INTERNATIONAL STANDARD

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Animal feeding stuffs — Determination of vitamin E content — Method using high-performance liquid chromatography

Aliments des animaux — Détermination de la teneur en vitamine E — Méthode par chromatographie liquide à haute performance

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Foreword

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International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 3.

Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

Attention is drawn to the possibility that some of the elements of this International Standard may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

International Standard ISO 6867 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 10, *Animal feeding stuffs*.

Annex A of this International Standard is for information only.

Animal feeding stuffs — Determination of vitamin E content — Method using high-performance liquid chromatography

1 Scope

This International Standard specifies a method for the determination of the vitamin E (DL-α-tocopherol) content of animal feeding stuffs and pet foods using high performance liquid chromatography.

2 Normative references

The following normative documents contain provisions which, through reference in this text, constitute provisions of this International Standard. For dated references, subsequent amendments to, or revisions of, any of these publications do not apply. However, parties to agreements based on this International Standard are encouraged to investigate the possibility of applying the most recent editions of the normative documents indicated below. For undated references, the latest edition of the normative document referred to applies. Members of ISO and IEC maintain registers of currently valid International Standards.

ISO 3696:1987, *Water for analytical laboratory use — Specifications and test methods*.

ISO 6498, *Animal feeding stuffs — Preparation of test samples*.

3 Principle

A test portion of the sample is saponified with ethanolic potassium hydroxide solution and the vitamin E is extracted into light petroleum. The light petroleum is removed by evaporation and the residue is dissolved in hexane. The vitamin E concentration in the hexane extract is determined by normal-phase liquid chromatography using conditions that separate DL-α-tocopherol from other tocopherols.

4 Reagents

Use only reagents of recognized analytical grade, unless otherwise stated.

4.1 Water, complying with at least grade 3 in accordance with ISO 3696.

4.2 Potassium hydroxide solution.

Dissolve 500 g of potassium hydroxide in water (4.1) and dilute to 1 litre.

4.3 Ethanol, $w(C_2H_5OH)$ = 95 % (by volume), or equivalent industrial methylated spirit.

4.4 Hexane, HPLC grade.

4.5 Light petroleum, boiling range 40 °C to 60 °C; the residue on evaporation shall be less than 20 mg/l.

4.6 Vitamin E standard substance: DL-α-tocopherol, minimum purity not less than 96,0 %.

The purity of the standard substance should be checked spectrophotometrically (see 8.5.2).

4.7 1,4-Dioxan, HPLC grade.

4.8 Sodium sulfate (Na_2SO_4), anhydrous.

4.9 Sodium ascorbate solution, ρ = 100 g/l.

4.10 Inert gas, e.g. nitrogen.

4.11 Mobile phase for liquid chromatography.

Mix 30 ml 1,4-dioxan (4.7) with 970 ml hexane (4.4).

Filter through a membrane filter (5.5) before use.

4.12 Ethanol, $w(C_2H_5OH)$ = 96 % (by volume).

4.13 Methanol (CH_3OH), HPLC grade.

5 Apparatus

Using laboratory apparatus and, in particular, the following.

5.1 High-performance liquid chromatograph, consisting of the following.

5.1.1 Pump, set to deliver a constant eluent volume flow rate of 1,5 ml/min.

5.1.2 HPLC injection device.

5.1.3 Column, length 250 mm, internal diameter 4,6 mm, packed with a stationary phase consisting of silica.

A column with at least 5 000 theoretical plates and a k' value of 0,8 m, both with respect to DL-α-tocopherol, has been found to be satisfactory. The particle size should not be smaller than 5 µm and not greater than 10 µm. Other systems may be used provided that a satisfactory separation of vitamin E from other co-extractives is achieved.

5.1.4 Detector, allowing the measurement of fluorescence emitted at a wavelength of 326 nm when the column eluent is irradiated with ultraviolet light at a wavelength of 293 nm, with integrator/recorder.

5.2 Boiling water bath.

5.3 Rotary vacuum evaporator, with water bath at 40 °C.

5.4 Extraction apparatus (see Figure 1) consisting of the following:

— a cylinder of 1 litre capacity fitted with a ground glass neck and stopper;

— a ground glass joint, fitting the cylinder and equipped with an adjustable tube passing through the centre; and

— a side-arm.

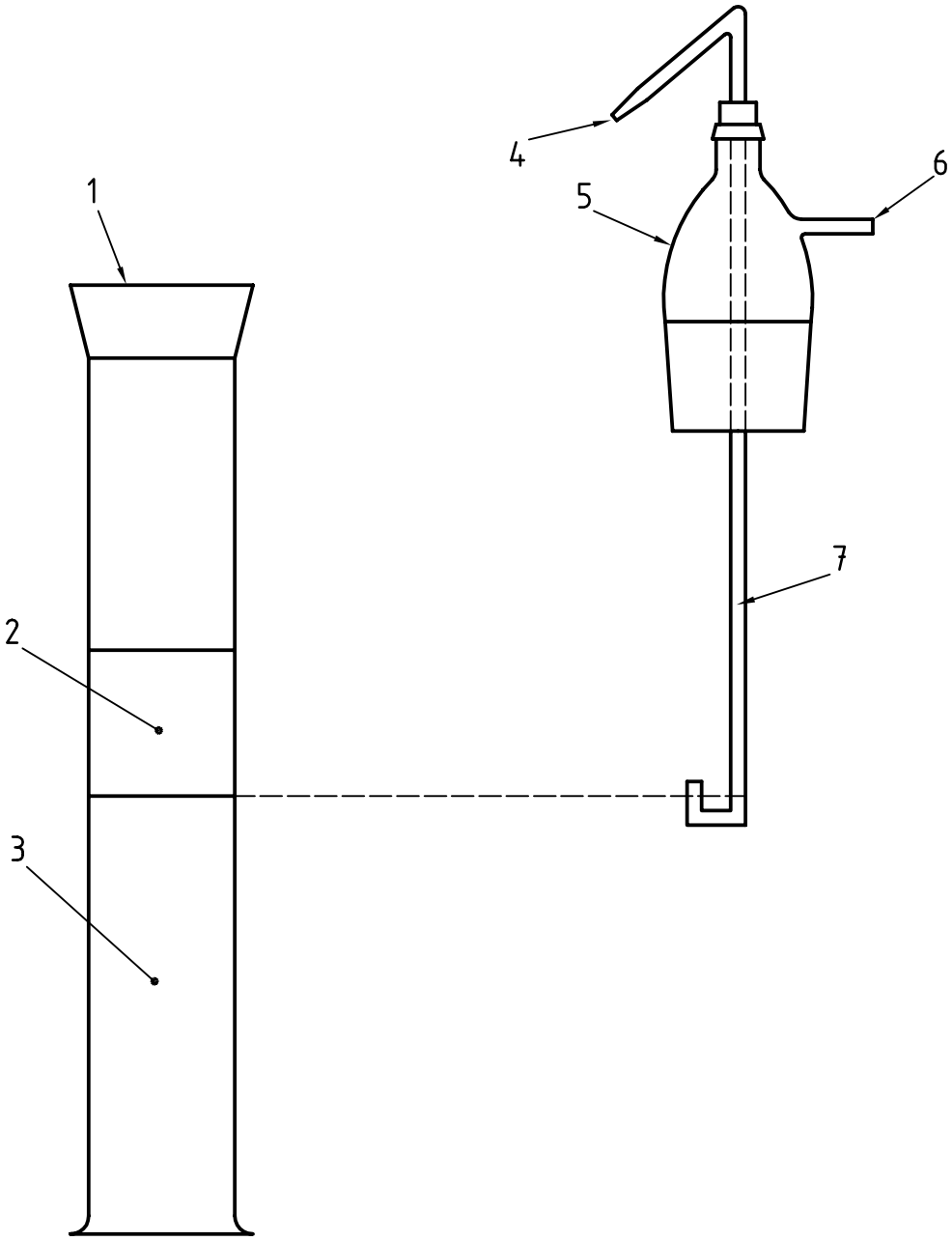
The adjustable tube should have a U-shaped lower end and a jet at the opposite end so that the upper liquid layer in the cylinder may be transferred to a separating funnel of 1 litre capacity.

Other extraction equipment such as conical flasks and separating funnels may be used in place of the apparatus shown in Figure 1, provided that satisfactory recoveries of vitamin E are achieved.

5.5 Membrane filter, 0,45 m pore size, for filtration of mobile phase (4.11) and sample test solutions.

5.6 Grinding apparatus, capable of grinding the sample so that it passes through a **sieve** with 1 mm apertures.

5.7 UV (or UV/Visible) spectrometer, capable of measuring absorbance at the wavelengths defined in 8.5.2, equipped with quartz cells of 10 mm path length.



Key

1 Cylinder, of capacity 1 litre, with ground-glass neck
2 Light petroleum layer
3 Aqueous layer + saponified feed
4 Jet
5 Bottle, of capacity 1 litre, with ground-glass joint
6 Side-arm
7 Adjustable tube

Figure 1 — Example of extraction apparatus

6 Sampling

It is important that the laboratory receive a sample which is truly representative and has not been damaged or changed during transport or storage.

Sampling is not part of the method specified in this International Standard. A recommended sampling method is given in ISO 6497 [1].

Store the sample in such a way that deterioration and change in its composition are prevented.

7 Preparation of test samples

Prepare the test sample in accordance with ISO 6498.

Just prior to starting the analysis, grind a portion of the well-mixed laboratory sample so that it passes through a sieve with 1 mm apertures. Mix thoroughly.

Homogenize canned pet foods. Pass semi-moist pet foods through a mincer with 4-mm apertures.

8 Procedure

8.1 General

Because of the sensitivity of vitamin E to UV radiation and air, perform all operations away from natural and strong fluorescent light and as rapidly as is consistent with accurate working. Use amber glassware where possible. Complete each assay within one working day.

8.2 Saponification

Weigh, to the nearest 0,1 g, approximately 50 g of the prepared sample (see clause 7) into a 1 litre conical flask.

Add to the test portion 200 ml of ethanol (4.3) whilst swirling the flask to disperse the sample. Add 2 ml of sodium ascorbate solution (4.9), mix by swirling and then add 50 ml of potassium hydroxide solution (4.2) and swirl again.

Fit a reflux condenser to the flask and immerse the flask in the boiling water bath (5.2).

Allow the contents of the flask to reflux for 30 min, swirling occasionally.

NOTE In exceptional cases some products may require a longer saponification time.

Cool the flask to room temperature under a stream of cold water.

Transfer the contents of the flask into the extraction cylinder (see 5.4).

8.3 Extraction of vitamin E

Rinse the saponification flask with two 25 ml portions of ethanol (4.3) and transfer the rinsings to the cylinder.

Repeat the rinsing of the flask with two 125 ml portions of light petroleum (4.5) and one 250 ml portion of water (4.1), each time transferring the rinsings to the cylinder.

Stopper the cylinder and shake well for 1 min, releasing the pressure from time to time.

Cool the cylinder under a stream of cold water while waiting for the two liquid phases to separate, before removing the stopper.

When the layers have separated, remove the stopper, wash the sides of the stopper with a few millilitres of light petroleum (4.5) and insert the adjustable tube (see 5.4), positioning the lower open end so that it is just above the level of the interface.

By application of a slight pressure of inert gas (4.10) to the side arm tube, transfer the upper, light petroleum layer to a 1 litre separating funnel (see 5.4).

Add 125 ml of light petroleum (4.5) to the cylinder, stopper and shake well for 1 min.

Allow the layers to separate and transfer the upper layer to the separating funnel using the adjustable tube (see 5.4) as before.

Again, add 125 ml of light petroleum (4.5) to the cylinder, stopper and shake well for 1 min.

Again, allow the layers to separate and transfer the upper layer to the separating funnel using the adjustable tube as before.

Wash the combined light petroleum extracts with four 100 ml portions of water using at first only gentle inversion then only gentle shaking in order to keep emulsion formation to a minimum.

Transfer the washed extract through a medium/fast filter paper containing 30 g of anhydrous sodium sulfate (4.8) into a 1 litre flask suitable for vacuum evaporation (5.3).

Rinse the separating funnel with two 20 ml portions of light petroleum (4.5) and add the rinsings through the filter to the evaporation flask.

Wash the filter further with two 25 ml portions of light petroleum (4.5) and collect the washings in the evaporation flask.

Evaporate the light petroleum extract to dryness under vacuum at a temperature not exceeding 40 °C.

Care should be taken to ensure that the flask is removed from the rotary evaporator immediately after reaching the point of dryness; prolonged drying may lead to loss of vitamin E from the extract residue.

If the vitamin E concentration of the light petroleum extract is sufficiently high, the extract may be made up to a fixed volume with light petroleum and an aliquot part taken for the rotary evaporation stage.

Restore atmospheric pressure by admitting inert gas (4.10).

8.4 Determination

8.4.1 Dissolve the residue from 8.3 in a minimum volume of hexane (4.4) and transfer quantitatively to a 25 ml volumetric flask.

Rinse the evaporation flask with three small portions of hexane (4.4), transferring the rinsings to the volumetric flask. Dilute to volume with hexane and mix.

If necessary, filter the sample extract through a membrane filter (5.5) or centrifuge.

8.4.2 Inject 20 µl of the sample extract onto the column of the liquid chromatograph (5.1) and measure the area of the DL-α-tocopherol peak. The following HPLC conditions are offered for guidance; other conditions may be used provided that they give equivalent results:

— liquid chromatographic column (5.1.3): 250 mm × 4,6 mm, silica 5 µm or 10 µm packing, or equivalent;

— mobile phase (4.11): mixture of hexane (4.4) and 1,4-dioxan (4.7), 970:30 (by volume);

— flow rate: 1,5 ml/min;

— detector (5.1.4): fluorescence detector (excitation 295 nm, emission 330 nm).

Reverse-phase chromatography may also be used provided that the efficiency of the column is sufficient to allow the separation of DL-α-tocopherol from other tocopherols and sample co-extractives. If reverse-phase chromatography is used, sample and standard solutions should be made up in an appropriate solvent, for example methanol (4.13).

8.4.3 Calculate the mean peak area from replicate injections of the sample extract and determine the DL-α-tocopherol concentration in micrograms per millilitre of the extract, either

a) by reference to the mean peak areas obtained from replicate injections of DL-α-tocopherol standard solution of concentration within 5 % of the concentration in the sample extract, or

b) by reference to a calibration curve prepared as in 8.5.

8.5 Calibration

8.5.1 Preparation of DL-α-tocopherol standard solutions

8.5.1.1 DL-α-tocopherol stock standard solution

Dissolve approximately 100 mg, weighed to the nearest 0,1 mg, of DL-α-tocopherol (4.6) in 100 ml of hexane (4.4). The stock standard solution is stable for 1 week when stored at $\leqslant$ 4 °C in an airtight amber glass flask.

8.5.1.2 DL-α-tocopherol working standard solution: single-point calibration

Prepare a working standard by diluting the stock standard (8.5.1.1) with hexane (4.4) to give a concentration approximately equal to that expected in the sample extract. Alternatively, proceed as in 8.5.1.3.

8.5.1.3 DL-α-tocopherol working standard solution: multi-point calibration

Prepare a range of calibration working standards containing 2 µg/ml, 4 µg/ml, 6 µg/ml, 8 µg/ml and 10 µg/ml of DL-α-tocopherol by diluting the stock standard solution (8.5.1.1) with hexane (4.4).

Prepare working standards daily.

8.5.2 UV check of DL-α-tocopherol standard substance

Weigh, to the nearest 0,1 mg, 100 mg of DL-α-tocopherol (4.6) in a 100 ml volumetric flask. Dissolve in ethanol (4.12). Dilute to the mark with the same solvent and mix.

Dilute 2,0 ml of this solution to 25,0 ml with ethanol (4.12) and measure the UV spectrum of the resulting solution against ethanol (4.12) in the spectrometer (5.7) at wavelengths of between 250 nm and 320 nm. The absorption maximum should be at 292 nm:

$$E^{1\%}_{1\,cm} = 75{,}8 \text{ at 292 nm in ethanol.}$$

At this dilution an extinction value of 0,6 should be obtained.

9 Expression of results

Calculate the vitamin E content of the test sample by the equation:

$$w_E = \frac{25 \times c \times 1{,}1}{m}$$

where

w_E is the numerical value of the vitamin E content of the test sample, in International Units per kilogram;

c is the numerical value of the DL-α-tocopherol concentration of the extract, in micrograms per millilitre;

m is the numerical value of the mass of the test sample, in grams;

1,1 is the correction factor for DL-α-tocopherol acetate.

10 Precision

10.1 Interlaboratory test

Details of a laboratory test on the precision of the method are summarized in annex A. The values derived from these tests may not be applicable to concentration ranges and matrices other than those given.

10.2 Repeatability

The absolute difference between two independent single test results, obtained using the same method on identical test material in the same laboratory by the same operator using the same equipment within a short interval of time, will in not more than 5 % of cases exceed the repeatability limit (r) mentioned in or derived from Table 1.

10.3 Reproducibility

The absolute difference between two single test results, obtained using the same method on identical test material in different laboratories by different operators using different equipment, will in not more than 5 % of cases exceed the reproducibility limit (R) mentioned in or derived from Table 1.

Table 1 — Repeatability limit (r) and reproducibility limit (R)

Sample	Vitamin E content IU/kg	r IU/kg	R IU/kg
Cattle ration	23,1	1,36	3,51
Poultry ration	29,7	2,07	10,02
Pig ration	64,9	4,06	17,44
Animal feed A [a] (Ration 722)	78,0	4,94	18,31
Animal feed B [a] (Ration 748)	140,6	15,73	43,91
Semi-moist pet food	20,6	0,98	7,46
Canned pet food	180,4	15,23	29,70
Dry pet food	78,7	4,22	16,97

[a] Calculated on dry matter basis.

11 Test report

The test report shall specify:

— all information necessary for the complete identification of the sample;

— the sampling method used, if known;

— the test method used, with reference to this International Standard;

— all operating details not specified in this International Standard, or regarded as optional, together with details of any incidents which may have influenced the test result(s);

— the test results obtained, or the two test results obtained if the repeatability has been checked.

Annex A
(informative)

Results of interlaboratory tests

The precision of the method was established by interlaboratory tests carried out in accordance with ISO 5725 [2][1)]. The results of these tests have been published (see reference [5]). In the tests 10 to 12 laboratories participated and samples of pet foods, cattle, poultry and pig feeds were investigated.

Table A.1 — Statistical results of an interlaboratory test for determination of vitamin E in feeding stuffs

Parameter	Sample[a]				
	1	2	3	4[b]	5[b]
Number of laboratories	10	10	10	12	12
Number of individual results	20	20	20	24	24
Number of accepted results	14	16	16	18	18
Mean vitamin E content, IU/kg	23,11	29,66	64,94	78,03	140,63
Repeatability standard deviation (s_r), IU/kg	0,44	0,69	1,35	1,66	5,27
Repeatability coefficient of variation, %	1,92	2,32	2,07	2,12	3,75
Repeatability limit (r) [$r = 2{,}8 \times s_r$], IU/kg	1,36	2,07	4,06	4,94	15,73
Reproducibility standard deviation (s_R), IU/kg	1,15	3,33	5,79	6,13	14,72
Reproducibility coefficient of variation, %	4,97	11,21	8,91	7,86	10,46
Reproducibility limit (R) [$R = 2{,}8 \times s_R$], IU/kg	3,51	10,02	17,44	18,31	43,91

a 1: cattle ration
2: poultry ration
3: pig ration
4: animal feed A (ration 722)
5: animal feed B (ration 748)

b Calculated on dry matter basis.

1) ISO 5725:1986 (now withdrawn) was used to obtain the precision data.

Table A.2 — Statistical results of an interlaboratory test for the determination of vitamin E in pet foods

Parameter	Sample[a]		
	6	7	8
Number of laboratories	11	11	11
Number of individual results	22	22	22
Number of accepted results	14	14	14
Mean vitamin E content, IU/kg	20,55	180,39	78,66
Repeatability standard deviation (s_r), IU/kg	0,32	4,98	1,38
Repeatability coefficient of variation, %	1,56	2,76	1,76
Repeatability limit (r) [$r = 2{,}8 \times s_r$], IU/kg	0,98	15,23	4,22
Reproducibility standard deviation (s_R), IU/kg	2,44	9,72	5,55
Reproducibility coefficient of variation, %	11,89	5,39	7,06
Reproducibility limit (R) [$R = 2{,}8 \times s_R$], IU/kg	7,46	29,70	16,97

[a] 6: canned cat food
7: semi-moist dog food
8: dry dog food

Bibliography

[1] ISO 6497, *Animal feeding stuffs — Sampling*.

[2] ISO 5725:1986, *Precision of test methods — Determination of repeatability and reproducibility for a standard test method by inter-laboratory tests*.

[3] ISO 5725-1:1994, *Accuracy (trueness and precision) of measurement methods and results — Part 1: General principles and definitions*.

[4] ISO 5725-2:1994, *Accuracy (trueness and precision) of measurement methods and results — Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method*.

[5] Analytical Methods Committee, *Analyst*, **116**, 1991, pp. 421-430.

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