



Institut luxembourgeois de la normalisation
de l'accréditation, de la sécurité et qualité
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ILNAS-EN 17547:2021

Animal feeding stuffs: Methods of sampling and analysis - Determination of vitamin A, E and D content - Method using solid phase extraction (SPE)

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

Aliments des animaux - Méthodes
d'échantillonnage et d'analyse -
Détermination de la teneur en vitamines
A, E et D - Méthode utilisant la

11/2021



National Foreword

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English Version

**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)**

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)

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

This European Standard was approved by CEN on 27 September 2021.

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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of an identical text or by endorsement, at the latest by May 2022, and conflicting national standards shall be withdrawn at the latest by May 2022.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.

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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 D₃ (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 D₂ but with the use of another internal standard. The method is fully validated only for vitamin D₃.

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 D₃: 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 μ g of all-trans-retinol or 0,344 μ g all-trans-retinol acetate or 0,546 μ g all-trans-retinol palmitate or 0,359 μ g 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 D₃ content

cholecalciferol

the content of cholecalciferol determined in accordance with this document

Note 1 to entry: The content of vitamin D₃ is expressed in International Units per kg (IU/kg). 1 IU corresponds to an activity of 0,025 μ g vitamin D₃ (cholecalciferol).

Note 2 to entry: For feeding stuffs, only vitamin D₃ is authorized as feed additive pursuant to Regulation (EC) No 1831/2003 [1]. Addition of vitamin D₂ is not allowed. Therefore, the vitamin D₂ 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 D₂ than that added as internal standard.

4 Principle

The sample is saponified with ethanolic potassium hydroxide solution. In case that vitamin D₃ (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 D₃ (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 D₃ 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 D₂ and vitamin D₃. Quantification of vitamin D₃ 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.