

 <p>FOOD SAFETY AND STANDARDS AUTHORITY OF INDIA Inspiring Trust, Assuring Safe & Nutritious Food Ministry of Health and Family Welfare, Government of India</p>	Method for Determination of Vitamin D2 and D3 in Edible Oil and Fats		
Method No.	NA	Revision No. & Date	NA
Scope	<ul style="list-style-type: none"> ▪ Applicable for the determination of Vitamin D2 and D3 in Edible oils and Fats. The limit of Quantification is 10 µg/kg 		
Caution	<ul style="list-style-type: none"> ▪ Refer to the Material Safety Data sheets for all chemicals prior to use. ▪ Wear eye protection, gloves, and lab coat. Use only with adequate ventilation. Keep away from heat, sparks, and open flames. ▪ Vitamin D is sensitive to light, perform all steps under UV- shielded lighting. 		
Principle	<ul style="list-style-type: none"> ▪ Samples are saponified at high temperature, and then lipid-soluble components are extracted to isooctane. ▪ A portion of 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 higher-molecular-mass, easily ionizable adduct. ▪ The vitamin D adduct is subsequently extracted into a small volume of acetonitrile and analyzed by reversed-phase liquid chromatography (RPLC). ▪ Detection is by tandem mass spectrometry (MS/MS) using multiple reaction monitoring (MRM). Stabled isotope-labeled <i>d6</i>- vitamin D2 & D3 are used for quantitation to correct for losses in extraction and any variation in derivatization and ionization efficiencies. ▪ 		
Apparatus/Instruments	<ul style="list-style-type: none"> ▪ Ultra-high-performance LC (UHPLC) system- Agilent 1290 II ▪ Triple-quadrupole mass spectrometer. - Agilent 6470 ▪ Column. - Infinity Lab Poroshell 120 EC- C18 (3.0 × 50 mm × 2.7 µm) ▪ Balance – Capable of accurately measuring weights from 0.05 to 100 g within ± 0.01 g. ▪ Vortex mixer/ rotary shaker ▪ Centrifuge tube (15/50 ml capacity) ▪ Cooling Centrifuge –Capable of holding 50 ml & 15 ml centrifuge tubes with constant temperature. ▪ Pipettes- graduated 100 - 1000 µl and 20 – 200 µl ▪ Water baths. - hot 70 °C ▪ Syringe with 0.2 µm syringe filter ▪ Microcentrifuge vials. - 2 ml 		

<p>Materials and Reagents/Standard</p>	<ul style="list-style-type: none"> ▪ Vitamin D2 (ergocalciferol) purity: ≥ 99%. ▪ Vitamin D3 (cholecalciferol) purity: ≥ 99%. ▪ <i>d6</i>-Vitamin D2: purity: ≥ 99%. ▪ <i>d6</i>-Vitamin D3: purity: ≥ 99%. ▪ PTAD- Reagent grade. ▪ Formic acid. - LC-MS grade. ▪ Potassium hydroxide. - Reagent grade. ▪ Pyrogallol. - Reagent grade. ▪ Ethanol. - LC grade. ▪ Isooctane. – LC grade. ▪ Acetone. - LC grade. ▪ Acetonitrile - Quality of sufficient purity that is free of interfering compounds. LC-MS grade. ▪ Methanol - Quality of sufficient purity that is free of interfering compounds. ▪ Millipore water - Quality of sufficient purity that is free of interfering compounds in LCMS/MS.
<p>Preparation of Standards/Reagents</p>	<ul style="list-style-type: none"> ▪ PTAD solution (10 mg/ml). - To a 5 ml volumetric flask add 50 mg PTAD, then add 4 ml acetone, and dissolve; dilute to volume with acetone. Expiry 1 day. ▪ Potassium hydroxide solution (50% w/v). - Dissolve 100 g potassium hydroxide in 200 ml water. Expiry 1 month. ▪ Ethanolic pyrogallol solution (1% w/v). - Dissolve 5 g pyrogallol in 500 ml ethanol. Expiry 1 day. ▪ Mobile phase A (formic acid: 0.1% v/v). - To 500 ml water, add 0.5 ml formic acid. Expiry 1 week. ▪ Mobile phase B (methanol; 100%, v/v). - 500 ml of methanol. Expiry 1 month. ▪ All standard solutions should be prepared in Methanol. ▪ Transfer 10 mg of Vitamin D2, D3 and isotope-label Standard into 10 ml volumetric flask and dissolve in Methanol, make sure that the standard is completely dissolved in Methanol. ▪ Make up to 10 ml. Label with name of the standard, Concentration, date of preparation, date of expiry. Keep at -20°C. ▪ Intermediate solution prepared in Methanol. The working standard solution of µg/kg levels for calibration curve is prepared by dilution in Methanol on the day of analysis.

- Matrix match linearity prepared from stock standard solution in Methanol. Dilutions of following conc. were prepared: - 1 µg/kg, 5 µg/kg, 10 µg/kg, 20 µg/kg, 50 µg/kg and 100 µg/kg. Plot the standard curve.

Sample Weight (gm.)	Concentration (mg/kg)	Standard volume(µL)	Final concentration (µg/kg)	IS Conc. (µg/kg)	Label
2.00	1	200	100	10	CC6
2.00	1	100	50	10	CC5
2.00	0.1	400	20	10	CC4
2.00	0.1	200	10	10	CC3
2.00	0.1	100	5	10	CC2
2.00	0.1	20	1	10	CC1

- All standard solutions should be prepared in Methanol.
- Transfer 10 mg of Vitamin D2, D3 and isotope-label Standard into 10 ml volumetric flask and dissolve in Methanol, make sure that the standard is completely dissolved in Methanol.
- Make up to 10 ml. Label with name of the standard, Concentration, date of preparation, date of expiry. Keep at - 20°C. The stock standard solution is stable up to six months.
- Intermediate solution prepared in Methanol is stable for three months. The working standard solution of µg/kg levels for calibration curve is prepared by dilution in Methanol on the day of analysis.

Sample Preparation and Method of analysis	<ul style="list-style-type: none"> ▪ Sample of 2 g ± 0.01 g was for the oil or fat samples into a 50 ml centrifuge tube. ▪ Add 10 ml ethanolic pyrogallol solution; then spiked with standard Vitamin D2, D3 and isotope-label at different concentrations (IS same concentration 10 µg/kg), cap and mix on vortex mixer 2 min. ▪ Add 2 ml potassium hydroxide solution to CF tube cap and vortex 2 min. ▪ Place the tube in a water bath at 70 °C for 1 hr. mix on vortex mixer every 5 min. ▪ Place the tube in a water bath at room temperature until cool. ▪ Add 10 ml isooctane to the tube cap tightly and place on a shaker or hand shake for 10 min. Thereafter, add 20 ml water to the tube and invert the tube 10 times; place in centrifuge at ≥ 250 × g for 15 min. ▪ Transfer 5 ml aliquot of the upper isooctane layer into 15 ml centrifuge tube using pipet, take care not to transfer any of the lower layer. ▪ Add 5 ml water to the centrifuge tube, cap and vortex for 2 min and place in a centrifuge at 2000 × g for 5 min. ▪ Transfer 4-5 ml upper isooctane layer to a new centrifuge tube, taking care not to transfer any of the lower layer. ▪ Add 75 µl PTAD solution to the centrifuge tube, cap and immediately vortex mix. ▪ Allow to stand in the dark for 5 min to allow the derivatization reaction to complete. ▪ Add 1 ml of ACN to the centrifuge tube, cap and vortex mix. ▪ Centrifuge at 2000 × g for 5 min. Using a variable volume pipet, transfer 500 µl lower layer into a microcentrifuge tube, taking care not to transfer any of the upper layer. ▪ Add 167 µl water to the microcentrifuge tube, cap and vortex mix. ▪ Filter the eluent with 0.2 µm syringe filter, fill in LC-MS vial and inject.
Calculation with units of expression	$Conc, mg/Kg = \frac{(Concentration\ From\ calibration \times Volume\ made\ up)}{Wt.\ of\ the\ sample}$
Inference (Qualitative Analysis)	<p style="text-align: center;">Quantitative Analysis</p>
References	<ul style="list-style-type: none"> • 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 2017, 100(1). • AOAC 2016.05

Approved by	Scientific Panel on Methods of Sampling and Analysis

The following ‘note’ need to be added in all manuals:

Note: The test methods given in the manual are standardised/ validated/ taken from national or international methods or recognised specifications, however it would be the responsibility of the respective testing laboratory to verify the performance of these methods onsite and ensure that it gives proper results before putting these methods in to use”.

Editorials (For Reference purpose while writing methods)

Abbreviations to be used

Microgram		µg
Milligram		mg
Gram		g
Kilogram		kg
Milliliter		mL
Litre		L
Second	sec	
Minute	min	
Hour		h
Celsius	°C	
Kelvin		°K
Centimeter		cm
Millimeter		mm
Molar		M
Millimolar		mM
Micromolar		µM
Mole		mol
Normal	N	
Wavelength		nm

Some Editorials for the manuals

Space between numbers and units

- Mass and volume need spaces 12 g not 12g, 100 mL not 100mL
- Time also needs space 10 h not 10h, 15 min not 15min
- Temperatures need spaces
 - between value and degree sign: **37 °C**, not 37° C or 37°C
 - but the degree sign for angles goes with the number: 90° angle
- Centrifugal forces need spaces
 - on both sides of the "×" (remember not x)
 - 10,000 × g, not 10,000g or 10,000xg
- Other "places for spaces"
 - around equals sign: **n = 3**, not n=3
 - also around >, <, ~, etc
 - around plus/minus: 29 ± 7, not 29±7
- Percentages is the only exception
 - **5%** serum, **0.01%** bromophenol blue
 - This is because % is not really a unit, just an indication that the value is presented as the "ratio to 100"
 - **a space is required:** 10 mM or 6 M, never 10mM or 6M
- Use numerals to express numbers 10 and above.
- Use words to express numbers below 10.
- Use numerals when you have 3 or more numbers in a series, even if each of the numbers is below 10.
- When numbers begin a sentence, you must write them out in words.
- Situations in which Numbers Should be Given as Numerals

General Guideline	Examples
All numbers 10 and above	Trial 14; 35 animals; 16 genera of legumes
All numbers that immediately precede a unit of measurement	A wing 10 cm long; 5 mg of drug; 21days
Numbers with decimals; fractions that include whole numbers	7.38 mm; 4 ¹ / ₂ hours
Numbers that represent statistical or mathematical functions or results, percentages, ratios	Multiply by 5; fewer than 6%; 3.75 times as many; the 2nd quartile
Numbers that represent exact times or dates; ages; size of samples, subsamples or populations; specific numbers of subjects in an experiment; scores and points on a scale; exact sums of money; and numerals as numerals	About 3 weeks ago, at 1:00 a.m. on January 25, 2000, the 25-year-old patients with IQ scores above 125 all awoke simultaneously in the nursing home at 125 Oak Street. They were paid \$25 apiece to go back to sleep
Numbers below 10 that are grouped for comparison with numbers 10 and above in the same paragraph	4 of 16 analyses, the 1st and 15th of the 25 responses; lines 2 and 21
Numbers that denote a specific place in a numbered series, parts of books and tables, and each number in a list of four or more numbers	Trial 6; Grade 9 (but the ninth grade); the groups consisted of 5, 9, 1, and 4 animals respectively

(to be written depending upon concerned manual)

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2.00	0.1	400	20	10	CC4
2.00	0.1	200	10	10	CC3
2.00	0.1	100	5	10	CC2
2.00	0.1	20	1	10	CC1