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NIST/NIH Vitamin D Metabolites Quality Assurance Program Report of Participant Results: Winter 2014 Comparability Study (Exercise 8)

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National Institute of Standards and Technology
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ABSTRACT

The National Institute of Standards and Technology (NIST) has established a Vitamin D Metabolites Quality Assurance Program (VitDQAP) in collaboration with the National Institutes of Health (NIH) Office of Dietary Supplements. Participants in the eighth exercise of this program, the Winter 2014 Comparability Study, were asked to use the methodology of their choice to measure concentrations of 25-hydroxyvitamin D in pooled human serum control and study materials distributed by NIST. The study materials consisted of Standard Reference Material (SRM) 909c Human Serum and VitDQAP-III (a material designed for the VitDQAP). SRM 968d Fat-Soluble Vitamins, Carotenoids, and Cholesterol in Human Serum Level 1 was provided as a control material. Participants provided their data to NIST, where it was compiled and evaluated for trueness relative to the NIST value and concordance within the participant community. A report of results was provided to all participants of the study, and laboratories were identified by code numbers known only to them. The results from this eighth study are reported along with a summary of the analytical methods used.

OVERVIEW OF THE WINTER 2014 COMPARABILITY STUDY

For the Winter 2014 comparability study of the collaborative National Institute of Standards and Technology and National Institutes of Health (NIST/NIH) Vitamin D Metabolites Quality Assurance Program (VitDQAP), human serum control and study materials were distributed to participants for evaluation. Standard Reference Material (SRM) 968d Fat-Soluble Vitamins, Carotenoids, and Cholesterol in Human Serum Level 1 (SRM 968d L1) was provided as a control material for assay validation. For SRM 968d L1 (Control), the participants were provided the NIST target values within the data reporting sheet so that they could qualify their methods prior to analyzing the study samples. The study materials consisted of two vials, each containing a sample of pooled human serum. In this study, Vial A was VitDQAP-III, and Vial B was SRM 909c Frozen Human Serum, both of which contain endogenous levels of the vitamin D metabolites. Participants were asked to determine 25-hydroxyvitamin D in each of the human serum control and study samples. Individual concentration values for 25-hydroxyvitamin D_3 ($25(OH)D_3$), 25-hydroxyvitamin D_2 ($25(OH)D_2$), and 3-epi-25-hydroxyvitamin D_3 (3-epi-$25(OH)D_3$) were requested along with a total concentration of 25-hydroxyvitamin D ($25(OH)D_{Total} = 25(OH)D_2 + 25(OH)D_3$).

There were a total of 58 participants and 71 datasets (12 participants provided data from two or more methods) in the Winter 2014 comparability study. Twenty-eight of the datasets originated from immunoassay (IA) techniques, including 19 from chemiluminescence immunoassay (CLIA), four from enzyme immunoassay (EIA), and five from radioimmunoassay (RIA). **Appendix A-1** summarizes the IA methods used by the participants. Forty-three of the datasets originated from liquid chromatographic (LC) methods; of those, 37 were from LC with tandem mass spectrometric detection ($LC\text{-}MS^n$), and six were from LC with ultraviolet absorbance detection (LC-UV). A summary of the LC methods used by the participants may be found in **Appendices A-2** and **A-3**.

The raw data received from all participants are summarized in **Appendix B**. The IA methods do not distinguish between $25(OH)D_3$ and $25(OH)D_2$, and IA participants reported single values for $25(OH)D_{Total}$ in the control and study materials. The LC methods measure the vitamin D metabolites separately, and the majority of the LC participants reported values for $25(OH)D_3$ and $25(OH)D_2$ in addition to $25(OH)D_{Total}$; eleven LC participants also reported results for 3-epi-$25(OH)D_3$ in at least one of the study materials.

Appendix B also provides the summarized NIST results for each of the serum materials. A detailed description of the NIST methods is provided in the next section of this report.

SUMMARY OF THE NIST METHODS USED TO EVALUATE THE CONTROL AND STUDY MATERIALS

NIST used isotope dilution LC-MS/MS (ID-LC-MS/MS) [1] or a combination of ID-LC-MS/MS and ID-LC-MS [2] procedures to determine the vitamin D metabolites ($25(OH)D_3$, $25(OH)D_2$, and 3-epi-$25(OH)D_3$) in the control and study materials evaluated in this comparability study. The ID-LC-MS/MS approach is a reference measurement procedure (RMP) for $25(OH)D_3$ and $25(OH)D_2$ that is recognized by the Joint Committee for Traceability in Laboratory Medicine (JCTLM).

For SRM 909c (Vial B), NIST determined $25(OH)D_3$ using both ID-LC-MS and the ID-LC-MS/MS RMP. The result for $25(OH)D_3$ is a combination of results from the two methods and is a certified value. A NIST certified value is a value for which NIST has the highest confidence in its accuracy in that all known or suspected sources of bias have been investigated or taken into account [3] and are reflected in the expanded uncertainty (*U*). Note that even though the NIST value for $25(OH)D_3$ is certified, this value does not currently appear on the Certificate of Analysis for SRM 909c. The $25(OH)D_2$ concentration was below the quantitation limit (≈ 0.5 ng/mL) in SRM 909c, and thus the NIST value for $25(OH)D_{Total}$ includes only the certified result for $25(OH)D_3$.

The NIST values for $25(OH)D_3$, $25(OH)D_2$, and 3-epi-$25(OH)D_3$ in VitDQAP-III (Vial A) and SRM 968d L1 (Control) were determined solely with the ID-LC-MS/MS method. For VitDQAP-III (Vial A), the NIST values for $25(OH)D_3$, $25(OH)D_2$, and 3-epi-$25(OH)D_3$ are reported with expanded uncertainties that incorporate components for measurement variability and measurement uncertainty associated with the density of the materials and the purity of the reference standards. In addition, the measurements include an additional 1% type B uncertainty for unknown systematic errors, which is consistent with the practice used at NIST for clinical measurements [1]. For SRM 968d L1 (Control), the NIST values for $25(OH)D_3$ and 3-epi-$25(OH)D_3$ are reported as described for VitDQAP-III (Vial A), but the value for $25(OH)D_2$ was well below the limit of quantitation and was estimated to be 0.1 ng/mL based on one measurement.

The NIST values for $25(OH)D_{Total}$ in VitDQAP-III (Vial A) and SRM 968d L1 (Control) are the sum of the individual values for $25(OH)D_3$ and $25(OH)D_2$, and the expanded uncertainties (*U*) incorporate the measurement uncertainties for the two analytes.

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[1] Tai, S. S.-C.; Bedner, M.; Phinney, K.W.; *Anal. Chem.* **2010** *82*, 1942-1948.
[2] Bedner, M.; Phinney, K.W.; *J. Chromatogr. A* **2012** *1240*, 132–139.
[3] May, W.; Parris, R.; Beck II, C.; Fassett, J.; Greenberg, R.; Guenther, F.; Kramer, G.; Wise, S.; Gills, T.; Colbert, J.; Gettings, R.; MacDonald, B.; NIST Special Publication 260-136 **2000**; http://www.nist.gov/srm/publications.cfm

WINTER 2014 COMPARABILITY STUDY RESULTS AND DISCUSSION

Results for 25(OH)D_{Total}

A summary of the individual participant data for total 25-hydroxyvitamin D (25(OH)D_{Total}) in VitDQAP-III (Vial A), SRM 909c (Vial B), and SRM 968d L1 (Control) is provided in **Table 1**.

The community results are summarized at the bottom of **Table 1** for all reported methods, the IA methods only, the LC methods only, and the LC-MS^n methods only. The community results include the total number of quantitative values reported (N), the median value for each analyte, the MADe (the median absolute deviation estimate, a robust estimate of the standard deviation), and the percent coefficient of variation (CV %).

Table 1 also presents the NIST results for 25(OH)D_{Total} in the control and the two study materials.

Table 1. Summary of participant and NIST results for 25(OH)D_{Total} (ng/mL) in VitDQAP-III (Vial A), SRM 909c (Vial B), and SRM 968d L1 (Control).

		VitDQAP-III	SRM 909c	SRM 968d L1
Lab	Method	Vial A	Vial B	Control
017	CLIA	31.0	16.2	13.9
026	LC-MS/MS	33.7	21.7	13.4
030a	RIA	40.5	24.8	15.3
030b	LC-MS/MS	24.5	23.9	13.2
056a	LC-MS/MS	34.5	20.3	12.8
056b	LC-MS/MS	34.0	20.3	12.7
060	LC-MS/MS	33.4	22.0	13.0
110	LC-UV	21.8	79.6	12.8
116	LC-MS/MS	34.2	20.7	12.8
119	LC-MS/MS	39.5	27.3	15.6
139	LC-UV	24.7	51.9	13.1
150	LC-MS/MS	31.2	17.7	12.1
161	CLIA	30.6	14.4	11.8
180	RIA	29.1	19.5	14.9
185a	LC-MS/MS	38.4	36.4	17.3
185b	CLIA	16.4	33.3	14.5
187	LC-MS/MS	30.3	19.0	13.1
188	CLIA	36.1	18.4	14.1
189	LC-UV	27.6	38.2	16.5
193	EIA	33.9	22.3	18.6
194	LC-MS/MS	33.0	15.8	13.0
196	CLIA	30.7	15.1	14.1
197	LC-MS/MS	34.2	22.0	13.1
198a	LC-MS/MS	30.8	18.5	12.6
198c	CLIA	29.1	19.7	8.3
199	LC-MS/MS	38.2	29.6	13.7
200	RIA	27.7	18.0	13.8
204a	CLIA	27.5	14.0	12.2
204b	LC-MS/MS	33.8	20.2	12.8
209	LC-MS/MS	33.9	22.9	12.5
210a	RIA	31.4	17.9	12.4
210b	CLIA	38.7	25.0	9.1
211	LC-MS/MS	36.0	18.3	13.6
212	LC-MS/MS	35.7	22.3	12.3
213a	CLIA	30.4	19.4	6.5
213b	EIA	26.3	17.3	12.9
214a	RIA	27.5	31.3	11.5
214b	CLIA	31.2	14.4	13.0
214c	LC-MS/MS	31.7	20.5	12.2
215	LC-MS/MS	37.2	21.2	13.6
216	LC-MS/MS	32.9	21.9	12.8
218a	CLIA	33.7	14.5	14.5
218b	LC-MS/MS	31.8	23.8	12.2
220	LC-MS/MS	36.0	23.0	15.0
221a	LC-MS/MS	36.7	31.7	15.0
221b	LC-UV	37.5	36.3	10.7
222	CLIA	35.2	23.5	11.1
225	LC-MS/MS	31.0	14.8	10.4
228a	LC-MS/MS	35.4	21.5	12.7
231	LC-UV	30.2	30.8	8.5
241	LC-MS/MS	28.7	17.2	12.3
242	LC-MS/MS	31.1	18.7	12.2
243a	LC-UV	27.8	19.6	11.5
243b	LC-MS/MS	28.2	21.1	12.4
244	LC-MS/MS	33.0	25.0	13.0
247a	CLIA	31.9	16.7	11.7
247b	EIA	31.0	22.3	15.7
249	LC-MS/MS	33.3	21.7	12.6
251	LC-MS/MS	34.4	21.6	n/r
253	LC-MS/MS	35.5	21.0	13.1
255	LC-MS/MS	32.9	20.2	12.8
256	CLIA	30.4	17.3	18.5
257	CLIA	31.9	22.4	6.2
258	CLIA	32.8	17.5	11.4
259	LC-MS/MS	26.1	25.5	14.8
260	EIA	32.0	22.0	18.2
261	CLIA	32.3	16.2	10.3
262	CLIA	34.1	52.1	16.5
263	CLIA	28.7	13.4	12.5
264	LC-MS/MS	41.4	29.0	11.4
265	LC-MS/MS	39.0	24.0	14.0

n/r = not reported or not determined

		VitDQAP-III	SRM 909c	SRM 968d L1
		Vial A	Vial B	Control
All methods	N	71	71	70
	Median	32.3	21.2	12.8
	MADe	3.1	4.3	1.4
	CV%	9.5	20	11
IA methods	N	28	28	28
	Median	31.1	18.2	13.0
	MADe	3.0	5.6	2.3
	CV%	9.5	31	18
LC methods	N	43	43	42
	Median	33.4	21.7	12.8
	MADe	3.6	3.3	0.7
	CV%	11	15	5.8
LC-MS^n	N	37	37	36
	Median	33.8	21.6	12.8
	MADe	3.1	2.1	0.7
	CV%	9.0	9.6	5.3
	NIST Value	32.7	20.7	12.5
	U	0.7	0.7	0.4

For all participant datasets, the single reported values for 25(OH)D_{Total} in VitDQAP-III (Vial A), SRM 909c (Vial B), and SRM 968d L1 (Control) are plotted in **Figure 1**, **Figure 2**, and **Figure 3**, respectively. The results from immunoassay methods are displayed with open dark blue circles (○), and the results from the LC-based methods are displayed with open light blue circles (○). The results from the individual methods were sorted separately, as indicated by the x-axis labels.

From the single reported values for all datasets for a given technique (IA or LC), the consensus median and the consensus variability (2 × MADe) were determined. For both of the major techniques (IA or LC) in each figure, the solid lines (——) and (——) represent the consensus median, and the dashed lines (- - - - -) and (- - - - -) represent the expanded uncertainty interval (2 × MADe). The laboratories with results that fall between the two dashed lines are within the consensus variability area for their technique (IA or LC).

For the IA data for SRM 909c (Vial B), the consensus variability based on MADe is an overestimation of the expanded uncertainty interval about the median (**Figure 2**). The large MADe is a result of the non-Gaussian data distribution that contributes to a relatively wide distribution of the central 50 % of the data.

The red lines (——) in each figure (**Figures 1 – 3**) represent the NIST value and its associated uncertainty (i.e., value ± *U*). NIST has confidence that the "true" value for each material lies within this interval. When these lines are not within the consensus ranges for each technique (IA or LC), then there may be method bias.

Specific results for each of the three study materials are summarized below. Note that the assessment is based on the actual reported values, not the lines and symbols, which have been enlarged to show detail and the laboratory number.

VitDQAP-III (Vial A): **Figure 1**

- For the IA results, three reported values are outside of the consensus variability range (two CLIA, one RIA).
- For the LC results, five reported values are outside of the consensus variability range (three LC-MS^n, two LC-UV).
- The consensus median value for the IA results is slightly lower than the NIST expanded uncertainty range (red lines).
- The consensus median value for the LC results is comparable to the NIST expanded uncertainty range (red lines).
- The NIST expanded uncertainty range (red lines) falls within the consensus variability ranges for both IA and LC.

SRM 909c (Vial B): **Figure 2**

- For the IA results, the data appear to be non-normally distributed, and the consensus variability is not well-described with a MADe estimation as described above.
- For the IA results, three reported values are outside the consensus variability range (two CLIA, one RIA).
- For the LC results, 10 reported values are outside the consensus variability range, including five from LC-MS^n and five of the six LC-UV results.
- The consensus median value for the IA results is slightly lower than the NIST expanded uncertainty range (red lines).
- The consensus median value for the LC results is comparable with the NIST expanded uncertainty range (red lines).
- The NIST expanded uncertainty range (red lines) falls within the consensus variability ranges for both IA and LC.

SRM 968d L1 (Control): **Figure 3**

- For the IA results, six reported values are outside of the consensus variability range (four CLIA, two EIA).
- For the LC results, nine reported values are outside of the consensus variability range (six LC-MS^n, three LC-UV).
- The consensus median values for both the IA and LC results are comparable with the NIST expanded uncertainty range (red lines).
- The NIST expanded uncertainty range (red lines) falls within the consensus variability range for both IA and LC.

Figure 1. Participant and NIST results for 25(OH)D_{Total} in VitDQAP-III (Vial A) as determined by immunoassay (CLIA, EIA, and RIA) and LC (LC-MS^n and LC-UV) methods.

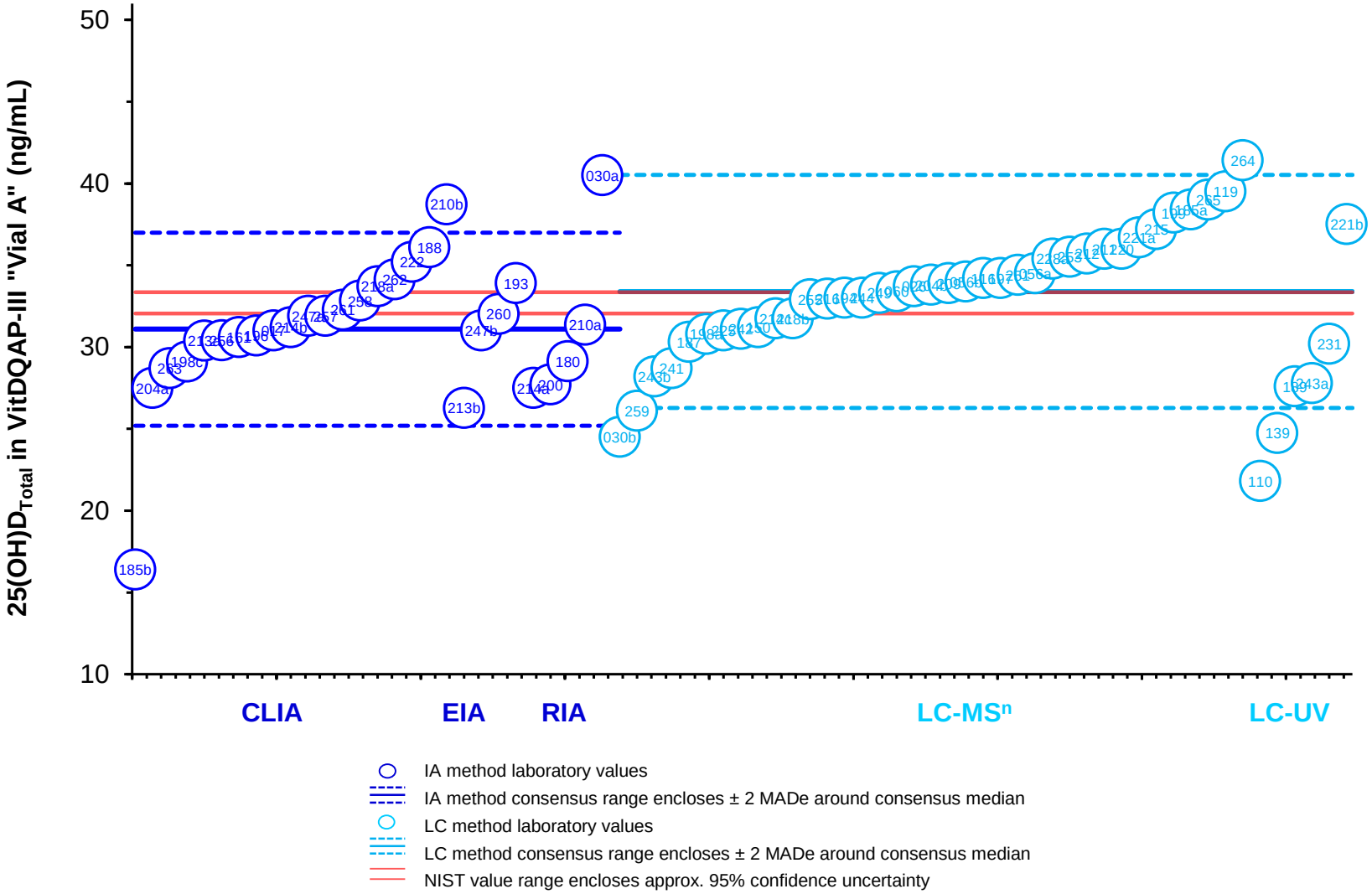


Figure 2. Participant and NIST results for 25(OH)D_{Total} in SRM 909c (Vial B) as determined by immunoassay (CLIA, EIA, and RIA) and LC (LC-MS^n and LC-UV) methods.

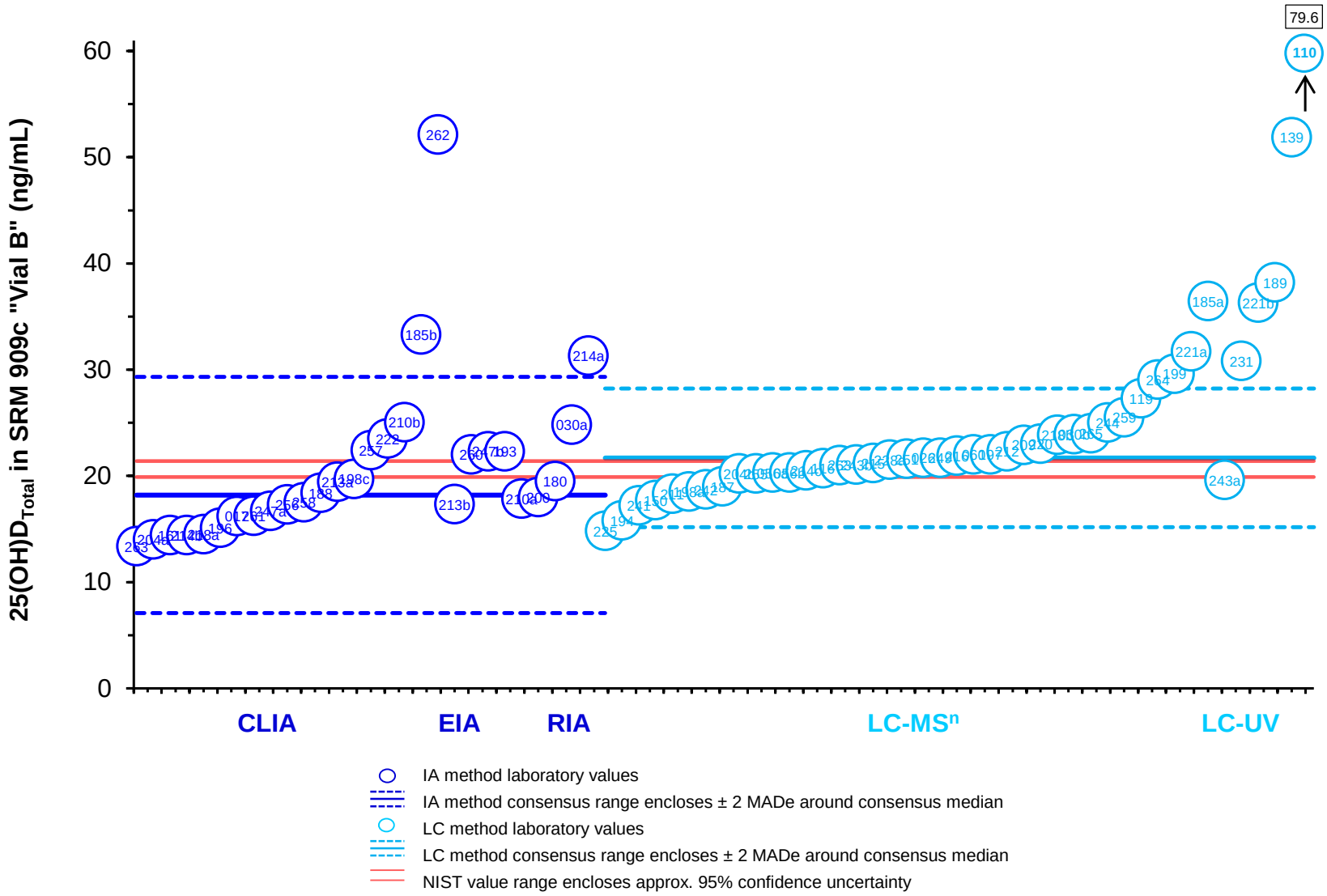


Figure 3. Participant and NIST results for 25(OH)D_{Total} in SRM 968d Level 1 (Control) as determined by immunoassay (CLIA, EIA, and RIA) and LC (LC-MS^n and LC-UV) methods.

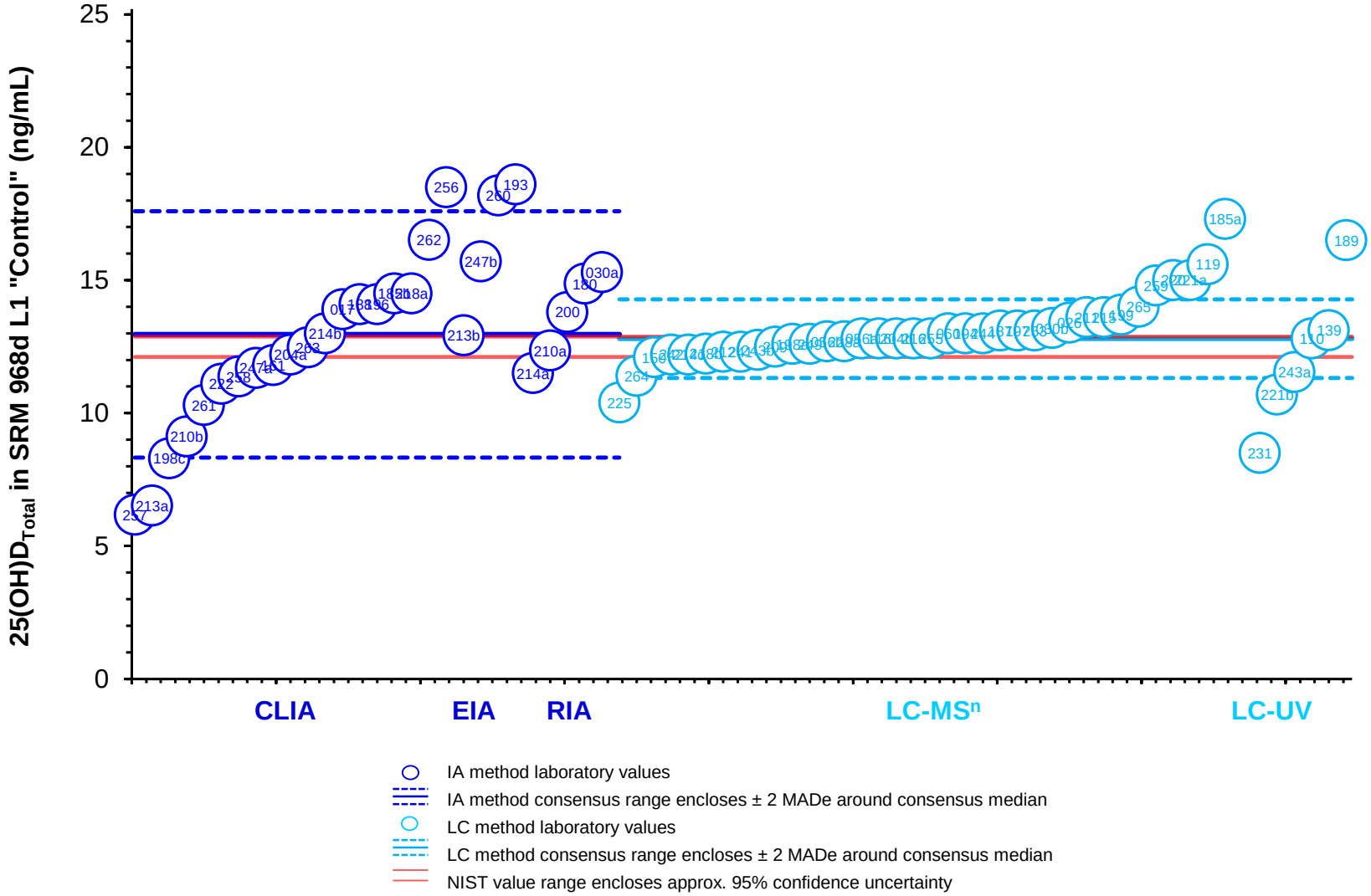


Figure 4 presents direct graphical comparisons of the 25(OH)D_{Total} results for a) VitDQAP-III (Vial A) and SRM 909c (Vial B), and b) SRM 909c (Vial B) and SRM 968d L1 (Control). In each plot, there are two blue consensus boxes, one for IA methods and one for LC methods (as indicated). Laboratory results that are within the consensus range for both study materials are within the blue consensus boxes. Conversely, laboratory results that fall outside of (or on the edge of) either of the consensus boxes are not included in the consensus ranges and are highlighted with their laboratory code numbers. In each plot, the NIST values for the materials are denoted with a red diamond symbol (◆), and the Youden line (y=x) centered on the NIST value is illustrated by a red line (——) across the magnitude of the y-axis and x-axis, respectively.

Specific results as assessed from the Youden comparison plots are summarized below.

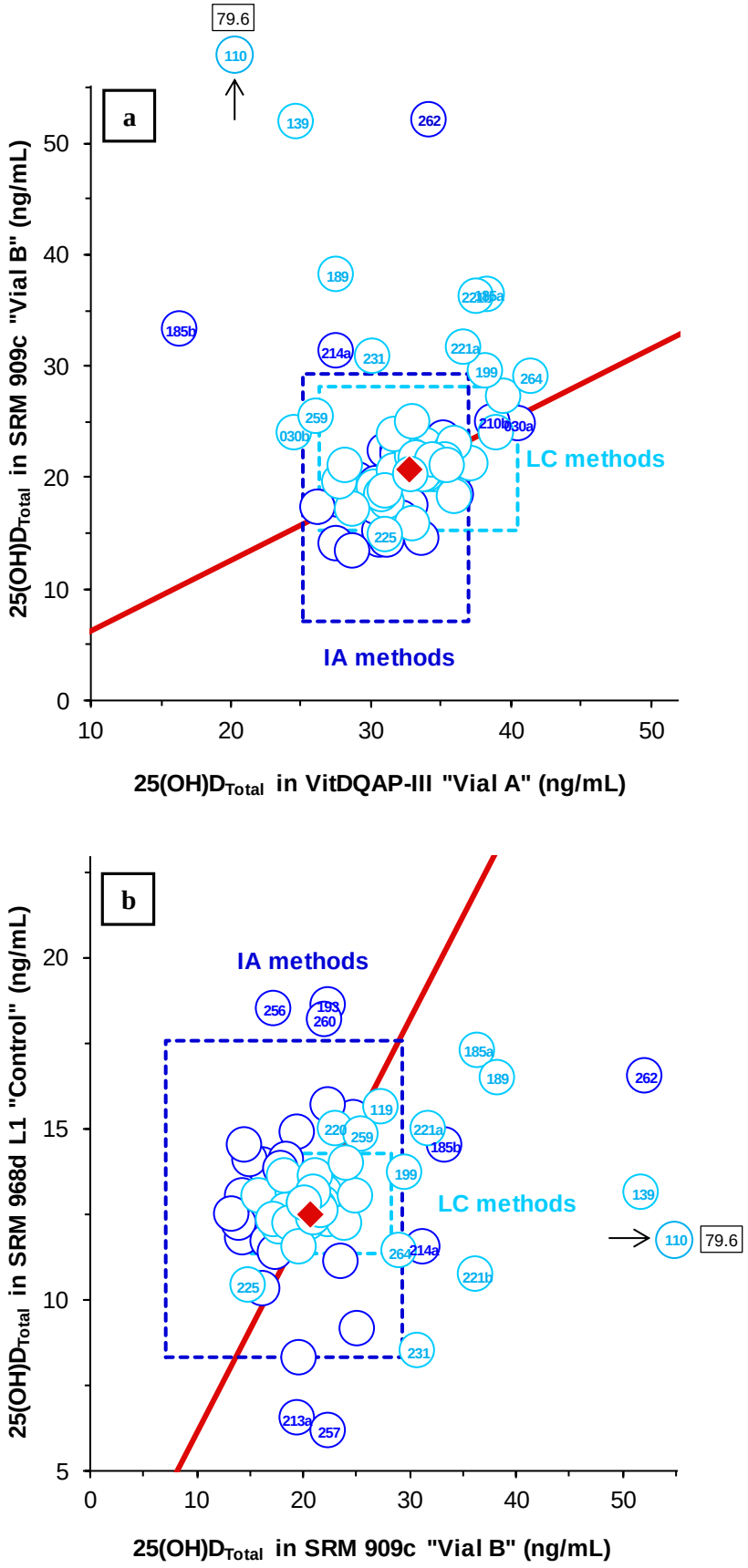
VitDQAP-III (Vial A) and SRM 909c (Vial B): **Figure 4 a**

- IA results that are not included in the consensus ranges include numbers 030a, 185b, 210b, 214a, and 262
- LC results that are not included in the consensus ranges include numbers 030b, 110, 139, 185a, 189, 199, 221a, 221b, 225, 231, 259, and 264
- The Youden line runs through the center of both the IA and LC consensus boxes, illustrating that both the IA and LC results are in agreement with each other and with the NIST results for these materials.

SRM 909c (Vial B) and SRM 968d L1 (Control): **Figure 4 b**

- IA results that are not included in the consensus ranges include numbers 185b, 193, 213a, 214a, 256, 257, 260, and 262
- LC results that are not included in the consensus ranges include numbers 110, 119, 139, 185a, 189, 199, 220, 221a, 221b, 225, 231, 259, and 264
- The Youden line runs through the center of both the IA and LC consensus boxes, illustrating that both the IA and LC results are in agreement with each other and with the NIST results for these materials.

Figure 4. Youden comparison plot of the results for 25(OH)D_{Total} in a) VitDQAP-III (Vial A) and SRM 909c (Vial B) and b) 909c (Vial B) and SRM 968d L1 (Control) for all methods.



Discussion of Results for 25(OH)D_{Total}

The consensus variability of 11 % (all methods) for SRM 968d L1 (Control) is higher than the 7 % CV % obtained for this material in the last comparability study (Summer 2013), but it is consistent with participant performance for other materials containing predominantly 25(OH)D_3 that were evaluated in previous comparability studies of the VitDQAP.

The VitDQAP-III material (Vial A) is different from SRM 909c (Vial B) and SRM 968d L1 (Control) because it contains significant amounts of both 25(OH)D_2 and 25(OH)D_3 that contribute to 25(OH)D_{Total}. The metabolite 25(OH)D_2 represents 20 % of the 25(OH)D_{Total} concentration in VitDQAP-III (Vial A), based on the NIST values of 6.5 ng/mL ± 0.2 ng/mL for 25(OH)D_2 and 32.7 ng/mL ± 0.7 ng/mL for 25(OH)D_{Total}. When materials containing appreciable amounts of 25(OH)D_2 were evaluated in previous comparability studies of the VitDQAP, the results were bimodal, with the IA methods underrepresenting the 25(OH)D_{Total} concentration. In addition, the CV % (all methods) for those materials was relatively large (approximately 17 % to 28 %). The results for VitDQAP-III (Vial A), do not reveal those same trends: the CV % (all methods) is relatively low (9.5 %), and the IA method results overlap almost completely with the LC results. The difference in the observed results for the VitDQAP-III material (Vial A) is most likely attributable to both the relatively high concentration of 25(OH)D_{Total} and the relatively low concentration of 25(OH)D_2, which causes any effect from the 25(OH)D_2 contribution to be lost in the overall variability of the results. However, the median IA result for VitDQAP-III (Vial A) is biased 5 % and 7 % lower than the NIST and the LC median results, respectively, which could be partially attributable to the nonequivalent response of many IA methods to 25(OH)D_2.

Like SRM 968d L1, SRM 909c (Vial B) contains predominantly 25(OH)D_3. However, the all-method consensus variability of 20 % for SRM 909c is somewhat large for a material with a relatively high 25(OH)D_3 concentration (NIST value 20.7 ng/mL ± 0.7 ng/mL). When SRM 909c was evaluated at NIST using the ID-LC/MS methodology, interferences from the matrix were observed near the retention times for both 25(OH)D_3 and the labeled internal standard that required method modifications to avoid a potential bias [1]. However, when SRM 909c was evaluated at NIST using the ID-LC-MS/MS methodology, interferences were observed only at the retention time for 3-epi-25(OH)D_3. Depending on the separation and detection parameters used by participants, the matrix peaks could interfere in the determination of 25(OH)D_3 and/or 3-epi-25(OH)D_3. The IA results had the largest variability (CV % of 31 %) for SRM 909c, but some of this is attributable to the non-normal distribution of the results; non-Gaussian distributions have also been observed for several materials previously evaluated in the VitDQAP. It is unclear if the IA methods are susceptible to the matrix interferences observed in SRM 909c or if these interferences contribute to the non-normal distribution. However, it is probable that the LC-UV methods were affected by the matrix interferences, as five of the six LC-UV results for 25(OH)D_{Total} were outliers and biased high (**Figure 2**), indicating potential coelution of the matrix peaks with the peak for 25(OH)D_3.

Both of the Youden plots in **Figure 4** incorporate the results for SRM 909c (Vial B) and reveal a large number of outliers, more than has been typically observed in Youden comparisons in previous comparability studies. The large number of outliers is mostly attributable to the variability of the results for SRM 909c, further illustrating that there is something unique about the SRM 909c matrix that is influencing the results.

LC method results for 25(OH)D_3, 25(OH)D_2, and 3-epi-25(OH)D_3 in VitDQAP-III (Vial A)

Of the two major techniques IA and LC, only the LC methods can measure the individual vitamin D metabolites. Given that 25(OH)D_{Total} is the sum of 25(OH)D_2 and 25(OH)D_3, LC methods require accurate, unbiased measurements of both 25(OH)D_2 and 25(OH)D_3 to obtain the correct values for 25(OH)D_{Total}. In the Winter 2014 comparability study, only VitDQAP-III (Vial A) contained a significant concentration of the 25(OH)D_2 metabolite, and the results for the individual metabolites in that study material are detailed below.

Of the 43 LC participants in the Winter 2014 comparability study, all but one of the LC participants reported values for 25(OH)D_3, and all but three reported values for 25(OH)D_2 in VitDQAP-III (Vial A); the study results for 25(OH)D_3 and 25(OH)D_2 are presented in **Table 2**. Since VitDQAP-III (Vial A) contains appreciable amounts of 25(OH)D_3 (NIST value 26.2 ng/mL ± 0.7 ng/mL), the 3-epi-25(OH)D_3 metabolite is also measureable in this material. Ten LC participants reported values for the 3-epi-25(OH)D_3 metabolite, and the results are also presented in **Table 2.**

The community results are summarized at the bottom of **Table 2** for all LC methods and for the LC-MS^n methods only. These summarized results include N, the median value, the MADe, and the CV %. **Table 2** also presents the NIST values and the expanded uncertainties (*U*) for 25(OH)D_3, 25(OH)D_2, and 3-epi-25(OH)D_3 in VitDQAP-III (Vial A).

The single reported values for 25(OH)D_3 and 25(OH)D_2 in VitDQAP-III (Vial A) are plotted in **Figure 5 a** and **b**, respectively. The results from LC-MS^n and LC-UV were sorted separately, as indicated by the x-axis labels. In each plot, the consensus median is represented by the solid line (——), and the expanded uncertainty interval (2 × MADe), is represented by the dashed lines (- - - -). The laboratories with results that fall between the two dashed lines are within the consensus variability range. The red lines (——) in **Figures 5 a** and **b** represent the NIST value and its associated uncertainty (i.e., value ± *U*). NIST has confidence that the "true" value for each metabolite lies within this interval. When these lines are not within the consensus range, then there may be method bias.

Specific results for 25(OH)D_3 and 25(OH)D_2 in VitDQAP-III (Vial A) are summarized below:

25(OH)D_3 in VitDQAP-III (Vial A): **Figure 5 a**

- Four reported values are outside of the consensus variability range (two LC-MS^n, two LC-UV).
- The consensus median value is slightly higher than the NIST expanded uncertainty range (red lines).
- The NIST expanded uncertainty range (red lines) falls within the consensus variability range.

25(OH)D_2 in VitDQAP-III (Vial A): **Figure 5 b**

- Five reported values are outside of the consensus variability range including two from LC-MS^n and three of the four LC-UV results.
- The consensus median value is in good agreement with the NIST expanded uncertainty range (red lines).
- The NIST expanded uncertainty range (red lines) falls within the consensus variability range.

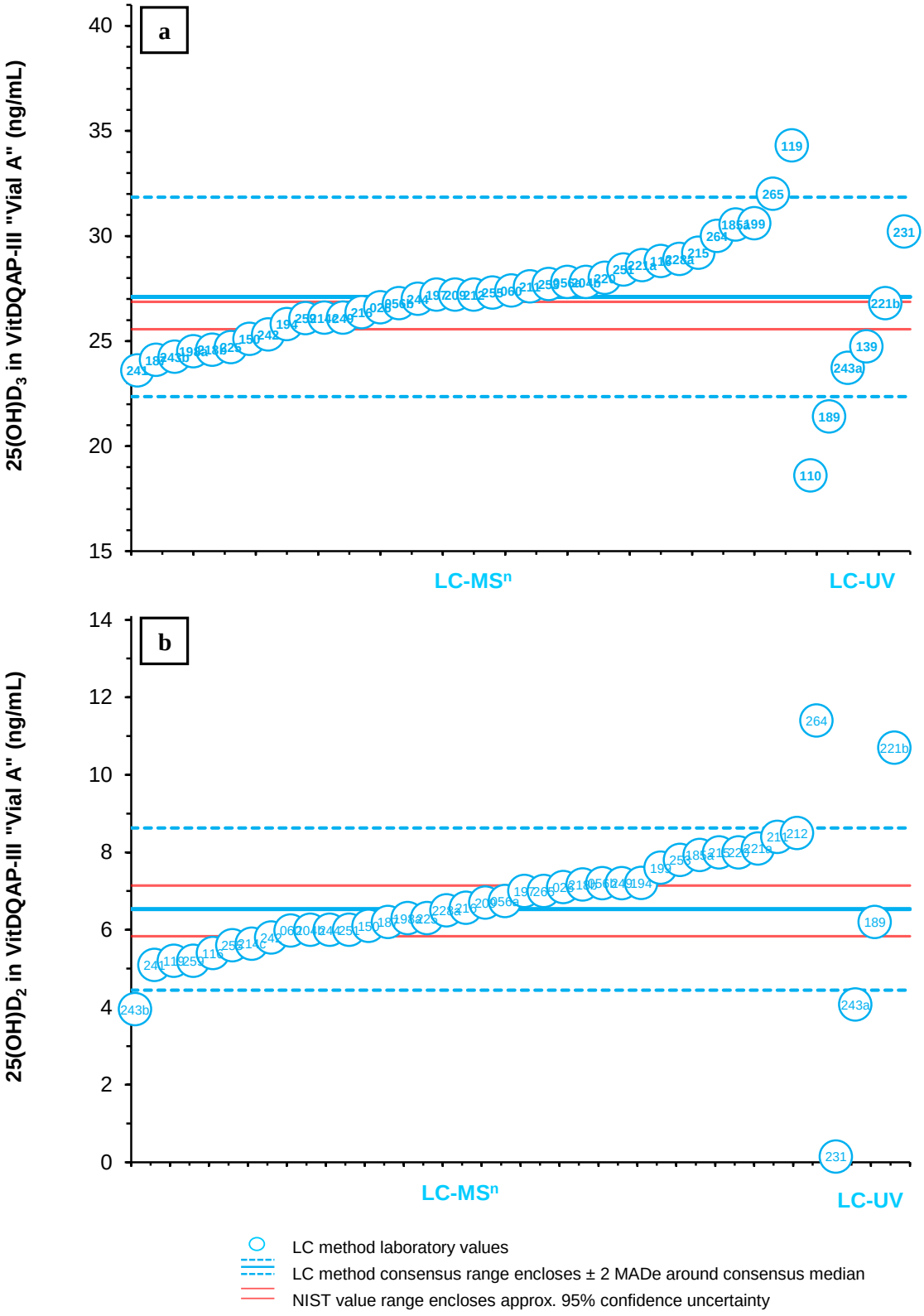
Table 2. Summary of LC participant and NIST results for 25(OH)D_3, 25(OH)D_2, and 3-epi-25(OH)D_3 (ng/mL) in VitDQAP-III (Vial A).

		25(OH)D_3	25(OH)D_2	3-epi-25(OH)D_3
Lab	Method	Vial A	Vial A	Vial A
026	LC-MS/MS	26.6	7.1	1.5
056a	LC-MS/MS	27.8	6.7	0.5
056b	LC-MS/MS	26.8	7.2	n/r
060	LC-MS/MS	27.4	6.0	1.1
110	LC-UV	18.6	<2	n/r
116	LC-MS/MS	28.8	5.4	<4.0
119	LC-MS/MS	34.3	5.2	n/r
139	LC-UV	24.7	n/d	n/r
150	LC-MS/MS	25.1	6.1	<2
185a	LC-MS/MS	30.5	7.9	n/r
187	LC-MS/MS	24.1	6.2	n/r
189	LC-UV	21.4	6.2	n/r
194	LC-MS/MS	25.8	7.2	n/r
197	LC-MS/MS	27.2	7.0	n/r
198a	LC-MS/MS	24.5	6.3	n/r
199	LC-MS/MS	30.6	7.6	n/r
204b	LC-MS/MS	27.8	6.0	<2.5
209	LC-MS/MS	27.2	6.7	n/r
211	LC-MS/MS	27.6	8.4	n/r
212	LC-MS/MS	27.2	8.5	n/r
214c	LC-MS/MS	26.1	5.6	n/r
215	LC-MS/MS	29.2	8.0	n/r
216	LC-MS/MS	26.4	6.6	1.4
218b	LC-MS/MS	24.6	7.2	n/r
220	LC-MS/MS	28.0	8.0	n/r
221a	LC-MS/MS	28.6	8.1	n/r
221b	LC-UV	26.8	10.7	n/r
225	LC-MS/MS	24.7	6.3	n/r
228a	LC-MS/MS	28.9	6.5	n/r
231	LC-UV	30.2	0.0	n/r
241	LC-MS/MS	23.6	5.1	0.8
242	LC-MS/MS	25.3	5.8	1.3
243a	LC-UV	23.7	4.1	1.5
243b	LC-MS/MS	24.3	4.0	1.6
244	LC-MS/MS	27.0	6.0	n/r
249	LC-MS/MS	26.1	7.2	1.4
251	LC-MS/MS	28.4	6.0	n/r
253	LC-MS/MS	27.7	7.8	1.5
255	LC-MS/MS	27.3	5.6	n/r
259	LC-MS/MS	26.1	5.2	n/r
264	LC-MS/MS	30.0	11.4	n/r
265	LC-MS/MS	32.0	7.0	n/r
LC methods	N	42	40	10
	Median	27.1	6.5	1.4
	MADe	2.4	1.0	0.1
	CV%	8.8	16	11
LC-MS^n	N	36	36	9
	Median	27.2	6.6	1.4
	MADe	1.9	1.0	0.1
	CV%	7.1	14	11
	NIST Value	26.2	6.5	1.6
	U	0.7	0.2	0.1

n/r = not reported or not determined; n/d = not detected

< X = less than a reported quantitation limit of X

Figure 5. Participant LC and NIST results for a) 25(OH)D_3 and b) 25(OH)D_2 in VitDQAP-III (Vial A).



Conclusions from the Winter 2014 Comparability Study of the VitDQAP

In the seven previous comparability studies of the VitDQAP, participant performance was consistent for study materials that contain predominantly $25(OH)D_3$; the CV was in the range from 7 % to 19 %, and the median values were biased slightly high relative to the NIST values. In the Winter 2014 comparability study, both SRM 909c (Vial B) and SRM 968d L1 (Control) also contain predominantly $25(OH)D_3$. Of these materials, SRM 968d (Control) follows the previously-observed trends. However, the results for SRM 909c (Vial B) exhibited larger variability than expected (all-method CV of 20 %) given the relatively high concentration of $25(OH)D_3$. In addition, the median IA result for $25(OH)D_{Total}$ in this material was biased 12 % lower than the NIST median value. It is unclear to what extent these observations are due to the potential matrix interferences that are present in SRM 909c (Vial B).

VitDQAP-III (Vial A) was the first study material evaluated in the VitDQAP that had an 'intermediate' concentration of $25(OH)D_2$ (NIST value 6.5 ng/mL ± 0.2 ng/mL) in addition to a significant concentration of $25(OH)D_3$ (NIST value 26.3 ng/mL ± 0.7 ng/mL). It was anticipated that the IA methods would underrepresent the $25(OH)D_{Total}$ concentration due to nonequivalent response to the $25(OH)D_2$ metabolite, which is the trend observed for other materials with relatively high concentrations of $25(OH)D_2$ that had been previously evaluated in the VitDQAP. To the contrary, the IA results overlapped almost completely with the LC results, and any effect from the $25(OH)D_2$ metabolite was lost in the overall variability of the results for the VitDQAP-III study material (Vial A). The median IA result for VitDQAP-III (Vial A) was biased lower than the median LC and NIST results, which is the only indication of potential non-equivalent response to the $25(OH)D_2$ metabolite. However, the IA median result for SRM 909c (Vial B), which does not contain significant $25(OH)D_2$, was also biased somewhat low in this comparability study, further blurring any conclusions about the IA response to $25(OH)D_2$ in the VitDQAP-III (Vial A) material.

Appendix A-1. Summary of immunoassay methods as reported by the study participants.

Laboratory Number	*IA Method*	*Sample Preparation*	*Vendor/kit**
17	CLIA	n/r	A
30a	RIA	Samples were extracted with acetonitrile	B
161	CLIA	n/r	A
180	RIA	Samples were extracted with acetonitrile	B
185b	CLIA	n/r	A
188	CLIA	n/r	C
193	EIA	n/r	D
196	CLIA	No sample preparation required	A
198c	CLIA	n/r	E
200	RIA	Samples were extracted	B
204a	CLIA	n/r	A
210a	RIA	Sample was extracted with acetonitrile	B
210b	CLIA	n/r	E
213a	CLIA	Sample was thawed and gently mixed prior to analysis	E
213b	EIA	Samples, calibrators, and controls processed per manufacturer's protocol	D
214a	RIA	n/r	F
214b	CLIA	n/r	A
218a	CLIA	Direct analysis	n/r
222	CLIA	n/r	C
247a	CLIA	No pretreatment was used	C
247b	EIA	No pretreatment was used	D
256	CLIA	n/r	A
257	CLIA	Sample was thawed at room temperature until analysis	E
258	CLIA	n/r	G
260	EIA	n/r	n/r
261	CLIA	No sample preparation required	G
262	CLIA	n/r	H
263	EIA	On board displacement	I

n/r = not reported

*NIST cannot endorse or recommend commercial products, therefore individual vendors/kits are indicated with a unique letter but not identified

Appendix A-2. Summary of LC-MS^n methods as reported by the study participants.

Laboratory Number	*Internal Standard (IS)*	*Sample Preparation*	*Chromatographic Conditions*	*Detection: MRM ions*
26	25(OH)D_2-d_6 and 25(OH)D_3-d_6	Liquid-liquid extraction method	PFP column (100 mm × 3.2 mm); isocratic elution with 82 % methanol/18 % water; flow 0.4 mL/min	25(OH)D_3 401/365; 25(OH)D_2 413/355; 3-epi-25(OH)D_3 401/365
30b	25(OH)D_3 -d_6	Samples were prepared with disposable pipette extraction	C18 column; isocratic elution with 85 % acetonitrile/15 % methanol; flow 0.5 mL/min	25(OH)D_3 383/211
56a	25(OH)D_2-d_3; 25(OH)D_3-d_6; 3-epi-25(OH)D_3-d_3	Samples were extracted with hexane, evaporated, then reconstituted with 69 % methanol	PFP column (100 mm × 2.1 mm; 1.9 µm); isocratic elution; flow 0.4 mL/min	25(OH)D_3 383/365; 25(OH)D_3-d_6 389/371; 25(OH)D_2 395/377; 25(OH)D_2-d_3 398/380; 3-epi-25(OH)D_3 383/365; 3-epi-25(OH)D_3-d_3 386/368
56b	n/r	n/r	n/r	n/r
60	25(OH)D_3 -d_6	IS was added, and then samples were extracted with acetonitrile, evaporated, and reconstituted with 90 % methanol/10 % water	PFP column (100 mm × 3.0 mm; 2.6 µm); gradient with water, methanol and acetonitrile (0.05 % formic acid)	APPI 25(OH)D_3 383/211; 25(OH)D_3-d_6 389/211; 25(OH)D_2 413/355; 3-epi-25(OH)D_3 401/383
116	25(OH)D_3 -d_6	Serum proteins were precipitated with methanol	Online SPE; reversed-phase column; isocratic elution with 95 % methanol/5 % water; flow 0.6 mL/min	25(OH)D_3 383/211; 25(OH)D_3-d_6 389/211; 25(OH)D_2 395/269
119	25(OH)D_3 -d_6	Samples were mixed with ethanol containing the IS, equilibrated, mixed, extracted with hexane, evaporated, and reconstituted in methanol	C18 column (150 mm × 3.0 mm; 2.7 µm); Gradient with water and methanol (0.1 % formic acid)	25(OH)D_3 401/383; 25(OH)D_3-d_6 407/371 and 407/389; 25(OH)D_2 395/209 and 395/251
150	25(OH)D_2-d_6 and 25(OH)D_3-d_3	Sample (200 µL) was mixed with IS solution, liquid-liquid extracted, centrifuged, supernatant evaporated, and reconstitued in mobile phase	PFP column (100 mm × 3.0 mm; 2.6 µm); isocratic separation with 74 % methanol/26 % water (2 mmol/L ammonium acetate, 0.1 % formic acid); flow 0.5 mL/min	25(OH)D_3 401/383, 401/365; 25(OH)D_2 413/395, 413/365
185a	n/r	Liquid-liquid extraction	PFP column (50 mm × 2.1 mm)	n/r
187	n/r	SPE	C18 column (50 mm × 2.1 mm; 3 µm); gradient with methanol and water	25(OH)D_2 413/395; 25(OH)D_3 401/383
194	25(OH)D_3 -d_6	Proteins precipitated with acetonitrile, top layer removed, evaporated, and reconstituted with methanol	C8 column (50 mm × 2 mm); isocratic elution with 70 % acetonitrile/ 30 % water; flow 0.7 mL/min	25(OH)D_2 395/119; 25(OH)D_3 383/211
197	25(OH)D_3 -d_6	Precipitating agent added (200 µL with 20 ng IS) to each serum sample (200 µL), calibrator and control sample followed by mixing, centrifugation, and analysis	C18 column (50 mm × 4.6 mm; 5 µm); column temp 45 °C; gradient with water and methanol; flow 1.0 mL/min	n/r
198a	25(OH)D_3 -d_6	Proteins precipitated with methanol, followed by hexane extraction, centrifugation, evaporation under N_2, and reconstitution in methanol with 0.1 % formic acid	C18 column (50 mm × 2.1 mm; 3.5 µm); isocratic elution with 85 % methanol (0.1 % formic acid); flow 0.5 mL/min	25(OH)D_3 401/383, 401/365; 25(OH)D_2 413/395, 413/355; 25(OH)D_3-d_6 407/389, 407/371
199	proprietary	proprietary	proprietary	proprietary

204b	25(OH)D_2-d_3; 25(OH)D_3-d_6; 3-epi-25(OH)D_3-d_3	Protein crash with 73 % methanol followed by liquid-liquid extraction with hexane, centrifugation, evaporation, and reconstitution in mobile phase	PFP column (100 mm × 2.1 mm; 1.9 µm); column temperature 30 °C; isocratic elution with 73 % methanol/27 % water; flow 0.4 mL/min	APCI 25(OH)D_3 383/365, 383/257; 25(OH)D_2 395/377, 395/209; 3-epi-25(OH)D_3 383/365, 383/257
209	25(OH)D_3 -d_6	Proteins were precipitated with 5 % $ZnSO_4$ in methanol	C8 column (50 mm × 2 mm; 5 µm); gradient with water/methanol; flow 0.7 mL/min	APCI 25(OH)D_3 383/229,383/211; 25(OH)D_3-d_6 389/211; 25(OH)D_2 395/269, 395/119
211	25(OH)D_3-d_6	Proteins precipitated with acetonitrile containing IS followed by centrifugation	Turbulent flow column (32 mm x 4.6 mm; 3 µm)	25(OH)D_3 383/365 (quant), 383/257 (qual); 25(OH)D_2 395/209 (quant), 395/377 (qual)
212	25(OH)D_3-d_6	Serum (100 µL) proteins precipitated using 5 % methanol/95 % acetonitrile containing the IS (350 µL)	C8 column (50 mm × 2 mm; 3 µm); gradient of 60% to 98% acetonitrile (0.1% formic acid)	25(OH)D_3 383/229, 383/211; 25(OH)D_2 395/269, 395/119
214c	25(OH)D_3-d_6	Samples were extracted with hexane, centrifuged, evaporated, and filtered	Column (50 mm × 2.1 mm); isocratic elution with 85 % methanol/ 15 % water/ 0.1 % formic acid; flow 0.3 mL/min	25(OH)D_3 401/383; 25(OH)D_3-d_6 407/389; 25(OH)D_2 413/395
215	25(OH)D_3-d_6	Protein precipitation with methanol/isopropanol and $ZnSO_4$; supernatant extracted using SPE	C18 column (50 mm × 2.1 mm; 2.6 µm) column; gradient with water (0.1 % formic acid, 5 mmol/L ammonium formate) and methanol (0.05 % formic acid)	ESI 25(OH)D_3 401/383; 25(OH)D_2 413/395; 25(OH)D_3-d_6 407/389
216	Derivatized deuteriated standard	Samples extracted using liquid-liquid extraction then labeled with a derivatization reagent	Revered-phase column (150 mm × 2.1 mm); gradient from 25 % water (0.05 % formic acid) to 50 % acetonitrile (0.05 % formic acid); flow 0.2 mL/min	n/r
218b	25(OH)D_2-d_3 and 25(OH)D_3-d_3	Sample was extracted, filtered, centrifuged, etc.	Phenyl column (50 mm × 2.1 mm; 1.7 µm); flow 0.45 mL/min	25(OH)D_3 401; 25(OH)D_2 413
220	25(OH)D_2-d_3 and 25(OH)D_3-d_6	Protein crash with 90 % methanol/ 10 % $ZnSO_4$ and then acetonitrile/ 1 % formic acid; sample filtered; phospholipids removed with SPE	C18 column (20 mm × 2.1mm, 2.7 µm); gradient with water and acetonitrile; flow 1 mL/min; column 40 °C	MRM with dehydrated precursor and product ions
221a	25(OH)D_3-d_6	Protein crash with 1% methanol in acetonitrile containing the IS	CN column (50 mm × 3.0 mm; 1.8 µm); methanol/water gradient at 50 °C	APCI 25(OH)D_3 383/211; 25(OH)D_3-d_6 389/211; 25(OH)D_2 395/209
225	25(OH)D_3-d_6	Liquid-liquid extraction	C8 column (50 mm × 2.1 mm; 1.7 µm); gradient with methanol/water; flow 0.4 mL/min	25(OH)D_3 401/107; 25(OH)D_2 413/83
228a	D8-labeled compound	Proteins precipitated	n/r	n/r
241	25(OH)D_3-d_6	Acetonitrile containing the IS (100 µL) added to sample (200 µL) to precipate proteins, followed by extraction with hexane, centrifugation, removal of supernatant, evaporation, and reconstitution in methanol solution	PFP column (100 mm × 2.1 mm; 2.6 µm); gradient starting with 50 % methanol (0.1 % formic acid), 50 % water (0.1 % formic acid)	APCI 25(OH)D_3 383/211 (quant), 383/229 (qual); 25(OH)D_2 395/119 (quant), 395/211 (qual); 25(OH)D_3-d_6 389/211

242	25(OH)D_3-d_6	Water with 0.1 % formic acid (500 µL) and the IS (400 µL) were added to the sample (400 µL), followed by centrifugation and dilution of supernatant with water	PFP column (150 mm × 2 mm; 3 µm); isocratic elution with 18 % water/ 82 % methanol/ 0.1 % formic acid; flow 0.35 mL/min	APCI 25(OH)D_3 383/257; 25(OH)D_2 395/269; 25(OH)D_3-d_6 389/263; 3-epi-25(OH)D_3 383/257; 3-epi-25(OH)D_2 395/269
243b	25(OH)D_3-d_6	Samples (400 µL) were mixed with solution containing the IS (400 µL), precipitation reagent was added (500 µL), and portion of upper layer (50 µL) was injected	PFP column (150 mm × 2 mm); isocratic separation with 85 % methanol/15 % water; flow 0.3 mL/m	25(OH)D_3 383/257; 25(OH)D_3-d_6 389/269; 25(OH)D_2 395/269
244	25(OH)D_3-d_6	Protein precipitation followed by filtration	CN column; mobile phase consisting of distilled water (formic acid) and methanol	25(OH)D_3 383/211; 25(OH)D_3-d_6 389/211; 25(OH)D_2 395/269
249	25(OH)D_2-d_3; 25(OH)D_3-d_6; 3-epi-25(OH)D_3-d_3	Serum was deproteinated with NaOH and 90 % acetonitrile/ 10 % methanol followed by SPE	PFP column (100 mm × 2.1 mm; 1.8 µm); gradient separation with water (2 mmol/L ammonium acetate) and methanol; flow 0.35 mL/min	25(OH)D_3 401/159; 25(OH)D_2 413/159
251	25(OH)D_2-d_3 and 25(OH)D_3-d_3	Protein precipitation followed by SPE	Phenyl column (50 mm × 2.1 mm; 1.7 µm); gradient with water and methanol (0.1 % formic acid, 2 mmol/L ammonium acetate); flow 0.45 mL/min	25(OH)D_3 401/159 (quant), 401/365 (qual); 25(OH)D_2 413/83 (quant), 413/355 (qual); 25(OH)D_3-d_3 404/162; 25(OH)D_2-d_3 416/358
253	25(OH)D_2-d_3 and 25(OH)D_3-d_3	The sample was extracted, centrifuged, and derivatized	C18 column (150 mm × 2.1 mm); isocratic separation with 77.5 % methanol/22.5 % water; flow 0.2 mL/min	25(OH)D_2 588; 25(OH)D_3 576
255	deuterium labeled compound	Samples were extracted and derivatized with 4-phenyl-1,2,4-triazoline-3,5-dione	Reversed-phase column (50 mm × 2.1 mm); gradient with methanol; flow 0.5 mL/min	25(OH)D_3 607/298; 25(OH)D_2 619/298
259	deuterium labeled 25(OH)D_3	n/r	C8 column; gradient with methanol/water/0.1 % formate	n/r
264	clozapine	Samples were extracted with tert-butyl methyl ether, evaporated, and derivatized with 4-phenyl-1,2,4-triazoline-3,5-dione	Reversed-phase column (150 mm × 4.6 mm); isocratic separation with 85 % methanol/15 % water/0.3 % formic acid; flow 1.4 mL/min	n/r
265	n/r	n/r	n/r	n/r

C18 = octadecyl; C8 = octyl; PFP = pentafluorophenyl; SPE = solid phase extraction; CN = cyano;

MRM = multiple reaction monitoring; quant/qual = quantitative/qualitative ions; n/r = not reported;

APPI = atmospheric pressure photoionization; APCI = atmospheric pressure chemical ionization; ESI = electrospray ionization

Appendix A-3. Summary of LC-UV methods as reported by the study participants.

Laboratory Number	*Internal Standard (IS)*	*Sample Preparation*	*Chromatographic Conditions*	*Wavelength*
110	n/a	Samples (500 µL) were mixed with ethanol (500 µL), extracted twice with hexane/methylene chloride (5:1), evaporated, and reconstituted	C18 column (2.1 mm × 100 mm; 1.8 µm); gradient with acetonitrile/methanol (85:15) and isopropanol (100 %)	267 nm
139	proprietary	The sample was extracted, centrifuged, and injected	Reversed-phase column heated to 40 °C, isocratic separation with proprietary mobile phase; flow 1 mL/min	264 nm
189	unidentified	Protein precipitation followed by SPE	Reversed-phase column (150 mm × 4.6 mm); isocratic separation; flow 0.7 mL/min	265 nm
221b	laurophenone	Protein crash with acetonitrile solution containing IS, followed by SPE, elution with methanol/acetonitrile solution, evaporation, and reconstitution with acetonitrile	CN column (150 mm × 5 mm; 3.5 µm); elution with methanol/water/formic acid; column temperature 47 °C	275 nm
231	1-alpha-hydroxy-vitamin D_3	Samples were extracted with hexane/dichloromethane, evaporated, and reconstituted with mobile phase (phosphate buffer/acetonitrile)	Reversed-phase column (250 mm × 4.5 mm; 5µm), isocratic separation with 14 % phosphate buffer, 86 % acetonitrile; flow 1.2 mL/min	265 nm
243a	laurophenone	Samples (400 µL) were mixed with solution containing the IS (400 µL), precipitation reagent was added (500 µL), and portion of upper layer (50 µL) was injected	Reversed-phase column (150 mm × 3 mm); isocratic separation with 63 % acetonitrile/37 % water; flow 1 mL/min	264 nm

n/a = not applicable; SPE = solid phase extraction

Appendix B. Raw participant data and NIST results for $25(OH)D_2$, $25(OH)D_3$, $25(OH)D_{Total}$, and 3-epi-$25(OH)D_3$, in VitDQAP-III (Vial A), SRM 909c (Vial B), and SRM 968d L1 (Control).

		$25(OH)D_2$ (ng/mL)			$25(OH)D_3$ (ng/mL)			$25(OH)D_{Total}$ (ng/mL)			3-epi-$25(OH)D_3$ (ng/mL)		
		VitDQAP-III	SRM 909c	SRM 968d L1	VitDQAP-III	SRM 909c	SRM 968d L1	VitDQAP-III	SRM 909c	SRM 968d L1	VitDQAP-III	SRM 909c	SRM 968d L1
Lab	Method	Vial A	Vial B	Control	Vial A	Vial B	Control	Vial A	Vial B	Control	Vial A	Vial B	Control
017	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	31.0	16.2	13.9	n/a	n/a	n/a
026	LC-MS/MS	7.1	0.4	0.2	26.6	21.3	13.2	33.7	21.7	13.4	1.5	1.7	1.0
030a	RIA	n/a	n/a	n/a	n/a	n/a	n/a	40.5	24.8	15.3	n/a	n/a	n/a
030b	LC-MS/MS	n/r	n/r	n/r	n/r	n/r	n/r	24.5	23.9	13.2	n/r	n/r	n/r
056a	LC-MS/MS	6.7	0.6	0.6	27.8	19.7	12.2	34.5	20.3	12.8	0.5	0.5	0.5
056b	LC-MS/MS	7.2	0.3	0.1	26.8	20.0	12.6	34.0	20.3	12.7	n/r	n/r	n/r
060	LC-MS/MS	6.0	0.9	0.2	27.4	21.1	12.8	33.4	22.0	13.0	1.1	0.9	0.8
110	LC-UV	<2	<2	<2	18.6	76.0	11.9	21.8	79.6	12.8	n/r	n/r	n/r
116	LC-MS/MS	5.4	<3.3	<3.3	28.8	20.7	12.8	34.2	20.7	12.8	<4.0	<4.0	<4.0
119	LC-MS/MS	5.2	n/d	n/d	34.3	27.3	15.6	39.5	27.3	15.6	n/r	n/r	n/r
139	LC-UV	n/d	n/d	n/d	24.7	51.9	13.1	24.7	51.9	13.1	n/r	n/r	n/r
150	LC-MS/MS	6.1	<2	<2	25.1	17.7	12.1	31.2	17.7	12.1	<2	<2	<2
161	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	30.6	14.4	11.8	n/a	n/a	n/a
180	RIA	n/a	n/a	n/a	n/a	n/a	n/a	29.1	19.5	14.9	n/a	n/a	n/a
185a	LC-MS/MS	7.9	0.0	0.0	30.5	36.4	17.3	38.4	36.4	17.3	n/r	7.0	n/r
185b	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	16.4	33.3	14.5	n/a	n/a	n/a
187	LC-MS/MS	6.2	0.0	0.0	24.1	19.0	13.1	30.3	19.0	13.1	n/r	n/r	n/r
188	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	36.1	18.4	14.1	n/a	n/a	n/a
189	LC-UV	6.2	23.4	0.0	21.4	14.8	16.5	27.6	38.2	16.5	n/r	n/r	n/r
193	EIA	n/a	n/a	n/a	n/a	n/a	n/a	33.9	22.3	18.6	n/a	n/a	n/a
194	LC-MS/MS	7.2	<7.0	<7.0	25.8	15.8	13.0	33.0	15.8	13.0	n/r	n/r	n/r
196	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	30.7	15.1	14.1	n/a	n/a	n/a
197	LC-MS/MS	7.0	<5	0.1	27.2	22.0	13.0	34.2	22.0	13.1	n/r	n/r	n/r
198a	LC-MS/MS	6.3	<5	<5	24.5	18.5	12.6	30.8	18.5	12.6	n/r	n/r	n/r
198c	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	29.1	19.7	8.3	n/a	n/a	n/a
199	LC-MS/MS	7.6	2.1	<2	30.6	27.5	13.7	38.2	29.6	13.7	n/r	n/r	n/r
200	RIA	n/a	n/a	n/a	n/a	n/a	n/a	27.7	18.0	13.8	n/a	n/a	n/a
204a	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	27.5	14.0	12.2	n/a	n/a	n/a
204b	LC-MS/MS	6.0	n/d	n/d	27.8	20.2	12.8	33.8	20.2	12.8	<2.5	<2.5	<2.5
209	LC-MS/MS	6.7	<1.0	<1.0	27.2	22.9	12.5	33.9	22.9	12.5	n/r	n/r	n/r
210a	RIA	n/a	n/a	n/a	n/a	n/a	n/a	31.4	17.9	12.4	n/a	n/a	n/a
210b	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	38.7	25.0	9.1	n/a	n/a	n/a
211	LC-MS/MS	8.4	<5	<5	27.6	18.3	13.6	36.0	18.3	13.6	n/r	n/r	n/r
212	LC-MS/MS	8.5	0.0	0.0	27.2	22.3	12.3	35.7	22.3	12.3	n/r	n/r	n/r
213a	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	30.4	19.4	6.5	n/a	n/a	n/a
213b	EIA	n/a	n/a	n/a	n/a	n/a	n/a	26.3	17.3	12.9	n/a	n/a	n/a
214a	RIA	n/a	n/a	n/a	n/a	n/a	n/a	27.5	31.3	11.5	n/a	n/a	n/a
214b	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	31.2	14.4	13.0	n/a	n/a	n/a
214c	LC-MS/MS	5.6	<0.5	<0.5	26.1	20.5	12.2	31.7	20.5	12.2	n/r	n/r	n/r
215	LC-MS/MS	8.0	<2	<2	29.2	21.2	13.6	37.2	21.2	13.6	n/r	n/r	n/r
216	LC-MS/MS	6.6	0.3	0.1	26.4	21.5	12.7	32.9	21.9	12.8	1.4	1.1	0.6
218a	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	33.7	14.5	14.5	n/a	n/a	n/a
218b	LC-MS/MS	7.2	0.1	0.0	24.6	23.7	12.2	31.8	23.8	12.2	n/r	n/r	n/r
220	LC-MS/MS	8.0	n/d	n/d	28.0	23.0	15.0	36.0	23.0	15.0	n/r	n/r	n/r
221a	LC-MS/MS	8.1	<5.0	<5.0	28.6	31.7	15.0	36.7	31.7	15.0	n/r	n/r	n/r
221b	LC-UV	10.7	15.1	<5.0	26.8	21.2	10.7	37.5	36.3	10.7	n/r	n/r	n/r
222	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	35.2	23.5	11.1	n/a	n/a	n/a
225	LC-MS/MS	6.3	<5	<5	24.7	14.8	10.4	31.0	14.8	10.4	n/r	n/r	n/r
228a	LC-MS/MS	6.5	n/d	n/d	28.9	21.5	12.7	35.4	21.5	12.7	n/r	n/r	n/r
231	LC-UV	0.0	0.0	0.0	30.2	30.8	8.5	30.2	30.8	8.5	n/r	n/r	n/r
241	LC-MS/MS	5.1	0.2	0.1	23.6	17.0	12.2	28.7	17.2	12.3	0.8	0.4	0.6
242	LC-MS/MS	5.8	n/d	n/d	25.3	18.7	12.2	31.1	18.7	12.2	1.3	2.2	0.6
243a	LC-UV	4.1	n/d	n/d	23.7	19.6	11.5	27.8	19.6	11.5	1.5	2.4	n/d
243b	LC-MS/MS	4.0	n/d	n/d	24.3	21.1	12.4	28.2	21.1	12.4	1.6	2.5	n/d
244	LC-MS/MS	6.0	<5	<5	27.0	25.0	13.0	33.0	25.0	13.0	n/r	n/r	n/r
247a	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	31.9	16.7	11.7	n/a	n/a	n/a
247b	EIA	n/a	n/a	n/a	n/a	n/a	n/a	31.0	22.3	15.7	n/a	n/a	n/a
249	LC-MS/MS	7.2	0.3	0.2	26.1	21.4	12.4	33.3	21.7	12.6	1.4	1.4	0.4
251	LC-MS/MS	6.0	<4	n/r	28.4	21.6	n/r	34.4	21.6	n/r	n/r	n/r	n/r
253	LC-MS/MS	7.8	0.3	0.1	27.7	20.7	13.0	35.5	21.0	13.1	1.5	1.1	0.6
255	LC-MS/MS	5.6	0.3	0.1	27.3	19.9	12.7	32.9	20.2	12.8	n/r	n/r	n/r
256	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	30.4	17.3	18.5	n/a	n/a	n/a
257	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	31.9	22.4	6.2	n/a	n/a	n/a
258	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	32.8	17.5	11.4	n/a	n/a	n/a
259	LC-MS/MS	5.2	<2	<2	26.1	20.3	14.8	26.1	25.5	14.8	n/r	n/r	n/r
260	EIA	n/a	n/a	n/a	n/a	n/a	n/a	32.0	22.0	18.2	n/a	n/a	n/a
261	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	32.3	16.2	10.3	n/a	n/a	n/a
262	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	34.1	52.1	16.5	n/a	n/a	n/a
263	CLIA	n/a	n/a	n/a	n/a	n/a	n/a	28.7	13.4	12.5	n/a	n/a	n/a
264	LC-MS/MS	11.4	0.7	0.1	30.0	28.3	11.3	41.4	29.0	11.4	n/r	n/r	n/r
265	LC-MS/MS	7.0	0.0	0.0	32.0	24.0	14.0	39.0	24.0	14.0	n/r	n/r	n/r

n/a = not applicable (for immunoassay methods); n/r = not reported or not determined; n/d = not detected; < X = less than a reported quantitation limit of X

	NIST Value	6.49	n/r	0.10	26.2	20.7	12.4	32.7	20.7	12.5	1.6	n/r	0.65
	U	0.17	n/r	---	0.6	0.7	0.4	0.7	0.7	0.4	0.1	n/r	0.03

*estimated value (no uncertainty determined)