



Analysis of Vitamin D3 in animal feed by LC-MS/MS

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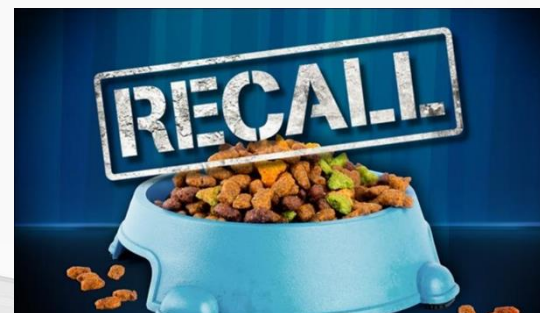
Vitamin D

- ❑ Fat soluble secosteroids
- ❑ Two major forms, vitamin D2 (ergocalciferol) and vitamin D3 (cholecalciferol).
- ❑ An essential nutrient for animals as well as people.
- ❑ VitD3 is the predominant form of VitD used in the manufacturing of pet foods .
- ❑ Vitamin D deficiency/overdoes in feed can cause animal sickness or death.



2018 Dog Food Recalls

- ❑ December 2018, eight brands of dry dog foods were recalled after the FDA said the food could contain toxic levels of vitamin D. The FDA also urged owners to stop using the recalled products.
- ❑ January 2019, Hill's Pet Nutrition notified the FDA about a report of vitamin D toxicity in a dog that ate a canned Hill's dog food and initiated a recall of 25 products.
- ❑ May 2019, Hill's expanded the recall to include 44 varieties.
- ❑ AAFCO recommends between 12.5 and 75 µg/kg vitamin D in dog food



Hill's Prescription Diet and Science Diet Dog Food Recall Expands to Include 44 Varieties

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Recall Updated 5/22/2019

March 20, 2019 — [Hill's Pet Nutrition is expanding its voluntary recall](#) of canned dog food products due to **elevated levels of vitamin D**.

This recall expansion relates to the same vitamin premix that led to [the January 31 voluntary recall previously announced on The Dog Food Advisor website](#).

Update: [Additional expansion announced by the FDA May 20, 2019](#).

Vitamin D, when consumed at very high levels, can lead to **serious health issues** in dogs including **kidney dysfunction**.

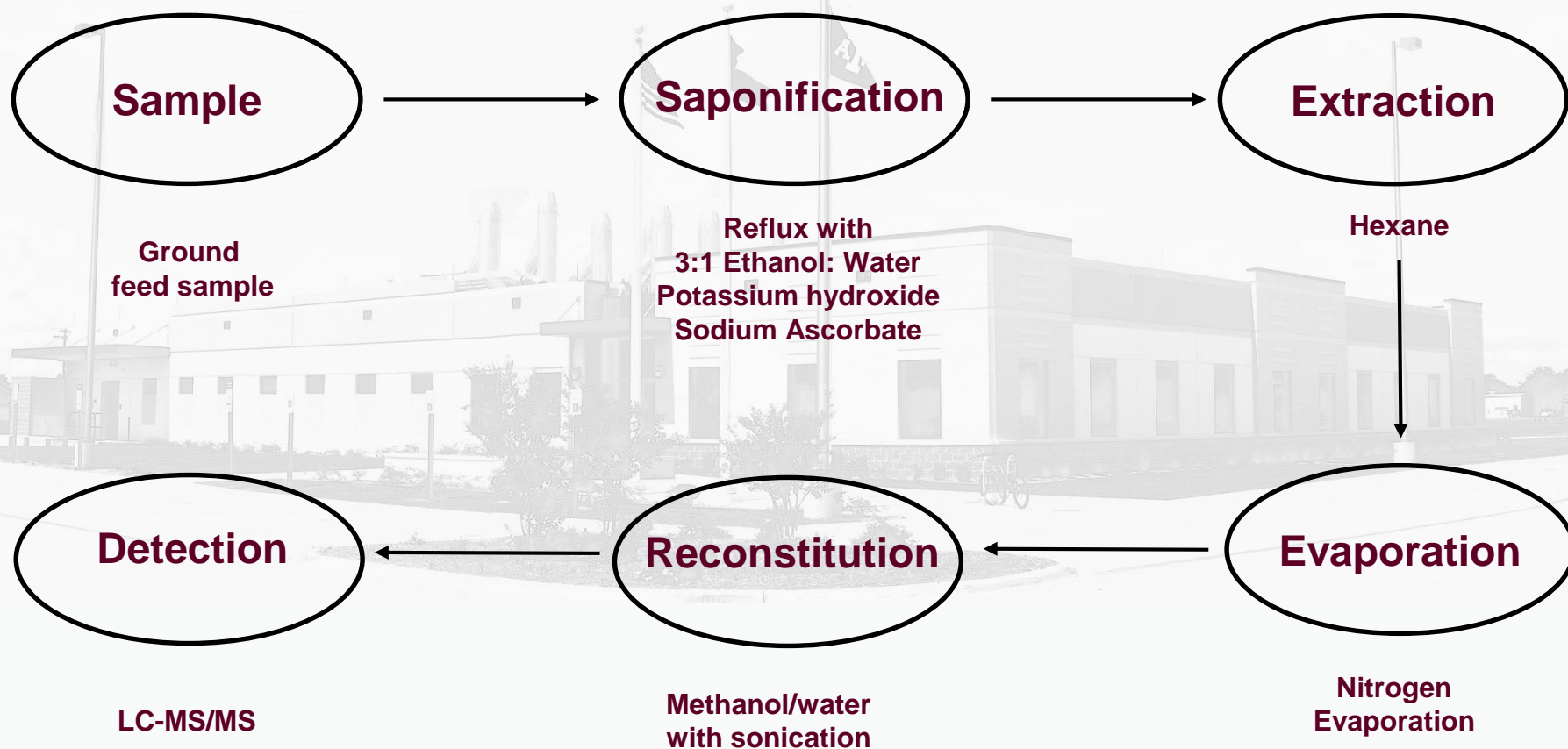


Vitamin D analysis

- ❑ Most studies of vitamin D analysis are done in serum, human foods or supplements. Methods include immunoassay, liquid chromatography coupled with UV or mass spectrometry.
- ❑ Only a few publications regarding analysis in feed matrices.
- ❑ Challenging in analysis of vitamin D: interferences from sample matrices.



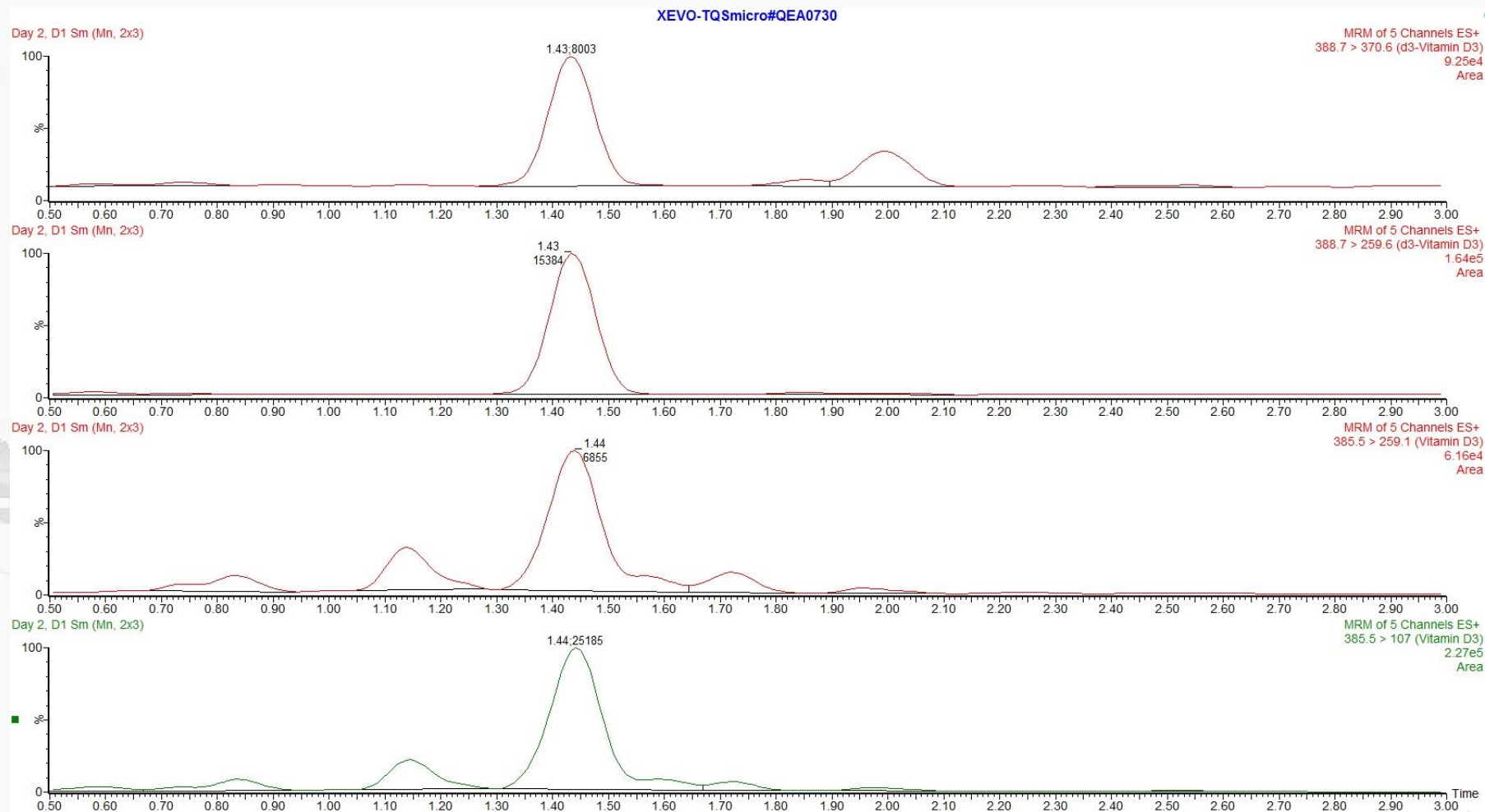
OTSC Method 1 for VitD3 analysis



The method was adapted from AOAC 974.29 "Vitamin A in Feeds, Premixes, a Pet and Human Foods", and OTSC SOP 13003 "Vitamin A in feeds and premixes by HPLC".



MRMs of VitD3 and IS VitD3



LC-MS/MS Chromatography of VitD3 and d3-VitD3 in a work control.



Method 1 Validation

	Spiking level (ng/g)	Recovery(%)	RSD (%)
Spiking recovery	10	110	11.5
	20	107	8.6
	40	105	6.2
Linearity	0.5 - 80 ng/mL		
LOD	1.27 µg/kg		
LOQ	4.23 µg/kg or 0.17 KU/kg		

* AAFCO recommends between 12.5 and 75 µg/kg vitamin D in dog food.



Method Performance

OTSC Method 1 for VitD3 analysis

Results of AAFCO Proficiency Samples

AAFCO ID	Matrix	OTSC results (KU/kg)	AAFCO mean (KU/kg)	Robust SD (KU/kg)	Z score
201523	Milk Replacer	20.0593	21.1560	5.0416	-0.22
201632	Lamb Feed	4.0374	4.2921	1.5147	-0.17
201723	Milk Replacer	8.7787	9.3532	3.4732	-0.17
201724	Beef Feed	5.4983	5.9667	0.8729	-0.54
201728	Dairy Beef Feed	4.7469	4.1383	0.9887	0.62
201822	Swine Feed	3.7649	3.1710	0.8811	0.67
201823	Pig feed	3.1932	3.6590	0.6064	-0.77
201825	Dry Cat Food	2.6284	3.4650	1.1236	-0.74
201827	Lamb Feed	4.8749	4.1363	1.1245	0.66
201829	Milk Replacer	12.3705	11.3640	2.8455	0.35
201832	Chicken Starter	5.1459	5.2490	0.9191	-0.11
201921	Equine Feed	4.5305	4.9333	0.5685	-0.71

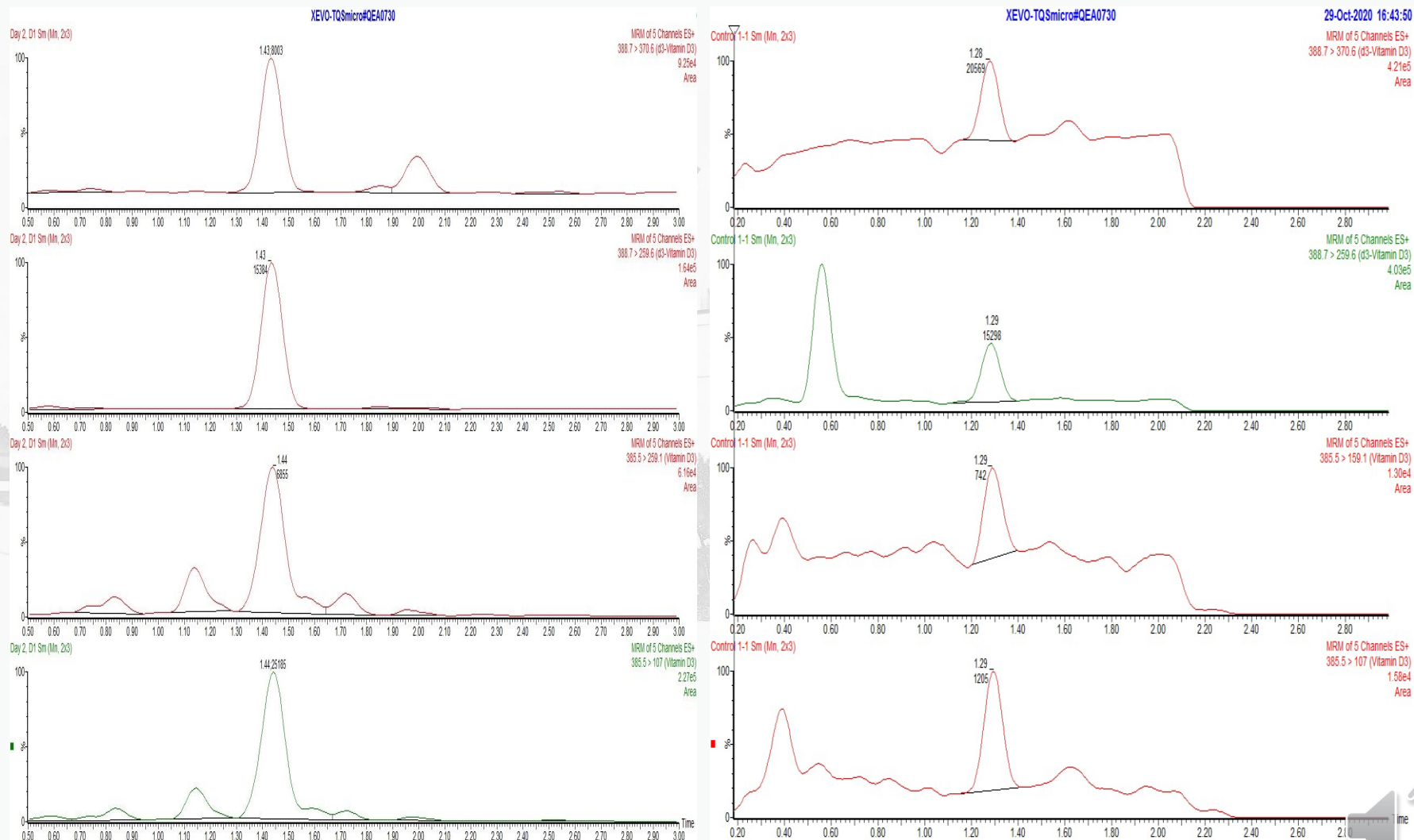


Issues of OTSC Method 1 for VitD3

- ❑ Resolution and signal intensity of VitD3 drops after ~ 1000 injections.
- ❑ Consequently, samples containing low level of VitD may not be quantitated; the results of replicates are not consistent. More replicates have to be run which causes longer turnaround time.
- ❑ Cost of sample testing will increase due to replacement of LC columns and extra replicates.

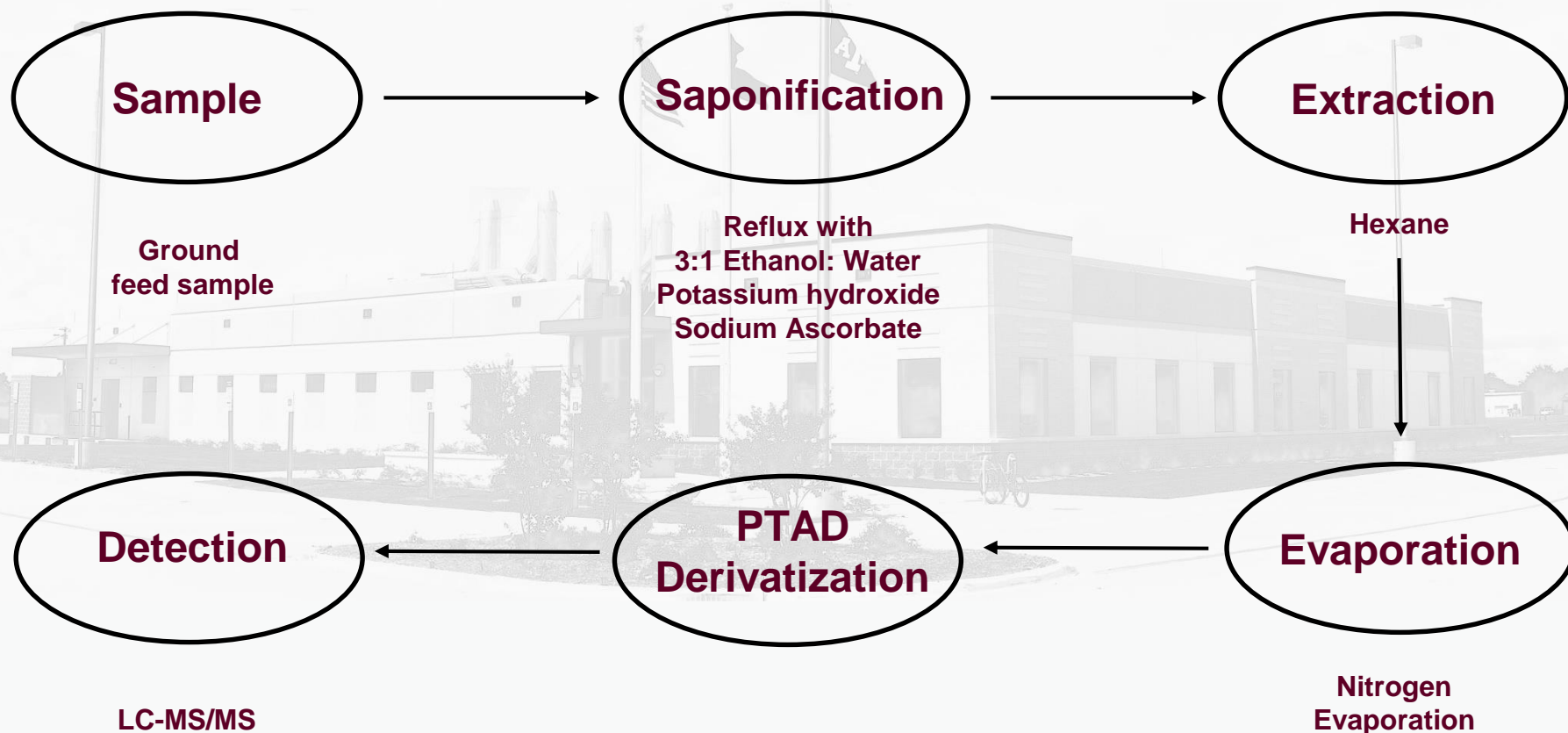


Comparison of before and after ~ 1000 injections



LC-MS/MS Chromatography of VitD3 and d3-VitD3 in work control.

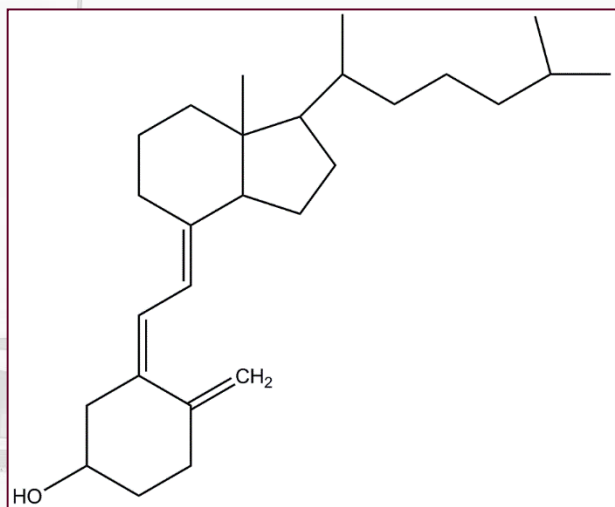
OTSC Method 2: VitD3 Method with PTAD derivatization



The method was adapted from AOAC OMA 2016.05 "Analysis of Vitamin D2 and Vitamin D3 in Fortified Milk Powders, Infant Formulas, and Adult/Pediatric Nutritional Formulas Liquid Chromatography–Tandem Mass Spectrometry" and Waters Application note 720006064en, "Determination of Vitamin D and Previtamin D in Food Products".

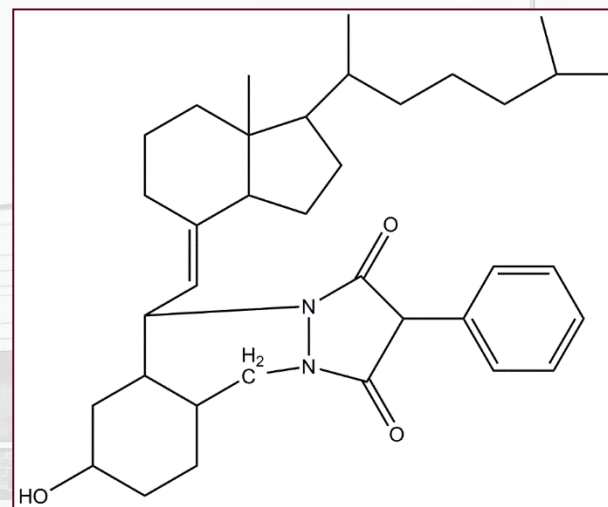


PTAD Derivatization



Vitamin D3

+ PTAD

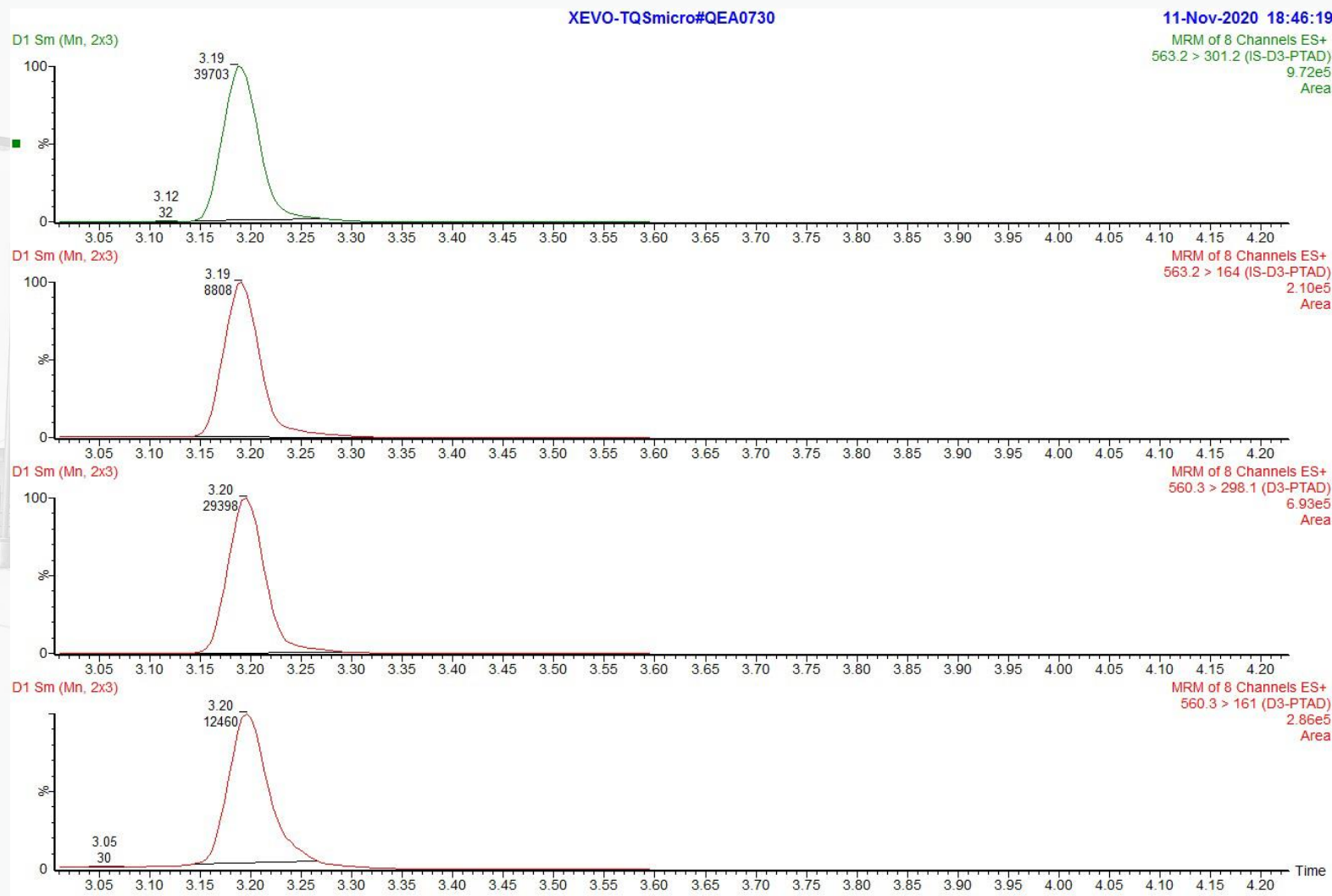


**Vitamin D3-PTAD
Derivative**

PTAD: 4-phenyl-1,2,4-triazoline-3,5-dione



MRMs of VitD3-PTAD and IS VitD3-PTAD



LC-MS/MS Chromatography of VitD3-PTAD and d3-VitD3-PTAD in work control.



Comparison of Method 1 and 2

Index	Unit: KU/kg	Method 2	Method 1	Difference	Ranked % Differences		
1	Control 1	1.53	1.49	2.32%		-27.09%	To test for a high end outlier use:
2	R21-118109	0.42	0.32	28.52%		-26.10%	The test value for the data point with the >diff is:
3	R21-118110	0.57	0.53	6.34%		-20.67%	Based upon this test, this result is:
4	R21-118114	0.49	0.46	7.87%		-20.15%	
5	R21-118115	0.28	0.37	-27.09%	not an outlier	-19.29%	To test for a low end outlier use:
6	Control 2	1.25	1.52	-20.15%		-13.23%	The test value for the data point with the <diff is:
7	R21-123099 P2	0.63	0.82	-26.10%		-9.67%	Based upon this test, this result is:
8	R21-106128	0.19	0.12	41.66%	not an outlier	-3.04%	
9	Control 1	1.50	1.26	17.57%		-2.91%	T-test performed using complete set of data
10	N2021-001948	1.26	1.21	3.54%		-2.19%	
11	R21-103080 P2	0.69	0.85	-20.67%		-2.05%	The critical value for data points is:
12	Control 2	1.50	1.39	7.76%		-0.71%	
13	R21-118143 P2	0.43	0.38	12.20%		1.60%	To test for a high end outlier use:
14	R21-118146 P2	0.56	0.48	14.56%		1.72%	The test value for the data pt. with the 2nd > diff is:
15	R21-121104 P3	0.82	0.85	-3.04%		2.32%	Based upon this test, this result is:
16	R21-121104 P4	0.55	0.54	1.72%		3.54%	
17	R21-108120	0.25	0.26	-2.19%		6.34%	To test for a low end outlier use:
18	R21-118137	16.21	17.86	-9.67%		6.83%	The test value for the data pt. with 2nd least diff is:
19	R21-116073	1.16	1.03	11.95%		7.76%	Based upon this test, this result is:
20	Control 1	1.64	1.53	6.83%		7.87%	
21	Control 2	1.54	1.57	-2.05%		8.10%	Sample # 21-10612 is not an outlier
22	R21-116074	1.81	1.86	-2.91%		11.95%	Sample # 21-11811 is not an outlier
23	R21-116081	0.28	0.28	1.60%		12.20%	
24	R21-123109 P2	11.78	13.45	-13.23%		14.56%	
25	R21-123110 P2	12.34	14.98	-19.29%		17.57%	
26	R21-106128 P3	0.18	0.14	23.46%		23.46%	
27	R21-123118	0.95	0.88	8.10%		28.52%	Paired t-test (All Data): P = 0.11679243
28	R21-106128 P4	0.22	0.22	-0.71%		41.66%	Mean Difference (All Data) = 0.20074345



Method Performance

OTSC Method 2 for VitD3 with PTAD Derivatization

Results of AAFCO Proficiency Samples

AAFCO ID	Matrix	AAFCO mean (KIU/kg)	AAFCO SD (KIU/kg)	Result of new method (KIU/Kg)	Z score
202022	Horse feed	4.307	1.806	3.544	-0.42
202022	Horse feed	4.307	1.806	4.424	0.06
202023	Dry cat feed	1.988	0.781	2.033	0.06
202023	Dry cat feed	1.988	0.781	1.902	-0.11
202029	Chicken Feed, Medicated	3.676	0.9227	3.612	-0.07
202029	Chicken Feed, Medicated	3.676	0.9227	3.715	0.04



Preliminary Validation Data of Method 2

	Spiking level (ng/g)	Recovery(%)	RSD (%)
Spiking recovery	10	-	-
	20	-	-
	40	-	-
Linearity	0.5 - 80 ng/mL		
LOD of vitD3	0.5 µg/kg		
LOQ of vitD3	1.67 µg/kg (method 1 is 4.23 µg/kg)		



Summary

- ❑ Two LC-MS/MS methods were developed for VitD3 analysis in feed matrices.
- ❑ Method 1 can be used for regulatory sample analysis but sensitivities and robustness may drop after long-term run.
- ❑ Method 2 using derivatization by PTAD can provide better sensitivity and has less interference from sample matrices than method 1 which may solve the issues existing in method 1.
- ❑ Method 2 will be expanded to Vitamin D2 analysis in feed matrices.



Acknowledgement

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Thank You for Your Attention!

