SLOVENSKI STANDARD
oSIST prEN 17547:2020

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

Animal feeding stuffs: Methods of sampling and analysis - Determination of vitamin A, E and D content - Method using solid phase extraction clean-up and High Performance Liquid Chromatography

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 et la chromatographie liquide à haute performance

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65.120	Krmila	Animal feeding stuffs

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

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European foreword

This document (prEN 17547:2020) 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 document is currently submitted to the CEN Enquiry.

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

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) content in animal feed using solid phase extraction (SPE) clean-up and high-performance liquid chromatography (HPLC).

NOTE 1 The procedure enables also 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.

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.

NOTE 2 The limits of quantification are minimum limits which were not determined within the validation study. Lower limits of quantification are possible but is 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)*

EN ISO 6498, *Animal feeding stuffs - Guidelines for sample preparation (ISO 6498)*

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:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at http://www.electropedia.org/

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

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 (α-tocopherol) content
content of α-tocopherol determined in accordance to this document

Note 1 to entry: The content of vitamin E (α-tocopherol) could 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 (cholecalciferol) content
the content of cholecalciferol determined in accordance with this standard

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.

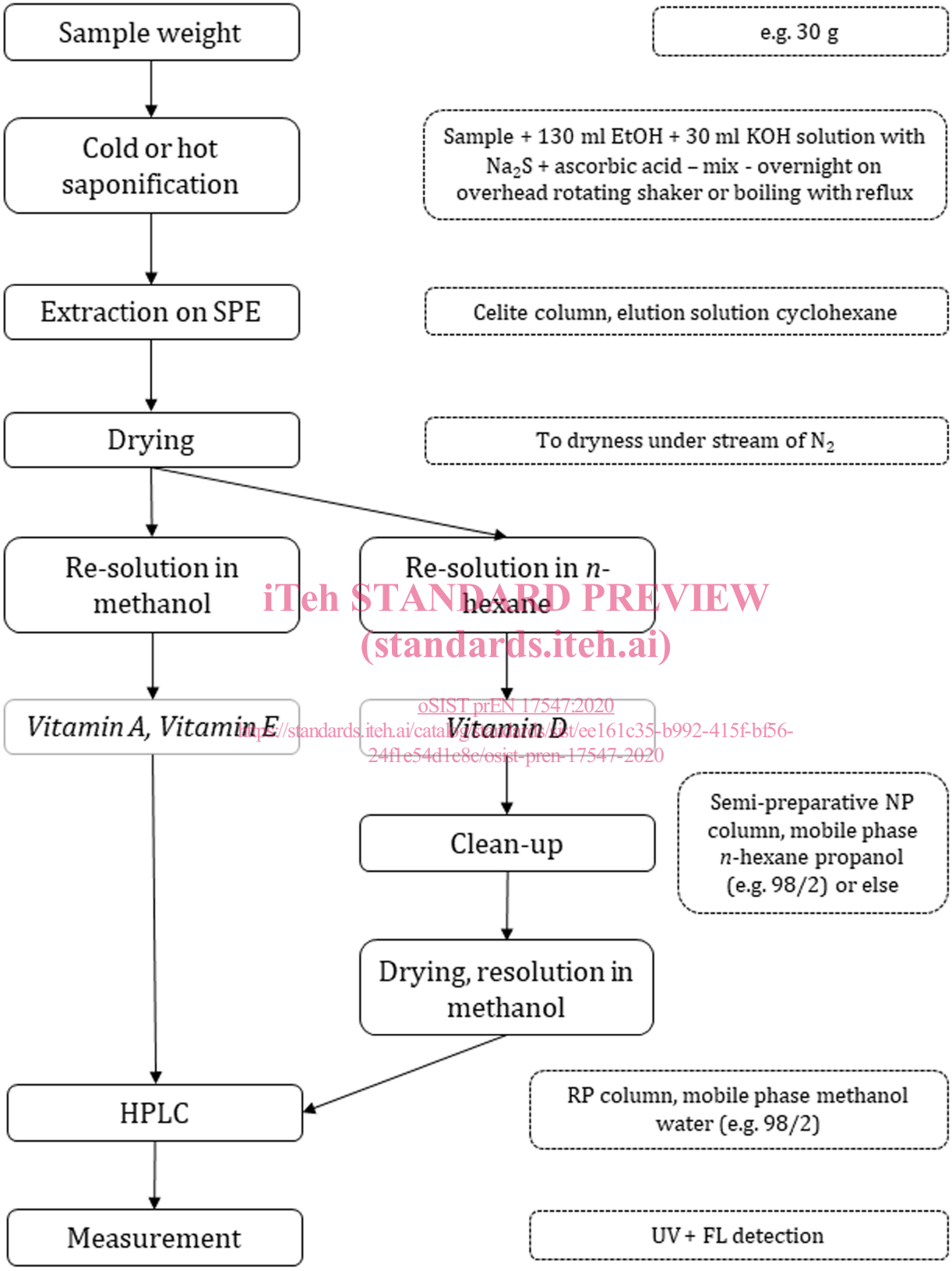

Figure 1 — Flowchart for the determination of vitamins A, D and E

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 ≅ 85 %.

5.3 Ethanol (C_2H_5OH), w = 95 % (by volume), 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 sulphide ($Na_2S \times 9\ H_2O$).

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

Dissolve 2 000 g of potassium hydroxide (5.2) in 1 200 ml of water (5.1). Dissolve 224 g of sodium sulphide (5.6) in 800 ml of water (5.1) in ultrasonic bath. Mix both solutions together.

NOTE Dissolution of KOH is a slow procedure. It is necessary to mix the solution until as much as possible of KOH is dissolved. After addition of sodium sulphide solution, the residuum of KOH is dissolved.

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. 98 + 2 (by volume). The ratio of the mixture shall 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 standard 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. $2{,}80 \times 10^6$ 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. $1{,}80 \times 10^6$ 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; $C_{27}H_{44}O$), 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 Overturning rotating stirrer (for cold saponification).

6.3 Amber glassware (see observation 11.3).

6.3.1 Flat bottom - or conical flasks, 250 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, 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 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 - 45/15 MS or suitable filter with smaller pore size.

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

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

6.9.2 HPLC injection device, injection volume of 500 µl.

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

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

6.9.5 UV-Detector

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

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

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

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

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

6.10.5 Detectors for UV- and fluorescent detection.

6.10.6 Integrator / data handling system.

6.11 UV (or UV-Visible) spectrophotometer, capable of measuring absorbance at the wavelengths defined in 9.2.1.3, 9.2.2.3 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 European Standard. 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.

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

Do not grind the sample(s) if the particle size distribution is adequate (e.g. premixtures and concentrates).

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 shall 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).