SLOVENSKI STANDARD
SIST EN 17547:2022

01-januar-2022

Krma: metode vzorčenja in analize - Določevanje vitaminov A, E in D - Metoda z ekstrakcijo na trdno fazo (SPE) in tekočinsko kromatografijo visoke ločljivosti (HPLC)

Animal feeding stuffs: Methods of sampling and analysis - Determination of vitamin A, E and D content - Method using solid phase extraction (SPE) clean-up and high-performance liquid chromatography (HPLC)

Futtermittel - Probenahme- und Untersuchungsverfahren - Bestimmung des Gehalts an Vitamin A, E und D - Verfahren mittels Reinigung durch Festphasenextraktion und Hochleistungs-Flüssigchromatographie

Aliments des animaux - Méthodes d'échantillonnage et d'analyse - Détermination de la teneur en vitamines A, E et D - Méthode utilisant la purification par extraction en phase solide (SPE) et la chromatographie liquide à haute performance (CLHP)

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EUROPEAN COMMITTEE FOR STANDARDIZATION
COMITÉ EUROPÉEN DE NORMALISATION
EUROPÄISCHES KOMITEE FÜR NORMUNG

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European foreword

This document (EN 17547:2021) has been prepared by Technical Committee CEN/TC 327 “Animal feeding stuffs - Methods of sampling and analysis”, the secretariat of which is held by NEN.

This European Standard shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by May 2022, and conflicting national standards shall be withdrawn at the latest by May 2022.

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

This document has been prepared under a Standardization Request given to CEN by the European Commission and the European Free Trade Association.

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Introduction

WARNING — The method described in this document implies the use of reagents that pose a hazard to health. The standard does not claim to address all associated safety problems. It is the responsibility of the user of this document to take appropriate measures for the health and safety protection of the personnel prior to use of the standard and to ensure that regulatory and legal requirements are complied with.

1 Scope

This document specifies a method for the determination of the content of the total vitamin A (retinol), vitamin E (α-tocopherol) and vitamin D_3 (cholecalciferol) in animal feed using solid phase extraction (SPE) clean-up and high-performance liquid chromatography (HPLC).

NOTE The procedure also enables determination of vitamin D_2 but with the use of another internal standard. The method is fully validated only for vitamin D_3.

The method has been successfully tested in collaborative trial for complete feed for broilers, pigs, and turkey, for premixture for broilers and piglets, for complementary feed for cows and mineral feed within the following ranges:

- vitamin A: 4 365 IU/kg – 4 118 352 IU/kg;
- vitamin E: 22 mg/kg – 13 800 mg/kg;
- vitamin D_3: 1 668 IU/kg – 1 638 150 IU/kg.

The limits of quantification were not determined within the validation study. Quantification limits of 1 100 IU for vitamin A/kg (using UV-detection), 4 mg for vitamin E/kg (using UV-detection), 2 mg for vitamin E/kg (using fluorescence detection) and 2 000 IU for vitamin D/kg (using UV-detection) should be normally achieved. Lower limits are possible provided they are validated by the user.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

EN ISO 3696:1995, *Water for analytical laboratory use - Specification and test methods (ISO 3696:1987)*

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

- IEC Electropedia: available at https://www.electropedia.org/
- ISO Online browsing platform: available at https://www.iso.org/obp

3.1
vitamin A content
retinol
content of all-trans- and cis-isomers of retinol determined in accordance with this document

Note 1 to entry: The vitamin A (retinol) content is expressed in International Units per kilogram (IU/kg).

Note 2 to entry: 1 IU of vitamin A (retinol) is equal to 0,300 µg of all-trans-retinol or 0,344 µg all-trans-retinol acetate or 0,546 µg all-trans-retinol palmitate or 0,359 µg all-trans-retinol propionate.

3.2
vitamin E content
α-tocopherol
content of α-tocopherol determined in accordance with this document

Note 1 to entry: The content of vitamin E (α-tocopherol) can be also expressed as mg α-tocopherol acetate per kg.

Note 2 to entry: 1 mg vitamin E (α-tocopherol acetate) corresponds to 0,91 mg vitamin E (α-tocopherol).

Note 3 to entry: In samples can also be present β-, γ-, δ-tocopherol and α-, β-, γ-, δ-tocotrienol. This method uses reverse phase separation which does not separate individual forms of tocopherol. Therefore, the content of vitamin E expressed as α-tocopherol or α-tocopherol acetate includes all forms without taking into account differences in vitamin activities and the respective proportions of each form. Using a normal phase-column the separation of α-, β-, γ- and δ-tocopherol is possible (see observation 11.6).

3.3
vitamin D_3 content
cholecalciferol
the content of cholecalciferol determined in accordance with this document

Note 1 to entry: The content of vitamin D_3 is expressed in International Units per kg (IU/kg). 1 IU corresponds to an activity of 0,025 µg vitamin D_3 (cholecalciferol).

Note 2 to entry: For feeding stuffs, only vitamin D_3 is authorized as feed additive pursuant to Regulation (EC) No 1831/2003 [1]. Addition of vitamin D_2 is not allowed. Therefore, the vitamin D_2 can be used as internal standard.

Note 3 to entry: For accurate calculation of the results it is important that the sample does not contain any other vitamin D_2 than that added as internal standard.

4 Principle

The sample is saponified with ethanolic potassium hydroxide solution. In case that vitamin D_3 (cholecalciferol) is to be determined the internal standard is added before saponification. The vitamins are extracted and purified by SPE column eluting with cyclohexane. The cyclohexane is removed by evaporation and the residue is dissolved in methanol (for determination of vitamin A (retinol) and vitamin E (α-tocopherol)) or in *n*-hexane (for determination of vitamin D_3 (cholecalciferol)).

The vitamin A (retinol) and vitamin E (α-tocopherol) concentrations in the methanolic extract are determined by reversed-phase liquid chromatography using external calibration and HPLC conditions that give a single peak for all retinol isomers as well as for all tocopherols.

The *n*-hexane extract for vitamin D_3 determination is purified by semi-preparative normal-phase HPLC on silica gel. The purified extract is separated by reversed phase HPLC using conditions that give a baseline separation between the vitamin D_2 and vitamin D_3. Quantification of vitamin D_3 is performed by external standard calibration taking into account the recovery of the internal standard.

NOTE Figure 1 contains a flowchart for the determination of vitamins A, D and E.

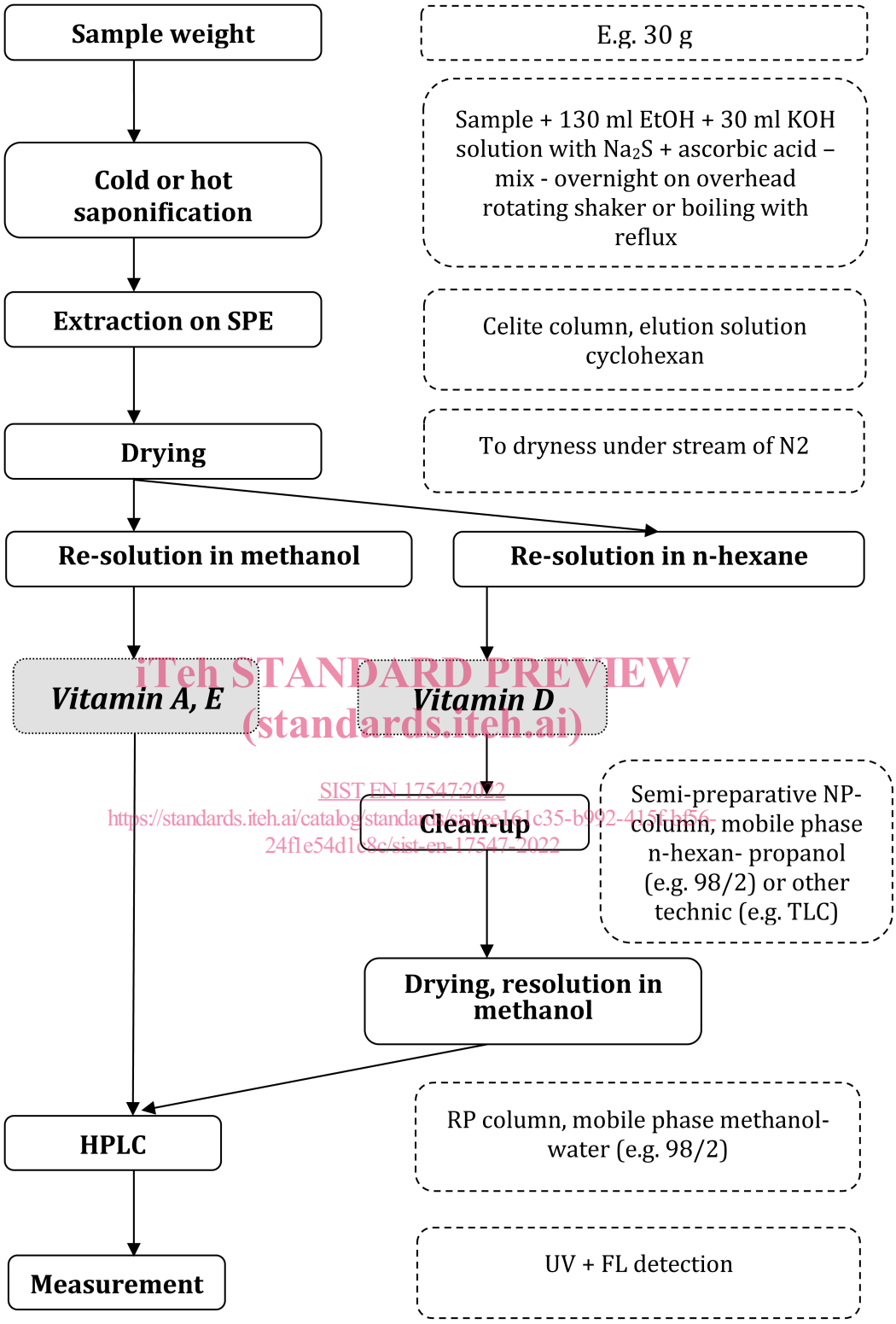

Figure 1 — Flowchart for the determination of vitamins A, D and E

5 Reagents and materials

Use only reagents of recognized analytical grade.

5.1 Water, complying with at least grade 3 in accordance with EN ISO 3696:1995.

5.2 Potassium hydroxide (KOH), w ≈ 850 g/kg.

5.3 Ethanol (C_2H_5OH), w = 950 ml/l, or equivalent industrial methylated spirit (ethanol denatured by methanol or hexane).

5.4 Ascorbic acid ($C_6H_8O_6$).

5.5 Ascorbic acid, solution, ρ = 200 g/l.

5.6 Sodium sulfide ($Na_2S \cdot 9\ H_2O$).

5.7 Sodium sulfide, alkali solution (see 11.1 observations).

Dissolve 2 000 g of potassium hydroxide (5.2) in 1 200 ml of water (5.1) until as much as possible of KOH is dissolved. In parallel dissolve 224 g of sodium sulfide (5.6) in 800 ml of water (5.1) in ultrasonic bath. Mix both solutions together and stir the mixture until the potassium hydroxide (5.2) is dissolved completely.

5.8 2,6-Di-tert-butyl-4-methylphenol (BHT), (see 11.2 observations).

5.9 Inert gas, e.g. nitrogen.

5.10 Methanol (CH_3OH), HPLC grade.

5.11 Ethanol (CH_3CH_2OH), HPLC grade.

5.12 Cyclohexane (C_6H_{12}), HPLC grade.

5.13 2-Propanol (C_3H_7OH), HPLC grade.

5.14 *n*-Hexane (C_6H_{14}), HPLC grade.

5.15 Mobile phase for semi-preparative HPLC-clean up of vitamin D_3.

Mixture of *n*-hexane (5.14) and propanol (5.13) in the proportions e.g. 980 + 20 (by volume). The ratio of the mixture must be adapted to the HPLC-column employed. If necessary, filter through a membrane filter (6.8).

5.16 Mobile phase for analytical HPLC.

Mix together methanol (5.10) and water (5.1) in the proportions 980 + 20 (by volume). The exact ratio will be determined by the characteristics of the column employed. The use of other mobile phase composition is allowed provided the separation of vitamins according the scope of this document is possible. If necessary, filter through a membrane filter (6.8).

5.17 Vitamin A standard substances.

5.17.1 All-*trans*-retinol acetate ($C_{22}H_{32}O_2$), CAS = 127-47-9, MW = 328,49 g/mol, extra pure, of certified activity, e.g. 2,80 × 10^6 IU/g.

5.17.2 All-*trans*-retinol palmitate ($C_{36}H_{60}O_2$), CAS = 79-81-2, MW = 524,86 g/mol, extra pure, of certified activity, e.g. 1,80 × 10^6 IU/g.

5.18 Vitamin E standard substance.

5.18.1 DL-α-tocopherol ($C_{29}H_{50}O_2$), CAS = 10191-41-0, MW = 430,72 g/mol, extra pure, of certified purity.

5.19 Vitamin D standard substances.

5.19.1 Vitamin D_2 (ergocalciferol; $C_{28}H_{44}O$), CAS = 50-14-6, MW = 384,62 g/mol, extra pure, of certified activity, e.g. 40 × 10^6 IU/g.

5.19.2 Vitamin D_3 (cholecalciferol; $C_{27}H_{44}O$), CAS = 67-97-0; MW = 384,62 g/mol, extra pure, of certified activity, e.g. 40 × 10^6 IU/g.

5.20 Celite for SPE column

Base material coarse-grained kieselguhr (also known as diatomaceous earth, hydromatrix, celite); particle size: max. 10 % < 100 µm, max. 90 % < 500 µm, max. 5 % > 800 µm; large pore size, high pore volume, constantly high batch-to-batch quality.

6 Apparatus

Usual laboratory equipment and, in particular, the following:

6.1 Boiling water bath with magnetic stirrer or electrical heating device with stirring (for hot saponification).

6.2 Overhead rotating shaker (for cold saponification).

6.3 Amber glassware (see observation 11.3).

6.3.1 Flat bottom - or conical flasks, 250 ml and 500 ml, with ground-glass socket.

6.3.2 Allihn condenser, jacket length 300 mm, with ground-glass joint, with adapter for gas feed pipe.

6.3.3 Graduated flasks with ground-glass stoppers, narrow-necked, 20 ml, 25 ml, 50 ml and 100 ml.

6.3.4 Pear shaped flask with ground-glass stoppers, 100 ml.

6.4 Vials, suitable for sample concentrator.

6.5 Column for SPE, filled with celite (e.g. Chromabond XTR[1], 70 ml volume) which is able to adsorb the water phase from the saponification solution (9.4.2) and release the vitamins A, E and D completely

[1] Chromabond XTR is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by CEN of this product.

by elution with organic solvents. The column shall have a capacity of not less than 20 ml aqueous solution and possibly closed by a valve at the outlet.

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

6.7 Sample nitrogen concentrator, heated to 50 °C.

6.8 Membrane filter, compatible with methanol, 0,45 µm pore size; e.g. Chromafil PET[2] - 45/15 MS or suitable filter with smaller pore size.

6.9 Syringe filter, with a Nylon or PVDF membrane, 0,2 µm (or 0,45 µm) pore size or equivalent, i.e. fully chemical compatibility with methanol and adaptable on 2-5ml syringes .

6.10 HPLC system semi-preparative, for the clean-up of vitamin D, consisting of:

6.10.1 HPLC pump, set to deliver a constant eluent volume flow rate of e.g. 2,5 ml/min.

6.10.2 HPLC injection device, injection volume of 500 µl.

6.10.3 HPLC semi-preparative normal phase column with guard column (see 9.7.2).

6.10.4 Column oven, set to provide a constant column temperature.

6.10.5 UV-Detector

6.11 HPLC-system for analytical separation, consisting of the following:

6.11.1 HPLC-pump, set to deliver a constant eluent volume flow rate of e.g. 1 ml/min.

6.11.2 HPLC injection devices, injection volume of 20 µl and 100 µl.

6.11.3 HPLC reversed-phase column, with guard column (see 9.8.1).

6.11.4 Column oven, set to provide a constant column temperature.

6.11.5 Detectors for UV- and fluorescent detection.

6.11.6 Integrator / data handling system.

6.12 UV (or UV-Visible) spectrophotometer, capable of measuring absorbance at the wavelengths defined in 9.2.1.4, 9.2.2.4 and 9.2.3.5, equipped with cells of 10 mm path length.

7 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 document. A recommended sampling method is given in EN ISO 6497 [2].

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

[2] Chromafil PET is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by CEN of this product.

8 Sample preparation

Samples are grinded at the day of analysis as recommended in the guidelines for sample preparation as in EN ISO 6498.

Grind a representative portion of the dry laboratory sample so that it passes through a sieve with 1 mm apertures. Prevent to heat up.

Grinding of sample(s) with adequate particle size distribution (e.g. premixtures and concentrates) may not be necessary if homogeneity is ensured.

Semi-moist pet foods (canned pet foods) can be homogenized by mincing.

Samples can be ground before the day of analysis. In this case the storage conditions must prevent any degradation, e.g. freeze the ground sample and defrost it in a fridge a night before analysis.

9 Procedure

9.1 General

Because of the sensitivity of vitamin A, E and D 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 (6.3) where possible (see observation 11.3).

9.2 Preparation and standardization of standard solutions

9.2.1 Vitamin A (retinol)

9.2.1.1 General

For preparation of vitamin A (retinol) standard solutions use all-trans-retinol acetate (5.17.1) or all-trans-retinol palmitate (5.17.2).

NOTE Standard substance of retinol itself is less stable then retinol palmitate or retinol acetate and therefor it is usual to use these esters for preparation of standard solution of vitamin A. Nevertheless, use of standard substance retinol is also possible.

9.2.1.2 Stock standard solution of vitamin A (retinol)

Weigh to the nearest 0,1 mg an amount of vitamin A (retinol acetate) (5.17.1) or vitamin A (retinol palmitate) (5.17.2) containing approximately 100 000 IU of vitamin A (retinol) into a 250 ml flat bottom or conical flask (6.3.1) and continue with saponification according to 9.4.2.1 or 9.4.2.2 and extraction according to 9.5.

Collect the eluate from the SPE column (6.5) in a 100 ml graduated flask (6.3.3) and fill up to the mark with cyclohexane (5.12).

The nominal concentration of stock standard solution of vitamin A (retinol) in cyclohexane is approximately 75 IU per ml.

The exact content has to be calculated from exact concentration of working standard solution of vitamin A (retinol) (9.2.1.3) determined according to 9.2.1.4.

The stock standard solution of vitamin A (retinol) is stable for 6 months in dark at 4°C and can be used for preparation of working standard solution according to 9.2.1.2 during this period.

9.2.1.3 Working standard solution of retinol

Pipette 10,0 ml of the vitamin A (retinol) stock standard solution (9.2.1.2) into a 100 ml graduated flask and fill up to the mark with cyclohexane (5.12).