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 olaquindox 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 [1], 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 [2], as last amended by Commission Directive 97/47/EC [3], and Council Directive 93/74/EEC of 13 September 1993 on feedingstuffs intended for particular nutritional purposes [4], as last amended by Directive 96/25/EC [5], 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 [6], as last amended by Commission Directive 98/19/EC [7], stipulates that the olaquindox 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 [8], as last amended by Commission Directive 84/4/EEC [9], 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 olaquindox 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.

[1] OJ L 170, 3. 8. 1970, p. 2.
[2] OJ L 86, 6. 4. 1979, p. 30.
[3] OJ L 211, 5. 8. 1997, p. 45.
[4] OJ L 237, 22. 9. 1993, p. 23.
[5] OJ L 125, 23. 5. 1996, p. 35.
[6] OJ L 270, 14. 12. 1970, p. 1.
[7] OJ L 96, 28. 3. 1998, p. 39.
[8] OJ L 279, 20. 12. 1971, p. 7.
[9] 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 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 $< 10\ \mu S$).

3.1. Hydrogen peroxide, w = 30 %.

3.2. Formic acid, w = 98-100 %.

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 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 mol/l:

Dissolve 300 g NaOH (3.5) in water and make up to 1 litre.

3.18. Sodium hydroxide solution, c = 1 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 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 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, ß = 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 mol Na^+/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 µmol/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 µmol/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 µmol/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 nmol/50 µl of cysteic acid and methionine sulphone and c = 10 nmol/50 µl 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 ± 2 °C.

4.4. pH-meter (three decimal places).

4.5. Membrane filter (0,2 μm).

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 ± 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.