APPLICATIONS

Determination of Vitamin D_2/D_3 and Pre-D_2/D_3 in Pet Food by LC/MS/MS

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The same challenges in determining Vitamin D_2/D_3 in foods and dietary supplements, such as matrix interferences, isomer conversions, and light sensitivity persist in pet food matrices. In this technical note, we describe an LC/MS/MS method with a simplified saponification and extraction procedure suitable for a variety of pet food samples.

Introduction

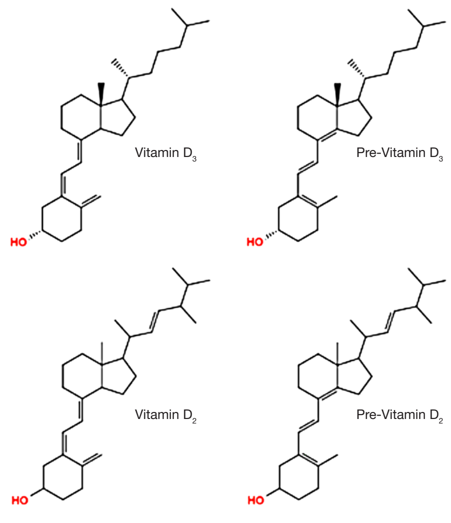
Vitamin D is an essential fat soluble vitamin, most known for its requirement in bone formation in animals amongst myriad other likely beneficial functions. Vitamin D_3 is typically produced naturally with exposure to sunlight, and may need to be supplemented in diets if not adequately exposed (both humans and animals, particularly under modern farming conditions). In animals, the consensus is that Vitamin D_3 is the most biologically active form, and thus needs to be distinguished apart from Vitamin D_2, periodically sourced from some plants and mushrooms. The isomeric forms, pre-vitamin D_2 and pre-vitamin D_3, are also important to quantify accurately without disrupting their natural equilibrium to accurately assess their source.

Experimental Conditions

Reagent and chemicals

All solvents and reagents were HPLC or analytical grade. Reference standards of Vitamin D_2/D_3 and its isotope internal standard of Vitamin D_2-d_3 and Vitamin D_3-d_3 were purchased from Cerilliant®; Pre-vitamin D_2/D_3 were from Toronto Research Chemicals. Samples from Purina®, Dog Chow®.

Figure 1.
Vitamin D and Pre-Vitamin D Structures



Equipment and materials

Agilent® 1260 SL pumps and autosampler were used along with a SCIEX 4500™, positive polarity, APCI for detection.

Sample Preparation

Matrix: Purina, Dog Chow (made with real chicken, for small dog), dry dog food.

Samples: Blank (n=1), Blank/IS (n=1), 4 Standards at concentration of STD (D_2/D_3): (Pre-D_2/Pre-D_3) 0/0 µg/mL, 0.5/0.25 µg/mL, 1/0.5 µg/mL, 5/2.5 µg/mL (n=1), and QC1 at 1.0/0.5 µg/mL, QC2 at 4/2 µg/mL (n=3 at each QC level).

Two stock solutions were prepared in 40 % ethanol at Conc. 40/40 µg/mL for Vitamin D_2/D_3, and 20/20 µg/mL for Pre-D_2/Pre-D_3

Saponification/Pre-extraction/LLE Extraction Procedure:

1) Weight ~2 g blended dog food powder + 0.2 g ascorbic acid + 0.2 g pyrogllic acid into 1st 50 mL centrifuge tube, mix, spike with corresponding standard stock solution to make STDs and QCs.
2) Add 100 µL of IS stock mix (10/10 µg/mL of Vit D_2-d_3/Vit D_3-d_3) and 8 mL ethanol to all samples except blank, mix/shake for the pre-extraction at 200 rpm for 15 min
3) Add 2 mL of 45% KOH to all samples, mix/shake/incubate at 200 rpm under room temperature for 1 hour
4) Add 5 mL DI water to 1st 50 mL centrifuge tube, shake at 200 rpm for 15 min, centrifuge at 4500 rpm for 5 min, transfer upper supernatant (dark brown color) to 2nd 50 mL centrifuge tube
5) Add 5 mL 40% ethanol to 1st 50 mL centrifuge tube, shake at 200 rpm for 15 min, centrifuge at 4500 rpm for 5 min, transfer/combine upper supernatant (dark brown color) to corresponding 2nd 50 mL centrifuge tube, discard 1st 50 mL centrifuge tube
6) Add 6 mL heptane to 2nd 50 mL centrifuge tube, shake at 200 rpm for 15 min, centrifuge at 4500 rpm for 5 min, transfer upper supernatant (light yellow color) to 100 X 16 mm glass tube by plastic transfer pipettes, dry samples in TurboVap at 30°C under N_2 for 20 min
7) Add 6 mL heptane to 2nd 50 mL centrifuge tube again, shake at 200 rpm for 15 min, centrifuge at 4500 rpm for 5 min, transfer upper supernatant (light yellow color) to corresponding 100 X 16 mm glass tube by plastic transfer pipettes, discard 2nd 50 mL centrifuge tube, dry samples in TurboVap at 30°C under N_2 about 10-15 min until liquid volume remaining is ~3 mL
(DO NOT DRY DOWN ALL LIQUID VOLUME)

Solid Phase Extraction (SPE) Procedures

SPE Cartridge:	Strata® NH_2 (55 µm, 70A), 2 g/12 mL Giga Tubes
Part No.:	8B-S009-KDG
Condition:	2X 6 mL Methylene chloride (DCM)
Equilibration:	2X 6 mL Heptane: Hexane 50:50
Load:	~3 mL supernatant from LLE (procedure step 7)
Wash:	2X 5 mL 10% DCM in Hexane
Dry:	4-5 min
Elute:	2x 6 mL Methylene chloride (DCM) into 10 X 16 mm glass tube

Dry down sample at 30 °C under N_2, then reconstitute the sample tube with 300 µL of 40% ethanol in water mix.

Transfer samples into amber autosampler vial with 300 µL glass insert. Inject on LC/MS/MS system.

LC/MS/MS Conditions

Column: Kinetex® 2.6 µm F5
Dimensions: 30 X 2.1 mm
Part No.: 00A-4723-AN
Mobile Phase: A: 0.1 % Formic Acid in Water
B: 0.1 % Formic Acid in Methanol
Gradient:

Time (min)	% B
0	80
6	80
6.01	100
8	100
8.01	80
9.5	80

Flow Rate: 300 µL/min
Injection Volume: 10-25 µL
Temperature: Ambient
Detection: MS/MS (APCI+) (SCIEX 4500™)
HPLC System: Agilent® 1100 HPLC with Quaternary Pump
Analytes: 1. Vitamin D_2
2. Pre-Vitamin D_3
3. Vitamin D_3
4. Pre-Vitamin D_3

Table 1.
Instrument Parameters

CUR:	20
TEM:	350
GS1:	70
GS2:	0
CAD:	8
NC:	2
DP	60

Table 2.
Mass Transitions

ID	Q1 Mass (Da)	Q3 Mass (Da)	Dwell (msec)	Param	CE	CXP
D_2 1	397.3	69.1	50	CE	20	15
D_2 2*	397.3	106.9	50	CE	20	15
D_3 1*	385.3	259.1	50	CE	21	15
D_3 2	385.3	107.1	50	CE	35	15
D_2-d_3 1	400.2	271.2	50	CE	25	13
D_2-d_3 2*	400.2	68.9	50	CE	28	13
D_3-d_3 1*	388.2	259.1	50	CE	28	13
D_3-d_3 2	388.2	110	50	CE	28	13
Pre-D_2 1	397.3	69	50	CE	37	13
Pre-D_2 2*	397.3	158.7	50	CE	35	13
Pre-D_3 1*	385.2	259.2	50	CE	19	13
Pre-D_3 2	385.2	106.9	50	CE	24	13

* Quantitation mass transition

Table 3.
Assay Recovery

Sample ID	Recovery (%)
Vitamin D_2	38.0
Vitamin D_3	33.7
Pre-Vitamin D_2	30.0
Pre-Vitamin D_3	31.4

Recovery calculation includes pre-treatment, LLE and SPE extractions, resulting in average recovery for each step of near 70% for all analytes.

Table 4.
Accuracy and Precision

	Vitamin D_2	Vitamin D_3	Pre-Vitamin D_2	Pre-Vitamin D_3
Sample ID	**Conc. Found (µg/mL) - QC 1**			
Norminal Conc. (µg/mL)	1	1	0.5	0.5
QC 1 1	1.08	0.979	0.439	0.490
QC 1 2	1.1	1.08	0.483	0.495
QC 1 3	0.936	0.917	0.526	0.514
Mean Conc. Found (µg/mL)	1.039	0.992	0.48	0.500
STDV	0.089	0.082	0.044	0.013
CV%	8.61	8.3	9.01	2.53
Accuracy (%)	103.9	99.2	96.5	99.9
n=	3	3	3	3
Sample ID	**Conc. Found (µg/mL) - QC 2**			
Norminal Conc. (µg/mL)	4	4	2	2
QC 2 1	4.05	4.50	1.75	2.15
QC 2 2	4.31	4.16	1.83	2.25
QC 2 3	4.12	4.11	1.81	2.22
Mean Conc. Found (µg/mL)	4.16	4.26	1.80	2.21
STDV	0.135	0.212	0.042	0.051
CV%	3.23	4.99	2.32	2.33
Accuracy (%)	104	106	89.8	110
n=	3	3	3	3

Figure 2.
Representative of chromatograms of neat solution (500 ng/mL)

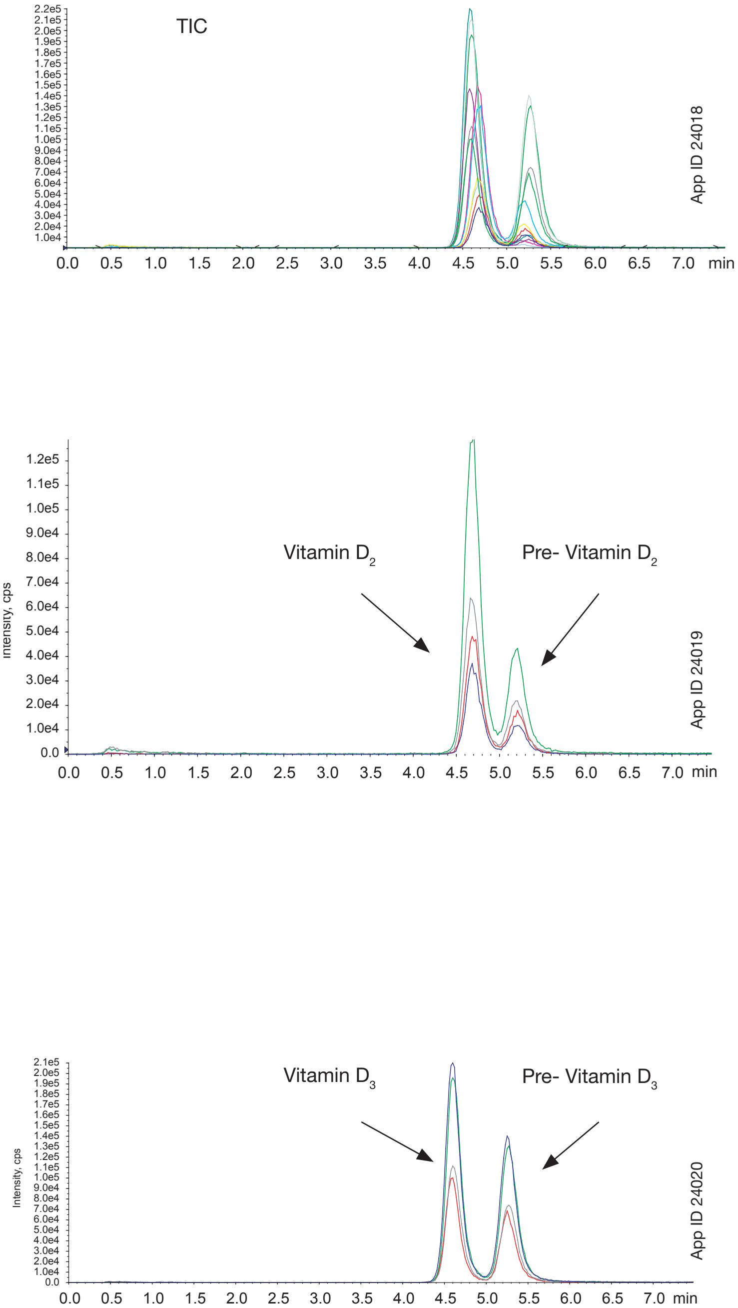


Figure 3.
Representative a chromatogram of STD 1 (0.5/0.25 µg/mL) in matrix

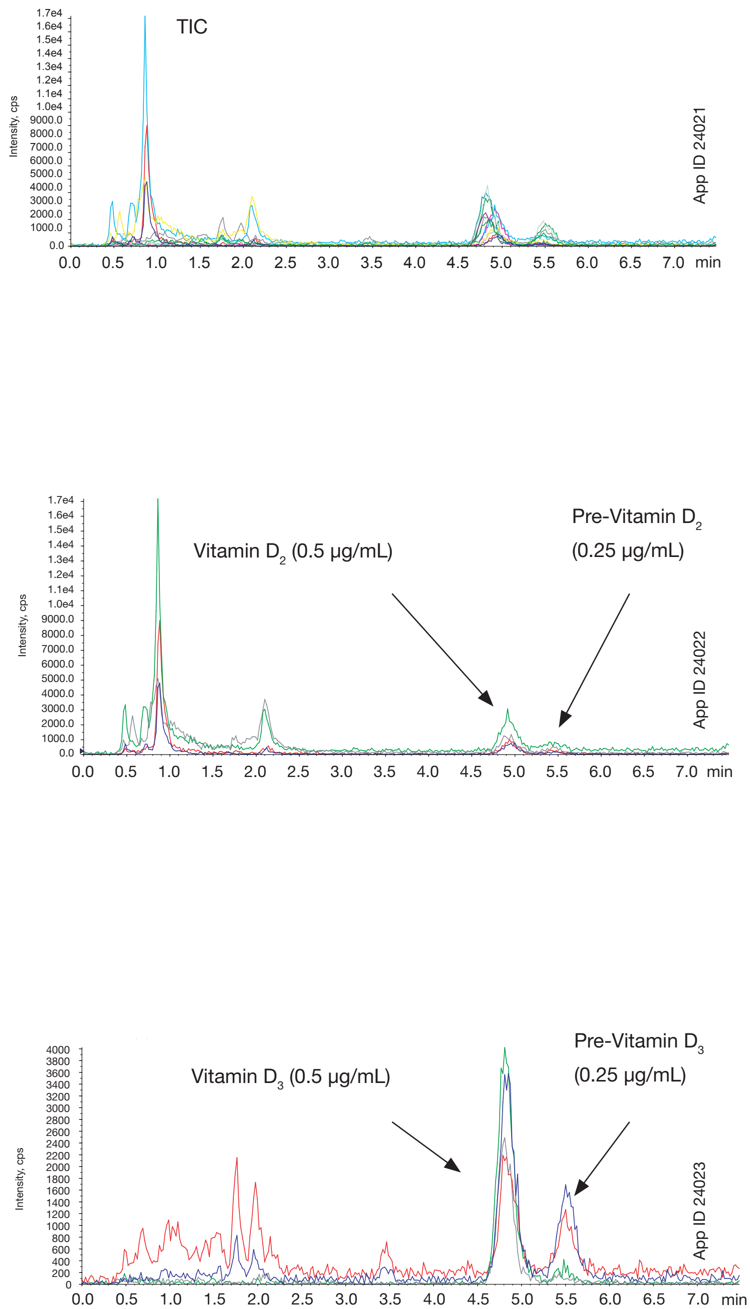
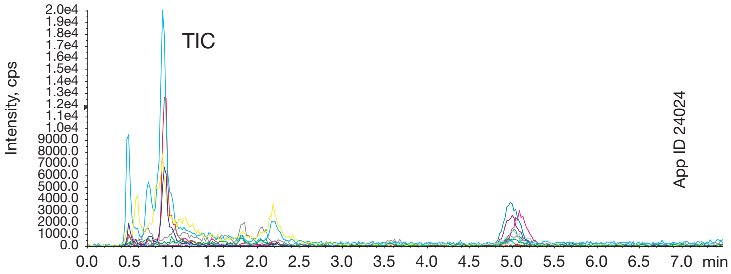
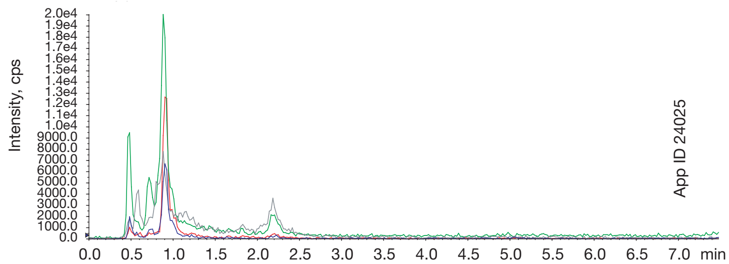


Figure 4.
Representative a chromatogram of Matrix Blank





Note: No Vitamin D_2 or Previtamin D_2

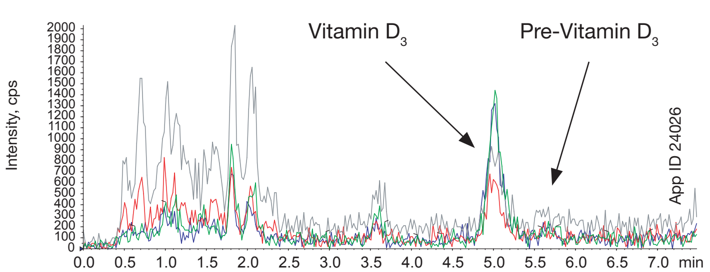
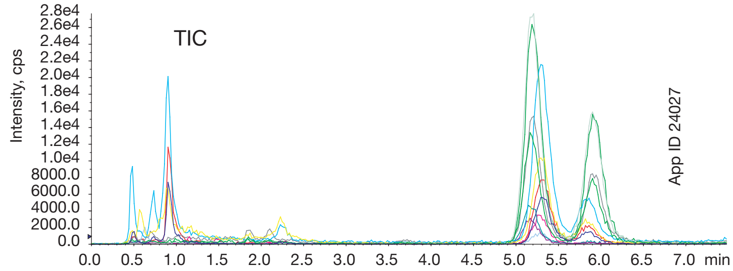
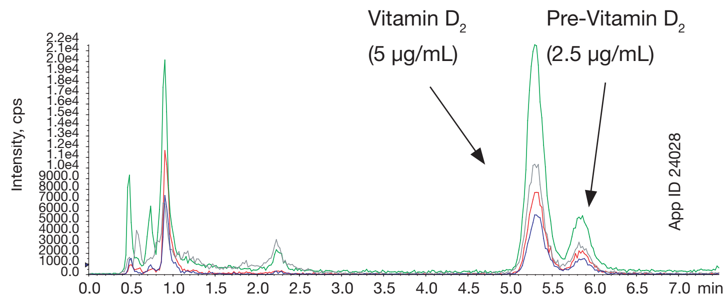


Figure 5.
Representative a chromatogram of STD 3 (5/2.5 µg/mL) in matrix





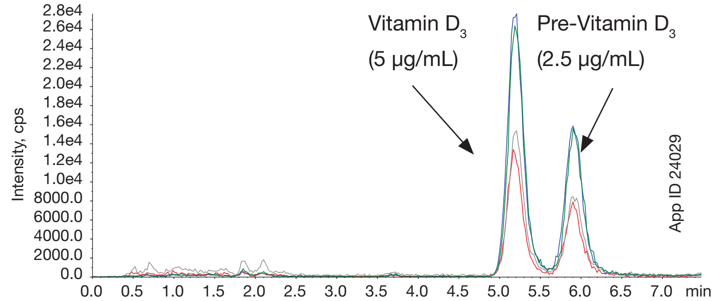
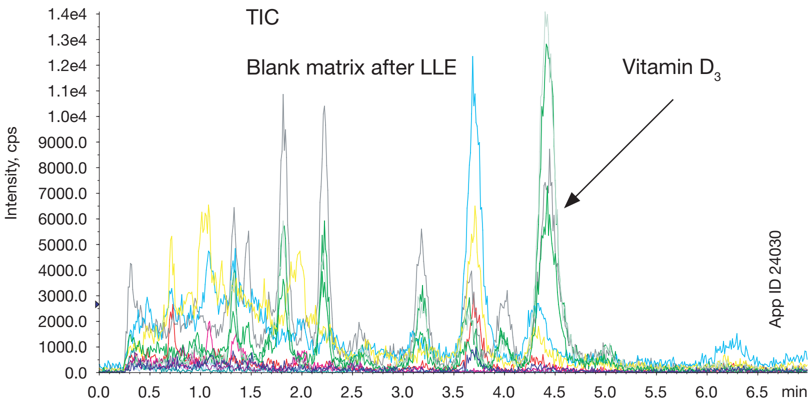


Figure 6.
Minimized matrix effect after SPE extraction



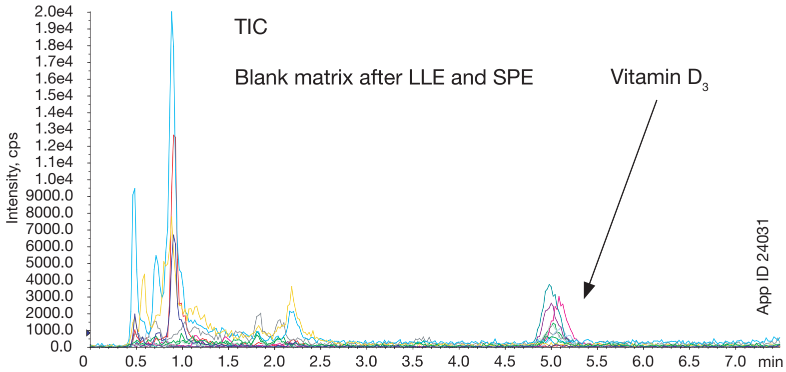


Figure 7.
Interference peaks after analytes

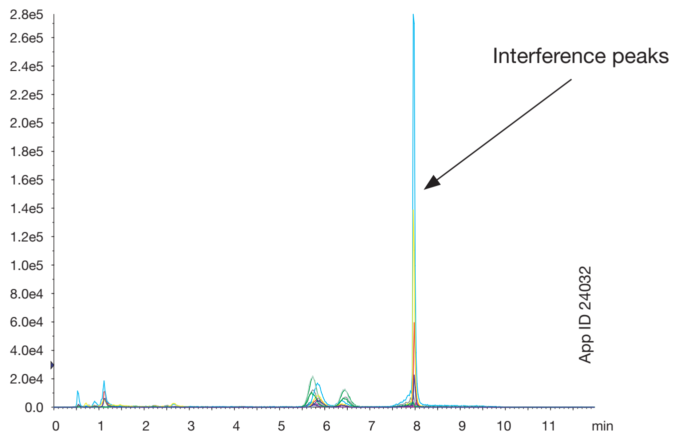
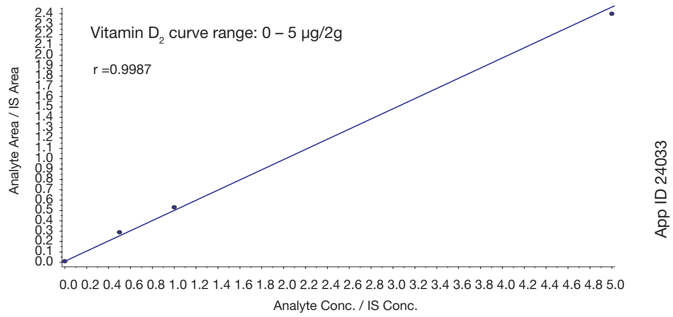


Figure 8.
Representative curve of Vitamin D_2 and Pre-Vitamin D_2



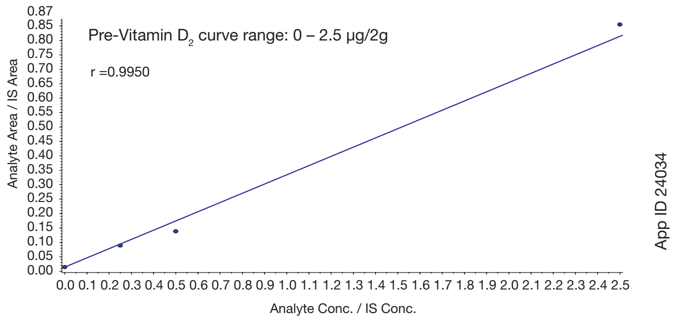
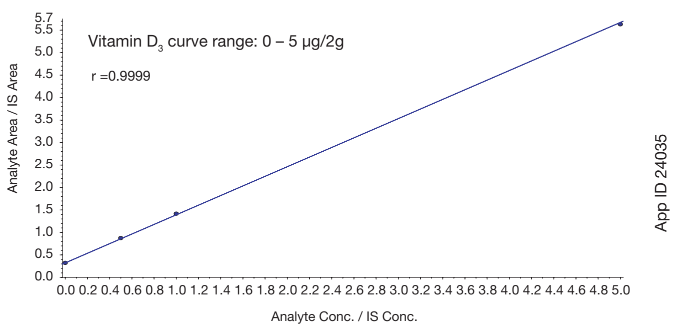


Figure 9.
Representative curve of Vitamin D_3 and Pre-Vitamin D_3



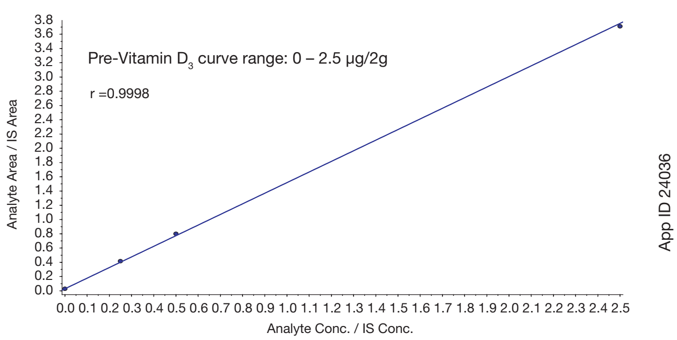
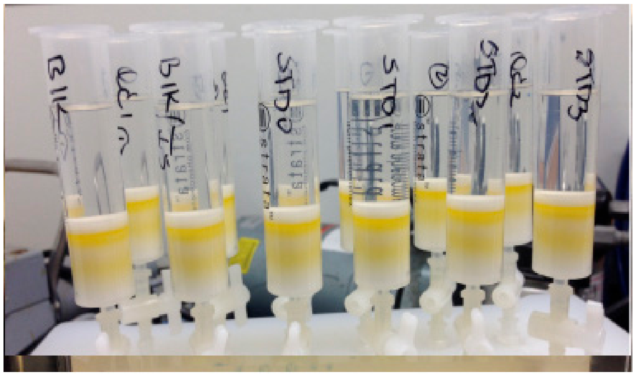
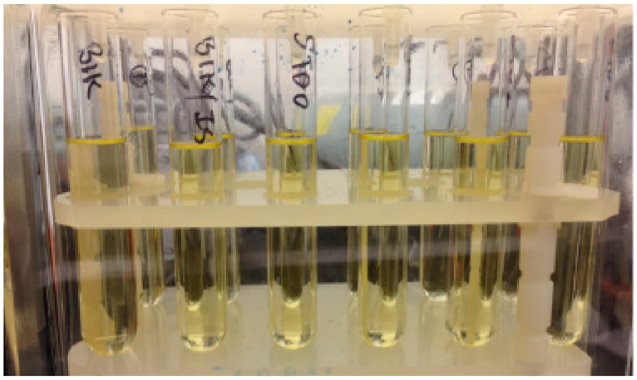


Figure 10.
Lab photos in SPE extraction procedures

Wash step after sample loading



After elution



Results and Discussion

Quantifying Vitamin D in complex food matrices is filled with a range of challenges, from isomer conversion to light sensitivity and saponification and extraction efficiency.

Samples were marginally protected from light with amber vials and dim lighting, and was completed in 1 hour, room temperature saponification was performed to minimize conversion to the pre-D forms for a more accurate quantification. After a double liquid-liquid extraction, a normal phase SPE protocol using Strata® NH_2 cartridge was used to further remove some matrix components (**Figure 6.**).

Near baseline resolution of vitamin D and pre-vitamin D (**Figure 2.**) was achieved with a fast run-time on a short (30 x 2.1mm) Kinetex® 2.6 µm F5, leveraging the polar and aromatic selectivity of the pentafluorophenyl ligand. The mass transitions of the analytes were listed in **Table 2**. The LC gradient was extended an additional two minutes (6.01-8 min in gradient table) with 100% organic solvent to elute some matrix components that might otherwise interfere in subsequent injections (**Figure 7.**).

Quantification was done using a standard addition curve, extrapolating from a back calculated blank sample. Accuracy and precision were evaluated at two QC levels (n=3); accuracy 99.2-106 % with CV % of 3.23-8.61 % for parent compounds of the vitamin D_2/

D_3, and accuracy 89.8-110 % with CV % 2.32-9.01 % for pre-vitamin D_2/D_3 (**Table 4.**). The assay overall recovery was shown in **Table 3**. The dynamic curve range of Vitamin D_2/D_3 is 0 – 5 µg/2g and pre-vitamin D_2/D_3 is 0 -2.5 µg/2 g (**Figures 8 and 9.**). The blank matrix, STD 1 (0.5/0.25 µg/mL) and ULQ (5/2.5 µg/mL) are seen in **Figures 3 – 5.**, respectively.

Conclusion

In this technical note, a quantitative method for the determination of Vitamins D_2 and D_3 and pre-vitamins D_2 and D_3 in dog food was developed with opportunities to explore in a wide range of food and pet food matrices.

Ordering Information

Kinetex® Core-shell Columns

2.6 µm Minibore Columns (mm)				SecurityGuard™ ULTRA Cartridges‡
Phase	50 x 2.1	100 x 2.1	150 x 2.1	3/pk
F5	00B-4723-AN	00D-4723-AN	00F-4723-AN	AJ0-9322
				for 2.1 mm ID

2.6 µm MidBore™ Columns (mm)				SecurityGuard ULTRA Cartridges‡
Phase	50 x 3.0	100 x 3.0	150 x 3.0	3/pk
F5	00B-4723-Y0	00D-4723-Y0	00F-4723-Y0	AJ0-9321
				for 3.0 mm ID

2.6 µm Analytical Columns (mm)				SecurityGuard ULTRA Cartridges‡
Phase	50 x 4.6	100 x 4.6	150 x 4.6	3/pk
F5	00B-4723-E0	00D-4723-E0	00F-4723-E0	AJ0-9320
				for 4.6 mm ID

1.7 µm Minibore Columns (mm)				SecurityGuard ULTRA Cartridges‡
Phase	50 x 2.1	100 x 2.1	150 x 2.1	3/pk
F5	00B-4722-AN	00D-4722-AN	00F-4722-AN	AJ0-9322
				for 2.1 mm ID

‡ SecurityGuard ULTRA cartridges require holder, Part No.: AJ0-9000.

guarantee

If Kinetex core-shell columns do not provide at least an equivalent separation as compared to a competing column of the same phase, return the column with comparative data within 45 days for a FULL REFUND.

Strata® NH_2/WAX

Format	Sorbent Mass	Part Number	Unit
Tube			
	100 mg	8B-S009-EAK	1 mL (100/box)
	200 mg	8B-S009-FBJ	3 mL (50/box)
	500 mg	8B-S009-HBJ	3 mL (50/box)
	500 mg	8B-S009-HCH	6 mL (30/box)
	1 g	8B-S009-JCH	6 mL (30/box)
Giga Tube			
	500 mg	8B-S009-HDG	12 mL (20/box)
	2 g	8B-S009-KDG	12 mL (20/box)
	5 g	8B-S009-LEG	20 mL (20/box)
	10 g	8B-S009-MFF	60 mL (16/box)
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