. Ethanolamine > 98 %

3.21. Solution of 1 g 1,1,1-trichloro-2-methyl-2-propanol (3.19) in 100 ml methanol (3.8)

3.22. Mobile phase for HPLC: 3,00 g acetic acid (3.18) + 900 ml water (3.1) + 50,0 ml solution (3.21) of 1,1,1-trichloro-2-methyl-2-propanol (3.19) in methanol (3.8) (1 g/100 ml). Adjust pH to 5,00 using ethanolamine (3.20). Make up to 1 000 ml with water (3.1)

4. **Apparatus**

4.1. HPLC equipment with a spectrofluorimetric detector

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

4.3. pH-meter

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

4.5. Membrane filter, 0,45 µm

4.6. Autoclave, 110 (± 2).°C, 1,4 (± 0,1) bar

4.7. Mechanical shaker or magnetic stirrer

4.8. Vortex mixer

5. **Procedure**

5.1. Preparation of samples

The sample is ground to pass through a 0,5 mm sieve. Samples high in moisture must be either air-dried at a temperature not exceeding 50.°C or freeze dried prior to grinding. Samples with high fat content should 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 (1-5 g) of the prepared sample (5.1), into a conical flask. Add 100,0 ml hydrochloric acid, c = 0,1 mol/l (3.13) and 5,00 ml 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 ortho-phosphoric acid, c = 0,5 mol/l (3.14). Adjust the pH to 3,0 using sodium hydroxide, c = 1,0 mol/l (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 to a volume necessary for the chromatography (approximately the same volume as the calibration standard solution (3.17)).

Filter a few ml 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 paragraph 5.4.

Protect 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 to 1 g of the prepared sample (5.1) into the polypropylene flask (4.4). The weighed sample portion should have a nitrogen content of about 10 mg. Add 8,4 g barium hydroxide octa-hydrate (3.4) and 10 ml water. 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 water. Put on the screw cap and close the flask loosely. Transfer to an autoclave (4.6) with boiling water and steam for 30-60 minutes. Close the autoclave and autoclave at 110 (± 2).°C for 20 hours.

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

Then, add 5 ml ortho-phosphoric acid, c = 0,5 mol/l (3.14). Keep the vessel in the cooling bath and neutralise with HCl, c = 6 mol/l (3.11) whilst stirring and adjust the pH to 3,0 using HCl, c = 1 mol/l (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 to the defined volume necessary for the chromatography (for example 100 ml). The addition of methanol should not cause precipitation.

Filter a few ml 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 paragraph 5.4.

Protect 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 9.1 and 9.2):

Liquid chromatographic column (4.2):	125 mm x 4 mm, C_{18}, 3 µm packing or equivalent
Column temperature:	Room temperature
Mobile phase (3.22):	3,00 g acetic acid (3.18) + 900 ml water (3.1) + 50,0 ml solution (3.21) of 1,1,1-trichloro-2-methyl-2-propanol (3.19) in methanol (3.8) (1 g/100 ml). Adjust pH to 5,00 using ethanolamine (3.20). Make up to 1 000 ml with water (3.1)
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**

$$\frac{A \times B \times C \times D \times E \times MW}{F \times G \times H \times 10\,000 \times W} = \text{g tryptophan per 100 g sample}$$

A = peak area of internal standard, calibration standard solution (3.17)

B = peak area of tryptophan, extract (5.2) or hydrolysate (5.3)

C = volume in ml (2 ml) of concentrated tryptophan solution (3.15) added to the calibration solution (3.17)

D = concentration in µmol/ml (= 2,50) of concentrated tryptophan solution (3.15) added to calibration solution (3.17)

E = volume in ml 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)

F = peak area of internal standard, extract (5.2) or hydrolysate (5.3)

G = peak area of tryptophan, calibration standard solution (3.17)

H = volume in ml (= 2,00 ml) of concentrated internal standard solution (3.16) added to calibration standard solution (3.17)

W = sample weight in g (corrected to original weight if dried and/or defatted)

MW = molecular weight of tryptophan (= 204,23)

7. **Repeatability**

The difference between the results of two parallel determinations carried out on the same sample must not exceed 10 % relative to the highest result.

8. **Results of a collaborative study**

A Community collaborative Study (Fourth intercomparison) was arranged in which three samples were analysed by up to 12 laboratories to certify the method for hydrolysis. Replicate (5) analyses were performed on each sample. The results are given in the following table:

	Sample 1 Pig feed	Sample 2 Pig feed supplemented with L-tryptophan	Sample 3 Feed concentrate for pigs
L	12	12	12
n	50	55	50
Mean [g/kg]	2,42	3,40	4,22
s_r [g/kg]	0,05	0,05	0,08
r [g/kg]	0,14	0,14	0,22
CV_r [%]	1,9	1,6	1,9
s_R [g/kg]	0,15	0,20	0,09
R [g/kg]	0,42	0,56	0,25
CV_R [%]	6,3	6,0	2,2

L: number of laboratories submitting results
n: number of single results retained eliminating outliers (identified by Cochran, dixon outlier test)
s_r: standard deviation of repeatability
s_R: standard deviation of reproducibility
r: repeatability
R: reproducibility
CV_r: coefficient of variation of repeatability, %
CV_R: coefficient of variation of reproducibility, %

Another Community collaborative study (third intercomparison) was arranged in which two samples were analysed by up to 13 laboratories to certify the method for extraction of free tryptophan. Replicate (5) analyses were performed on each sample. The results are given in the following table:

	Sample 4 Wheat and soya mixture	Sample 5 Wheat and soya mixture (= sample 4) with added tryptophan (0,457 g/kg)
L	12	12
n	55	60
Mean [g/kg]	0,391	0,931
s_r [g/kg]	0,005	0,012
r [g/kg]	0,014	0,034
CV_r [%]	1,34	1,34
s_R [g/kg]	0,018	0,048
R [g/kg]	0,050	0,134
CV_R [%]	4,71	5,11

L number of laboratories submitting results
n number of single results retained eliminating outliers (identified by Cochran, dixon outlier test)
s_r standard deviation of repeatability
s_R standard deviation of reproducibility
r repeatability
R reproducibility
CV_r coefficient of variation of repeatability, %
CV_R coefficient of variation of reproducibility, %

Another Community intercomparison study was arranged in which four samples were analysed by up to seven laboratories with the aim of a tryptophan certification for hydrolysis. The results are given below Replicate five analyses were performed on each sample.

	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)
L	7	7	7	7
n	25	30	30	30
Mean [g/kg]	2,064	8,801	6,882	5,236
s_r [g/kg]	0,021	0,101	0,089	0,040
r [g/kg]	0,059	0,283	0,249	0,112
CV_r [%]	1,04	1,15	1,30	0,76
s_R [g/kg]	0,031	0,413	0,283	0,221
R [g/kg]	0,087	1,156	0,792	0,619
CV_R [%]	1,48	4,69	4,11	4,22

L: number of laboratories submitting results
n: number of single results retained eliminating outliers (identified by Cochran, Dixon outlier test)
s_r: standard deviation of repeatability
s_R: standard deviation of reproducibility
r: repeatability
R: reproducibility
CV_r: coefficient of variation of repeatability, %
CV_R: coefficient of variation of reproducibility, %

9. **Observations**

9.1. Following special chromatographic conditions may give better separation between tryptophan and α-methyl-tryptophan.

Isocratic elution followed by gradient column cleaning:

Liquid chromatographic column:	125 mm x 4 mm, C_{18}, 5 µm packing or equivalent		
Column temperature:	32.°C		
Mobile phase:	A: 0,01 mol/l KH_2PO_4/Methanol, 95 + 5 (V + V).		
	B: Methanol		
Gradient program:	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 minutes		

9.2. The chromatography will vary according to the type of HPLC and column packing material used. The chosen system must 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 internal standard should be run in order to check the base line 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.

In the range of operation, the chromatographic system should give linear response. The linear response should be measured with a constant (the normal) concentration of the internal standard and varying concentrations of tryptophan. It is of importance 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.

9.3. Barium hydroxide

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.