

AOAC TDLM Method Verification Workshop
Example 3: Determination of Vitamin D3 in Food



122nd AOAC
ANNUAL MEETING & EXPOSITION

Example 3

Determination of Cholecalciferol (Vitamin D₃) in Selected Foods by Liquid Chromatography

Applicable to the determination of vitamin D₃ (0.4–12 mg/100 g) in fortified milk, infant formula, gruel, margarine, cooking oil, and fish oil. Materials tested must not contain measurable levels of endogenous vitamin D₂.

A. Principle

After the addition of an internal standard (vitamin D₂) and basic hydrolysis, vitamin D₃ is extracted with *n*-heptane. The fraction that contains vitamin D₂/D₃ is separated by preparative normal-phase liquid chromatography (LC). After evaporation and dilution in acetonitrile–methanol, vitamin D₃ is determined by reversed-phase LC with UV detection at 265 nm. A separate test portion is analyzed in parallel to confirm the absence of endogenous vitamin D₂.

This method is primarily intended for the routine determination of vitamin D₃ in fortified foods, but it can also be used for the determination of natural vitamin D₃ in foods. Alternatively, vitamin D₂ can be determined with vitamin D₃ as the internal standard. In either case, it is necessary to confirm that the natural content of internal standard in the foods is below the detection limit by assay of the test sample without the addition of internal standard.

The cooking oil, infant formula, and fish oil were analyzed for endogenous vitamin before addition of vitamin D₃, and the endogenous concentration was found to be below the detection limit of 0.1 mg/100 g in all 3 matrixes.

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Activity:

The lab is verifying the method for the determination of Vitamin D in food. Use the ALACC Method Verification Guide and information from the AOAC collaborative study to plan how the lab should verify the method.

Verification Purpose

The lab is verifying the method for confirming the absence of endogenous vitamin D₂ in infant formula.

AOAC Collaborative Study Information

Results are presented from an NMKL (Nordic Committee on Food Analysis) collaborative study of a method for the determination of cholecalciferol (vitamin D₃) in foods. The method is based on the addition of an internal standard (vitamin D₂), followed by saponification and extraction with n-heptane. The fraction that contains vitamin D₂/D₃ is separated by preparative normal-phase liquid chromatography (LC), and the analytes are determined by reversed-phase LC with UV detection at 265 nm. The method was tested by 8 participating laboratories. In this study 6 different matrixes were analyzed for cholecalciferol content: milk, liquid infant formula (gruel), cooking oil, margarine, infant formula, and fish oil. The contents varied from 0.4 to 12 mg/100 g. Three matrixes (milk, gruel, and margarine) were fortified with vitamin D₃. In the other matrixes, vitamin D₃ was added at 3 different levels at the Swedish National Food Administration. The milk was analyzed as a blind duplicate, whereas the other matrixes were analyzed as split-level pairs. The recoveries from the samples with vitamin D₃ added varied from 93 to 102%. The repeatability relative standard deviation (RSD_r) values for accepted results varied between 2.2% (fish oil) and 7.4% (cooking oil), whereas the reproducibility relative standard deviation (RSD_R) values varied between 6.8% (margarine) and 24% (cooking oil).

The cooking oil, infant formula, and fish oil were analyzed for endogenous vitamin before addition of vitamin D₃, and the endogenous concentration was found to be below the detection limit of 0.1 mg/100 g in all 3 matrixes.

[Applicable to the determination of vitamin D₃ (0.4–12 mg/100 g) in fortified milk, infant formula, gruel, margarine, cooking oil, and fish oil. Materials tested must not contain measurable levels of endogenous vitamin D₂.]

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A. Principle

After the addition of an internal standard (vitamin D2) and basic hydrolysis, vitamin D3 is extracted with n-heptane. The fraction that contains vitamin D2/D3 is separated by preparative normal-phase liquid chromatography (LC). After evaporation and dilution in acetonitrile–methanol, vitamin D3 is determined

by reversed-phase LC with UV detection at 265 nm. A separate test portion is analyzed in parallel to confirm the absence of endogenous vitamin D2.

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Table 2002.05. Interlaboratory study results^a for determination of vitamin D₃ in selected foods by liquid chromatography

Parameter	Milk	Gruel	Cooking oil	Margarine	Infant formula	Fish oil
Mean, µg/100 g	0.418	1.38	4.61	8.39	10.1	11.6
Recovery, %	—	—	102	—	93.9	92.9
s _p , µg/100 g	0.019	0.08	0.34	0.54	0.2	0.3
RSD _p , %	4.6	5.9	7.4	6.5	2.4	2.2
r, µg/100 g	0.054	0.23	0.96	1.52	0.7	0.7
s _R , µg/100 g	0.038	0.17	1.11	0.57	0.7	2.1
RSD _R , %	9.1	12.1	24.1	6.8	7.1	17.7
R, µg/100 g	0.106	0.47	3.11	1.60	2.0	5.8
N ^b	7(1)	8(0)	8(0)	7(1)	7(1)	8(0)

^a Outliers were excluded in the statistical analysis.

^b Each value is the number of laboratories retained after elimination of outliers; each value in parentheses is the number of laboratories removed as outliers.

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Answer

Method Verification Category

The method is Category 3. Determining if an analyte is present above or below a specified, low concentration (often called a Limit Test). The specified concentration is close to the LOQ.

Table 4. Category 3: Analyte is present above or below a specified, low concentration (Limit Test)

Performance Characteristic	Verification	Verification Activity	Reason for Verification
LOD	Yes	Run a sample close to LOD	LOD is very likely to be matrix and instrument specific
LOQ	Yes	Run a sample close to LOQ	LOQ is very likely to be matrix and instrument specific
Specificity	No/Yes	See Specificity in General Requirements	See Specificity in General Requirements

The following Performance Characteristics need verification:

LOD

LOQ

Possibly Specificity

Process

LOD and LOQ

The limit is 0.1 mg/100 g.

Verify by preparing and analyzing a solution at the LOD and LOQ levels. The results should meet the same requirements as for method validation, such as a specified %RSD, signal:noise ratio or rates of false positives/negatives and be acceptable for use.

Appendix D: Guidelines for Collaborative Study Procedures To Validate Characteristics of a Method of Analysis

This Guideline is found on the AOAC website at:

http://www.aoac.org/vmeth/Manual_Part_6.pdf

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3.4 Limit of Detection/Quantitation

If the limit of detection/quantitation is important, it is necessary to provide a design which gives special attention to the number of blanks, and to the necessity for interpreting false positives and false negatives. In all cases, the definition of limit of detection/quantitation used in the study must be given by the Study Director.

Eurachem Guide at
<http://www.eurachem.org/>

The Fitness for Purpose of Analytical Methods A Laboratory Guide to Method Validation and Related Topics

False Negatives / Positives:

For qualitative methods the false positives/negatives rate may be determined. Data from a confirmatory method comparison should be provided if such method(s) is applicable to the same matrix(es) and concentration range(s). In the absence of a method comparison, populations of negative and positive fortified samples must be analysed. False positives / negatives may be determined as follows:

False positive rate (%) = $\frac{\text{false positives}}{\text{total known negatives}} \times 100$

False negative rate (%) = $\frac{\text{false negatives}}{\text{total known positives}} \times 100$

[AOAC Research Institute - Performance tested Methods Programme, Procedure]

Specificity

The matrix is identical to that in the collaborative study, so no further assessment of specificity is needed.

The ALACC method verification guide states:

General Requirements—Specificity

For specificity in all categories of methods, if samples are identical to those for which the method is intended and validated, and the method is based on basic principles then no verification is needed. If the samples have the same matrix, the specificity which is based on basic principles, will not be impacted. Basic principles are chemical reactions, e.g. reaction of Ag with Cl to create a precipitate. For some methods, the specificity can be affected by the instrument used. In these cases the lab should assess if the instrument differences could affect the specificity, and if so, include specificity in the verification, e.g. the different resolution and/or detection systems in inductively coupled plasma optical emission spectrophotometers may result in different interferences.

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Continued Verification of Performance

The lab has a thorough QC program that sufficiently verifies precision and accuracy.

Conclusion

This study demonstrated that the LOD and LOQ are acceptable. The samples are the same as those in the collaborative study, such that there is no impact on specificity.

The method has been successfully verified.