Single Laboratory Validation for Vitamin D_2 and D_3 Analysis in Infant Formula and Adult Nutritionals: First Action 2011.04

Darryl Sullivan
Covance Laboratories, Madison, WI

Introduction

The purpose of this report is to document a single laboratory validation performed for the AOAC First Action Method **2011.11** for total vitamin D_2 and D_3 in infant formula and adult/pediatric nutritional formula. The SPIFAN (stakeholder panel on infant formula and adult nutritionals) test material kit was **used to determine that the method meets the standard method performance requirements (SMPRs; 1) for linearity, limit of quantification (LOQ), limit of detection (LOD), repeatability, and method accuracy. (AUTHOR: OKAY AS CHANGED?)**

Method

AOAC First Action Method **2011.11** was followed, without modification, as previously published (2).

(AUTHOR: DESCRIBE ANY MODIFICATIONS MADE TO METHOD AS PREVIOUSLY PUBLISHED.)

(AUTHOR: PLEASE CITE REFERENCE 4 AND 5 IN THE TEXT)

Materials

Samples

Determination of total vitamin D_2 and vitamin D_3 was conducted for the matrices included in the SPIFAN test material kit. The matrixes are listed in Table 1. **AUTHOR: IN TABLE 1 PLEASE CLARIFY WHAT IS MEANT BY "TARGET VALUE" AND HOW THIS RELATES TO ANALYTICAL RANGE. ALSO, NOTE THE ASTERISK REFERS TO mcg WHEREAS THE TABLE USES μg.**

All powdered samples with the exception of NIST SRM 1849a were reconstituted at approximately 25 g of material and diluted with approximately 250 mL of de-ionized water prior to sampling.

Validation Protocol

SLV guidelines.—SPIFAN SLV recommended guidelines were followed (3).

AUTHOR: PROVIDE A SENTENCE OR TWO TO DESCRIBE THE FOLLOWING:

System suitability.—

Linearity/calibration fit.—

LOD/LOQ.—

Precision studies.—

Accuracy (trueness).—

Reference sample.—

Statistical Analysis/Calculations

AUTHOR: PROVIDE A SENTENCE OR TWO TO DESCRIBE STATISTICAL ANALYSIS/CALCULATIONS

Validation Results

Specificity

Specificity was determined by analyzing the five placebo samples supplied with the SPIFAN test material kit. All placebo analyses show no levels of vitamin D_3 present and the mass spectra were free of chromatographic interferences. The placebo samples also showed no vitamin D_2 present, however, each placebo sample showed some degree of chromatographic interference that inhibited the methods ability to accurately fortify and determine recoveries.

Linearity

Five standards were prepared over a range of 0.2 IU/mL to 200 IU/mL. The peak responses were plotted against concentrations and regression analysis performed. The correlation coefficient (r^2) of all standard curves generated during this validation exceeds the minimum requirement of ≥0.998.

Accuracy

Accuracy was determined by fortifying the placebo samples at 50 and 100% of the typical level of vitamin D_3 found in a powdered infant formula. For this these experiments, a nominal level of 400 IU/100 grams was used to determine the amount of analyte added to each placebo sample. These recovery experiments were performed by two different analysts over multiple days. In addition, NIST SRM 1849a was analyzed during the course of this validation. Table 2 contains recovery results for both analysts on the five placebo samples.

The percent recovered ranged from a low of 89.4% to a high of 110%. The percent relative standard deviations (RSD) ranged from a low of 2.0% to a high of 6.7%. The overall mean recovery is 101% with a RSD of 4.2%. With the exception of one recovery value at 89.4%, all data meets the criteria specified in the SMPR.

Results on NIST SRM 1849a can be found in Table 3 under the precision section. The mean value of 11.7 mcg/100g (n = 32), falls within the certified range of 9.4–12.8 μg/100g. With the exception of one result (8.88 μg/100g), all values fall

Corresponding author's email: darryl.sullivan@covance.com

Table 1: SPIFAN Single Laboratory Validation (SLV) Test Materials Kit Information

Category	Sample Name	Container Size (g)	Lot No. Identification	Target Value*	
				IU/100g	µg/100g
Fortified	Infant Formula Powder Partially Hydrolyzed Milk Based	360	1172572116	300	7.50
	Infant Formula Powder Partially Hydrolyzed Soy Based	360	117257661Z	300	7.50
	Infant Elemental Powder	250	00403RF00	381	9.53
	Infant Formula Powder Milk Based	360	D04HTCVV09	Not Provided	Not Provided
	Infant Formula Powder Soy Based	730	E29JVL05	Not Provided	Not Provided
	Infant Formula RTF, Milk Based	60	SPIFAN Control Milk Form	60	1.50
	Child Formula Powder**	250	00412RF00	410	10.3
	Child Formula Powder**	200	00413RF00	Not Provided	Not Provided
	Adult Nutritional Powder Milk Protein Based	400	11750017V3	128	3.20
	Adult Nutritional Powder Low Fat	250	00394RF00	128	3.20
	Adult Nutritional RTF, High Protein	250	00414RF00	44.5	1.11
	Adult Nutritional RTF, High Fat	250	00406RF00	10.2	0.255
	NIST SRM 1849a	10	CLC10-b	376-512	9.40-12.8
Placebo	Infant Elemental Powder	250	00402RF00	Not Provided	Not Provided
	Infant Formula RTF, Milk Based	60	SPIFAN Blank Milk Form	Not Provided	Not Provided
	Child Formula Powder	250	00411RF00	Not Provided	Not Provided
	Adult Nutritional RTF, High Protein	250	00415RF00	Not Provided	Not Provided
	Adult Nutritional RTF, High Fat	250	00407RF00	Not Provided	Not Provided

* 1 mcg vitamin D = 40 IU
** This sample showed no quantifiable levels of vitamin D2 or D3.
RTF = Ready to Feed

Table 2: Accuracy Results (%) Vitamin D3

Sample	50% of Theoretical			100% of Theoretical		
	A1	A2		A1	A2	
	Day 1	Day 1	Day 2	Day 1	Day 1	Day 2
Infant Elemental Powder	97.2	102	89.4	98.0	101	102
	97.9	102	105	99.1	101	104
Infant Formula RTF, Milk Based	100	105	97.0	101	104	97.3
	101	102	97.7	101	100	102
Child Formula Powder	96.9	108	109	102	NR*	107
	100	107	103	98.5	104	101
Adult Nutritional RTF, High Protein	101	108	108	99.4	105	102
	98.8	103	108	101	102	105
Adult Nutritional RTF, High Fat	98.3	97.2	110	96.7	98.9	100
	94.7	96.9	110	90.5	98.6	102
Mean (n=10)	98.6	103.1	103.7	98.7	101.6*	102.2
SD	2.00	4.00	6.93	3.32	2.31*	2.68
%RSD	2.0	3.9	6.7	3.4	2.3*	2.6
	Overall Mean (n=59) 101 SD 4.28 %RSD 4.2					

A1 = Analyst 1
A2 = Analyst 2
NR* = No result due to laboratory error; therefore, the statistics were calculated using n=9.
RTF = Ready to Feed

Table 3. Precision Results for NIST (µg/100g) Vitamin D_3. Certified mean = 11.1 µg/100g. Certified range = 9.4–12.8 µg/100g

Statistics	SRM 1849a	
	A1	A2
Individual (µg/100g)	11.4	11.8
	12.0	11.2
	11.5	13.0
	11.1	10.6
	10.9	12.6
	11.3	12.7
	10.7	12.4
	11.1	12.3
	12.6	12.3
	12.4	12.4
	11.6	12.6
	12.4	8.88
	12.4	11.6
	12.4	10.1
	11.5	11.4
	11.3	11.5
Mean (n=16)	11.7	11.7
SD	0.616	1.099
%RSD	5.3	9.4
Mean (n=32)	11.7	
SD	0.877	
%RSD	7.5	

A1 = Analyst 1
A2 = Analyst 2

within the certified range. The RSD obtained utilizing all results is 7.5%. This is within the limits set in the SMPR.

Precision

Precision was determined by analyzing 12 different matrixes on multiple days for each product, including the NIST SRM 1849a. Two different analysts performed duplicate analyses on each of three days on the 11 fortified products. In addition, each analyst performed duplicate analyses on the NIST SRM 1849a sample one each of 8 days. Precision data for all samples can be found in Tables 3 and 4. Precision data was not included for the Child Formula Powders (Lot No.00412RF00 and 00413RF00) as no vitamin D_2 or D_3 was detected in these two samples.

All precision data obtained for vitamin D_3 meets the criteria specified in the SMPR. Percent relative standard deviations ranged from a low of 3.6% to a high of 11.0%.

Reproducibility

Reproducibility was determined in the precision experiments as two analysts conducted the testing over multiple days and involved different lots of reagents made fresh each analysis day. In addition, two different instruments were utilized during the course of this validation.

Table 4: Precision Results for Infant Formula and Adult/Pediatric Nutritional Formula (µg/100g) Vitamin D3

Statistics	Infant Formula Powder Partially Hydrolyzed Milk Based		Infant Formula Powder Partially Hydrolyzed Soy Based		Infant Elemental Powder		Infant Formula Powder Milk Based		Infant Formula Powder Soy Based	
	A1	A2	A1	A2	A1	A2	A1	A2	A1	A2
Individual (µg/100g)	9.19	9.27	11.5	11.0	8.50	8.66	9.66	9.39	9.40	9.55
	9.61	8.63	11.5	11.6	8.90	8.76	9.81	9.46	9.74	9.55
	10.1	9.12	11.3	11.1	8.33	8.68	10.8	10.8	10.5	10.6
	9.21	9.21	10.8	10.9	8.81	8.54	10.9	10.6	10.9	10.4
	10.2	9.89	12.3	11.9	9.32	9.12	10.6	10.3	10.8	10.3
	9.97	9.88	12.3	12.1	9.28	9.16	10.5	10.5	10.4	10.3
Mean (n=6)	9.7133	9.3333	11.617	11.433	8.8567	8.8200	10.378	10.175	10.290	10.117
SD	0.4450	0.4833	0.5879	0.5046	0.4006	0.2580	0.5202	0.6033	0.5972	0.4524
%RSD	4.6	5.2	5.1	4.4	4.5	2.9	5.0	5.9	5.8	4.5
Mean (n=12)	9.5233		11.525		8.8383		10.277		10.203	
SD	0.4854		0.5311		0.3218		0.5475		0.5131	
%RSD	5.1		4.6		3.6		5.3		5.0	
Statistics	**Infant Formula RTF, Milk Based**		**Adult Nutritional Powder Milk Protein Based**		**Adult Nutritional Powder Low Fat**		**Adult Nutritional RTF, High Protein**		**Adult Nutritional RTF, High Fat**	
	A1	A2	A1	A2	A1	A2	A1	A2	A1	A2
Individual (µg/100g)	1.18	1.30	3.32	3.75	3.74	3.51	1.04	1.03	1.25	1.28
	1.20	1.22	3.30	3.67	3.86	3.35	1.05	1.03	1.26	1.30
	1.35	1.32	2.75	3.48	3.73	3.53	1.18	1.26	1.44	1.33
	1.36	1.34	2.63	3.24	3.50	3.65	1.18	1.18	1.34	1.33
	1.37	1.29	3.00	3.10	4.26	3.73	1.15	1.14	1.32	1.34
	1.36	1.31	2.90	3.55	4.23	3.95	1.19	1.13	1.35	1.36
Mean (n=6)	1.3033	1.2967	2.9833	3.4650	3.8867	3.6200	1.1317	1.1283	1.3267	1.3233
SD	0.08824	0.04131	0.2829	0.2508	0.3012	0.2074	0.06853	0.08886	0.06919	0.02875
%RSD	6.8	3.2	9.5	7.2	7.7	5.7	6.1	7.9	5.2	2.2
Mean (n=12)	1.3000		3.2242		3.7533		1.1300		1.3250	
SD	0.06578		0.3581		0.2831		0.07568		0.05054	
%RSD	5.1		11.0		7.5		6.7		3.8	

A1 = Analyst 1
A2 = Analyst 2
RTF = Ready to Feed

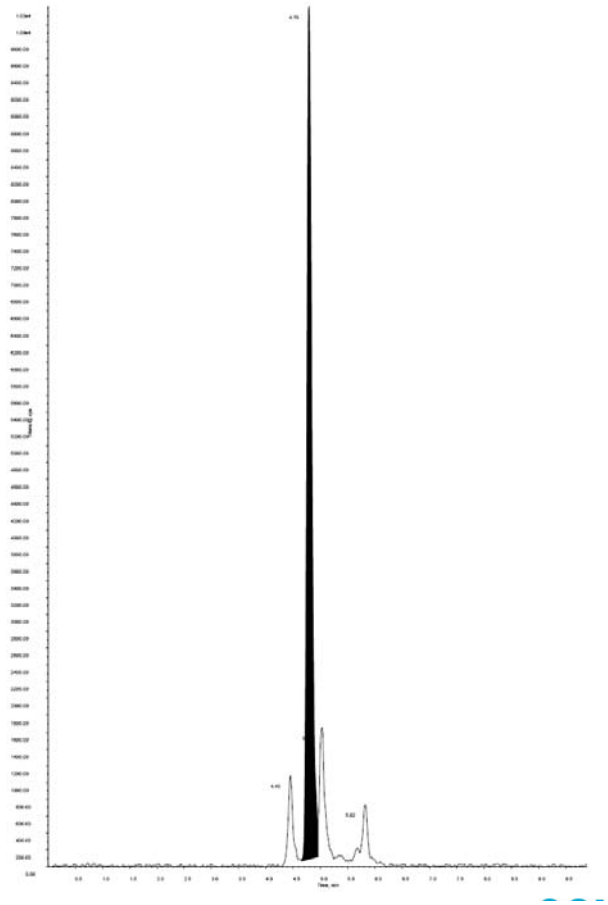

Figure 1. Infant formula powder partially hydrolyzed milk based-fortified. *Note*: **Previtamin D3 elutes at ~4.45 min, Vitamin D3 elutes at ~4.79 min.**

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The LOD and LOQ for the method were evaluated by dilution of a vitamin D standard to a known concentration, which for the purpose of this study was diluted to a level of 0.2 IU/mL. Using this low standard, the LOD was calculated at 3 times the signal to noise ratio, while the LOQ was calculated at 10 times the signal to noise ratio. The LOD and LOQ for Vitamin D_3 in the Ready to Feed form are 2.4 IU/100g and 8.1 IU/100g, respectively. The LOD and LOQ for Vitamin D_2 in the Ready to Feed form was determined but not included in the report at this time due to difficulties analyzing for this form on the vitamin.

Summary

The method met all criteria specified in the SMPR for precision for vitamin D_3. All accuracy data for vitamin D_3 met the criteria specified in the SMPR with the exception of one value obtained at the 50% fortification level on the Infant Elemental Powder. The limit of quantitation was estimated to be 0.06 IU/gram. The limit of detection was estimated to be 0.02 IU/gram. Acceptable linearity was demonstrated over a range of 0.2 IU/mL to 200 IU/mL.

The analyses of the fortified placebo samples for vitamin D_2 showed chromatographic interferences. While the mass spectra showed no vitamin D present in these five placebo samples, chromatographic separation did not demonstrate the necessary resolution of these interferences that co-eluted with the added vitamin D_2. The recoveries did not meet the specifications outlined in the SMPR. In addition, analyses of the Child Formula Powder showed no vitamin D_2 present in this matrix.

This method is found to be suitable for the analysis of vitamin D_3 in infant formula and adult nutritional formula. Further evaluation of the method would include: improved selectivity for vitamin D_2, resolution of possible chromatographic interferences, precision and accuracy would be necessary to facilitate a rugged and robust procedure for the analysis of vitamin D_2 in infant formula and adult nutritional products.

References

(1) AOAC SMPR **2011.004** (2012) *J. AOAC Int.* **95**, 292
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(3) SPIFAN SLV Recommended Guidelines (2012) *J. AOAC Int.* **95**, XXX–XXX
(4) Huang, M., & Winters, D. (2011) *J. AOAC Int*. **94**, 211–223
(5) Huang, M., LaLuzerne, P., Winters, D., & Sullivan, D. (2009) *J. AOAC Int.* **92**, 1327–1335