BUREAU OF INDIAN STANDARDS

DRAFT FOR COMMENTS ONLY

भारतीय मानक मसौदा

फोर्टिफाइड वनस्पति तेलों में विटामिन डी का निर्धारण अति-उच्च प्रदर्शन तरल क्रोमैटोग्राफी द्वारा और टेंडेम मास स्पेक्ट्रोमेट्री द्वारा - परीक्षण की पद्धति

Draft Indian Standard

Determination of Vitamin D in Fortified Vegetables Oils by ultra-high performance liquid chromatography and tandem mass spectrometry (UHPLC-MS/MS) — Method of Test

(*First Revision of IS 5835*)

ICS No 67.050

Test Methods for Food Products, Sectional Committee, FAD 28	Last date of comments 22/02/2025

FOREWORD

Foreword shall be updated later

Vitamin D is a fat-soluble vitamin essential for overall health, primarily known for its role in calcium and phosphorus absorption, which is vital for maintaining strong bones and teeth. It also supports immune system function, reduces inflammation, and helps regulate cell growth. The body produces vitamin D when exposed to sunlight, but it can also be obtained through certain foods like fatty fish, fortified dairy and oils, and supplements.

This standard was first published in 1970 with a title "*Method for estimation of vitamin D in foodstuffs*" in which microbiological method was described for estimation of Vitamin D. The microbiological method is old and there is a lot of variability in this method so a need was felt to revise the standard. In this revision, the microbiological method has been replaced by the method based on AOAC Official Method 2016.05 "*Analysis of Vitamin D2 and Vitamin D3 in Fortified Milk Powders, Infant Formulas, and Adult/Pediatric Nutritional Formulas by Liquid Chromatography-Tandem Mass Spectrometry.*" The current test method is designed to determine concentration of Vitamin D forms in fortified edible oils. The prescribed fortification range of these Vitamins in India is 0.11 µg– 0.16 µg per gm of oil.

For the revision of the standard, a collaborative Research & Development (R&D) project with an objective to validate test methods of Vitamins in identified food matrices was assigned to CSIR - Central Food Technological Research Institute (CFTRI), Mysuru along with five participating laboratories. A multi-lab validation of the method described in AOAC 2016.05 has been performed by Envirocare Labs (Thane), Eureka Analytical Services Pvt Ltd (Bengaluru), Eurofins Analytical Services India Pvt. Ltd. (Bengaluru), NDDB-CALF (Anand) and CSIR-CFTRI for determination of Vitamin D in vegetables oils. The performance characteristics obtained for these matrices have been given in Annex A. The HORRATr values are within recommended value of less than 2.

In reporting the result of a test or analysis made in accordance with this standard, is to be rounded off, it shall be done in accordance with IS 2 : 2022 'Rules for rounding off numerical values (second revision)'.

1 SCOPE

This document specifies a method for the quantitative determination of Vitamin D forms in vegetables oils by ultra-high performance liquid chromatography–tandem mass spectrometry (UHPLC-MS/MS). This document is not intended to be used for products which are not fortified by Vitamin D. This method is applicable for edible oils which contain Vitamin D2 or Vitamin D3 in the range of 10 to 200 µg/Kg.

2 REFERENCES

The standards given below contain provisions which, through reference in this text, constitute provisions of this standard. At the time of publication, the editions indicated were valid. All standards are subject to revision and parties to agreements based on this standard are encouraged to investigate the possibility of applying the most recent editions of these standards:

IS No.	*Title*
IS 1070 : 2023	Reagent Grade Water Specification (*Fourth Revision*)

3 DEFINITIONS

3.1 Procedural Standards

Procedural standards are the standards prepared by spiking a series of blank test portions with different amounts of analyte, prior to extraction. The procedural standards are then analyzed in exactly the same way as the samples.

4 PRINCIPLE

The samples are saponified at high temperature; then lipid soluble components are extracted into isooctane. A portion of the isooctane layer is transferred and washed, and an aliquot of 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) is added to derivatize Vitamin D to form a high-molecular-mass, easily ionizable adduct. The Vitamin D adduct is then re-extracted into a small volume of acetonitrile and analyzed by Reversed Phase Liquid Chromatography. Detection is by MS using multiple reaction monitoring (MRM). Stable isotope-labeled (SIL) d6-Vitamin D2 and d6-Vitamin D3internal standards are used for quantitation to correct for losses in extraction and any variation in derivatization and ionization efficiencies.

5 REAGENTS AND MATERIALS

During the analysis, unless otherwise stated, use only reagents of recognized analytical grade anddistilled or demineralized water or water of equivalent purity.

5.1 Vitamin D2 (ergocalciferol), CAS Registry Number® (CAS RN®): 50-14-6; purity: ≥99%.

5.2 Vitamin D3 (cholecalciferol), CAS Registry Number® (CAS RN®): 67-97-0; purity: ≥99%.

5.3 d6-Vitamin D2, (26,26,26,27,27,27-d6 ergocalciferol), CAS Registry Number® (CAS RN®): 1311259-89-8; enrichment: ≥99%,; purity: ≥99%.

5.4 d6-Vitamin D3, (26,26,26,27,27,27-d6 cholecalciferol), CAS Registry Number® (CAS RN®): 118584-54-6; enrichment: ≥99%; purity: ≥99%.

5.5 PTAD (4-phenyl-1,2,4-triazoline-3,5-dione), Reagent grade (store in desiccator at 2–8°C).

5.6 Formic acid, LCMS grade.

5.7 Potassium hydroxide, Reagent grade.

5.8 Magnesium chloride anhydrous, Reagent grade.

5.9 Pyrogallol, Reagent grade.

5.10 Ethanol, LC grade.

5.11 Methanol, LCMS grade.

5.12 Isooctane (2,2,4-trimethylpentane), LC grade.

5.13 Acetone, LC grade.

5.14 Acetonitrile, LCMS grade.

5.15 Reagent Grade Water, grade 1 as per IS 1070.

NOTE — CAS Registry Number® is a trademark of CAS corporation. This information is given for the convenience of users of this document and does not constitute an endorsement by BIS of the product named. Equivalent products may be used if they can be shown to lead to the same results.

6 APPARATUS

6.1 Ultra-HPLC (UHPLC) system, LC system consisting of a dual pump system, a sample injector unit, a degasser unit, and a column oven.

6.2 Triple-quadrupole mass spectrometer, Triple Quad tandem mass spectrometer with positive Electrospray Ionization (ESI) mode operating at unit resolution, or equivalent. The mass spectrometer shall be capable of detecting both the forms of Vitamins, D2 and D3 at concentration of 10 µg/L. The signal noise ratio of these two solvent standards shall be more than 10.

6.3 Column, Reversed phase LC column with a core shell technology C18, 4.6 mm x 100 mm, 2.7 µ.

6.4 UV spectrophotometer, Digital readout to three decimal places.

6.5 Centrifuge tubes, Polypropylene, 15 mL.

6.6 Boiling tubes, Glass, 60 mL.

6.7 Water baths, Cold 20°C, hot 70°C.

6.8 Disposable syringes, 1 mL.

6.9 Syringe filters, PTFE, 0.2 µm, 13 mm.

6.10 Centrifuge, Suitable for 60 mL boiling tubes and 15 mL centrifuge tubes.

6.11 Pasteur pipet, Glass, ~140 mm.

6.12 Horizontal shaker

6.13 Centrifuge vials,2 mL.

6.14 Filter membranes, 0.45 μm nylon.

6.15 Cryogenic vials, 2 mL.

6.16 Solvent glass bottles, 1 L, 100 mL.

6.17 HPLC vials, **septa, and caps.**

7 PROCEDURE

7.1 Standard and Solution Preparation

7.1.1 *Mobile phases and prepared solutions*

7.1.1.1 *Mobile phase A (formic acid; 0.1%, v/v),* To 500 mL water, add 0.5 mL formic acid. Expiry: 1 week.

7.1.1.2 *Mobile phase B (methanol; 100%, v/v),* 500 mL methanol. Expiry: 1 month.

7.1.1.3 *Acetone (dry),* To a 100 mL Schott bottle, add 50 mL acetone, then add ~10 g magnesium chloride to remove traces of moisture. Cap the bottle and seal with parafilm and wait for the magnesium chloride to settle before use (~24 h). Expiry: 1 month.

7.1.1.4 *PTAD solution (10 mg/mL),* To a 5 mL volumetric flask, add 50 mg PTAD, then add 4 mL dry acetone, and dissolve; dilute to volume with acetone. Expiry: 1 day

7.1.1.5 *Potassium hydroxide solution (50%, w/v),* Dissolve 100 g potassium hydroxide in 200 mL water. Expiry of the solution is 1 month.

7.1.1.6 *Ethanolic pyrogallol solution (1%, w/v),* Dissolve 5 g pyrogallol in 500 mL ethanol. Expiry: 1 day

7.1.2 *Stable isotope labelled compounds, individual, internal standard stock solutions*

Vitamin D is sensitive to light so perform all steps under UV-shielded lighting. If Vitamin D3 is exclusively required for analysis, then standards pertaining to Vitamin D2 need not be used and vice versa.

7.1.2.1 *Stable isotope-labeled Vitamin D2 or Vitamin D3 stock standard (SILD2SS or SILD3SS; ~10 μg/mL),*

7.1.2.1.1 Dispense the contents of a 1 mg vial of d6-Vitamin D2 or a 1 mg vial of d6-Vitamin D3 into separate 100 mL volumetric flasks.

7.1.2.1.2 Dissolve in ~90 mL ethanol. To promote dissolution, sonicate if necessary. Mix thoroughly; dilute to volume with ethanol.

7.1.2.1.3 Measure the absorbance of an aliquot of SILD2SS or SILD3SS at 265 nm. The spectrophotometer should be zeroed against an ethanol blank solution. Calculate and record the concentration.

7.1.2.1.4 Immediately dispense aliquots of SILD2SS or SILD3SS (~1.3 mL) into cryogenic vials and freeze at ≤15°C.

7.1.3 *Stable isotope-labeled internal standard (SILIS; ~1 μg/mL)*

7.1.3.1 Prepare an adequate volume of SILIS for the daily sample numbers. For every 15 samples (or part thereof) in an analytical run, remove one vial of SILD2SS and one vial of SILD3SS from the freezer and

allow to warm to room temperature. This solution should be made fresh daily.

7.1.3.2 Pipet 1.0 mL each of SILD2SS and SILD3SS into the same 10 mL volumetric flask (use a separate 10 mL volumetric flask for each set of 15 samples). Dilute to volume with acetonitrile and mix thoroughly.

7.1.3.3 Pool all 10 mL volumetric flasks together and mix thoroughly.

7.1.4 *Non labeled Vitamin D2 or Vitamin D3 stock standard (NLD2SS or NLD3SS; ~1 mg/mL)*

7.1.4.1 Accurately weigh approximately 50 mg Vitamin D2 or Vitamin D3 into separate 50 mL volumetric flasks.

7.1.4.2 Dissolve in ~40 mL ethanol. To promote dissolution, sonicate if necessary. Mix thoroughly; dilute to volume with ethanol. Store in a freezer at ≤15°C for a maximum of 3 months.

7.1.5 *Non labeled Vitamin D2 or Vitamin D3 purity standard (NLD2PS or NLD3PS; ~10 µg/mL)*

7.1.5.1 Pipette 1.0 mL NLD2SS or NLD3SS into separate 100 mL volumetric flasks. Dilute to volume with ethanol. This solution should be made fresh daily.

7.1.5.2 Measure the absorbance of an aliquot of each solution at 265 nm. The spectrophotometer should be zeroed against an ethanol blank solution. Record the absorbance and calculate the concentration.

7.1.6 *Non labeled working standard (NLWS; ~1 µg/mL)*

Pipette 1.0 mL NLD2PS and 1.0 mL NLD3PS into a single 10 mL volumetric flask. Dilute to volume with acetonitrile. This solution should be made fresh daily.

7.2 Sample preparation and Derivatization

Vitamin D is sensitive to light, perform all steps under UV-shielded lighting.

7.2.1 Take 0.5 g non fortified edible oil sample and to it add 0.5 mL SILIS.

7.2.2 Add 10 mL ethanolic pyrogallol solution and vortex mixture.

7.2.3 Add 2 mL potassium hydroxide solution to the boiling tube; cap and vortex mix.

7.2.4 Place the boiling tube in a water bath at 70°C for 1 hr; vortex mixes every 15 min.

7.2.5 Place the boiling tube in a water bath at room temperature until cool.

7.2.6 Add 10 ml isooctane to the boiling tube; cap the boiling tube tightly and place on a horizontal shaker for 10 min.

7.2.7 Add 20 mL water to the boiling tube and invert the tube 10 times; place in a centrifuge at 2500 rpm for 15 min.

7.2.8 Transfer a 5 ml aliquot of the upper isooctane layer into a 15 mL centrifuge tube using a micro pipette, taking care not to transfer any of the lower layer.

7.2.9 Add 5 mL water to the centrifuge tube; cap and vortex mix; then place in a centrifuge at 12000 rpm for 5 min.

7.2.10 Transfer 4-5 ml upper isooctane layer to a new 15 mL disposable centrifuge tube using a micro pipette, taking care not to transfer any of the lower layer.

7.2.11 Add 75 µL PTAD solution to the centrifuge tube; cap and immediately vortex mix.

7.2.12 Allow to stand in the dark for 5 min to allow the derivatization reaction to complete.

7.2.13 Add 1 mL acetonitrile to the centrifuge tube; cap and vortex mix; then place in a centrifuge at 12000 rpm for 5 min.

7.2.14 Using a variable volume pipette, transfer 500 µL lower layer into 2 ml centrifuge tube, taking care not to transfer any of the upper layer.

7.2.15 Add 167 µL water into 2 ml centrifuge tube; cap and vortex mix.

7.2.16 Using a syringe filter, transfer an aliquot from the 2 ml centrifuge tube to an amber vial; then cap.

7.3 Procedural Calibration Curve

7.3.1 Procedural standards fortify or spike the non-fortified edible oil blank samples at following levels 10 µg/kg, 50 µg/kg, 75 µg/kg, 100 µg/kg, 150 µg/kg and 200 µg/kg**.**

(a) Procedural Calibration Standard 1 (10 µg/kg): Weigh 0.5 g of blank edible oil in a 25 ml of volumetric flask. To the same flask, add 50 µl NLWS from 0.1 µg/ml and 500 µl SILIS from 1 µg/ml.
(b) Procedural Calibration Standard 2 (50 µg/kg): Weigh 0.5 g of blank edible oil in a 25 ml of volumetric flask. To the same flask add 250 µl NLWS from 0.1 µg/ml and 500 µl SILIS from 1 µg/ml.
(c) Procedural Calibration Standard 3 (75 µg/kg): Weigh 0.5 g of blank edible oil in a 25 ml of volumetric flask. To the same flask add 37.5 µl NLWS from 1.0 µg/ml and 500 µl SILIS from 1 µg/ml.
(d) Procedural Calibration Standard 4 (100 µg/kg): Weigh 0.5 g of blank edible oil in a 25 ml of volumetric flask. To the same flask add 50 µl NLWS from 1.0 µg/ml and 500 µl SILIS from 1 µg/ml.
(e) Procedural Calibration Standard 5 (150 µg/kg): Weigh 0.5 g of blank edible oil in a 25 ml of volumetric flask. To the same flask add 75 µl NLWS from 1.0 µg/ml and 500 µl SILIS from 1 µg/ml.
(f) Procedural Calibration Standard 6 (200 µg/kg): Weigh 0.5 g of blank edible oil in a 25 ml of volumetric flask. To the same flask add 100 µl NLWS from 1.0 µg/ml and 500 µl SILIS from 1 µg/ml.

7.3.2 Each of this matrix-based calibration point is then processed as a 'sample' and steps **7.2.2** to **7.2.16** mentioned under the title sample preparation and derivatization are carried out. The resulting vials are named as 10 µg/kg, 50 µg/kg, 75 ug/kg 100 µg/kg, 150 µg/kg and 200 µg/kg procedural calibration points respectively.

7.4 UHPLC-MS/MS ANALYSIS

7.4.1 UHPLC conditions

7.4.1.1 *Injection volume,* 5 µl

7.4.1.2 *LC column temperature,* 40°C

7.4.1.3 *System suitability test*
Equilibrate the chromatographic system for at least 15 min at the initial conditions. Inject a working standard solution three to six times and check peak retention times and responses. Inject working standard solutions

on a regular basis within a series of analyses. The gradient program for the column is given in Table 1.

7.4.1.4 *Analysis*
Make single injections of standard and test solutions. Measure chromatographic peak response (height or area). Identify the separation of Vitamin D2 and Vitamin D3 forms.

7.4.1.5 *Identification*
Identify Vitamin D2 and D3 peak in the chromatograms of the test solution by comparison with the retention time and multiple reaction monitoring of the corresponding peak obtained for the standard solution.

7.4.1.6 *Calibration*
Plot peak responses against concentrations (in ng/mL). Perform regression analysis.

Table 1 Gradient program
(*Clause 7.4.1.3*)

Time	Flow (ml/min)	% A	%B
0.01	0.5	30	70
1	0.5	30	70
3	0.5	00	100
6.5	0.5	00	100
8.0	0.5	30	70
12.0	0.5	30	70

7.4.2 Mass transitions

7.4.1 Mass transitions for each Vitamin and its corresponding internal standard along with the retention time windows are given in the table 2.

7.4.2 MS /MS conditions
The MS/MS conditions are as follows:

a) Nebulising gas flow, 3 L/min.
b) Heating gas flow,10 L/min.
c) Interface temperature, 300°C.
d) Desolvation temperature, 526 °C.
e) Desolvation Line temperature, 250 °C.
f) Heat block temperature, 400 °C.
g) Drying gas flow, 10 L/min.

NOTE — The above mass spectrometer parameters are valid for a particular model of mass spectrometer and may need optimization based upon the mass spectrometer model.

Table 2 Conditions for MS transitions
(*Clause 7.4.2*)

Analyte	Retention time, min		Precursor ion, (m/z)	Product ion, (m/z)
VitaminD2	7.553	Analyte quantifier	572.50	298.20
				280.1

Vitamin D2-D3	7.525	Internal Standard quantifier	578.20	298.10
				280.20
Vitamin D3	7.664	Analyte quantifier	560.50	298.30
				280.10
Vitamin D3-D6	7.618	Internal Standard quantifier	566.60	298.20
				280.30

8 CALCULATIONS

8.1 While selecting the quantification method in commercial software of LC MS/MS vendors, select 'Internal standard' method.
Concentration of unknown is found out using the following formula

Vitamin D (µg/kg) = $\boldsymbol{x} = \frac{((\textbf{Area of Analyte x IS Concentration})/\textbf{IS Area}) - \pm \textbf{Intercept x DF}}{\textbf{Slope}}$

Where
DF is the Dilution factor of the method

8.2 In case of matrix-based calibration curve, dilution factor is selected as 1.

Annex A
(foreword)

PERFORMANCE CHARACTERISTICS OF MULTI-LABORATORY VALIDATION STUDY

Table 3 Performance Characteristics of the Vitamin D method used for fortified edible oils

	Sunflower oil, mg/kg	**Sunflower and Rice bran oil (20:80) mg/kg**
Mean concentration of Vitamin D_2	0.160	0.112
SD_r (Standard deviation of repeatability)	0.019	0.014
Repeatability RSDr % (Observed)	13.500	8.526
$HORRAT_r$	1.936	1.666
SDR	2.129	10.635
Reproducibility RSDR % (Observed)	13.55	8.89
$HORRAT_R$ (Standard deviation of reproducibility)	1.28	1.14

Annex B
(informative)

QUALITY CONTROL

Quality control is an important aspect of a test method validation and implementation. To confirm that the method yields a linear response across the procedural steps and verify the performance of the method, bracketing standards are used. The Bracketing standards are calibration standards used at concentrations immediately above and below the expected concentration of analytes in samples.

The bracketing standards of 75 µg/kg and 150 µg/kg can be used for the LC MS/MS system. Run the matrix-based calibration standards of 75 µg/kg and 150 µg/kg after the calibration points and before beginning the sample sequence. Once the samples are analyzed a second set of bracketing standards of 75 µg/kg and 150 µg/kg shall be run.

The response of each bracketing standard should be within ±15% of the nominal concentration for standards within the calibration curve range.

The response factor of bracketing calibration standards at each level should not differ by more than 20%.

Recalibration or re-injection of the batch may be necessary if the bracketing standards fall outside the accuracy criteria.

Annex C
(informative)

Bibliography

AOAC 2016.05: Gill, B. D., Abernethy, G. A., Green, R. J., & Indyk, H. E. (2016). Analysis of Vitamin D2 and Vitamin D3 in fortified milk powders and infant and nutritional formulas by liquid chromatography–tandem mass spectrometry: single-laboratory validation, first action 2016.05. *Journal of AOAC International*, *99*(5), 1321-1330.

Appendix D AOAC: Guidelines for Collaborative Study Procedures

Guidance SANTE 11312/2021 – Analytical quality control and method validation procedures for pesticide residues analysis in food and feed.