Commission Regulation (EC) No 152/2009 of 27 January 2009 laying down the methods of sampling and analysis for the official control of feed (Text with EEA relevance)

ANNEX IV

METHODS OF ANALYSIS TO CONTROL THE LEVEL OF AUTHORISED ADDITIVES IN FEED

B.DETERMINATION OF VITAMIN E

1. Purpose and Scope

This method makes it possible to determine the level of vitamin E in feed 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 quantification is 2 mg vitamin E/kg. This limit of quantification is only achievable with fluorescence detector. With an UV detector the limit of quantification is 10 mg/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 ^{o}C-60 ^{o}C.

3.3. Methanol.

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

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

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

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

3.7. Phenolphthalein solution, c = 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. Stock solution of DL-α-tocopherol: 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. Rotary film 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 × 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 shall 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 shall 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 shall 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 sulphide 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 5 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 1 000 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 must be about 2:1. Shake vigorously for 2 min. and allow to settle for 2 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 ^{o}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 must 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 × 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, 9,1 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 shall be at 284 nm:

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

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

5.6.2. *DL-α-tocopherol standard*

5.6.2.1. *Preparation of the working standard solution*

Transfer by pipette 2 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. 2,2, 4,4, 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 shall be at 292 nm:

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

At this dilution an extinction value of 0,6 must 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:

$w = \frac{500 \times c \times V_2}{V_1 \times m}$
[mg/kg]

in which:

c	=	vitamin E concentration (as α-tocopherol acetate) 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	=	weight 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 shall 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 max. 4 weeks).

7.5. Hydroquinone can be used instead of BHT.

7.6. Using a normal phase-column the separation of α-, β-, γ- 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 sulphide solution.

7.9. Vitamin E acetate hydrolyses very fast under alkaline conditions and is therefore very sensitive to oxidation, especially in the presence of trace elements like iron or copper. In case of the determination of vitamin E in premixtures at levels higher than 5 000 mg/kg, a degradation of vitamin E could be the consequence. Therefore a HPLC method including an enzymatic digestion of the vitamin E formulation without an alkaline saponification step is to be recommended for confirmation.

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.

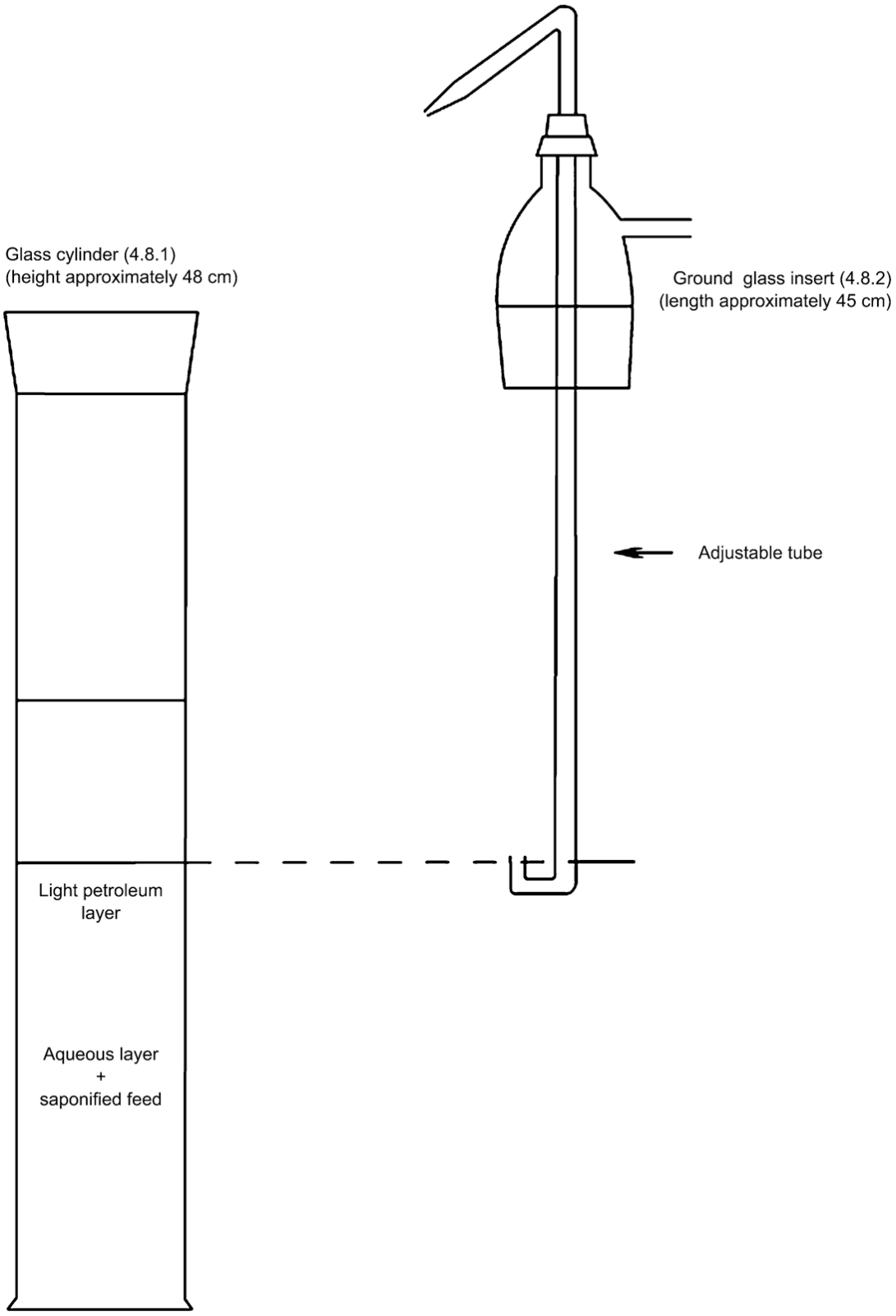
9. Results of a collaborative study[1]

	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

Figure 1: Extraction apparatus (4.8)



(**1**) Conducted by the Feed Working Group of Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten (VDLUFA).

Changes to legislation:
There are currently no known outstanding effects for the Commission Regulation (EC) No 152/2009, Division B..