

## COMMISSION DIRECTIVE 98/64/EC

of 3 September 1998

establishing Community methods of analysis for the determination of amino-acids, crude oils and fats, and olaquinox in feedingstuffs and amending Directive 71/393/EEC

(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<sup>(1)</sup>, as last amended by the Act of Accession of Austria, Finland and Sweden, and in particular Article 2 thereof,

Whereas 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;

Whereas Council Directive 79/373/EEC of 2 April 1979 on the marketing of compound feedingstuffs<sup>(2)</sup>, as last amended by Commission Directive 97/47/EC<sup>(3)</sup>, and Council Directive 93/74/EEC of 13 September 1993 on feedingstuffs intended for particular nutritional purposes<sup>(4)</sup>, as last amended by Directive 96/25/EC<sup>(5)</sup>, stipulate that amino-acids and crude oils and fats are to be set out on the feedingstuffs label;

Whereas Council Directive 70/524/EEC of 23 November 1970 concerning additives in feedingstuffs<sup>(6)</sup>, as last amended by Commission Directive 98/19/EC<sup>(7)</sup>, stipulates that the olaquinox content must be indicated on the labelling where this substance is added to compound feedingstuffs;

Whereas the second Commission Directive 71/393/EEC of 18 November 1971 establishing Community methods of analysis for the official control of feedingstuffs<sup>(8)</sup>, as last amended by Commission Directive 84/4/EEC<sup>(9)</sup>, sets

out methods for analysis for, *inter alia*, the determination of crude oils and fats; whereas it is appropriate to modify the method described;

Whereas Community methods of analysis must be established for checking these substances;

Whereas 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 amino-acid, crude oil and fat, and olaquinox content of feedingstuffs are carried out using the methods set out in the Annex hereto.

*Article 2*

In the Annex to Commission Directive 71/393/EEC, point '4. Determination of crude oils and fats' is replaced by part B of the annex to this Directive.

*Article 3*

1. Member States shall bring into force the laws, regulations or administrative provisions necessary to comply with this Directive by 31 December 1998. They shall immediately inform the Commission thereof.

They shall apply the measures from 1 January 1999.

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.

<sup>(1)</sup> OJ L 170, 3. 8. 1970, p. 2.

<sup>(2)</sup> OJ L 86, 6. 4. 1979, p. 30.

<sup>(3)</sup> OJ L 211, 5. 8. 1997, p. 45.

<sup>(4)</sup> OJ L 237, 22. 9. 1993, p. 23.

<sup>(5)</sup> OJ L 125, 23. 5. 1996, p. 35.

<sup>(6)</sup> OJ L 270, 14. 12. 1970, p. 1.

<sup>(7)</sup> OJ L 96, 28. 3. 1998, p. 39.

<sup>(8)</sup> OJ L 279, 20. 12. 1971, p. 7.

<sup>(9)</sup> OJ L 15, 18. 1. 1984, p. 28.

2. Member States shall communicate to the Commission the text of the main provisions of domestic law which they adopt in the field governed by this Directive.

*Article 4*

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

*Article 5*

This Directive is addressed to the Member States.

Done at Brussels, 3 September 1998.

*For the Commission*

Franz FISCHLER

*Member of the Commission*

---

## ANNEX

## PART A

## DETERMINATION OF AMINO ACIDS

## 1. Purpose and scope

This method is for the determination of free (synthetic and natural) and total (peptide bound and free) amino acids in feedingstuffs, using an amino acid analyzer. It is applicable to the following amino acids: cyst(e)ine, methionine, lysine, threonine, alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, isoleucine, leucine, phenylalanine, proline, serine, tyrosine and valine.

The method does not distinguish between the salts of amino acids and it cannot differentiate between D and L forms of amino acids. It is not valid for the determination of tryptophan or hydroxy analogues of amino acids.

## 2. Principle

## 2.1. Free amino acids

The free amino acids are extracted with diluted hydrochloric acid. Coextracted 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 must be oxidised to cysteic acid and methionine sulphone respectively prior to hydrolysis. Tyrosine must be determined in hydrolysates of unoxidized samples. All the other amino acids listed in paragraph 1 can be determined in either the oxidised or unoxidised sample.

Oxidation is performed at 0 °C with a performic acid/phenol mixture. Excess oxidation reagent is decomposed with sodium disulphite. The oxidised or unoxidised sample is hydrolysed with hydrochloric acid ( $c = 6 \text{ mol/l}$ ) for 23 hours. 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

Double distilled water or water of equivalent quality must be used (conductivity  $< 10 \mu\text{S}$ ).

- 3.1. Hydrogen peroxide,  $w = 30 \%$ .
- 3.2. Formic acid,  $w = 98\text{-}100 \%$ .
- 3.3. Phenol.
- 3.4. Sodium disulfite.
- 3.5. Sodium hydroxide.
- 3.6. 5-Sulfosalicylic acid dihydrate.
- 3.7. Hydrochloric acid, density approximately 1,18 g/ml.
- 3.8. tri-Sodium citrate dihydrate.
- 3.9. 2,2'-Thiodiethanol (thiodiglycol).
- 3.10. Sodium chloride.
- 3.11. Ninhydrin.
- 3.12. Light petroleum, boiling range 40-60 °C.
- 3.13. Norleucine, or other compound suitable for use as internal standard.
- 3.14. Nitrogen gas ( $< 10 \text{ ppm oxygen}$ ).

- 3.15. 1-Octanol.
- 3.16. Amino acids.
- 3.16.1. Standard substances listed under paragraph 1. Pure compounds containing no water of crystallisation. Dry under vacuum over  $P_2O_5$  or  $H_2SO_4$  for 1 week prior to use.
- 3.16.2. Cysteic acid.
- 3.16.3. Methionine sulphone.
- 3.17. Sodium hydroxide solution,  $c = 7,5 \text{ mol/l}$ :  
Dissolve 300 g NaOH (3.5) in water and make up to 1 litre.
- 3.18. Sodium hydroxide solution,  $c = 1 \text{ mol/l}$ :  
Dissolve 40 g NaOH (3.5) in water and make up to 1 litre.
- 3.19. Formic acid-phenol solution:  
Mix 889 g formic acid (3.2) with 111 g water and add 4,73 g phenol (3.3).
- 3.20. Hydrolysis mixture,  $c = 6 \text{ mol HCl/l}$  containing 1 g phenol/l:  
Add 1 g phenol (3.3) to 492 ml HCl (3.7) and make up to 1 litre with water.
- 3.21. Extraction mixture,  $c = 0,1 \text{ mol HCl/l}$  containing 2 % thiodiglycol:  
Take 8,2 ml HCl (3.7), dilute with approximately 900 ml water, add 20 ml thiodiglycol (3.9) and make up to 1 litre with water, (do not mix 3.7 and 3.9 directly).
- 3.22. 5-Sulfosalicylic acid,  $\beta = 6 \%$ :  
Dissolve 60 g 5-sulfosalicylic acid (3.6) in water and make up to 1 litre with water.
- 3.23. Oxidation mixture (Performic acid-phenol):  
Mix 0,5 ml hydrogen peroxide (3.1) with 4.5 ml formic acid-phenol solution (3.19) in a small beaker. Incubate at 20-30 °C for 1 hour in order to form performic acid, then cool on an ice-water bath (15 min) before adding to the sample.  
*Caution:* Avoid contact with skin and wear protective clothing.
- 3.24. Citrate buffer,  $c = 0,2 \text{ mol Na}^+/\text{l}$ , pH 2,20:  
Dissolve 19,61 g sodium citrate (3.8), 5 ml thiodiglycol (3.9), 1 g phenol (3.3) and 16,50 ml HCl (3.7) in approximately 800 ml water. Adjust pH to 2,20. Make up to 1 litre with water.
- 3.25. Elution buffers, prepared according to conditions for the analyzer used (4.9).
- 3.26. Ninhydrin reagent, prepared according to conditions for the analyzer used (4.9).
- 3.27. Standard solutions of amino acids. These solutions should be stored below 5 °C.
- 3.27.1. Stock standard solution of amino acids (3.16.1).  $c = 2,5 \mu\text{mol/ml}$  of each in hydrochloric acid. May be obtained commercially.
- 3.27.2. Stock standard solution of cysteic acid and methionine sulphone,  $c = 1,25 \mu\text{mol/ml}$ .  
Dissolve 0,2115 g cysteic acid (3.16.2) and 0,2265 g methionine sulphone (3.16.3) in citrate buffer (3.24) in a 1 litre graduated flask and make up to mark with citrate buffer. Store below 5 °C for not more than 12 months. This solution is not used if the stock standard solution (3.27.1) contains cysteic acid and methionine sulphone.
- 3.27.3. Stock standard solution of the internal standard e.g. norleucine,  $c = 20 \mu\text{mol/ml}$ .  
Dissolve 0,6560 g norleucine (3.13) in citrate buffer (3.24) in a graduated flask and make up to 250 ml with citrate buffer. Store below 5 °C for no more than 6 months.
- 3.27.4. Calibration solution of standard amino acids for use with hydrolysates,  $c = 5 \text{ nmol}/50 \mu\text{l}$  of cysteic acid and methionine sulphone and  $c = 10 \text{ nmol}/50 \mu\text{l}$  of the other amino acids.

Dissolve 2,2 g sodium chloride (3.10) in 100 ml beaker with 30 ml citrate buffer (3.24). Add 4,00 ml stock standard solution of amino acids (3.27.1), 4,00 ml stock standard solution of cysteic acid and methionine sulphone (3.27.2) and 0,50 ml stock standard solution of internal standard (3.27.3) if used. Adjust pH to 2,20 with sodium hydroxide (3.18).

Transfer quantitatively to a 50 ml graduated flask and make up to the mark with citrate buffer (3.24) and mix.

Store below 5 °C for not more than 3 months.

See also observations 9.1.

- 3.27.5. Calibration solution of standard amino acids for use with hydrolysates prepared according to paragraph 5.3.3.1. and for use with extracts (5.2). The calibration solution is prepared according to 3.27.4 omitting sodium chloride.

Store below 5 °C for not more than 3 months.

#### 4. Apparatus

- 4.1. 100 or 250 ml round bottomed flask fitted with a reflux condenser.
- 4.2. 100 ml borosilicate glass bottle 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 (three decimal places).
- 4.5. Membrane filter (0,2  $\mu$ m).
- 4.6. Centrifuge.
- 4.7. Rotary vacuum evaporator.
- 4.8. Mechanical shaker or magnetic stirrer.
- 4.9. Amino acid analyzer 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 analyzers hydrolysis procedures can 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 the volume of acid is reduced by evaporation to approx. 5 ml after the hydrolysis and prior to pH adjustment. The evaporation should be performed under vacuum at 40 °C maximum.

#### 5. Procedure

##### 5.1. Preparation of the sample

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 a high fat content should be extracted with light petroleum (3.12) prior to grinding.

##### 5.2. Determination of free amino acids in feedingstuffs and premixtures

Weigh to the nearest 0,2 mg an appropriate amount (1-5 g) of the prepared sample (5.1), into a conical flask and add 100,0 ml of extraction mixture (3.21). 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 (3.22), with stirring and continue to stir with the aid of magnetic stirrer for 5 minutes. 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 (3.18), transfer to a volumetric flask of appropriate volume using citrate buffer (3.24), and make up to the mark with the buffer solution (3.24).

If an internal standard is being used add 1,00 ml of internal standard (3.27.3) for each 100 ml final solution and make up to the mark with the buffer solution (3.24).

Proceed to the chromatography step according to paragraph 5.4.

If the extracts are not being examined the same day, they must 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 to 1 g of the prepared 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 (5.3.3.1) or,
- a 100 ml bottle fitted with a screw cap (4.2), (for closed hydrolysis 5.3.2.4).

The weighed sample portion should 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 (3.23) and mix using a glass spatula with a bent tip. Seal the flask/bottle containing the spatula with an air-tight film, place the ice-water bath containing the sealed container in a refrigerator at 0 °C and leave for 16 hours. After 16 hours remove from the refrigerator and decompose the excess oxidation reagent by the addition of 0,84 g of sodium disulfite (3.4).

Proceed to 5.3.2.1.

#### 5.3.2. Hydrolysis

##### 5.3.2.1. Hydrolysis of oxidised samples

To the oxidised sample prepared according to 5.3.1 add 25 ml of hydrolysis mixture (3.20) 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 unoxidised samples

Weigh into either a 100 ml or a 250 ml round-bottom flask (4.1) or a 100 ml bottle fitted with a screw cap (4.2), to the nearest 0,2 mg, from 0,1 to 1 g of the prepared sample (5.1). The weighed sample portion should have a nitrogen content of about 10 mg. Carefully add 25 ml of hydrolysis mixture (3.20) 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 3 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 hours. On completion of hydrolysis, wash the condenser down with 5 ml of citrate buffer (3.24). 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) 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 one hour close the vessel with the cap and leave in the oven (4.3) for 23 hours. 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 (3.24).

Proceed according to 5.3.3.

#### 5.3.3. Adjustment of pH

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

##### 5.3.3.1. For chromatographic systems (4.9) requiring a low sodium concentration:

It is advisable to use an internal stock standard solution (3.27.3) when amino acid analyzers requiring a low sodium concentration are employed (when the acid volume has to be reduced).

In this case add 2,00 ml of the internal stock standard solution (3.27.3) to the hydrolysate before the evaporation.

Add 2 drops of 1-octanol (3.15) to the hydrolysate obtained in accordance with paragraph 5.3.2.3 or 5.3.2.4.

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

Adjust the pH to 2,20 with sodium hydroxide solution (3.18) and proceed to paragraph 5.3.4.

#### 5.3.3.2. For all other Amino Acid Analyzers (4.9)

Take the hydrolysates obtained in accordance with 5.3.2.3 or 5.3.2.4 and partly neutralise them by carefully adding with stirring, 17 ml of sodium hydroxide solution (3.17), ensuring that the temperature is kept below 40 °C.

Adjust the pH to 2,20 at room temperature using sodium hydroxide solution (3.17) and finally sodium hydroxide solution (3.18). Proceed to 5.3.4.

#### 5.3.4. Sample solution for chromatography

Quantitatively transfer the pH adjusted hydrolysate (5.3.3.1 or 5.3.3.2) with citrate buffer (3.24) to a 200 ml graduated flask, and make up to the mark with buffer (3.24).

If an internal standard has not already been used, add 2,00 ml of internal standard (3.27.3) and make up to the mark with citrate buffer (3.24). Mix thoroughly.

Proceed to the chromatography step (5.4).

If the sample solutions are not being examined the same day they must be stored below 5 °C.

#### 5.4. *Chromatography*

Before 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). The resulting clear solution is subjected to ion exchange chromatography, using an amino acid analyzer (4.9).

The injection may be performed manually or automatically. It is important that the same quantity of solution  $\pm 0,5\%$  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 calibration runs depends on the stability of the ninhydrin reagent and the analytical system. The standard or sample is diluted with citrate buffer (3.24) to give a peak area of the standard of 30-200 % of the sample amino acid peak area.

The chromatography of amino acids will vary slightly according to the type of analyzer employed and resin used. The chosen system must be capable of separating the amino acids from each other and from the ninhydrin-positive materials. In the range of operation the chromatographic system should 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 analyzed. This equimolar solution must contain at least 30 % of the maximum load of each amino acid which 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 should 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 must be better than 1:10.

The system must 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, in g amino acid per kg sample, is calculated.

$$\frac{A \times E \times MW \times F}{B \times W \times 1\,000} = \text{g amino acid per kg sample}$$

If an internal standard is used multiply by:  $\frac{D}{C}$

- A = peak area, hydrolysate or extract  
 B = peak area, calibration standard solution  
 C = peak area, internal standard in hydrolysate or extract  
 D = peak area, internal standard, calibration standard solution  
 MW = molecular weight of the amino acid being determined  
 E = concentration of standard in  $\mu\text{mol/ml}$   
 W = sample weight (g) (corrected to original weight if dried or defatted)  
 F = ml total hydrolysate (5.3.4) or ml calculated total dilution volume of extract (6.1).

Cystine and cysteine are both determined as cysteic acid in hydrolysates of oxidized sample, but calculated as cystine ( $\text{C}_6\text{H}_{12}\text{N}_2\text{O}_4\text{S}_2$ , MW 240,30 by using MW 120,15 ( $= 0,5 \times 240,30$ ).

Methionine is determined as methionine sulphone in hydrolysates of oxidized sample, but calculated as methionine by using MW of methionine: 149,21.

Added free methionine is determined after extraction as methionine, for the calculation the same MW is used.

- 6.1. The total dilution volume of extracts (F) for determination of free amino acids (5.2) is calculated as following:

$$F = 100 \text{ ml} \times \frac{(10 \text{ ml} + 5 \text{ ml})}{10 \text{ ml}} \times \frac{V_{\text{ml}}}{10 \text{ ml}}$$

V = volume of final extract

## 7. Evaluation of the method

The method has been tested in an intercomparison made at international level in 1990 using four different feedstuffs (mixed pig feed, broiler compound, protein concentrate, premixture). The results, after elimination of outliers, of mean and standard deviation are given in the table below:

Means in g/kg

Reference Material	Amino acid			
	Threonine	Cyst(e)ine	Methionine	Lysine
Mixed pig feed	6,94 n = 15	3,01 n = 17	3,27 n = 17	9,55 n = 13
Broiler compound	9,31 n = 16	3,92 n = 18	5,08 n = 18	13,93 n = 16
Protein concentrate	22,32 n = 16	5,06 n = 17	12,01 n = 17	47,74 n = 15
Premixture	58,42 n = 16	—	90,21 n = 16	98,03 n = 16

n = number of participating laboratories

### 7.1. Repeatability

The repeatability expressed as 'within laboratory standard deviation' of the above mentioned intercomparison is given in the tables below:

Within laboratory standard deviation ( $S_i$ ) in g/kg

Reference material	Amino acid			
	Threonine	Cyst(e)ine	Methionine	Lysine
Mixed pig feed	0,13 n = 15	0,10 n = 17	0,11 n = 17	0,26 n = 13
Broiler compound	0,20 n = 16	0,11 n = 18	0,16 n = 18	0,28 n = 16
Protein concentrate	0,48 n = 16	0,13 n = 17	0,27 n = 17	0,99 n = 15
Premixture	1,30 n = 16	—	2,19 n = 16	2,06 n = 16

n = number of participating laboratories

Coefficient of variation (%) for within laboratory standard deviation ( $S_i$ )

Reference material	Amino acid			
	Threonine	Cyst(e)ine	Methionine	Lysine
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 = number of participating laboratories

## 7.2. Reproducibility

The results for between laboratory standard deviation by the above mentioned intercomparison are given in the table below:

Between laboratory standard deviation ( $S_R$ ) in g/kg

Reference material	Amino acid			
	Threonine	Cyst(e)ine	Methionine	Lysine
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 = number of participating laboratories

Coefficient of variation (%) for between laboratory standard deviation ( $S_R$ )

Reference material	Amino acid			
	Threonine	Cyst(e)ine	Methionine	Lysine
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 = number of participating laboratories

#### 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

- 9.1. Because of differences between amino acid analysers the final concentrations of the calibration solutions of standard amino acids (see 3.27.4 and 3.27.5) and of the hydrolysate (see 5.3.4) should be taken as a guideline.

The range of linear response of the apparatus has to 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 must be optimised in accordance with the manufacturer's recommendations.
- 9.3. By applying the method to feedingstuffs containing more than 1 % chloride (concentrate, mineral feeds, supplementary feeds) underestimation of methionine could occur and special treatment has to be done.

## PART B

### DETERMINATION OF CRUDE OILS AND FATS

#### 1. Purpose and scope

This method is for the determination of crude oils and fats in feedingstuffs. It does not cover the analysis of oil seeds and oleaginous fruit defined in Council Regulation 136/66/EEC of 22 September 1966.

The use of the two procedures described below depends on the nature and composition of the feedingstuff and the reason for carrying out the analysis.

##### 1.1. Procedure A — Directly extractable crude oils and fats

This method is applicable to feed materials of plant origin, except those included within the scope of Procedure B.

##### 1.2. Procedure B — Total crude oils and fats

This method is applicable to feed materials of animal origin and to all compound feeds. It is to be used for all materials from which the oils and fats cannot be completely extracted without prior hydrolysis (eg glutens, yeast, potato proteins and products subject to processes such as extrusion, flaking and heating).

### 1.3. *Interpretation of results*

In all cases where a higher result is obtained by using Procedure B than by Procedure A, the result obtained by Procedure B shall be accepted as the true value.

## 2. **Principle**

### 2.1. *Procedure A*

The sample is extracted with light petroleum. The solvent is distilled off and the residue dried and weighed.

### 2.2. *Procedure B*

The sample is treated under heating with hydrochloric acid. The mixture is cooled and filtered. The residue is washed and dried and submitted to the determination according to Procedure A.

## 3. **Reagents**

- 3.1. Light petroleum, boiling range: 40 to 60 °C. The bromine value must be less than 1 and the residue on evaporation less than 2 mg/100 ml.
- 3.2. Sodium sulfate, anhydrous.
- 3.3. Hydrochloric acid,  $c = 3 \text{ mol HCl/l}$
- 3.4. Filtration aid, e.g. Kieselguhr, Hyflo-supercel.

## 4. **Apparatus**

- 4.1. Extraction apparatus. If fitted with a siphon (Soxhlet apparatus), the reflux rate should be such as to produce about 10 cycles per hour; if of the non-siphoning type, the reflux rate should be about 10 ml per minute.
- 4.2. Extraction thimbles, free of matter soluble in light petroleum and having a porosity consistent with the requirements of point 4.1.
- 4.3. Drying oven, either a vacuum oven set at  $75 \text{ °C} \pm 3 \text{ °C}$  or an air-oven set at  $100 \text{ °C} \pm 3 \text{ °C}$ .

## 5. **Procedure**

### 5.1. *Procedure A (see point 8.1)*

Weigh 5 g of the sample to the nearest 1 mg, transfer it to an extraction thimble (4.2) and cover with a fat-free wad of cotton wool.

Place the thimble in an extractor (4.1) and extract for six hours with light petroleum (3.1). Collect the light petroleum extract in a dry, weighed flask containing fragments of pumice stone<sup>(1)</sup>.

Distil off the solvent. Dry the residue maintaining the flask for one and a half hours in the drying oven (4.3). Leave to cool in a desiccator and weigh. Dry again for 30 minutes to ensure that the weight of the oils and fats remains constant (loss in weight between two successive weighings must be less than 1 mg).

### 5.2. *Procedure B*

Weigh 2,5 g of the sample to the nearest 1 mg (see point 8.2), place in a 400 ml beaker or a 300 ml conical flask and add 100 ml of hydrochloric acid (3.3) and fragments of pumice stone. Cover the beaker with a watch glass or fit the conical flask with a reflux condenser. Bring the mixture to a gentle boil over a low flame or a hot-plate and keep it there for one hour. Do not allow the product to stick to the sides of the container.

Cool and add a quantity of filtration aid (3.4) sufficient to prevent any loss of oil and fat during filtration. Filter through a moistened, fat-free, double filter paper. Wash the residue in cold water until a neutral filtrate is obtained. Check that the filtrate does not contain any oil or fats. Their presence indicates that the sample must be extracted with light petroleum, using Procedure A, before hydrolysis.

Place the double filter paper containing the residue on a watch glass and dry for one and a half hours in the air oven (4.3)  $100 \text{ °C} \pm 3 \text{ °C}$ .

Place the double filter paper containing the dry residue in an extraction thimble (4.2) and cover with a fat-free wad of cotton wool. Place the thimble in an extractor (4.1) and proceed as indicated in the second and third paragraphs of point 5.1.

<sup>(1)</sup> Where the oil or fat has to undergo subsequent quality tests, replace the fragments of pumice stone by glass beads.

**6. Expression of result**

Express the weight of the residue as a percentage of the sample.

**7. Repeatability**

The difference between the results of two parallel determinations carried out on the same sample by the same analyst should not exceed:

- 0,2 % in absolute value, for contents of crude oils and fats lower than 5 %,
- 4,0 % relative to the highest result for contents of 5 to 10 %,
- 0,4 % in absolute value, for contents above 10 %.

**8. Observations**

- 8.1. For products with a high content of oils and fats, which are difficult to crush or unsuitable for drawing a homogeneous reduced test sample, proceed as follows.

Weigh 20 g of the sample to the nearest 1 mg and mix with 10 g or more of anhydrous sodium sulfate (3.2). Extract with light petroleum (3.1) as indicated in point 5.1. Make up the extract obtained to 500 ml with light petroleum (3.1) and mix. Take 50 ml of the solution and place in a small, dry, weighed flask containing fragments of pumice stone<sup>(1)</sup>. Distil off the solvent, dry and proceed as indicated in the last paragraph of point 5.1.

Eliminate the solvent from the extraction residue left in the thimble, crush the residue to a fineness of 1 mm, return it to the extraction thimble (do not add sodium sulfate) and proceed as indicated in the second and third paragraphs of point 5.1.

Calculate the content of oils and fats as a percentage of the sample by using the following formula:

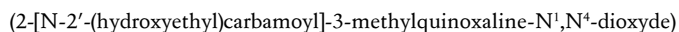
$$(10 a + b) \times 5$$

where:

a = mass in grams of the residue after the first extraction (aliquot part of the extract),

b = mass in grams of the residue after the second extraction.

- 8.2. For products low in oils and fats the test sample may be increased to 5 g.
- 8.3. Pet foods containing a high content of water may need to be mixed with anhydrous sodium sulfate prior to hydrolysis and extraction as per Procedure B.
- 8.4. In paragraph 5.2 it may be more effective to use hot water in place of cold water to wash the residue after filtration.
- 8.5. The drying time of 1,5 h may need to be extended for some feedingstuffs. Excessive drying should be avoided as this can lead to low results. A microwave oven can also be used.
- 8.6. Pre-extraction by Procedure A prior to hydrolysis and re-extraction by Procedure B is recommended if the crude oil/fat content is greater than 15 %. To some extent this depends on the nature of the feedingstuff and the nature of the oil-fat in the feedingstuff.

**PART C****DETERMINATION OF OLAQUINDOX****1. Purpose and scope**

This method is for the determination of olaquindox in feedingstuffs. The lower limit of determination is 5 mg/kg.

**2. Principle**

The sample is extracted by a water-methanol mixture. The content of olaquindox is determined by reversed-phase high-performance liquid chromatography (HPLC) using an UV detector.

<sup>(1)</sup> Where the oil or fat has to undergo subsequent quality tests, replace the fragments of pumice stone by glass beads.

### 3. Reagents

#### 3.1. Methanol.

#### 3.2. Methanol, HPLC grade

#### 3.3. Water, HPLC grade

#### 3.4. Mobile phase for HPLC:

Water (3.3) -methanol (3.2) mixture, 900 + 100 (V + V).

#### 3.5. Standard substance: pure olaquinox 2-[N-2'-(hydroxyethyl)carbamoyle]-3-methylquinoxaline-N<sup>1</sup>,N<sup>4</sup>-dioxyde, E 851.

##### 3.5.1. Olaquinox stock standard solution, 250 µg/ml

Weigh to the nearest 0,1 mg 50 mg of olaquinox (3.5) in a 200 ml graduated flask and add ca. 190 ml water. Then place the flask for 20 min into an ultrasonic bath (4.1). After ultrasonic treatment bring the solution to room temperature, make up to the mark with water and mix. Wrap the flask with aluminum foil and store in a refrigerator. This solution must be prepared fresh each month.

##### 3.5.2. Olaquinox intermediate standard solution, 25 µg/ml

Transfer 10,0 ml of the stock standard solution (3.5.1) into a 100 ml graduated flask, make up to the mark with the mobile phase (3.4) and mix. Wrap the flask with aluminium foil and store in a refrigerator. This solution must be prepared fresh each day.

##### 3.5.3. Calibration solutions:

Into a series of 50 ml graduated flask transfer 1,0, 2,0, 5,0, 10,0, 15,0 and 20,0 ml of the intermediate standard solution (3.5.2). Make up to the mark with the mobile phase (3.4) and mix. Wrap the flask with aluminium foil. These solutions correspond to 0,5, 1,0, 2,5, 5,0, 7,5 and 10,0 µg of olaquinox per ml respectively.

These solutions must be prepared fresh each day.

### 4. Apparatus

#### 4.1. Ultrasonic bath

#### 4.2. Mechanical shaker

#### 4.3. HPLC equipment with variable wavelength ultraviolet detector or diode array detector

##### 4.3.1. Liquid chromatographic column, 250 mm × 4 mm, C18, 10 µm packing, or equivalent

#### 4.4. Membrane filters, 0,45 µm

### 5. Procedure

*Note:* Olaquinox is light sensitive. Carry out all procedures under subdued light or use amber glassware.

#### 5.1. General

##### 5.1.1. A blank feed should be analysed to check that neither olaquinox nor interfering substances are present.

##### 5.1.2. A recovery test should be carried out by analysing the blank feed which has been fortified by addition of a quantity of olaquinox, similar to that present in the sample. To fortify at a level of 50 mg/kg, transfer 10,0 ml of the stock standard solution (3.5.1) to a 250 ml conical flask and evaporate the solution to ca. 0,5 ml. Add 50 g of the blank feed, mix thoroughly and leave for 10 min mixing again several times before proceeding with the extraction step (5.2).

*Note:* For the purpose of this method the blank feed should be similar in type to that of the sample and olaquinox should not be detected.

#### 5.2. Extraction

Weigh to the nearest 0,01 g, approximately 50 g of the sample. Transfer to a 1 000 ml conical flask, add 100 ml of methanol (3.1) and place the flask for 5 minutes in the ultrasonic bath (4.1). Add 410 ml water and leave in the ultrasonic bath for further 15 minutes. Remove the flask from the ultrasonic bath, shake it for 30 minutes on the shaker (4.2) and filter through a folded filter. Transfer 10,0 ml of the filtrate into a 20 ml graduated flask, make up to the mark with water and mix. An aliquot is filtered through a membrane filter (4.4). (see 9. Observation) Proceed to the HPLC determination (5.3).

### 5.3. HPLC determination

#### 5.3.1. Parameters:

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

Analytical column (4.3.1)

Mobile phase (3.4): water (3.3) — methanol (3.2) mixture, 900 + 100 (V + V)

Flow rate: 1,5-2 ml/min.

Detection wavelength: 380 nm

Injection volume: 20 µl-100 µl

Check the stability of the chromatographic system, injecting several times the calibration solution (3.5.3) containing 2,5 µg/ml, until constant peak heights and retention times are achieved.

#### 5.3.2. Calibration graph

Inject each calibration solution (3.5.3) several times and determine the mean peak height (areas) for each concentration. Plot a calibration graph using the mean peak heights (areas) of the calibration solutions as the ordinates and the corresponding concentrations in µg/ml as the abscissae.

#### 5.3.3. Sample solution

Inject the sample extract (5.2) several times using the same volume as taken for the calibration solutions and determine the mean peak height (area) of the olaquinox peaks.

## 6. Calculation of the results

From the mean height (area) of the olaquinox peaks of the sample solution determine the concentration of the sample solution in µg/ml by reference to the calibration graph (5.3.2).

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

$$w = \frac{c \times 1\,000}{m}$$

in which:

*c* = olaquinox concentration of the sample extract (5.2) in µg/ml

*m* = mass of the test portion in g (5.2).

## 7. Validation of the results

### 7.1. Identity

The identity of the analyte can be confirmed by co-chromatography, or by using a diode-array detector by which the spectra of the sample extract (5.2) and the calibration solution (3.5.3) containing 5,0 µg/ml are compared.

#### 7.1.1. Co-chromatography

A sample extract (5.2) is fortified by addition of an appropriate amount of calibration solution (3.5.3). The amount of added olaquinox should be similar to the amount of olaquinox found in the sample extract.

Only the height of the olaquinox peak should be enhanced after taking into account both the amount added and the dilution of the extract. The peak width, at half of its height, must be within  $\pm 10\%$  of the original width of the olaquinox peak of the unfortified sample extract.

#### 7.1.2. Diode array detection

The results are evaluated according to the following criteria:

- (a) The wavelength of maximum absorption of the sample and of the standard spectra, recorded at the peak apex on the chromatogram, must be within a margin determined by the resolving power of the detection system. For diode-array detection this is typically within  $\pm 2$  nm.

- (b) Between 220 and 400 nm, the sample and standard spectra recorded at the peak apex of the chromatogram, must not be different for those parts of the spectrum within the range 10-100 % of relative absorbance. This criterion is met when the same maxima are present and at no observed point the deviation between the two spectra exceeds 15 % of the absorbance of the standard analyte.
- (c) Between 220 and 400 nm, the spectra of the upslope, apex and downslope of the peak produced by the sample extract must not be different from each other for those parts of the spectrum within the range 10-100 % of relative absorbance. This criterion is met when the same maxima are present and when at all observed points the deviation between the spectra does not exceed 15 % of the absorbance of the spectrum of the peak apex.

If one of these criteria is not met the presence of the analyte has not been confirmed.

#### 7.2. *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 for olaquinox contents between 10 and 20 mg/kg.

#### 7.3. *Recovery*

For a fortified blank sample the recovery should be at least 90 %.

### 8. **Results of a collaborative study**

An EC collaborative study was arranged in which four piglet feed samples including one blank feed were analysed by up to 13 laboratories. The results are given below:

	Sample 1	Sample 2	Sample 3	Sample 4
L	13	10	11	11
n	40	40	44	44
mean (mg/kg)	—	14,6	48,0	95,4
(S <sub>r</sub> ) (mg/kg)	—	0,82	2,05	6,36
(S <sub>R</sub> ) (mg/kg)	—	1,62	4,28	8,42
CV <sub>r</sub> (%)	—	5,6	4,3	6,7
CV <sub>R</sub> (%)	—	11,1	8,9	8,8
Nominal content (mg/kg)	—	15	50	100
recov. (%)	—	97,3	96,0	95,4

L : number of laboratories  
n : number of single values  
S<sub>r</sub> : standard deviation of repeatability  
S<sub>R</sub> : standard deviation of reproducibility  
CV<sub>r</sub> : coefficient of variation of repeatability  
CV<sub>R</sub> : coefficient of variation of reproducibility.

### 9. **Observation**

Although the method has not been validated for feeds containing more than 100 mg/kg of olaquinox, it may be possible to obtain satisfactory results by taking a smaller sample weight and/or diluting the extract (5.2) to reach a concentration within the range of the calibration graph (5.3.2).