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

A.DETERMINATION OF VITAMIN A

1. Purpose and Scope

This method makes it possible to determine the level of vitamin A (retinol) in feed 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 0,3 µg all-*trans*-vitamin A alcohol or 0,344 µg all-*trans*-vitamin A acetate or 0,55 µg all-*trans*-vitamin A palmitate.

The limit of quantification 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 ^{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, ß = 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. 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. 2,8 x 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. 1,8 x 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 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 A 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 A.

5.2. Saponification

Depending on the vitamin A content weigh, to the nearest 1 mg, 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 sulphide 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 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 (3.10) 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 must 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 × 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:

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

(
$E_{1\,cm}^{1\%}$
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:

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

(
$E_{1\,cm}^{1\%}$
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:

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:

$$w = \frac{500 \times c \times V_2 \times 1\,000}{V_1 \times m}$$

[IU/kg]

in which:

c	=	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	=	weight 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 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. With cod-liver oil and other pure fats the saponification time shall 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. But in that case, the heights (areas) of all cis and trans isomers peaks have to be summed for calculations.

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. In cases of analysis of vitamin A in milk replacers, specific attention has to be paid

— at saponification (5.2): due to the amount of fat present in the sample, increasing of potassium hydroxide solution amount (3.4) may be necessary,

— at extraction (5.3): due to the presence of emulsions, adaptation of the water/ethanol 2:1 ratio may be necessary.

To check if the applied method of analysis generates reliable results on this specific matrix (milk replacer), a recovery test shall be applied on an additional test portion. If the recovery rate is lower than 80 %, the analytical result has to be corrected for recovery.

8. Repeatability

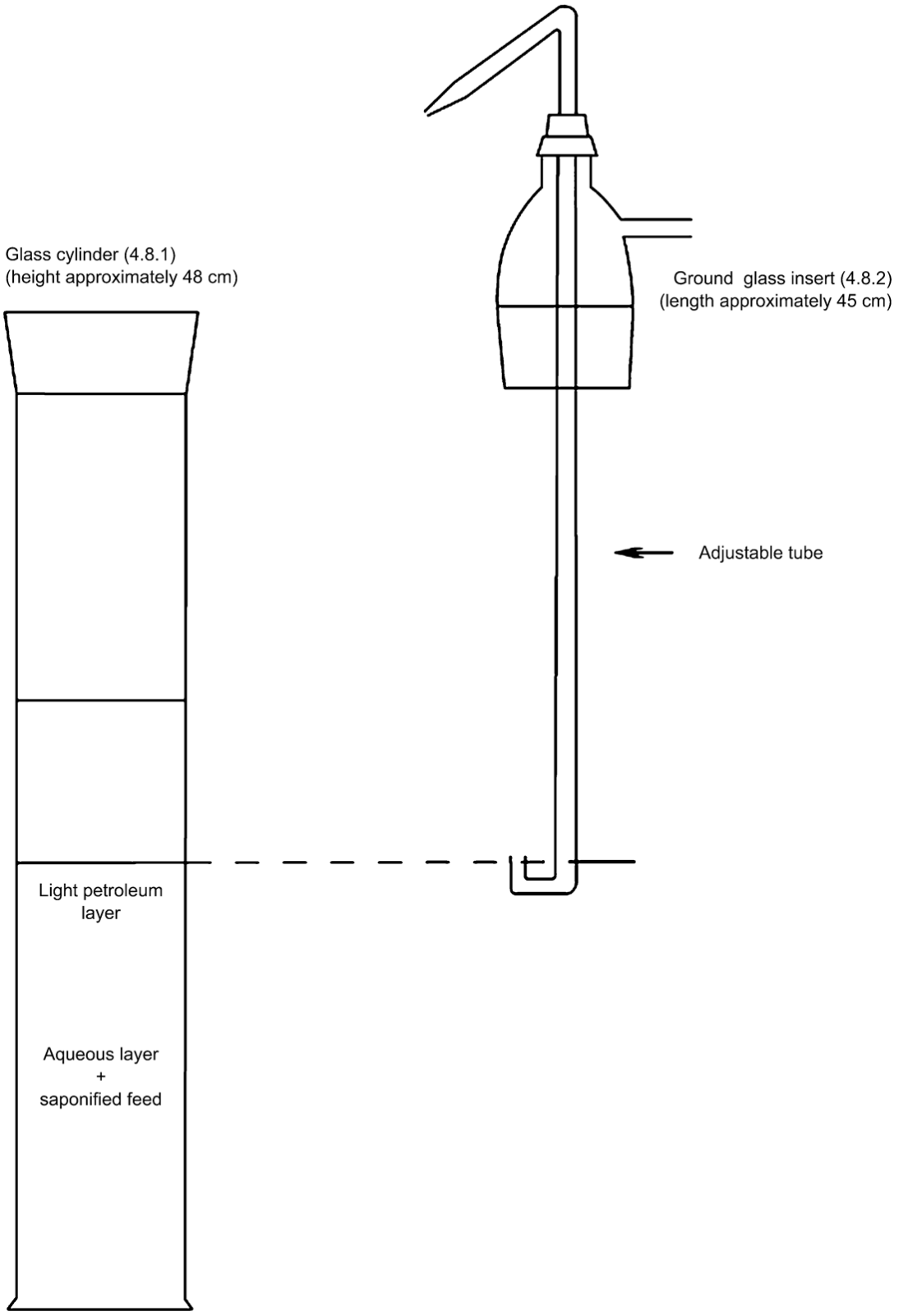
The difference between the results of two parallel determinations carried out on the same sample must not exceed 15 % relative to the higher result.

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 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)



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 °C-60 °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.

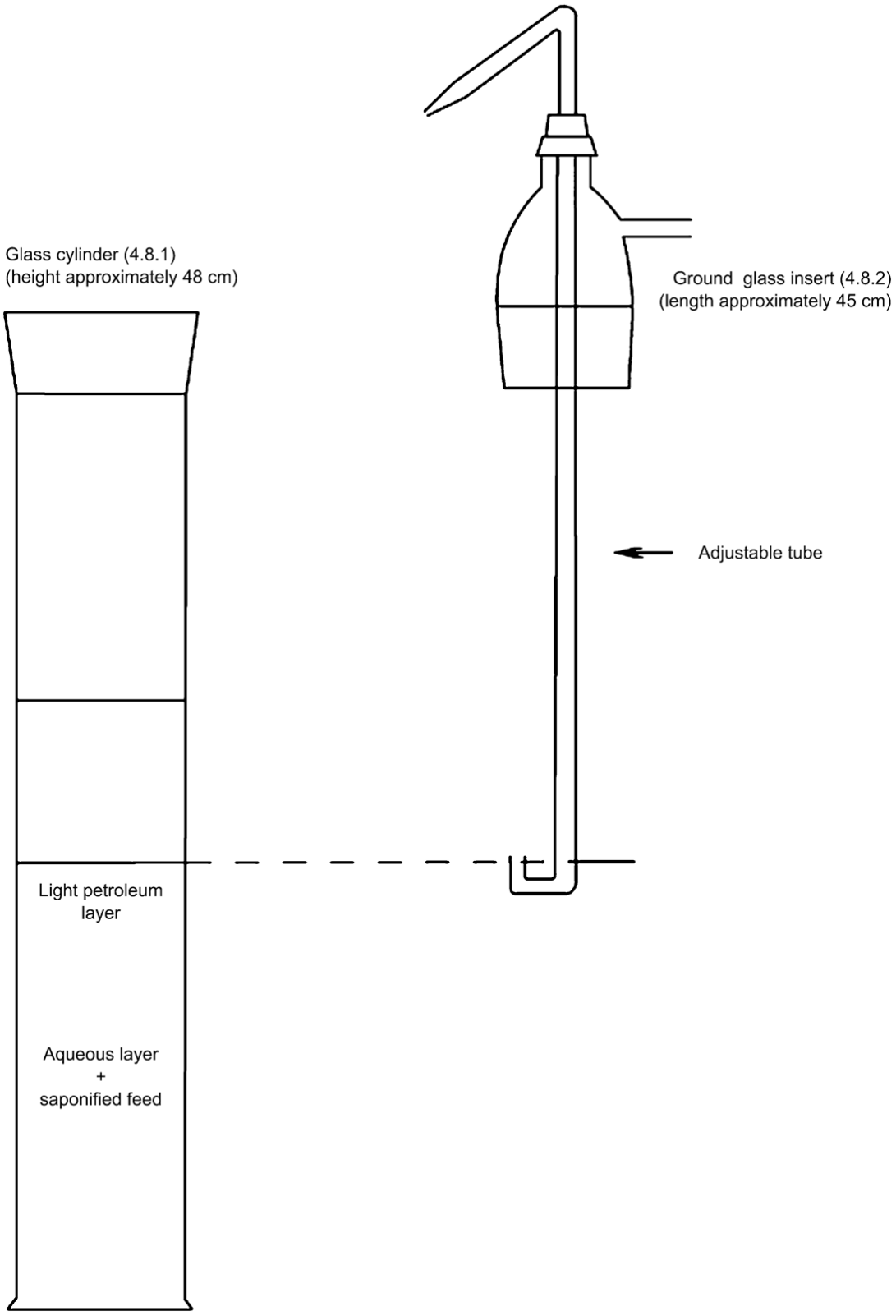
9. Results of a collaborative study[(2)]

	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)



C. DETERMINATION OF THE TRACE ELEMENTS IRON, COPPER, MANGANESE AND ZINC

1. Purpose and scope

The method makes it possible to determine the trace elements iron, copper, manganese and zinc in feed. The limits of quantification are:

— iron (Fe): 20 mg/kg
— copper (Cu): 10 mg/kg
— manganese (Mn): 20 mg/kg
— zinc (Zn): 20 mg/kg.

2. Principle

The sample is brought into solution in hydrochloric acid after destruction of organic matter, if any. The elements iron, copper, manganese and zinc are determined, after appropriate dilution, by atomic absorption spectrometry.

3. Reagents

Introductory comments

For preparation of the reagents and analytical solutions use water free from the cations to be determined, obtained either by double distilling water in a borosilicate glass or quartz still or by double treatment on ion exchange resin.

The reagents must be of at least analytical grade. Freedom from the element to be determined must be checked in a blank experiment. If necessary, the reagents must be further purified.

In place of the standard solutions described below, commercial standard solutions may be used provided that they are guaranteed and have been checked before use.

3.1. Hydrochloric acid (d:1,19 g/ml).

3.2. Hydrochloric acid (6 mol/litre).

3.3. Hydrochloric acid (0,5 mol/litre).

3.4. Hydrofluoric acid 38 % to 40 % (v/v) having an iron (Fe) content of less than 1 mg/litre and a residue after evaporation of less than 10 mg (as sulphate)/litre.

3.5. Sulphuric acid (d: 1,84 g/ml).

3.6. Hydrogen peroxide (approximately 100 volumes of oxygen (30 % by weight)).

3.7. Standard iron solution (1 000 µg Fe/ml) prepared as follows or equivalent commercially available solution: dissolve 1 g of iron wire in 200 ml of 6 mol/litre hydrochloric acid (3.2), add 16 ml of hydrogen peroxide (3.6) and make up to one litre with water.

3.7.1. Working standard iron solution (100 µg Fe/ml) prepared by diluting one part of the standard solution (3.7) with 9 parts of water.

3.8. Standard copper solution (1 000 µg Cu/ml) prepared as follows or equivalent commercially available solution:

— dissolve 1 g of copper in powder form in 25 ml of 6 mol/litre hydrochloric acid (3.2), add 5 ml of hydrogen peroxide (3.6) and make up to one litre with water.

3.8.1. Working standard copper solution (10 µg Cu/ml) prepared by diluting 1 part of the standard solution (3.8) with 9 parts of water and then diluting 1 part of the resulting solution with 9 parts of water.

3.9. Standard manganese solution (1 000 µg Mn/ml) prepared as follows or equivalent commercially available solution:

— dissolve 1 g of manganese in powder form in 25 ml of 6 mol/litre hydrochloric acid (3.2) and make up to one litre with water.

3.9.1. Working standard manganese solution (10 µg Mn/ml) prepared by diluting 1 part of the standard solution (3.9) with 9 parts of water and then diluting 1 part of the resulting solution with 9 parts of water.

3.10. Standard zinc solution (1 000 µg Zn/ml) prepared as follows or equivalent commercially available solution:

— dissolve 1 g of zinc in strip or leaf form in 25 ml of 6 mol/litre hydrochloric acid (3.2) and make up to one litre with water.

3.10.1. Working standard zinc solution (10 µg Zn/ml) prepared by diluting 1 part of the standard solution (3.10) with 9 parts of water and then diluting 1 part of the resulting solution with 9 parts of water.

3.11. Lanthanum chloride solution: dissolve 12 g of lanthanum oxide in 150 ml of water, add 100 ml of 6 mol/litre hydrochloric acid (3.2) and make up to one litre with water.

4. Apparatus

4.1. Muffle furnace with temperature regulation and preferably recorder.

4.2. Glassware must be of resistant borosilicate type and it is recommended to use apparatus which is reserved exclusively for trace element determinations.

4.3. Atomic absorption spectrophotometer meeting the requirements of the method with regard to sensitivity and precision in the required range.

5. Procedure[3]

5.1. Samples containing organic matter

5.1.1. *Ashing and preparation of the solution for analysis*[4]

5.1.1.1. Place 5 to 10 g of sample weighed to the nearest 0,2 mg in a quartz or platinum crucible (see Note (b)), dry in an oven at 105 ^{o}C and introduce the crucible into the cold muffle furnace (4.1). Close the furnace (see Note (c)) and gradually raise the temperature to 450 to 475 ^{o}C over about 90 minutes. Maintain this temperature for 4 to 16 hours (e.g. overnight) to remove carbonaceous material and then open the furnace and allow to cool (see Note (d)).

Moisten the ashes with water and transfer these in a beaker of 250 ml. Wash the crucible out with a total of about 5 ml of hydrochloric acid (3.1) and add the latter slowly and carefully to the beaker (there may be a vigorous reaction due to CO_2 formation). Add hydrochloric acid (3.1) dropwise with agitation until all effervescence has stopped. Evaporate to dryness, occasionally stirring with a glass rod.

Next add 15 ml of 6 mol/litre hydrochloric acid (3.2) to the residue followed by about 120 ml of water. Stir with the glass rod, which shall be left in the beaker, and cover the beaker with

a watch-glass. Bring gently to the boil and maintain at boiling point until no more ash can be seen to dissolve. Filter on ash-free filter paper and collect the filtrate in a 250 ml volumetric flask. Wash the beaker and filter with 5 ml of hot 6 mol/litre hydrochloric acid (3.2) and twice with boiling water. Fill the volumetric flask up to the mark with water (HCl concentration about 0,5 mol/litre).

5.1.1.2. If the residue in the filter appears black (carbon), put it back in the furnace and ash again at 450 to 475 ^{o}C. This ashing, which only requires a few hours (about three to five hours), is complete when the ash appears white or nearly white. Dissolve the residue with about 2 ml of hydrochloric acid (3.1), evaporate to dryness and add 5 ml of 6 mol/litre hydrochloric acid (3.2). Heat, filter the solution into the volumetric flask and make up to the mark with water (HCl concentration about 0,5 mol/litre.

Notes:

(a) In determining trace elements it is important to be alert to the risks of contamination, particularly by zinc, copper and iron. For this reason, the equipment used in preparing the samples must be free of these metals.

To reduce the general risk of contamination, work in a dust-free atmosphere with scrupulously clean equipment and carefully washed glassware. The determination of zinc is particularly sensitive to many types of contamination, e.g. from glassware, reagents, dust, etc.

(b) The weight of sample to be ashed is calculated from the approximate trace element content of the feed in relation to the sensitivity of the spectrophotometer used. For certain feed low in trace elements it may be necessary to start with a 10 to 20 g sample and make up the final solution to only 100 ml.

(c) Ashing must be carried out in a closed furnace without injection of air or oxygen.

(d) The temperature indicated by the pyrometer must not exceed 475 ^{o}C.

5.1.2. *Spectrophotometric determination*

5.1.2.1. *Preparation of calibration solutions*

For each of the elements to be determined, prepare from the working standard solutions given in points 3.7.1, 3.8.1, 3.9.1 and 3.10.1 a range of calibration solutions, each calibration solution having an HCl concentration of about 0,5 mol/litre (and (in the cases of iron, manganese and zinc) a lanthanum chloride concentration equivalent to 0,1 % La (w/v).

The trace element concentrations selected must lie within the range of sensitivity of the spectrophotometer used. The tables below show, by way of example, the compositions of typical ranges of calibration solutions; depending, however, on the type and sensitivity of spectrophotometer used it may be necessary to select other concentrations.
Iron

µg Fe/ml	0	0,5	1	2	3	4	5
ml working standard solution (3.7.1) (1 ml = 100 µg Fe)	0	0,5	1	2	3	4	5

ml HCl (3.2)	7	7	7	7	7	7	7
+ 10 ml of lanthanum chloride solution (3.11) and make up to 100 ml with water							

Copper

µg Cu/ml	0	0,1	0,2	0,4	0,6	0,8	1,0
ml working standard solution (3.8.1) (1 ml = 10 µg Cu)	0	1	2	4	6	8	10
ml HCl (3.2)	8	8	8	8	8	8	8

Manganese

µg Mn/ml	0	0,1	0,2	0,4	0,6	0,8	1,0
ml working standard solution (3.9.1) (1 ml = 10 µg Mn)	0	1	2	4	6	8	10
ml HCl (3.2)	7	7	7	7	7	7	7
+ 10 ml of lanthanum chloride solution (3.11) and make up to 100 ml with water							

Zinc

µg Zn/ml	0	0,05	0,1	0,2	0,4	0,6	0,8
ml working standard solution (3.10.1) (1 ml = 10 µg Zn)	0	0,5	1	2	4	6	8
ml HCl (3.2)	7	7	7	7	7	7	7
+ 10 ml of lanthanum chloride solution (3.11) and make up to 100 ml with water							

5.1.2.2. *Preparation of solution for analysis*

For the determination of copper, the solution prepared from point 5.1.1 can normally be used directly. If necessary to bring its concentration within the range of the calibration solutions, an aliquot portion may be pipetted into a 100 ml volumetric flask and made up to the mark with 0,5 mol/litre hydrochloric acid (3.3).

For the determination of iron, manganese and zinc, pipette an aliquot portion of the solution prepared from point 5.1.1 into a 100 ml volumetric flask, add 10 ml of lanthanum chloride solution (3.11) and make up to the mark with 0,5 mol/litre hydrochloric acid (3.3) (see also point 8 'Observation').

5.1.2.3. *Blank experiment*

The blank experiment must include all the prescribed steps of the procedure except that the sample material is omitted. The calibration solution '0' must not be used as the blank.

5.1.2.4. *Measurement of the atomic absorption*

Measure the atomic absorption of the calibration solutions and of the solution to be analysed using an oxidising air-acetylene flame at the following wavelengths:

Fe: 248,3 nm
Cu: 324,8 nm
Mn: 279,5 nm
Zn: 213,8 nm

Carry out each measurement four times.

5.2. Mineral feed

If the sample contains no organic matter, prior ashing is unnecessary. Proceed as described in point 5.1.1.1 starting from the second paragraph. Evaporation with hydrofluoric acid may be omitted.

6. Calculation of results

Using a calibration curve, calculate the trace element concentration in the solution to be analysed and express the result in milligrams of trace element per kilogram of sample (ppm).

7. Repeatability

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

— 5 mg/kg, in absolute value, for contents of the trace element concerned up to 50 mg/kg,
— 10 % of the higher result for contents of the trace element concerned from 50 and up to 100 mg/kg,
— 10 mg/kg, in absolute value, for contents of the trace element concerned from 100 and up to 200 mg/kg,
— 5 % of the higher result for contents of the trace element concerned above 200 mg/kg.

8. Observation

The presence of large quantities of phosphates may interfere with the determination of iron, manganese and zinc. Such interference must be corrected by addition of lanthanum chloride solution (3.11). If, however, in the sample the weight ratio Ca + Mg/P is > 2, addition of

lanthanum chloride solution (3.11) to the solution for analysis and to the calibration solutions may be omitted.

D. DETERMINATION OF HALOFUGINONE

DL-trans-7-bromo-6-chloro-3- [3-(3-hydroxy-2-piperidyl)acetonyl]-quinazolin-4-(3H)-one hydrobromide

1. Purpose and scope

The method makes it possible to determine the level of halofuginone in feed. The limit of quantification is 1 mg/kg.

2. Principle

After treatment with hot water, halofuginone is extracted as the free base into ethyl acetate and subsequently partitioned as the hydrochloride into an aqueous acid solution. The extract is purified by ion-exchange chromatography. The content of halofuginone is determined by reversed-phase high performance liquid chromatography (HPLC) using an UV detector.

3. Reagents

3.1. Acetonitrile, equivalent to HPLC grade.

3.2. Amberlite XAD-2 resin.

3.3. Ammonium acetate.

3.4. Ethyl acetate.

3.5. Acetic acid, glacial.

3.6. Halofuginone standard substance (DL-trans-7-brome-6-chloro-3-[3-hydroxy-2-piperidyl)acetonyl] quinazoline-4-(3H)-one hydrobromide, E 764).

3.6.1. *Halofuginone stock standard solution, 100 µg/ml*

Weight to the nearest 0,1 mg, 50 mg of halofuginone (3.6) in a 500 ml graduated flask, dissolve in ammonium acetate buffer solution (3.18), make up to the mark with the buffer solution and mix. This solution is stable for three weeks at 5 ^{o}C if stored in the dark.

3.6.2. *Calibration solutions*

Into a series of 100 ml graduated flasks transfer 1,0, 2,0, 3,0, 4,0 and 6,0 ml of the stock standard solution (3.6.1). Make up to the mark with mobile phase (3.21) and mix. These solutions have concentrations of 1,0, 2,0, 3,0, 4,0 and 6,0 µg/ml of halofuginone respectively. These solutions must be freshly prepared before use.

3.7. Hydrochloric acid (ρ_{20} approximately 1,16 g/ml).

3.8. Methanol.

3.9. Silver nitrate.

3.10. Sodium ascorbate.

3.11. Sodium carbonate.

3.12. Sodium chloride.

3.13. EDTA (ethylenediaminetetraacetic acid, disodium salt).

3.14. Water, equivalent to HPLC grade.

3.15. Sodium carbonate solution, c = 10 g/100 ml.

3.16. Sodium chloride-saturated sodium carbonate solution, c = 5 g/100 ml.

Dissolve 50 g of sodium carbonate (3.11) in water, dilute to 1 litre and add sodium chloride (3.12) until the solution is saturated.

3.17. Hydrochloric acid, approximately 0,1 mol/l.

Dilute 10 ml of HCI (3.7) with water to 1 litre.

3.18. Ammonium acetate buffer solution, approximately 0,25 mol/l.

Dissolve 19,3 g of ammonium acetate (3.3) and 30 ml of acetic acid (3.5) in water (3.14) and dilute to 1 1itre.

3.19. Amberlite XAD-2 resin preparation.

Wash an appropriate quantity of Amberlite (3.2) with water until all chloride ions have been removed, as indicated by a silver nitrate (3.20) test performed on the discarded aqueous phase. Then wash the resin with 50 ml of methanol (3.8), discard the methanol and store the resin under fresh methanol.

3.20. Silver nitrate solution, approximately 0,1 mol/l.

Dissolve 0,17 g of silver nitrate (3.9) in 10 ml of water.

3.21. HPLC Mobile phase.

Mix 500 ml of acetonitrile (3.1) with 300 ml of ammonium acetate buffer solution (3.18) and 1 200 ml of water (3.14). Adjust the pH to 4,3 using acetic acid (3.5). Filter through a 0,22 μm filter (4.8) and degas the solution (e.g. by ultrasonification for 10 minutes). This solution is stable for one month, if stored in the dark in a closed container.

4. Apparatus

4.1. Ultrasonic bath

4.2. Rotary film evaporator

4.3. Centrifuge

4.4. HPLC equipment with variable wavelength ultraviolet detector or diode-array detector

4.4.1. Liquid chromatographic column, 300 mm x 4 mm, C_{18}, 10 μm packaging, or an equivalent column

4.5. Glass column (300 mm x 10 mm) fitted with a sintered-glass filter and a stopcock

4.6. Glass-fibre filters, diameter 150 mm

4.7. Membrane filters, 0,45 μm

4.8. Membrane filters, 0,22 μm

5. Procedure

Note: Halofuginone as the free base is unstable in alkaline and ethyl acetate solutions. It shall not remain in ethyl acetate for more than 30 minutes.

5.1. General

5.1.1. A blank feed shall be analysed to check that neither halofuginone nor interfering substances are present.

5.1.2. A recovery test shall be carried out by analysing the blank feed which has been fortified by addition of a quantity of halofuginone, similar to that present in the sample. To fortify at a level of 3 mg/kg, add 300 µl of the stock standard solution (3.6.1) to 10 g of the blank feed, mix and wait for 10 minutes before proceeding with the extraction step (5.2).

Note: for the purpose of this method, the blank feed shall be similar in type to that of the sample and on analysis halofuginone shall not be detected.

5.2. Extraction

Weigh to the nearest 0,1 g, 10 g of the prepared sample, into a 200 ml centrifuge tube, add 0,5 g of sodium ascorbate (3.10), 0,5 g of EDTA (3.13) and 20 ml of water and mix. Place the tube for 5 minutes in a water bath (80 ^{o}C). After cooling down to room temperature, add 20 ml of sodium carbonate solution (3.15) and mix. Add immediately 100 ml of ethyl acetate (3.4) and shake vigorously by hand for 15 seconds. Then place the tube for three minutes in the ultrasonic bath (4.1) and loosen the stopper. Centrifuge for two minutes and decant the ethyl acetate phase through a glass fibre filter (4.6), into a 500 ml separating funnel. Repeat the extraction of the sample with a second portion of 100 ml of ethyl acetate. Wash the combined extracts for one minute with 50 ml of sodium chloride saturated sodium carbonate solution (3.16) and discard the aqueous layer.

Extract the organic layer for 1 min. with 50 ml of hydrochloric acid (3.17). Run the lower acid layer into a 250 ml separating funnel. Re-extract the organic layer for 1,5 minutes with a further 50 ml of hydrochlorid acid and combine with the first extract. Wash the combined acid extracts by swirling for approximately 10 seconds with 10 ml of ethyl acetate (3.4).

Quantitatively transfer the aqueous layer into a 250 ml round-bottomed flask and discard the organic phase. Evaporate all the remaining ethyl acetate from the acid solution using a rotary film evaporator (4.2). The temperature of the water bath must not exceed 40 ^{o}C. Under a vacuum of approximately 25 mbar all of the residual ethyl acetate will be removed within 5 minutes at 38 ^{o}C.

5.3. Clean up

5.3.1. *Preparation of the Amberlite column*

An XAD-2 column is prepared for each sample extract. Transfer 10 g of prepared Amberlite (3.19) into a glass column (4.5) with methanol (3.8). Add a small plug of glass-wool to the top of the resin bed. Drain the methanol from the column and wash the resin with 100 ml of water, stopping the flow as the liquid reaches the top of the resin bed. Allow the column to equilibrate for 10 minutes before use. Never allow the column to run dry.

5.3.2. *Sample clean up*

Transfer the extract (5.2) quantitatively to the top of the prepared Amberlite column (5.3.1) and elute, discarding the eluate. The rate of elution must not exceed 20 ml/min. Rinse the round-bottomed flask with 20 ml of hydrochlorid acid (3.17) and use this to wash the resin column. Blow through any remaining acid solution with a stream of air. Discard the washings. Add 100 ml of methanol (3.8) to the column and allow 5 to 10 ml to elute, collecting the eluate in a 250 ml round-bottomed flask. Leave the remaining methanol for 10 minutes to equilibrate with

the resin and continue the elution at a rate not exceeding 20 ml/min. collecting the eluate in the same round-bottomed flask. Evaporate the methanol on the rotary film evaporator (4.2), the temperature of the water bath must not exceed 40 ^{o}C. Transfer the residue quantitatively into a 10 ml calibrated flask using the mobile phase (3.21). Make up to the mark with mobile phase and mix. An aliquot is filtered through a membrane filter (4.7). Reserve this solution for the HPLC determination (5.4).

5.4. HPLC determination

5.4.1. *Parameters*

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

Liquid chromatographic column (4.4.1)

HPLC Mobile phase (3.21)

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

Detection wavelength: 243 nm

Injection volume: 40 to 100 µl.

Check the stability of the chromatographic system, injecting the calibration solution (3.6.2) containing 3,0 µg/ml several times, until constant peak heights (or areas) and retention times are achieved.

5.4.2. *Calibration graph*

Inject each calibration solution (3.6.2) several times and measure the peak heights (areas) for each concentration. Plot a calibration graph using the mean peak heights or areas of the calibration solutions as the ordinates and the corresponding concentrations in µg/ml as the abscissae.

5.4.3. *Sample solution*

Inject the sample extract (5.3.2) several times, using the same volume as taken for the calibration solutions and determine the mean peak height (area) of the halofuginone peaks.

6. Calculation of results

Determine the concentration of the sample solution in µg/ml, from the mean height (area) of the halofuginone peaks of the sample solution by reference to the calibration graph (5.4.2).

The content of halofuginone w (mg/kg) of the sample is given by the following formula:

$$w = \frac{c \times 10}{m}$$

in which:

c = halofuginone concentration of the sample solution in µg/ml,
m = weight of the test portion in grams.

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 and the calibration solution (3.6.2) containing 6,0 µg/ml are compared.

7.1.1. *Co-chromatography*

A sample extract is fortified by addition of an appropriate amount of a calibration solution (3.6.2). The amount of added halofuginone must be similar to the estimated amount of halofuginone found in the sample extract.

Only the height of the halofuginone peak shall be enhanced after taking into account both the amount added and the dilution of the extract. The peak width, at half of its maximum height, must be within ± 10 % of the original width.

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 the same within a margin determined by the resolving power of the detection system. For diode-array detection, this is typically within ± 2 nm;

(b) between 225 and 300 nm, the sample and standard spectra recorded at the peak apex on the chromatogram, must not be different for those parts of the spectrum within the range 10 % to 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 225 and 300 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 % to 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 apex.

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

7.2. Repeatability

The difference between results of two parallel determinations carried out on the same sample must not exceed 0,5 mg/kg for halofuginone contents up to 3 mg/kg.

7.3. Recovery

For the fortified blank sample the recovery shall be at least 80 %.

8. Results of a collaborative study

A collaborative study[(5)] was arranged in which three samples were analysed by eight laboratories.

RESULTS

	Sample A(blank)On receipt	**Sample B (Meal)**		**Sample C (Pellets)**	
		On receipt	**After two months**	**On receipt**	**After two months**

Mean [mg/kg]	ND	2,8	2,42	2,89	2,45
S_R [mg/kg]	—	0,45	0,43	0,4	0,42
CV_R [%]	—	16	18	14	17
Rec. [%]		86	74	88	75

ND = not detected
S_R = standard deviation of reproducibility
CV_R = coefficient of variation of reproducibility (%)
Rec. = recovery (%)

E. DETERMINATION OF ROBENIDINE

1,3-bis [(4-chlorobenzylidene)amino]guanidine — hydrochloride

1. Purpose and scope

This method makes it possible to determine the levels of robenidine in feed. The limit of quantification is 5 mg/kg.

2. Principle

The sample is extracted with acidified methanol. The extract is dried and an aliquot portion subjected to a clean-up on an aluminium oxide column. Robenidine is eluted from the column with methanol, concentrated, and made up to a suitable volume with mobile phase. The content of robenidine is determined by reversed-phase high-performance liquid chromatography (HPLC) using an UV detector.

3. Reagents

3.1. Methanol.

3.2. Acidified methanol.

Transfer 4,0 ml hydrochloric acid (ρ20 = 1,18 g/ml) into a 500 ml graduated flask, make up to the mark with methanol (3.1) and mix. This solution shall be freshly prepared before use.

3.3. Acetonitrile, equivalent to HPLC grade.

3.4. Molecular sieve.

Type 3A, 8 to 12 mesh beads (1,6-2,5 mm beads, crystalline alumino-silicate, diameter of pores 0,3 mm).

3.5. Aluminium oxide acidic activity grade I for column chromatography.

Transfer 100 g aluminium oxide into a suitable container and add 2,0 ml of water. Stopper and shake for approximately 20 minutes. Store in a well stoppered container.

3.6. Potassium dihydrogen phosphate solution, c = 0,025 mol/l.

Dissolve 3,4 g of potassium dihydrogen phosphate in water (HPLC grade) in a 1 000 ml graduated flask, make up to the mark and mix.

3.7. Di-sodium hydrogen phosphate solution, c = 0,025 mol/l.

Dissolve 3,55 g of anhydrous (or 4,45 g of dihydrate or 8,95 g of dodecahydrate) di-sodium hydrogen phosphate in water (equivalent to HPLC grade) in a 1 litre graduated flask, make up to the mark and mix.

3.8. HPLC mobile phase.

Mix together the following reagents:

650 ml acetonitrile (3.3),
250 ml water (equivalent to HPLC-grade),
50 ml potassium di-hydrogen phosphate solution (3.6),
50 ml di-sodium hydrogen phosphate solution (3.7).

Filter through a 0,22 μm filter (4.6) and degas the solution, (e.g. by ultrasonification for 10 minutes).

3.9. Standard substance.

Pure robenidine: 1,3-bis [(4-chlorobenzylidene)amino]guanidine — hydrochloride.

3.9.1. *Robenidine stock standard solution: 300 μg/ml*

Weigh to the nearest 0,1 mg, 30 mg of robenidine standard substance (3.9). Dissolve in acidified methanol (3.2) in a 100 ml graduated flask, make up to the mark with the same solvent and mix. Wrap the flask with aluminium foil and store in a dark place.

3.9.2. *Robenidine intermediate standard solution: 12 μg/ml*

Transfer 10,0 ml of the stock standard solution (3.9.1) into a 250 ml graduated flask, make up to the mark with the mobile phase (3.8) and mix. Wrap the flask with aluminium foil and store in a dark place.

3.9.3. *Calibration solutions*

Into a series of 50 ml calibrated flasks, transfer 5,0, 10,0, 15,0, 20,0 and 25,0 ml of the intermediate standard solution (3.9.2). Make up to the mark with mobile phase (3.8) and mix. These solutions correspond to 1,2, 2,4, 3,6, 4,8 and 6,0 μg/ml of robenidine respectively. These solutions must be freshly prepared before use.

3.10. Water equivalent to HPLC grade.

4. Apparatus

4.1. Glass column.

Constructed of amber glass fitted with a stopcock and a reservoir of approximately 150 ml capacity, internal diameter 10 to 15 mm, length 250 mm.

4.2. Mechanical shaker or magnetic stirrer.

4.3. Rotary film evaporator.

4.4. HPLC equipment with variable wavelength ultraviolet detector or diode array detector operating in the range of 250 to 400 nm.

4.4.1. Liquid chromatographic column: 300 mm x 4 mm, C_{18} 10 μm packing or equivalent.

4.5. Glass fibre filter paper (Whatman GF/A or equivalent).

4.6. Membrane filters, 0,22 μm.

4.7. Membrane filters, 0,45 µm.

5. Procedure

Note: Robenidine is light-sensitive. Amber glassware shall be used in all operations.

5.1. General

5.1.1. A blank feed shall be analysed to check that neither robenidine nor interfering substances are present.

5.1.2. A recovery test shall be carried out by analysing the blank feed (5.1.1) which has been fortified by addition of a quantity of robenidine, similar to that present in the sample. To fortify at a level of 60 mg/kg, transfer 3,0 ml of the stock standard solution (3.9.1) to a 250 ml conical flask. Evaporate the solution to ca. 0,5 ml in a stream of nitrogen. Add 15 g of the blank feed, mix and wait for 10 minutes before proceeding with the extraction step (5.2).

Note: For the purpose of this method, the blank feed shall be similar in type to that of the sample and on analysis robenidine shall not be detected.

5.2. Extraction

Weigh to the nearest 0,01 g, approximately 15 g of the prepared sample. Transfer to a 250 ml conical flask and add 100,0 ml of acidified methanol (3.2), stopper and shake for one hour on the shaker (4.2). Filter the solution through a glass fibre filter paper (4.5) and collect the whole filtrate in a 150 ml conical flask. Add 7,5 g molecular sieve (3.4), stopper and shake for five minutes. Filter immediately through a glass-fibre filter paper. Retain this solution for the purification step (5.3).

5.3. Purification

5.3.1. *Preparation of the aluminium-oxide column*

Insert a small glass-wool plug into the lower end of a glass column (4.1) and tamp it down using a glass rod. Weigh out 11,0 g of the prepared aluminium oxide (3.5) and transfer to the column. Care shall be taken to minimise the exposure to the atmosphere during this stage. Gently tap the loaded column at its lower end to settle the aluminium oxide.

5.3.2. *Sample purification*

Transfer onto the column by pipette 5,0 ml of the sample extract prepared in (5.2) Rest the pipette tip close to the column wall and allow the solution to be absorbed onto the aluminium oxide. Elute the robenidine from the column using 100 ml methanol (3.1), at a flow rate of 2 to 3 ml/minute and collect the eluate in a 250 ml round bottomed flask. Evaporate the methanol solution to dryness under reduced pressure at 40 ^{o}C by means of a rotary film evaporator (4.3). Re-dissolve the residue in 3 to 4 ml of mobile phase (3.8) and transfer quantitatively to a 10 ml graduated flask. Rinse the flask with several 1 to 2 ml portions of mobile phase and transfer these rinsings to the graduated flask. Make up to the mark with the same solvent and mix. An aliquot is filtered through a 0,45 µm membrane filter (4.7). Reserve this solution for HPLC determination (5.4).

5.4. HPLC determination

5.4.1. *Parameters*

The following conditions are offered for guidance, other conditions may be used provided they yield equivalent results:

Liquid chromatographic column (4.4.1),

HPLC mobile phase (3.8),

Flow rate: 1,5 to 2 ml/minute,

Detector wavelength: 317 nm,

Injection volume: 20 to 50 µl.

Check the stability of the chromatographic system, injecting the calibration solution (3.9.3) containing 3,6 µg/ml several times, until constant peak heights and retention times are achieved.

5.4.2. *Calibration graph*

Inject each calibration solution (3.9.3) several times and measure the peak heights (areas) for each concentration. Plot a calibration curve using the mean peak heights or areas of the calibration solutions as the ordinates and corresponding concentrations in µg per ml as abscissae.

5.4.3. *Sample solution*

Inject the sample extract (5.3.2) several times, using the same volume as taken for the calibration solutions and determine the mean peak height (area) of the robenidine peaks.

6. Calculation of results

From the mean height (area) of the robenidine peaks of the sample solution determine the concentration of the sample solution in µg/ml by reference to the calibration graph (5.4.2).

The content of robenidine w (mg/kg) in the sample is given by the following formula:

$$w = \frac{c \times 200}{m}$$

in which:

c = robenidine concentration of the sample solution in µg/ml,
m = weight of the test portion in grams.

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 and the calibration solution (3.9.3) containing 6 µg/ml are compared.

7.1.1. *Co-chromatography*

A sample extract is fortified by addition of an appropriate amount of calibration solution (3.9.3). The amount of added robenidine must be similar to the estimated amount of robenidine found in the sample extract.

Only the height of the robenidine peak shall be enhanced after taking into account both the amount added and the dilution of the extract. The peak width, at half of its maximum height, must be within approximately 10 % of the original width.

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 the same within a margin determined by the resolving power of the detection system. For diode-array detection, this is typically within approximately 2 nm;

(b) between 250 and 400 nm, the sample and standard spectra recorded at the peak apex on the chromatogram, must not be different for those parts of the spectrum within the range 10 % to 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 250 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 % to 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 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 10 % of the higher result for robenidine content higher than 15 mg/kg.

7.3. Recovery

For a fortified blank sample the recovery shall be at least 85 %.

8. Results of a collaborative study

An EC collaborative study was arranged in which four samples of poultry and rabbit feed, in meal or pelleted form were analysed by 12 laboratories. Duplicate analyses were performed on each sample. The results are given in the table below:

	Poultry		**Rabbit**	
	Meal	**Pellet**	**Meal**	**Pellet**
Mean [mg/kg]	27,0	27,99	43,6	40,1
s_r [mg/kg]	1,46	1,26	1,44	1,66
CV_r [%]	5,4	4,5	3,3	4,1
S_R [mg/kg]	4,36	3,36	4,61	3,91
CV_R [%]	16,1	12,0	10,6	9,7
Recovery [%]	90,0	93,3	87,2	80,2

s_r = standard deviation of repeatability,
CV_r = coefficient of variation of repeatability, %
S_R = standard deviation of reproducibility,
CV_R = coefficient of variation of reproducibility. %

F. DETERMINATION OF DICLAZURIL

(+)-4-chlorphenyl [2,6-dichloro-4-(2,3,4,5-tetrahydro-3,5-dioxo-1,2,4-triazin-2-yl)phenyl] acetonitrile

1. Purpose and scope

The method makes it possible to determine the level of diclazuril in feed and premixtures. The limit of detection is 0,1 mg/kg, the limit of quantification is 0,5 mg/kg.

2. Principle

After addition of an internal standard, the sample is extracted with acidified methanol. For feed, an aliquot of the extract is purified on a C_{18} solid phase extraction cartridge. Diclazuril is eluted from the cartridge with a mixture of acidified methanol and water. After evaporation, the residue is dissolved in DMF/water. For premixtures, the extract is evaporated and the residue is dissolved in DMF/water. The content of diclazuril is determined by ternary gradient reversed-phase high-performance liquid chromatography (HPLC) using a UV detector.

3. Reagents

3.1. Water, equivalent to HPLC-grade

3.2. Ammonium acetate

3.3. Tetrabutylammonium hydrogen sulphate (TBHS)

3.4. Acetonitrile, equivalent to HPLC grade

3.5. Methanol, equivalent to HPLC grade

3.6. N, N-dimethylformamide (DMF)

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

3.8. Standard substance: diclazuril II-24: (+)-4-chlorphenyl [2,6-dichloro-4-(2,3,4,5-tetrahydro-3,5-dioxo-1,2,4-triazin-2-yl) phenyl] acetonitrile with guaranteed purity, E771

3.8.1. *Diclazuril stock standard solution, 500 µg/ml*

Weigh to the nearest 0,1 mg, 25 mg of diclazuril standard substance (3.8) in a 50 ml graduated flask. Dissolve in DMF (3.6), make up to the mark with DMF (3.6) and mix. Wrap the flask with aluminium foil or use amber flask and store in the refrigerator. At a temperature of ≤ 4 °C the solution is stable for 1 month.

3.8.2. *Diclazuril standard solution, 50 µg/ml*

Transfer 5,0 ml of the stock standard solution (3.8.1) into a 50 ml graduated flask, make up to the mark with DMF (3.6) and mix. Wrap the flask with aluminium foil or use amber flask and store in the refrigerator. At a temperature of ≤ 4 °C the solution is stable for 1 month.

3.9. Internal standard substance: 2,6 dichloro-α-(4-chlorophenyl)-4-(4,5 dihydro-3,5-dioxo-1,2,4-triazine-2 (3H) — yl) α-methylbenzene-acetonitrile

3.9.1. *Internal stock standard solution, 500 µg/ml*

Weigh to the nearest 0,1 mg 25 mg of internal standard substance (3.9) in a 50 ml graduated flask. Dissolve in DMF (3.6), make up to the mark with DMF (3.6) and mix. Wrap the flask with aluminium foil or use amber flask and store in the refrigerator. At a temperature of ≤ 4 °C the solution is stable for 1 month.

3.9.2. *Internal standard solution, 50 µg/ml*

Transfer 5,0 ml of the internal stock standard solution (3.9.1) into a 50 ml graduated flask, make up to the mark with DMF (3.6) and mix. Wrap the flask with aluminium foil or use amber flask and store in the refrigerator. At a temperature of ≤ 4 °C the solution is stable for 1 month.

3.9.3. *Internal standard solution for premixtures, p/1 000 mg/ml*

(p = nominal content of diclazuril in the premixture in mg/kg)

Weigh to the nearest 0,1 mg p/10 mg of the internal standard substance in a 100 ml graduated flask, dissolve in DMF (3.6) in a ultrasonic bath (4.6), make up to the mark with DMF and mix. Wrap the flask with aluminium foil or use amber flask and store in a refrigerator. At a temperature of ≤ 4 °C the solution is stable for 1 month.

3.10. Calibration solution, 2 µg/ml.

Pipet 2,0 ml diclazuril standard solution (3.8.2) and 2,0 ml internal standard solution (3.9.2) into a 50 ml graduated flask. Add 16 ml DMF (3.6), make up to the mark with water and mix. This solution must be prepared freshly before use.

3.11. C_{18} solid phase extraction cartridge, e.g. Bond Elut, size: 1 cc, sorbent weight: 100 mg.

3.12. Extraction solvent: acidified methanol.

Pipet 5,0 ml hydrochloric acid (3.7) into 1 000 ml of methanol (3.5), and mix.

3.13. Mobile phase for HPLC

3.13.1. Eluent A: ammonium acetate — tetrabutylammonium hydrogen sulphate solution.

Dissolve 5 g ammonium acetate (3.2) and 3,4 g TBHS (3.3) in 1 000 ml water (3.1) and mix.

3.13.2. Eluent B: acetonitrile (3.4).

3.13.3. Eluent C: methanol (3.5).

4. Apparatus

4.1. Mechanical shaker

4.2. Equipment for ternary gradient HPLC

4.2.1. Liquid chromatographic column, Hypersil ODS, 3 µm packing, 100 mm x 4,6 mm, or equivalent

4.2.2. UV detector with variable wavelength adjustment or diode array detector

4.3. Rotary film evaporator

4.4. Membrane filter, 0,45 µm

4.5. Vacuum manifold

4.6. Ultrasonic bath

5. Procedure

5.1. General

5.1.1. *Blank feed*

A blank feed shall be analysed to check that neither diclazuril nor interfering substances are present. The blank feed shall be similar in type to that of the sample and on analysis diclazuril or interfering substances shall not be detected.

5.1.2. *Recovery test*

A recovery test shall be carried out by analysing the blank feed which has been fortified by addition of a quantity of diclazuril similar to that present in the sample. To fortify at a level of 1 mg/kg add 0,1 ml of the stock standard solution (3.8.1) to 50 g of a blank feed, mix thoroughly and leave for 10 min. mixing again several times before proceeding (5.2).

Alternatively, if a blank feed similar in type to that of the sample is not available (see 5.1.1), a recovery test can be performed by means of the standard addition method. In this case, the sample to be analysed is fortified with a quantity of diclazuril, similar to that already present in the sample. This sample is analysed, together with the unfortified sample and the recovery can be calculated by subtraction.

5.2. Extraction

5.2.1. *Feed*

Weigh to the nearest 0,01 g approximately 50 g of the sample. Transfer to a 500 ml conical flask, add 1,0 ml internal standard solution (3.9.2), 200 ml extraction solvent (3.12) and stopper the flask. Shake the mixture on the shaker (4.1) overnight. Allow to settle for 10 minutes. Transfer a 20 ml aliquot of the supernatant to a suitable glass container and dilute with 20 ml water. Transfer this solution on an extraction cartridge (3.11), and pass through by applying vacuum (4.5). Wash the cartridge with 25 ml of a mixture of extraction solvent (3.12) and water, 65 + 35 (V + V). Discard the collected fractions and elute the compounds with 25 ml of a mixture of extraction solvent (3.12) and water, 80 + 20 (V + V). Evaporate this fraction until it had just reached dryness by means of the rotary evaporator (4.3) at 60 ^{o}C. Dissolve the residue in 1,0 ml DMF (3.6), add 1,5 ml of water (3.1) and mix. Filter through a membrane filter (4.4). Proceed to the HPLC determination (5.3).

5.2.2. *Premixtures*

Weigh to the nearest 0,001 g approximately 1 g of the sample. Transfer to a 500 ml conical flask, add 1,0 ml internal standard solution (3.9.3), 200 ml extraction solvent (3.12) and stopper the flask. Shake the mixture overnight on the shaker (4.1). Allow to settle for 10 minutes. Transfer an aliquot of 10 000/p ml (p = nominal content of diclazuril in the premix in mg/kg) of the supernatant to a round bottomed flask of suitable size. Evaporate until it had just reached dryness, under reduced pressure at 60 ^{o}C by means of the rotary evaporator (4.3). Redissolve the residue in 10,0ml DMF (3.6), add 15,0 ml water (3.1) and mix. 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.

Liquid chromatographic column (4.2.1)	100 mm × 4,6 mm, Hypersil ODS, 3 µm packing, or equivalent	
Mobile phase:	Eluent A (3.13.1):	Aqueous solution of ammonium acetate and tetrabutyl-ammonium hydrogen sulphate
	Eluent B (3.13.2):	acetonitrile
	Eluent C (3.13.3):	methanol
Elution mode:	— linear gradient — initial conditions: A + B + C = 60 + 20 + 20 (V + V + V) — after 10 min. gradient elution during 30 min. to: A + B + C = 45 + 20 + 35 (V + V + V) Flush with B during 10 min.	
Flow rate:	1,5-2 ml/min.	
Injection volume:	20 µl	
Detector wavelength:	280 nm.	

Check the stability of the chromatographic system, injecting several times the calibration solution (3.10), containing 2 µg/ml, until constant peak heights and retention times are achieved.

5.3.2. *Calibration solution*

Inject 20 µl of the calibration solution (3.10) several times and determine the mean peak height (area) of the diclazuril and internal standard peaks.

5.3.3. *Sample solution*

Inject 20 µl of the sample solution (5.2.1 or 5.2.2) several times and determine the mean peak height (area) of the diclazuril and internal standard peaks.

6. Calculation of the results

6.1. Feeds

The diclazuril content w (mg/kg) in the sample is given by the following formula:

$$w = \frac{h_{d,s} \times h_{i,c}}{h_{i,s} \times h_{d,c}} \times \frac{c_{d,c} \times 10\ V}{m}$$

[mg/kg]

where:

$h_{d,s}$ = peak height (area) of diclazuril in the sample solution (5.2.1)
$h_{i,s}$ = peak height (area) of the internal standard in the sample solution (5.2.1)
$h_{d,c}$ = peak height (area) of diclazuril in the calibration solution (3.10)
$h_{i,c}$ = peak height (area) of the internal standard in the calibration solution (3.10)
$c_{d,c}$ = diclazuril concentration in the calibration solution in µg/ml (3.10)
m = weight of the test portion in g
V = volume of the sample extract according to 5.2.1 (i.e. 2,5 ml)

6.2. Premixtures

The diclazuril content w (mg/kg) in the sample is given by the following formula:

$$w = \frac{h_{d,s} \times h_{i,c}}{h_{i,s} \times h_{d,c}} \times \frac{c_{d,c} \times 0{,}02V \times p}{m}$$

[mg/kg]

where:

$h_{d,c}$	=	peak height (area) of diclazuril in the calibration solution (3.10)
$h_{i,c}$	=	peak height (area) of the internal standard in the calibration solution (3.10)
$h_{d,s}$	=	peak height (area) of diclazuril in the sample solution (5.2.2)
$h_{i,s}$	=	peak height (area) of the internal standard in the sample solution (5.2.2)
$c_{d,c}$	=	diclazuril concentration in the calibration solution in µg/ml (3.10)
m	=	weight of the test portion in g
V	=	volume of the sample extract according to 5.2.2 (i.e. 25 ml)
p	=	nominal content of diclazuril in mg/kg in the premixture

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 (5.2.1 or 5.2.2) and the calibration solution (3.10) are compared.

7.1.1. *Co-chromatography*

A sample extract (5.2.1 or 5.2.2) is fortified by addition of an appropriate amount of calibration solution (3.10). The amount of added diclazuril must be similar to the amount of diclazuril found in the sample extract.

Only the height of the diclazuril peak and the internal standard peak shall 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 ± 10 % of the original width of the diclazuril peak or the internal standard 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 the same within a margin determined by the resolving power of the detection system. For diode-array detection this is typically within ± 2 nm.

(b) Between 230 and 320 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 230 and 320 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:

— 30 % relative, to the higher value for diclazuril contents from 0,5 mg/kg to 2,5 mg/kg,

— 0,75 mg/kg for diclazuril contents between 2,5 mg/kg and 5 mg/kg,

— 15 % relative to the higher value for diclazuril contents of more than 5 mg/kg.

7.3. Recovery

For a fortified (blank) sample the recovery shall be at least 80 %.

8. Results of a collaborative study

A collaborative study was arranged in which 5 samples were analysed by 11 laboratories. These samples consisted of two premixtures; one was mixed with an organic matrix (O 100) and the other with an inorganic matrix (A 100). The theoretical content is 100 mg diclazuril per kg. The three mixed feeds for poultry were made by 3 different producers (NL) (L1/Z1/K1). The theoretical content is 1 mg diclazuril per kg. The laboratories were instructed to analyse each of the samples once or in duplicate. (More detailed information on this collaborative study can be found in the *Journal of AOAC International, Volume 77, No 6, 1994, p. 1359-1361)*. The results are given in the following table.

	Sample 1A 100	**Sample 2O 100**	**Sample 3L1**	**Sample 4Z1**	**Sample 5K1**
L	11	11	11	11	6
n	19	18	19	19	12
Mean	100,8	103,5	0,89	1,15	0,89
S_r (mg/kg)	5,88	7,64	0,15	0,02	0,03
CV_r (%)	5,83	7,38	17,32	1,92	3,34
S_R (mg/kg)	7,59	7,64	0,17	0,11	0,12
CV_R (%)	7,53	7,38	18,61	9,67	13,65
Nominal content (mg/kg)	100	100	1	1	1

L = number of laboratories
n = number of single values
S_r = standard deviation of repeatability
CV_r = coefficient of variation of repeatability
S_R = standard deviation of reproducibility
CV_R = coefficient of variation of reproducibility

9. Observations

The diclazuril response must have been previously demonstrated to be linear over the range of concentrations being measured.

G. DETERMINATION OF LASALOCID SODIUM

Sodium salt of a polyether monocarboxylic acid produced by Streptomyces lasaliensis

1. Purpose and scope

The method makes it possible to determine the level of lasalocid sodium in feed and premixtures. The limit of detection is 5 mg/kg, the limit of quantification is 10 mg/kg.

2. Principle

Lasalocid sodium is extracted from the sample into acidified methanol and determined by reversed-phase high performance liquid chromatography (HPLC) using a spectrofluorometric detector.

3. Reagents

3.1. Potassium dihydrogen phosphate (KH_2PO_4).

3.2. Orthophosphoric acid, w (w/w) = 85 %.

3.3. Orthophosphoric acid solution, c = 20 %.

Dilute 23,5 ml of orthophosphoric acid (3.2) to 100 ml with water.

3.4. 6-Methyl-2-heptylamine (1,5-dimethylhexylamine), w (w/w) = 99 %.

3.5. Methanol, equivalent to HPLC grade.

3.6. Hydrochloric acid, density = 1,19 g/ml.

3.7. Phosphate buffer solution, c = 0,01 mol/l.

Dissolve 1,36 g of KH_2PO_4 (3.1) in 500 ml of water (3.11), add 3,5 ml of orthophosphoric acid (3.2) and 10,0 ml of 6-methyl-2-heptylamine (3.4). Adjust the pH to 4,0 with orthophosphoric acid solution (3.3) and dilute to 1 000 ml with water (3.11).

3.8. Acidified methanol.

Transfer 5,0 ml of hydrochloric acid (3.6) into a 1 000 ml graduated flask, make up to the mark with methanol (3.5) and mix. This solution must be prepared freshly before use.

3.9. HPLC mobile phase, phosphate buffer-methanol solution 5 + 95 (V + V).

Mix 5 ml of phosphate buffer solution (3.7) with 95 ml of methanol (3.5).

3.10. Lasalocid sodium standard substance with guaranteed purity, $C_{34}H_{53}O_8Na$ (sodium salt of a polyether monocarboxylic acid produced by *Streptomyces lasaliensis*), E763.

3.10.1. *Lasalocid sodium stock standard solution, 500 µg/ml*

Weigh to the nearest 0,1 mg, 50 mg of lasalocid sodium (3.10) into a 100 ml graduated flask, dissolve in acidified methanol (3.8), make up to the mark with the same solvent and mix. This solution must be freshly prepared before use.

3.10.2. *Lasalocid sodium intermediate standard solution, 50 µg/ml*

Pipette 10,0 ml of stock standard solution (3.10.1) into a 100 ml graduated flask, make up to the mark with acidified methanol (3.8) and mix. This solution must be prepared freshly before use.

3.10.3. *Calibration solutions*

Into a series of 50 ml graduated flasks transfer 1,0, 2,0, 4,0, 5,0 and 10,0 ml of the intermediate standard solution (3.10.2). Make up to the mark with acidified methanol (3.8) and mix. These solutions correspond to 1,0, 2,0, 4,0, 5,0 and 10,0 µg of lasalocid sodium per ml respectively. These solutions must be prepared freshly before use.

3.11. Water, equivalent to HPLC grade.

4. Apparatus

4.1. Ultrasonic bath (or shaking water-bath) with temperature control.

4.2. Membrane filters, 0,45 µm.

4.3. HPLC equipment with injection system, suitable for injecting volumes of 20 µl.

4.3.1. Liquid chromatographic column 125 mm x 4 mm, reversed-phase C_{18}, 5 µm packing or equivalent.

4.3.2. Spectrofluorometer with variable wavelength adjustment of excitation and emission wavelengths.

5. Procedure

5.1. General

5.1.1. *Blank feed*

For the performance of the recovery test (5.1.2) a blank feed shall be analysed to check that neither lasalocid sodium nor interfering substances are present. The blank feed shall be similar in type to that of the sample and lasalocid sodium or interfering substances shall not be detected.

5.1.2. *Recovery test*

A recovery test shall be carried out by analysing the blank feed which has been fortified by addition of a quantity of lasalocid sodium, similar to that present in the sample. To fortify at a level of 100 mg/kg, transfer 10,0 ml of the stock standard (3.10.1) to a 250 ml conical flask and evaporate the solution to approximately 0,5 ml. Add 50 g of the blank feed, mix thoroughly and leave for 10 minutes mixing again several times before proceeding with the extraction step (5.2).

Alternatively, if a blank feed similar in type to that of the sample is not available (see 5.1.1), a recovery test can be performed by means of the standard addition method. In this case the sample to be analysed is fortified with a quantity of lasalocid sodium similar to that already present in the sample. This sample is analysed together with the unfortified sample and the recovery calculated by subtraction.

5.2. Extraction

5.2.1. *Feed*

Weigh to the nearest 0,01 g, from 5 g to 10 g of the sample into a 250 ml conical flask with stopper. Add 100,0 ml of acidified methanol (3.8) by pipette. Stopper loosely and swirl to disperse. Place the flask in an ultrasonic bath (4.1) at approximately 40 ^{o}C for 20 minutes, then remove and cool to room temperature. Allow to stand for about 1 hour until the suspended

matter has settled, then filter an aliquot portion through a 0,45 μm membrane filter (4.2) into a suitable vessel. Proceed to the HPLC determination (5.3).

5.2.2. *Premixtures*

Weigh to the nearest 0,001 g about 2 g of the unground premix into a 250 ml graduated flask. Add 100,0 ml of acidified methanol (3.8) and swirl to disperse. Place the flask and contents in an ultrasonic bath (4.1) at approximately 40 ^{o}C for 20 minutes, then remove and cool to room temperature. Dilute to the mark with acidified methanol (3.8) and mix thoroughly. Allow to stand for 1 hour until the suspended matter has settled, then filter an aliquot portion through a 0,45 μm membrane filter (4.2). Dilute an appropriate volume of the clear filtrate with acidified methanol (3.8) to produce a final test solution containing about 4 μg/ml of lasalocid sodium. 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 they yield equivalent results:

Liquid chromatographic column (4.3.1):	125 mm × 4 mm, reversed-phase C_{18}, 5 μm packing or equivalent
Mobile phase (3.9):	Mixture of phosphate buffer solution (3.7) and methanol (3.5), 5+95 (V+V)
Flow rate:	1,2 ml/min.
Detection wavelengths:	
Excitation:	310 nm
Emission:	419 nm
Injection volume:	20 μl

Check the stability of the chromatographic system, injecting the calibration solution (3.10.3) containing 4,0 μg/ml several times, until constant peak heights (or areas) and retention times are achieved.

5.3.2. *Calibration graph*

Inject each calibration solution (3.10.3) several times and determine the mean peak heights (areas) for each concentration. Plot a calibration graph using the mean peak heights (areas) as the ordinates and the corresponding concentrations in μg/ml as the abscissae.

5.3.3. *Sample solution*

Inject the sample extracts obtained in 5.2.1 or 5.2.2 several times, using the same volume as taken for the calibration solution and determine the mean peak heights (areas) of the lasalocid sodium peaks.

6. Calculation of results

From the mean peak height (area) produced by injection of the sample solution (5.3.3) determine the concentration of lasalocid sodium (μg/ml) by reference to the calibration graph.

6.1. Feed

The lasalocid sodium content, w (mg/kg) in the sample is given by the following formula:

$$w = \frac{c \times V_1}{m}$$

[mg/kg]

where:

c	=	lasalocid sodium concentration of the sample solution (5.2.1) in µg/ml
V_1	=	volume of the sample extract according to 5.2.1 in ml (i.e. 100)
m	=	weight of the test portion in g

6.2. Premixtures

The lasalocid sodium content, w (mg/kg) in the sample is given by the following formula:

$$w = \frac{c \times V_2 \times f}{m}$$

[mg/kg]

where:

c	=	lasalocid sodium concentration of the sample solution (5.2.2) in µg/ml
V_2	=	volume of the sample extract according to 5.2.2 in ml (i.e. 250)
f	=	dilution factor according to 5.2.2
m	=	weight of the test portion in g

7. Validation of the results

7.1. Identity

Methods based on spectrofluorometry are less subject to interference than those in which UV detection is used. The identity of the analyte can be confirmed by co-chromatography.

7.1.1. *Co-chromatography*

A sample extract (5.2.1 or 5.2.2) is fortified by the addition of an appropriate amount of a calibration solution (3.10.3). The amount of added lasalocid sodium must be similar to the amount of lasalocid sodium found in the sample extract. Only the height of the lasalocid sodium peak shall be enhanced after taking into account the amount of lasalocid sodium added and the dilution of the extract. The peak width, at half height, must be within ± 10 % of the original peak width produced by the unfortified sample extract.

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 value for lasalocid sodium contents from 30 mg/kg to 100 mg/kg,
— 15 mg/kg for lasalocid sodium contents from 100 mg/kg to 200 mg/kg,
— 7,5 % relative to the higher value for lasalocid sodium contents of more than 200 mg/kg.

7.3. Recovery

For the fortified (blank) feed sample, the recovery shall be at least 80 %. For the fortified premixture samples, the recovery shall be at least 90 %.

8. Results of a collaborative study

A collaborative study[6] was arranged in which 2 premixtures (samples 1 and 2) and 5 feeds (samples 3-7) were analysed by 12 laboratories. Duplicate analyses were performed on each sample. The results are given in the following table:

	Sample 1Chicken premix	**Sample 2Turkey premix**	**Sample 3Turkey pellets**	**Sample 4Chicken crumbs**	**Sample 5Turkey Feed**	**Sample 6Poultry Feed A**	**Sample 7Poultry Feed B**
L	12	12	12	12	12	12	12
n	23	23	23	23	23	23	23
Mean [mg/kg]	5 050	16 200	76,5	78,4	92,9	48,3	32,6
s_r [mg/kg]	107	408	1,71	2,23	2,27	1,93	1,75
CV_r [%]	2,12	2,52	2,24	2,84	2,44	4,0	5,37
s_R [mg/kg]	286	883	3,85	7,32	5,29	3,47	3,49
CV_R [%]	5,66	5,45	5,03	9,34	5,69	7,18	10,7
Nominal content [mg/kg]	5 000[a]	16 000[a]	80[a]	105[a]	120[a]	50[b]	35[b]

a Content declared by manufacturer.

b Feed prepared in the laboratory.

L = number of laboratories
n = number of single results
s_r = standard deviation of repeatability
s_R = standard deviation of reproducibility
CV_r = coefficient of variation of repeatability, %
CV_R = coefficient of variation of reproducibility, %.

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

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

(**3**) Other methods of digestion may be used provided they have been demonstrated to have similar results (such as microwave pressure digestion).

(**4**) Green fodder (fresh or dried) is liable to contain large amounts of vegetable silica, which may retain trace elements and must be removed. For samples of these feed, therefore, the following modified procedure must be followed. Carry out operation 5.1.1.1. as far as the filtration. Wash the filter paper containing the insoluble residue twice with boiling water and place it in a quartz or platinum crucible. Ignite in the muffle furnace (4.1) at a temperature below 550 ^{o}C until all carbonaceous material has completely disappeared. Allow to cool, add a few drops of water followed by 10 to 15 ml of hydrofluoric acid (3.4) and evaporate to dryness at about 150 ^{o}C. If any silica remains in the residue, redissolve it in a few millilitres of hydrofluoric acid (3.4) and evaporate to dryness. Add five drops of sulphuric acid (3.5) and heat until no more white fumes are given off. After the addition of 5 ml of 6 mol/litre hydrochloric acid (3.2) and about 30 ml of water, heat, filter the solution into the 250 ml volumetric flask and make up to the mark with water (HCl concentration about 0,5 mol/l). Proceed then with the determination from point 5.1.2.

(**5**) The Analyst 108, 1983, pp. 1252 to 1256.

(**6**) *Analyst*, 1995, 120, 2175-2180.

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