Method for the Determination of Vitamin D_2 and D_3 in Foods, Feeds, Pet Foods, and Ingredients Using HPLC-UV with Prep-LC Cleanup

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Outline

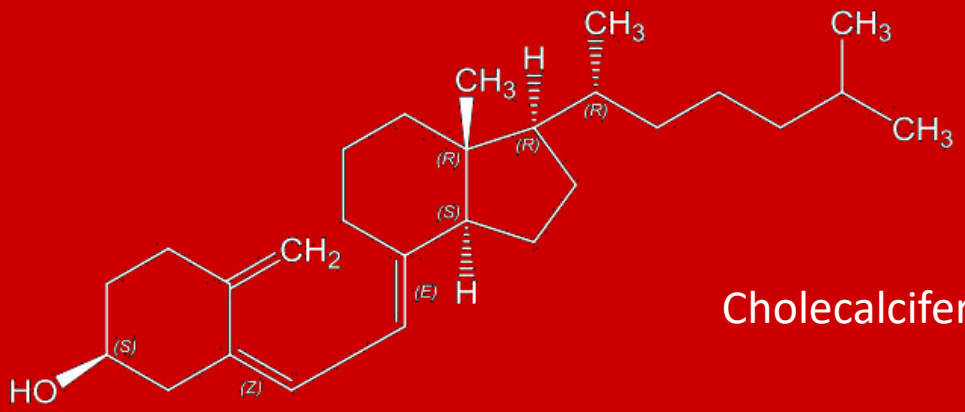
- Introduction
- Method Walk-Through
- Method Performance
- Conclusions

Vitamin D

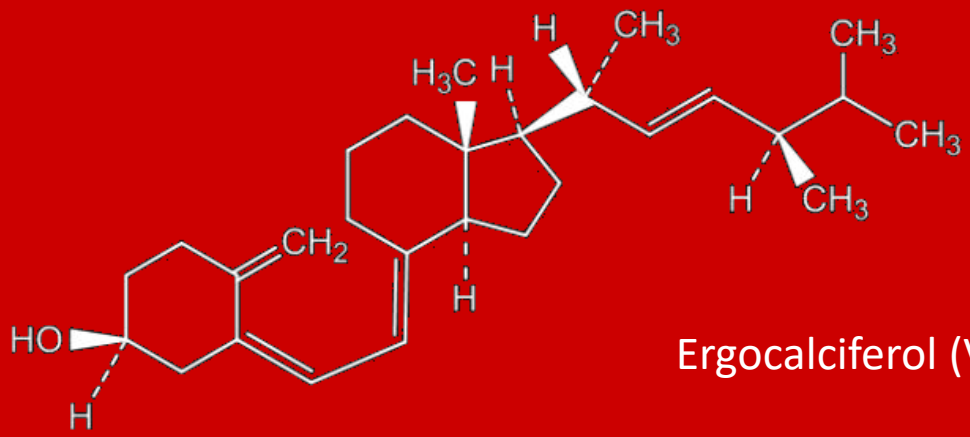
- **Nutrition** – Balance and Retention of Calcium and Phosphorus
- **Toxicity** – Kidney Failure and Death from hypercalcemia

Merck Manual

Vitamin D



Cholecalciferol (Vitamin D3)



Ergocalciferol (Vitamin D2)

United States Pharmacopeia

Vitamin D

Compound	IU/g
Cholecalciferol (D3)	40,000,000
Ergocalciferol (D2) *	40,000,000

*No acitivity for birds

Methods of Vitamin Assay, 1985

Vitamin Stability

Vitamin	Temperature	Oxygen	Humidity	Light	pH	
					Acid	Base
A	XX	XX	X	XX	X	O
D	X	XX	X	X	X	O
E	X	O	X	X	X	X

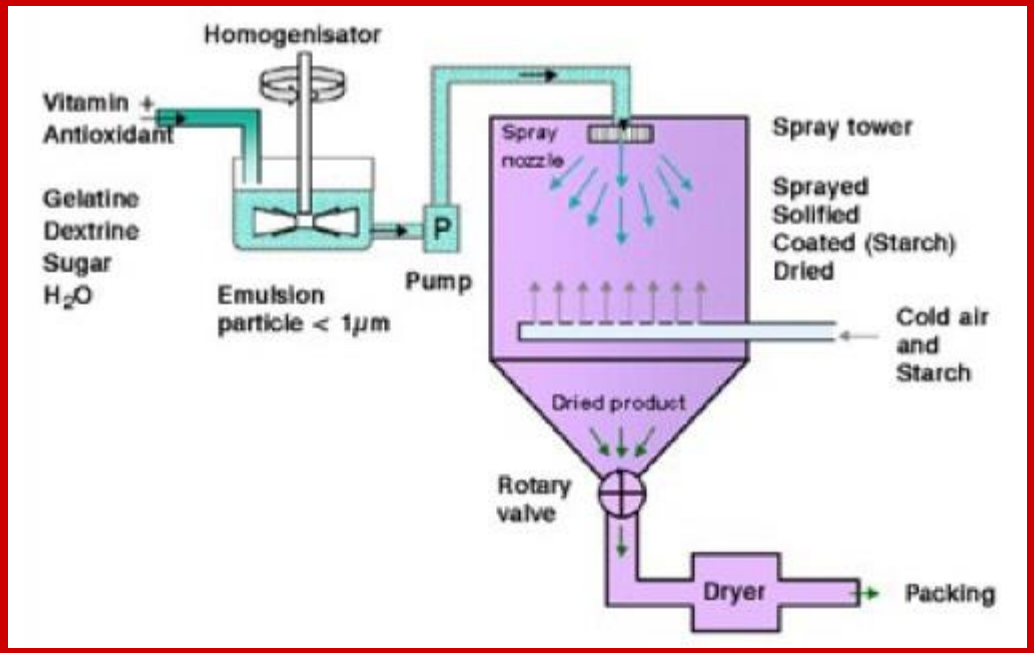
O - Stable

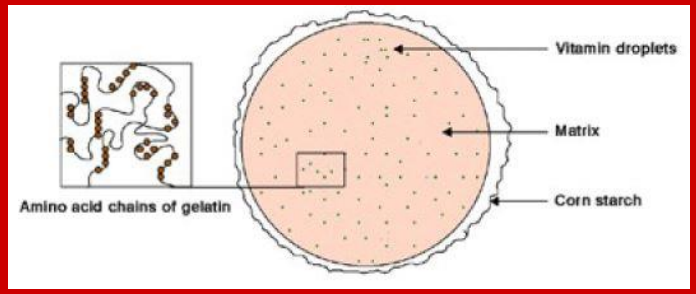
X - Slightly Sensitive to Sensitive

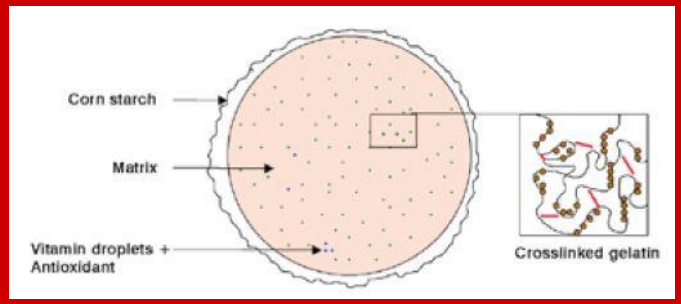
XX - Very Sensitive

Gadient, 1986

Vitamin Encapsulation

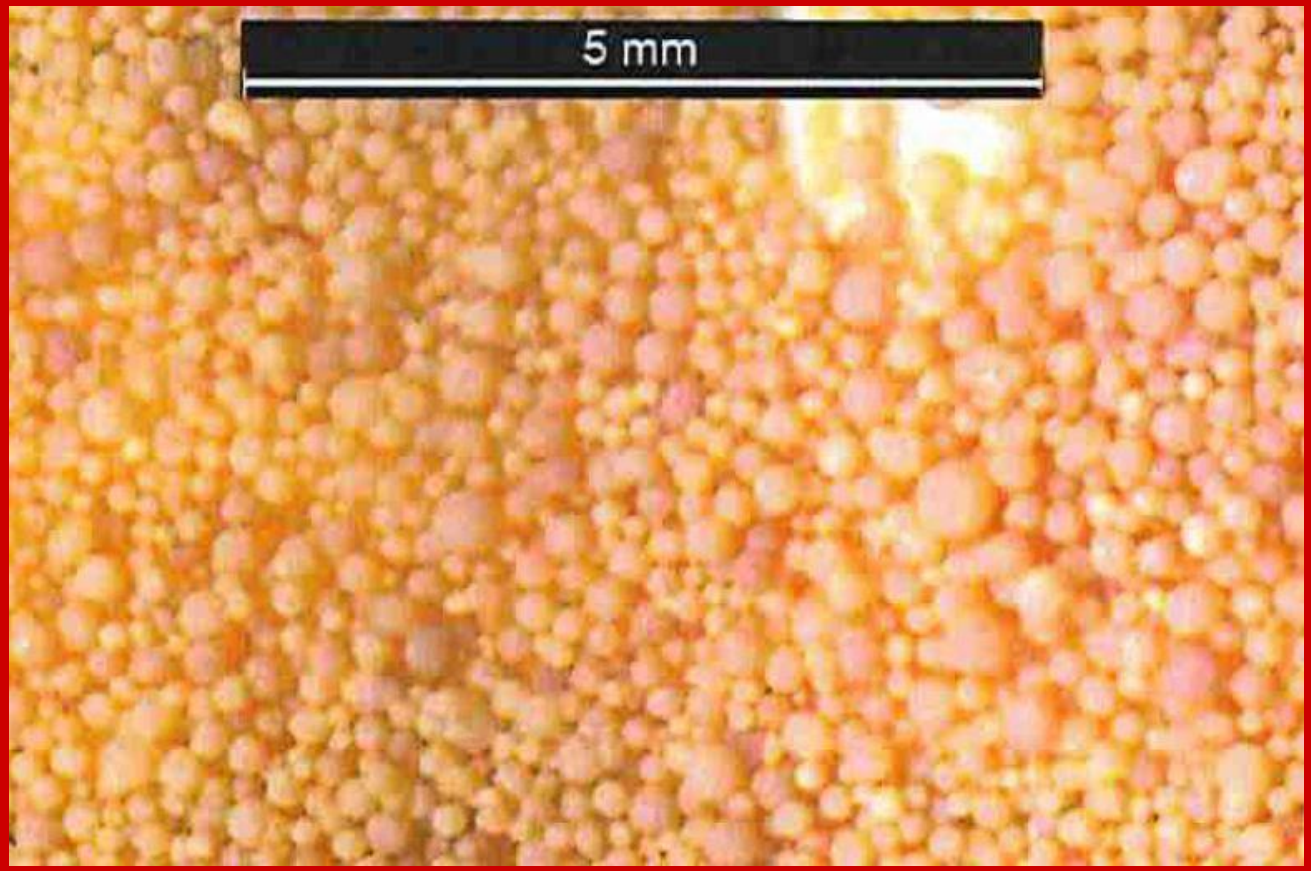






DSM Website

Vitamin Encapsulation



Michele Swarbrick, Minnesota Dept. Ag.

NPAL Method

- Overnight Room Temperature Saponification
- Liquid-Liquid Partitioning
- Reverse-Phase Semi-Preparative Liquid Chromatography (Prep-LC) – Separate D2 If Desired
- Normal-Phase HPLC
- Similar to AOAC 2012.11

Extraction

- 10 gram Test Portion
- 250 mL Erlenmeyer
- Ethanolic KOH
- Pyrogallol
- Room Temperature
- Overnight
- Orbital Shaker
- Protect From Light

Liquid-Liquid Partition

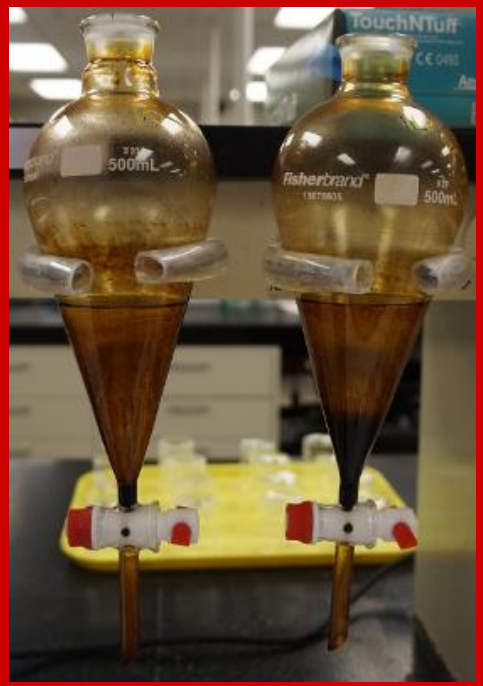
- Allow Solids To Settle
- 500 mL Sep Funnel
- Transfer Saponicate
- 50 mL 1N KOH
- 50 mL Hexane
- 50 mL Hexane

Liquid-Liquid Partition

Shake

Settle

Drain



Liquid-Liquid Partition 2

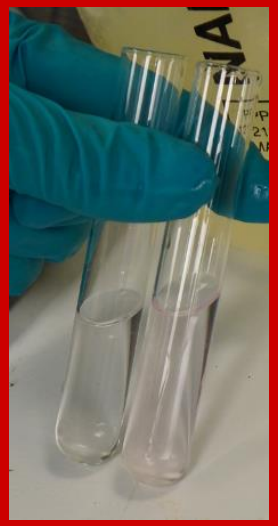
- 1 – 2 grams NaCl
- 50 mL 0.5 N KOH
- Shake
- Settle
- Drain

Liquid-Liquid Partition 3

- 10 - 15 grams NaCl
- 400 - 500 mL Water
- Shake
- Settle
- Drain

Liquid-Liquid Partition 4

- 10 - 15 grams NaCl
- 400 - 500 mL Water
- Shake
- Settle
- Drain/Check pH
- pH Neutral to Phenolphthalein
- 5th Partition Typically Needed



Dry Hexane Layer

- Funnel w/ Glass Wool Plug
- 2 cm Sodium Sulfate
- Drain Hexane Layer Through Sodium Sulfate Into 100 mL Graduated Cylinder
- Record Volume

Evaporate

- Transfer to 250 mL Flat-Bottom Boiling Flasks
- Evaporate Under Nitrogen

Evaporate 2

- Redissolve in 6 mL Hexane
- Pipet 5 mL Extract Into 50 mL Conical Tubes
- Evaporate Under Nitrogen
- Redissolve in 500 µL of 1:1 Methanol:Acetonitrile
- Centrifuge and Transfer to Autosampler Vials

Prep LC

- Inject 10 – 180 µL (180 µL Typical)
- Mobile Phase – 1:1 MeOH:ACN, 4 mL/min
- Column: Microsorb 5µ C18 Dynamax, 10x250 mm; 45°C
- UV = 264 nm; Run Time = 35 min

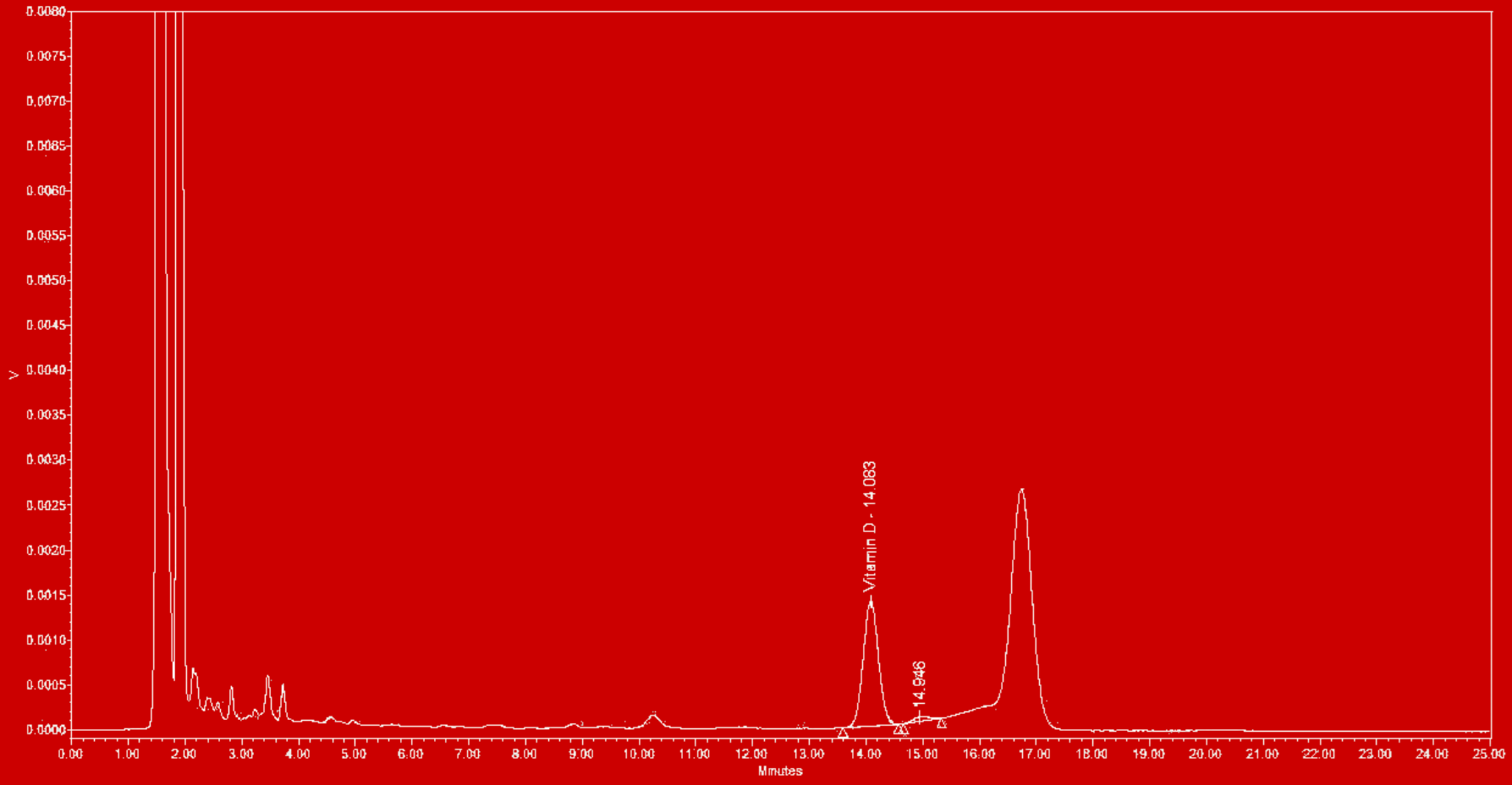
Evaporate

- Evaporate Collected Fractions Under Nitrogen
- Rinse Down Sides of Tubes with 2 mL Hexane
- Evaporate Under Nitrogen
- Redissolve in 300 µL of Heptane w/ 0.1% IPA
- Transfer to Autosampler Vials and Crimp to Seal

HPLC

- Mobile Phase: Hexane w/ 0.4% IPA
- Flow Rate: 2 mL/min
- Column: Microsorb 100-5 Si, 5µ, 4.6x250 mm, 35°C
- UV = 264 nm, 50 µL Injection
- Run Time = 25 min

Standard


Vitamin D - 14.083
14.946
V
Minutes
0.0080
0.0075
0.0070
0.0065
0.0060
0.0055
0.0050
0.0045
0.0040
0.0035
0.0030
0.0025
0.0020
0.0015
0.0010
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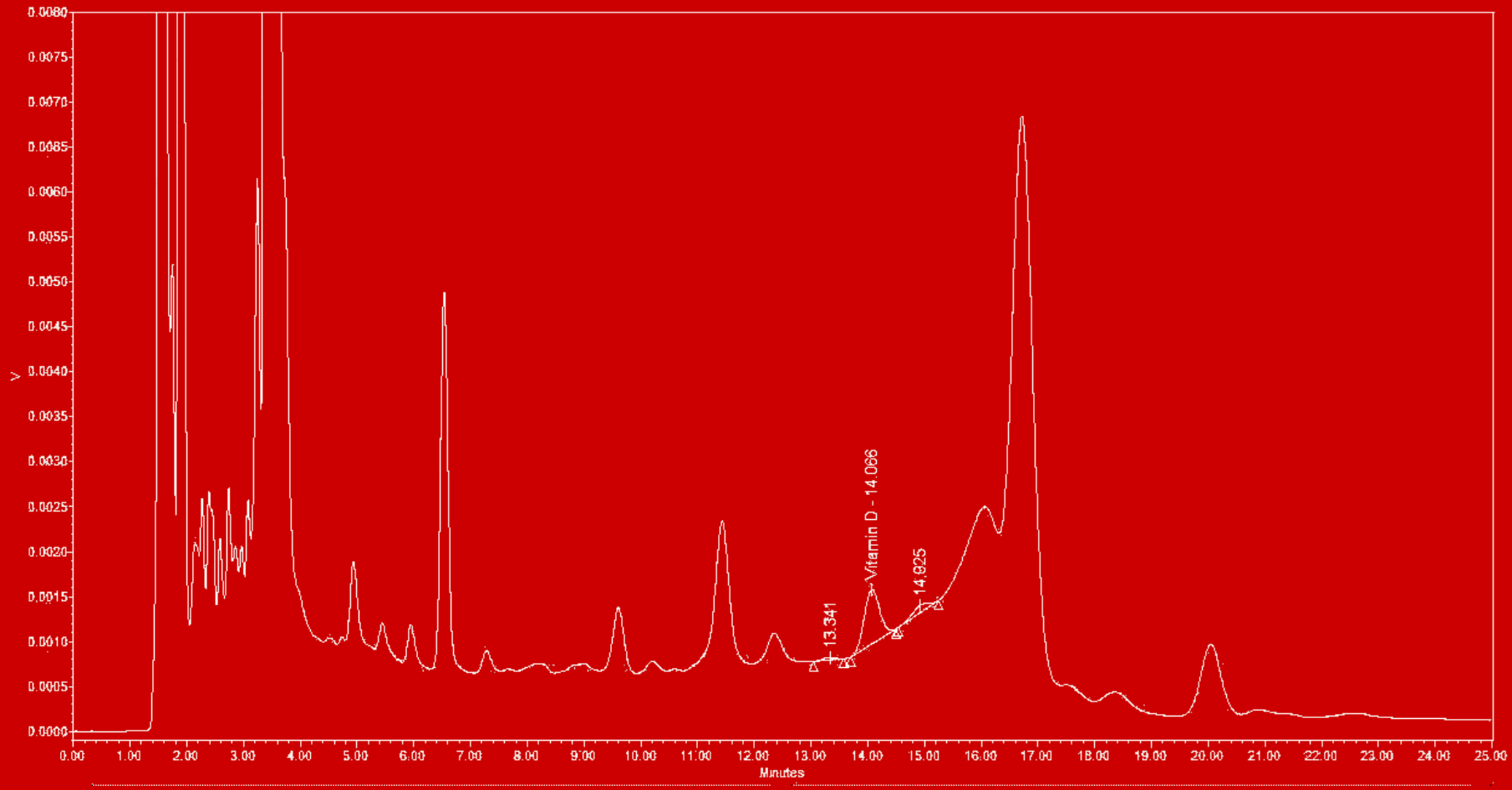
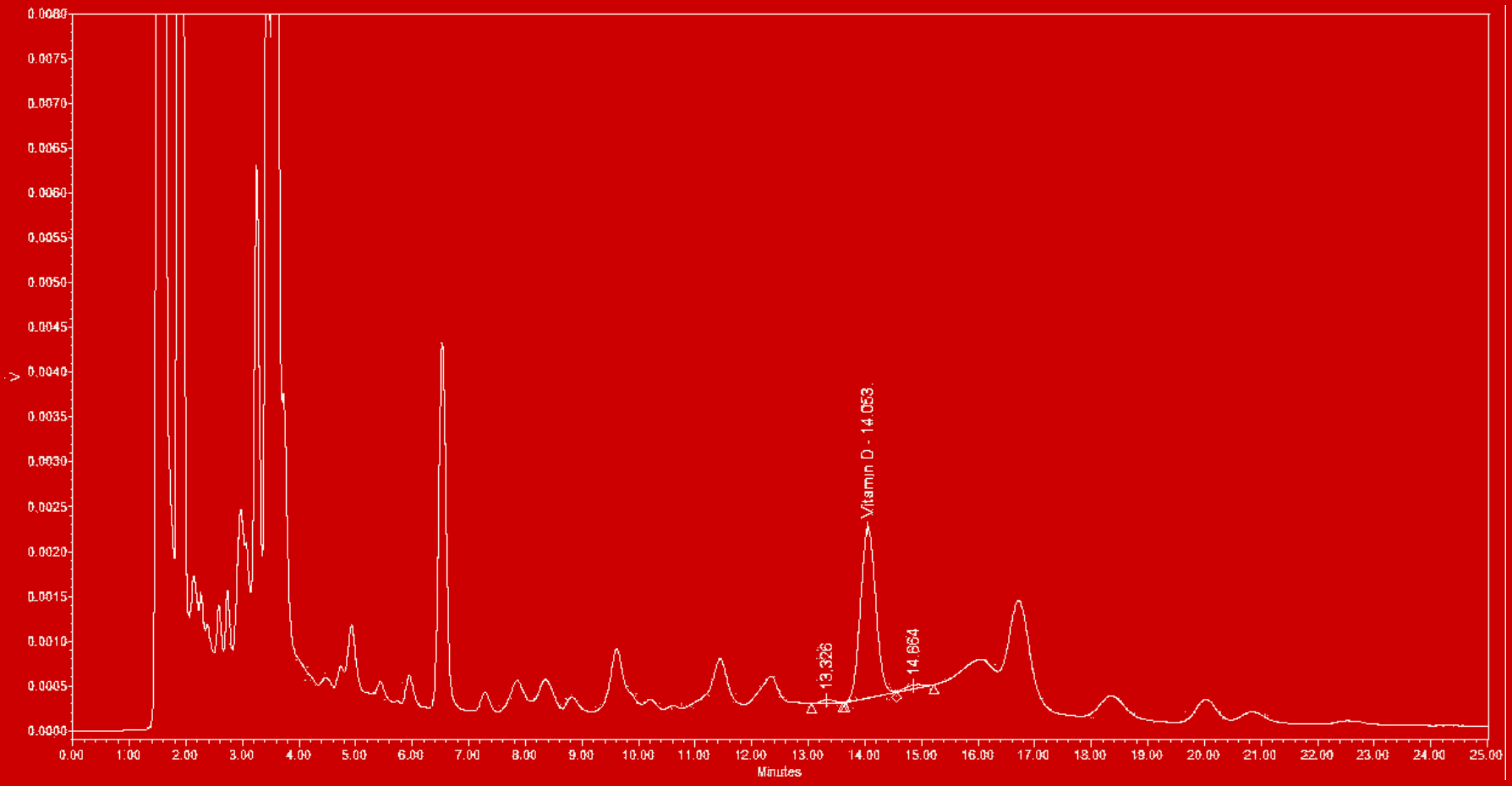
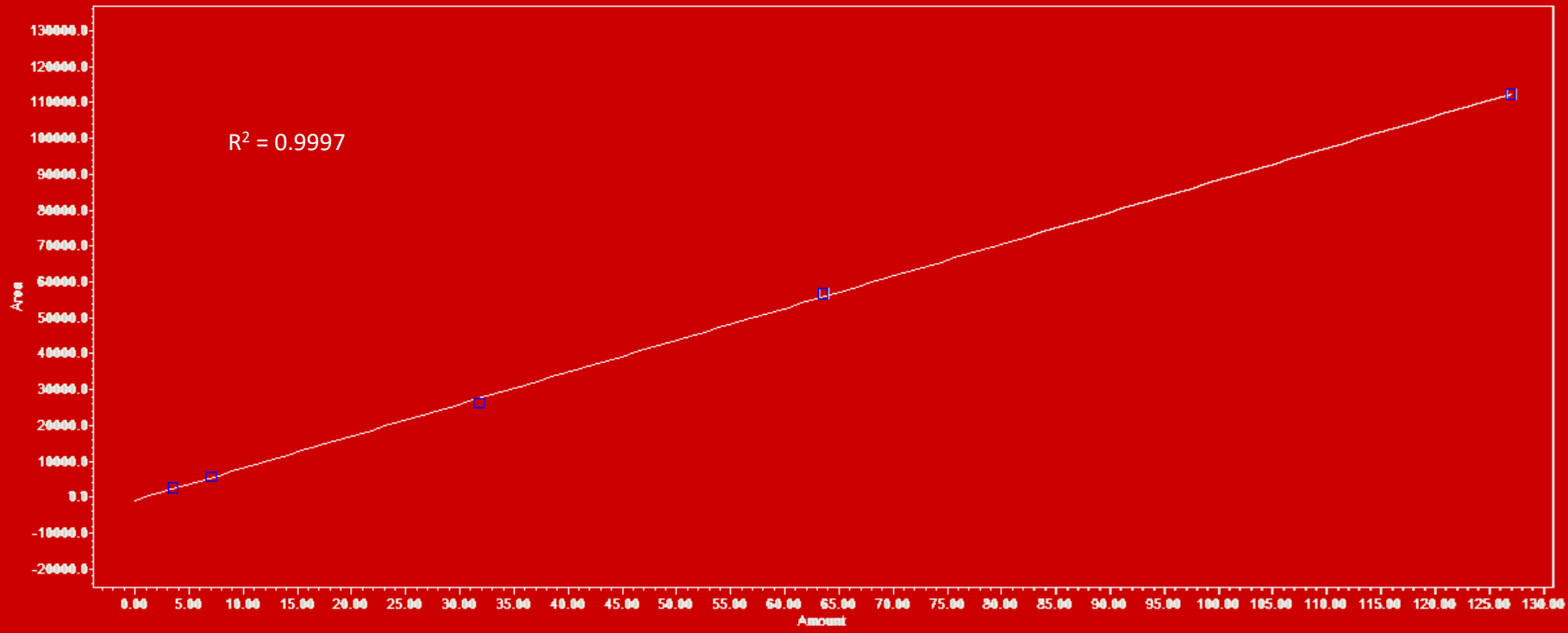
Control Sample



Other Sample



Linearity



Performance

- Reproducibility: 5 – 30 %RSD Depending on Level of Vitamin D and Sample
- Accuracy: AAFCO PT Sample and Internal PT Sample both within 1 Standard Deviation of Mean (Consensus)
- LOQ: 0.5 IU/g

Method Advantages

- No Expensive Instrumentation Needed (LC-MS/MS)
- No Expensive Internal Standard Needed (Isotopically Labeled)
- No Special Expertise Needed (SPE, LCMS)

Method Disadvantages

- Throughput: 10 - 12 Samples per Run; 2 Runs per Week
- Labor-Intensive
- Sensitivity (0.5 IU/g)
- Selectivity – UV Detection Susceptible to Interferences

Summary

- Good Option If You Do Not Have a Large Number of Samples per Week
- Good If You Do Not Have the Budget for an LC-MS/MS Instrument
- Steady Performance and Reliable Results

Acknowledgements

- Nicole Konsdorf

Questions?

- Ken.Riter@purina.nestle.com

References

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- Augustin, Klein, et al, eds., *Methods of Vitamin Assay*, 1985.
- Gadient, M “Effect of Pelleting on Nutritional Quality of Feed”, presented at Maryland Nutrition Conference, 1986.