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).