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ANALYTICAL REPORT

DETERMINATION OF CHOLECALCIFEROL (VITAMIN D_3) IN XXXXXXXXX

1. Samples:

The samples were received on xxxxxxxx, 2009, for Vitamin D3 (cholecalciferol) determination.

Sample ID	Description
XXXX	Vitamin D
XXXX	Vitamin D
XXXX	Vitamin D
XXXX	Vitamin D

The testing was performed using an Agilent 1200RR series HPLC system with isocratic elution.

2. Summary of procedure:

AOAC Method 2002.05 – Cholecalciferol (Vitamin D_3) in Selected Foods

After the addition of an internal standard (Vitamin D_2) and basic hydrolysis, Vitamin D_3 is extracted with *n*-heptane. After evaporation and dilution in acetonitrile-methanol, Vitamin D_3 is determined by reversed phase HPLC with UV detection at 265 nm.

3. Standards and reagents:

Cholecalciferol (Vitamin D_3): ChromaDex – ASB-00022774, Lot #: 22774-869, Purity: 98.2%
Ergocalciferol (Vitamin D_2): ChromaDex – ASB-00022751, Lot #: 22751-427, Purity: 96.9%
Acetonitrile: Sigma-Aldrich – ChromaSolv for HPLC
Ascorbic acid: Spectrum Chemical - USP grade
BHT: Alfa Aesar - 99%
Ethanol: Acros Organics – abs., 99.5%
n-heptane: Alfa Aesar - HPLC grade
Methanol: B&J – HPLC grade
Phenolphtalein: Alfa Aesar - ACS grade

Potassium hydroxide:	EMD Chemicals - ACS grade
Water:	Purified by Nanopure water purification system to >18 M-ohm resistivity

4. Preparation of solutions:

40% Ethanol:	Diluted 400 mL ethanol to 1L with Nanopure water.
50% Potassium hydroxide:	Dissolved 500 g pellets in 500 mL Nanopure water.
1% Phenolphtalein:	Dissolved 1 g phenolphthalein in ethanol and dilute to 100 mL.
HPLC mobile phase:	Diluted 200 mL methanol to 1L with acetonitrile.

5. Equipment:

HPLC system:	Agilent 1200RR binary pump, thermostatted column compartment, autosampler and diode array detector (Agilent Technologies, Santa Clara, CA)
HPLC column:	Synergy Hydro-RP, 4µ, 250x4.6 mm (Phenomenex, Torrance, CA)

6. Procedure:

6.1. Standard preparation:

- *Vitamin D_3 stock standard solution*: Using a weigh boat, 16.8 mg Vitamin D_3 standard was weighed and transferred to a 10 mL glass volumetric flask and diluted to mark with abs. ethanol. Concentration (corrected for purity): 1649.76 µg/mL.
- *Vitamin D_3 working standard solution*: 1 mL of the D_3 stock standard solution was transferred into a 50 mL glass volumetric flask and diluted to mark with abs. ethanol. Concentration (corrected for purity): 32.9952 µg/mL.
- *Vitamin D_2 stock standard solution*: Using a weigh boat, 8.9 mg Vitamin D_2 standard was weighed and transferred to a 10 mL glass volumetric flask and diluted to mark with abs. ethanol. Concentration (corrected for purity): 862.41 µg/mL.
- *Vitamin D_2 working standard solution*: 1 mL of the D_2 stock standard solution was transferred into a 50 mL glass volumetric flask and diluted to mark with abs. ethanol. Concentration (corrected for purity): 17.2482 µg/mL.
- *Vitamin D_2 internal standard solution*: 2 mL of the D_2 working standard solution was transferred into a 50 mL glass volumetric flask and diluted to mark with abs. ethanol. Concentration (corrected for purity): 0.689928 µg/mL.

6.2. Calibration standards:

- Vitamin D_3 calibration standards were prepared by diluting specified volumes of Vitamin D_3 stock and working standard solution with HPLC mobile phase.

Calibration level	Calibration standard preparation		Vitamin D3 concentration (µg/mL)
	Standard	**Dilution**	
Level 1	1 mL working standard	5 mL	6.599
Level 2	1 mL working standard	2 mL	16.498
Level 3	working standard	No dilution	32.995
Level 4	0.5 mL stock standard	10 mL	82.488
Level 5	1 mL stock standard	10 mL	164.976

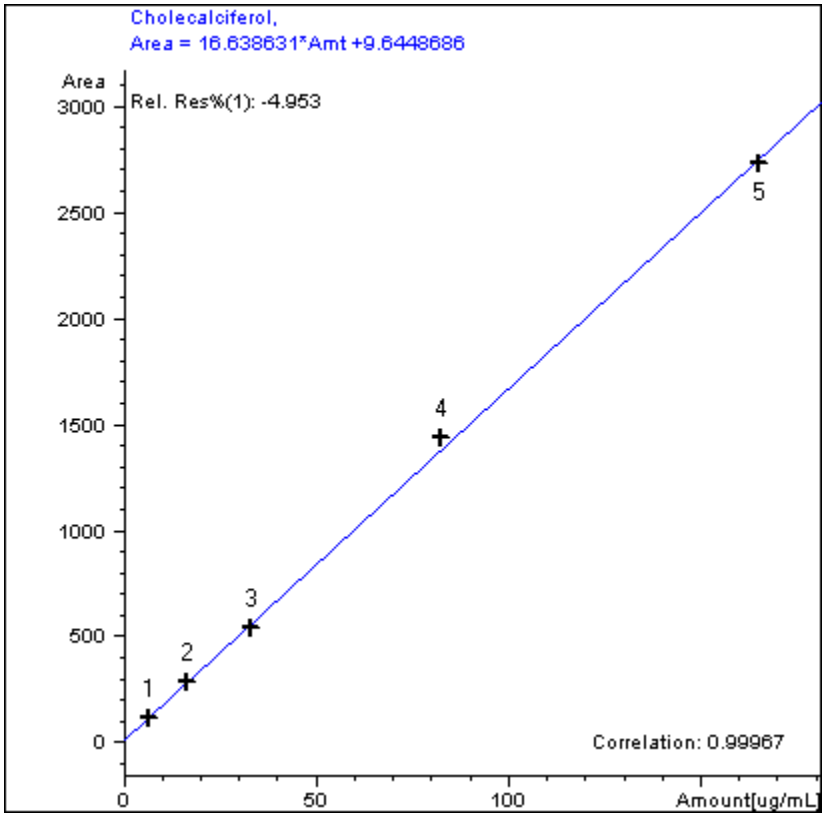

Figure 1.
Calibration curve of cholecalciferol (Vitamin D_3)

6.3. Sample preparation:

- *Saponification*: 1 mL sample, 0.5 g ascorbic acid, 100 mL abs ethanol, 2 mL Vitamin D2 internal standard solution and 25 mL 50% potassium-hydroxide was heated at 65-70ºC for 30 minutes.
- *Extraction*: The cooled samples were extracted with 2x50 mL *n*-heptane and washed with 50 mL 1M potassium-hydroxide, 2x50 mL 40% ethanol and water until the pH of the rinse was neutral.

- The heptane extracts were treated with ethanol and a few granules of BHT, and then the solvent was evaporated.
- The residue was dissolved in 1 mL HPLC mobile phase, transferred into a HPLC vial and injected onto the column.

7. Chromatographic conditions (isocratic method):

HPLC system:	Agilent 1200RR binary pump, thermostatted column compartment, autosampler and diode array detector (Agilent Technologies, Santa Clara, CA)
Column:	Synergy Hydro-RP, 4µ, 250x4.6 mm (Phenomenex, Torrance, CA)
Column temperature:	25ºC
Mobile phase:	Acetonitrile:methanol (8:2)
Flow rate:	2.0 ml/min
Injection volume:	20 µl
Detection:	265 and 280 nm
Data acquisition software:	ChemStation for LC 3D, Rev. B. 03.02 (341)

8.Instrumental analysis and calculations:

The column was equilibrated with the mobile phase for 30 min. The sequence of injection was: mobile phase blank, calibration standards, blank, unknown samples, blank, unknown samples, blank, calibration standards The peaks were integrated, and manually corrected if baseline-to-baseline integration was not achieved. The calibration curve did not include the origin.

9. Chromatograms:

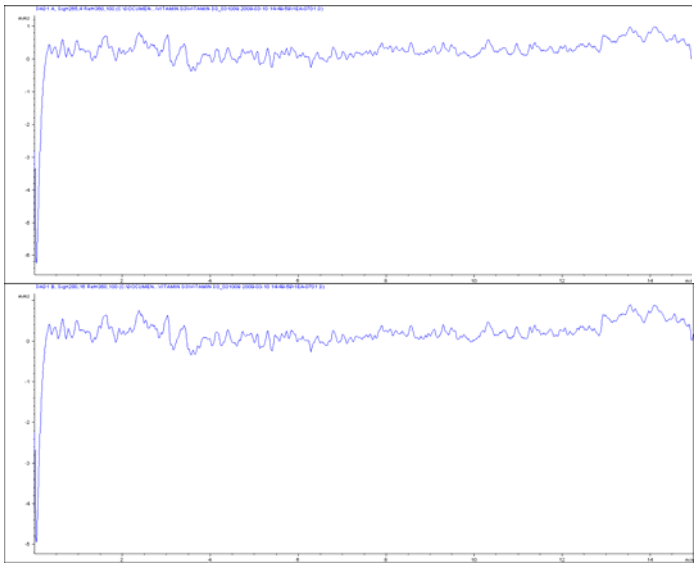

Figure 2.
Representative HPLC chromatogram of the "Mobile phase blank injection" at 265 nm (upper panel) and 280 nm (lower panel)

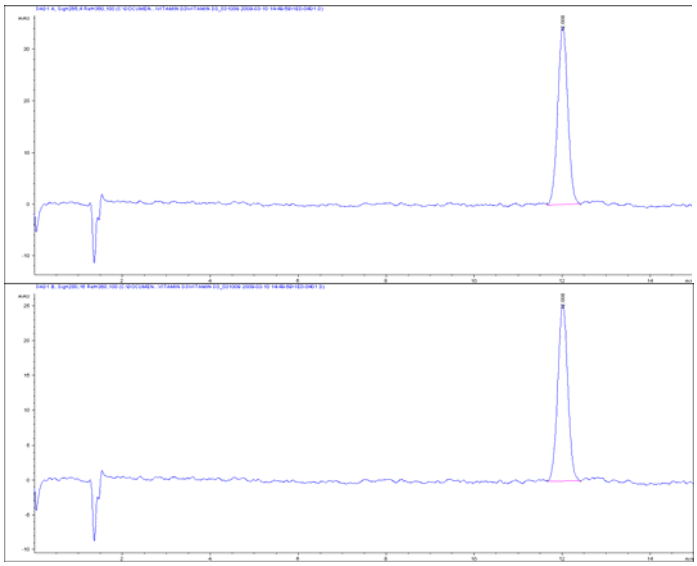

Figure 3.
Representative HPLC chromatogram of the "Level 3 calibration standard" at 265 nm (upper panel) and 280 nm (lower panel)

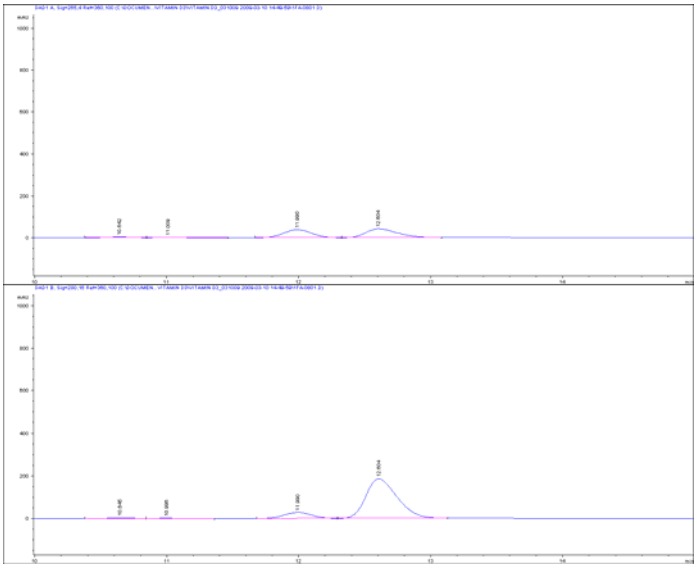

Figure 4.
Representative HPLC chromatograms of the XXXXX sample at 265 nm (upper panel) and 280 nm (lower panel)

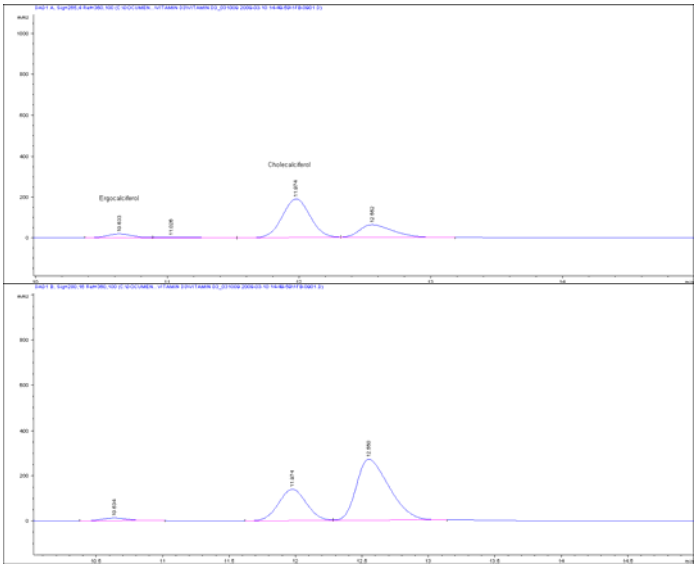

Figure 5.
Representative HPLC chromatograms of the XXXXX sample at 265 nm (upper panel) and 280 nm (lower panel)

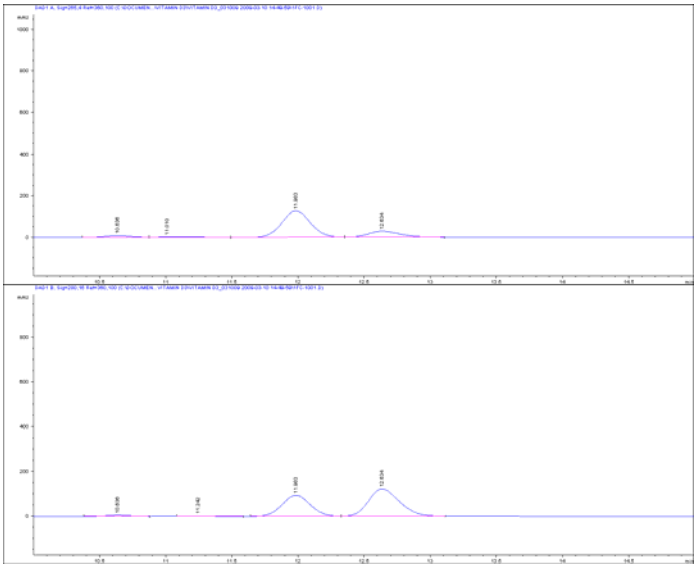

Figure 6.
Representative HPLC chromatograms of the XXXXX sample at 265 nm (upper panel) and 280 nm (lower panel)

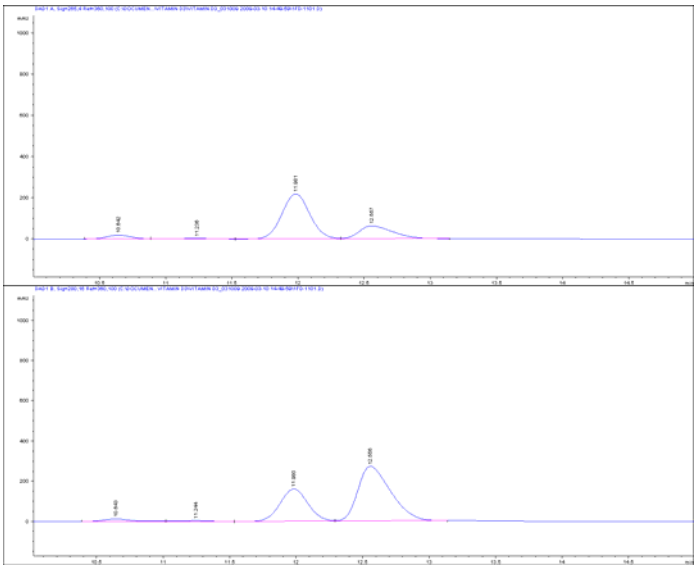

Figure 7.
Representative HPLC chromatograms of the XXXXX sample at 265 nm (upper panel) and 280 nm (lower panel)

10. Results:

Sample ID	#1 µg/mL	#2 µg/mL	Average conc. µg/mL	Average conc. IU/mL
XXXXX	33.08	33.17	33.13	**1325**
XXXXX	168.53	168.79	168.66	**6746**
XXXXX	114.13	114.22	114.18	**4567**
XXXXX	193.78	194.44	194.11	**7764**

11. References:

AOAC Official Method 2002.05 Cholecalciferol (Vitamin D_3) in Selected Foods (First Action 2002)
GAAS Analytical's Laboratory Notebook S-1-008-014
GAAS Analytical's Laboratory Notebook ST-1-16, 17, 19 and 20

We, the undersigned, acknowledge that the laboratory work performed on this project was executed in accordance to the GAAS Analytical Standard Operating Procedures (SOPs).

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Anikó M. Sólyom, Ph.D.
CSO

Date

_____ _____

George Z. Angeli, Ph.D.
Quality Assurance Director

Date
