CCQM-K132
Low-Polarity Analytes in a Biological Matrix: Vitamin D Metabolites in Human Serum

Key Comparison
Track C

Final Report
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SUMMARY

Vitamin D is a fat-soluble vitamin that occurs primarily in two forms, vitamin D_2 and vitamin D_3. Vitamin D_3 is produced naturally when skin is exposed to UV radiation, is naturally-occurring in foods (generally of animal origin), and is fortified in some foods and dietary supplements. Vitamin D_2 occurs in food (generally plant sources) and until recently was the form most often used in dietary supplements. Vitamin D is metabolized in the body to produce several closely related, hydroxylated species (metabolites), with 25-hydroxyvitamin D_3 [25(OH)D_3] and 25-hydroxyvitamin D_2 [25(OH)D_2] as the most common metabolites measured in human serum. Concentrations of total vitamin D in human serum, calculated as the sum of 25(OH)D_2 and 25(OH)D_3, are typically in the 16 ng/g to 30 ng/g (40 nmol/L to 75 nmol/L) range, with 25(OH)D_3 usually accounting for more than 90 % of the total. An epimer of 25(OH)D_3, 3-*epi*-25(OH)D_3, can be present at levels up to 10 % of 25(OH)D_3 concentration.

Seven National Metrology Institutions participated in the Track C Key Comparison CCQM-K132 Low-Polarity Analytes in a Biological Matrix: Vitamin D Metabolites in Human Serum. Participants were requested to evaluate the mass fractions, expressed in ng/g, of 25(OH)D_3, 25(OH)D_2, and 3-*epi*-25(OH)D_3 in two human serum materials, termed Serum Pool I and Serum Pool II. Due to the known low levels of 3-*epi*-25(OH)D_3 in both materials and the very low level of 25(OH)D_2 in Serum Pool I, the study protocol stated that Key Comparison Reference Values (KCRVs) would be assigned only to 25(OH)D_3 in both materials and 25(OH)D_2 in Serum Pool II. Results for 3-*epi*-25(OH)D_3 were requested to evaluate the separation technologies employed; 3-*epi*-25(OH)D_3 needs to be chromatographically separated from 25(OH)D_3 for proper quantification of 25(OH)D_3. Results for 25(OH)D_2 in Serum Pool I were requested to explore measurement performance at its low level. All participants used isotope dilution liquid chromatography with tandem mass spectrometry detection (ID LC-MS/MS) for the measurement of the vitamin D metabolites.

Successful participation in CCQM-K132 demonstrates capabilities in analysis of low molecular mass (100 g/mol to 500 g/mol) and low-polarity (nonpolar, $pK_{ow} < -2$) analytes at the 1 ng/g to 500 ng/g mass fraction range in complex biological matrixes with core competencies for sample preparation and analysis using ID LC-MS/MS. This study extends the mass fraction capability range to 10^5 to 10^6 times lower than that demonstrated in previous CCQM Key Comparisons for cholesterol in serum, another nonpolar clinical analyte.

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ACRONYMS

3-*epi*-25(OH)D_3	an epimer of 25-hydroxyvitamin D_3
25(OH)D	25-hydroxyvitamin D, vitamin D, the sum of 25(OH)D_2 and 25(OH)D_3
25(OH)D_2	25-hydroxyvitamin D_2, vitamin D_2
25(OH)D_3	25-hydroxyvitamin D_3, vitamin D_3
CCQM	Consultative Committee for Amount of Substance: Metrology in Chemistry and Biology
CMC	Calibration and Measurement Capability
CRM	certified reference material
CV	coefficient of variation, expressed in %: CV = $100 \cdot s/\bar{x}$
DI	designated institute
DoE	degrees of equivalence
GC-MS	gas chromatography with mass spectrometry detection
HSA	Health Sciences Authority, Singapore
ID	isotope dilution
JCTLM	Joint Committee for Traceability in Laboratory Medicine
KC	Key Comparison
KCRV	Key Comparison Reference Value
KRISS	Korea Research Institute of Standards and Science, Republic of Korea
LC-MS	liquid chromatography with mass spectrometry detection
LC-MS/MS	liquid chromatography with tandem mass spectrometry detection

MAD_E	median absolute deviation from the median (MAD)-based estimate of *s*: $MAD_E = 1.4826 \cdot MAD$, where MAD = median($\lvert x_i$-median(x_i)$\rvert$)
NIM	National Institute of Metrology, China
NIMT	National Institute of Metrology (Thailand), Thailand
NIST	National Institute of Standards and Technology, USA
NMI	national metrology institute
NMIA	National Measurement Institute Australia
OAWG	Organic Analysis Working Group
pK_{ow}	logarithm of the octanol-water partition coefficient
RMP	Reference Measurement Procedure
SRM	Standard Reference Material, a NIST CRM
UME	National Metrology Institute of Turkey, Turkey
VitDQAP	Vitamin D Metabolites Quality Assurance Program

SYMBOLS

d_i	degree of equivalence: x_i - KCRV
$\%d_i$	percent relative degree of equivalence: $100 \cdot d_i$/KCRV
k	coverage factor: $U(x) = k \cdot u(x)$
n	number of quantity values in a series of quantity values
s	standard deviation of a series of quantity values: $s = \sqrt{\sum_{i=1}^{n}(x_i - \bar{x})^2/(n-1)}$
t_s	Student's *t*-distribution expansion factor
$u(x_i)$	standard uncertainty of quantity value x_i
$\bar{u}(x)$	pooled uncertainty: $\bar{u}(x) = \sqrt{\sum_{i=1}^{n} u^2(x_i)/n}$
$U(x)$	expanded uncertainty
$U_{95}(x)$	expanded uncertainty defined such that $x \pm U_{95}(x)$ is asserted to include the true value of the quantity with an approximate 95 % level of confidence
$U_{k=2}(x)$	expanded uncertainty defined as $U_{k=2}(x) = 2 \cdot u(x)$
x	a quantity value
x_i	the i^{th} member of a series of quantity values
$\bar{x}$	mean of a series of quantity values: $\bar{x} = \sum_{i=1}^{n} x_i/n$
z_i	z-score, a standardized quantity value: $z_i = (x_i - \bar{x})/s$

INTRODUCTION

Vitamin D is a fat-soluble vitamin that occurs primarily in two forms, vitamin D_2 and vitamin D_3. Vitamin D_3 is produced naturally when skin is exposed to UV radiation, is naturally-occurring in foods (generally of animal origin), and is fortified in some foods and dietary supplements. Vitamin D_2 occurs in food (generally plant sources) and until recently was the form most often used in dietary supplements. Vitamin D is metabolized in the body to produce several closely related, hydroxylated species (metabolites), with 25-hydroxyvitamin D_3 [$25(OH)D_3$] and 25-hydroxyvitamin D_2 [$25(OH)D_2$] as the most common metabolites measured in human serum. Figure 1 displays the structure of these metabolites.

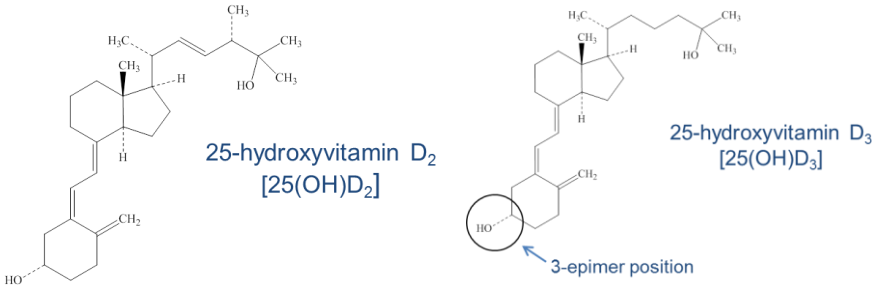

Figure 1: Structures of 25-hydroxyvitamin D_2 and 25-hydroxyvitamin D_3

Vitamin D levels in serum are typically reported as total 25-hydroxyvitamin D [25(OH)D], which is the sum of $25(OH)D_2$ and $25(OH)D_3$. Measurement of 25(OH)D in serum is generally considered a reliable indicator of vitamin D status and is used in the investigation of bone health as well as a variety of emerging non-skeletal conditions (i.e., immunity, cancer). Vitamin D deficiency (25(OH)D < 30 nmol/L [1]) can lead to bone pain and weakness and compromised immunity.

Total 25(OH)D concentrations in human serum are typically in the 16 ng/g to 30 ng/g (40 nmol/L to 75 nmol/L) range, with $25(OH)D_3$ usually accounting for more than 90 % of the total. An epimer of the $25(OH)D_3$, 3-*epi*-$25(OH)D_3$, can be present at levels up to 10 % of the $25(OH)D_3$ concentration in adults and may be higher in infants. For accurate quantification 3-*epi*-$25(OH)D_3$ needs to be chromatographically separated from $25(OH)D_3$. 3-*epi*-$25(OH)D_2$ has been reported; however, it is expected to be less than 10 % of the $25(OH)D_2$ and is not typically measured in serum samples.

An isotope dilution liquid chromatography with tandem mass spectrometry (ID LC-MS/MS) method for the determination of $25(OH)D_2$ and $25(OH)D_3$ was published by Tai et al. [2] in 2010 and subsequently recognized by the Joint Committee on Traceability in Laboratory Medicine (JCTLM) as a Reference Measurement Procedure (RMP). A second RMP for $25(OH)D_2$ and $25(OH)D_3$ based on ID LC-MS/MS by was reported by Stepman et al. [3] from the University of Ghent (Belgium). An ID LC-MS/MS-based candidate RMP for vitamin D metabolites in human serum was recently published by Mineva et al. [4] from the U.S. Centers for Disease Control and Prevention and is now recognized as a RMP by the JCTLM.

At least three National Metrology Institutes (NMIs) and one designated institute (DI) are currently providing measurement services in the determination of vitamin D metabolites in human serum. In 2009 the National Institute of Standards and Technology (NIST) issued the first Certified Reference Material (CRM) for determination of vitamin D metabolites in human serum, Standard Reference Material (SRM) 972 Vitamin D in Human Serum [5,6]. SRM 972 consisted of four levels of human serum containing endogenous levels of $25(OH)D_2$, $25(OH)D_3$, and 3-*epi*-$25(OH)D_3$ in two level and two levels with fortified levels of $25(OH)D_2$ and 3-*epi*-$25(OH)D_3$. Due to the widespread use of SRM 972, the supply was depleted in 2011 and a second material, SRM 972a Vitamin D Metabolites in Frozen Human Serum, was issued in 2013 [7]. SRM 972a consisted of four levels of $25(OH)D_2$, $25(OH)D_3$, and 3-*epi*-$25(OH)D_3$ (one level was fortified with 3-*epi*-$25(OH)D_3$; the other three were endogenous levels). In 2015 National Metrology Institute of Turkey (UME) issued UME CRM 1308, 25-Hydroxy Vitamin D_2 and 25-Hydroxy Vitamin D_3 in Lyophilized Serum, which was prepared by adding $25(OH)D_2$ and $25(OH)D_3$ standards to horse serum prior to lyophilization [8].

$25(OH)D_2$ and $25(OH)D_3$ are low-polarity analytes (molar mass of 412 g/mol and 400 g/mol, respectively) in the same pK_{ow} range as cholesterol (molar mass of 386 g/mol); however, the concentrations in serum of $25(OH)D_2$ and $25(OH)D_3$ are lower than cholesterol levels by a factor of more than 10^5. While the OAWG has coordinated Key Comparisons (KCs) for cholesterol, CCQM-K132 is the first KCs for this measurand class at this low concentration range.

Table 1 lists the timeline for CCQM-K132 Vitamin D Metabolites in Human Serum.

Table 1: Timeline for CCQM-K132

Date	Action
Oct 2014	Draft protocol presented to OAWG as potential Track C Key Comparison
Apr 2015	OAWG authorized CCQM-K132 as a Track C Key Comparison; protocol approved
May 2015	Call for participation to OAWG members
Jun 2015	Study samples shipped to participants
Sep 2015	Results due to coordinating laboratory
Oct 2015	First report of results to OAWG
Mar 2016	Draft A report distributed to OAWG
Jun 2016	Draft B report distributed to OAWG
TBD	Final report delivered to OAWG Chair

Appendices A to E reproduce as distributed the Call for Participation, the study Protocol, the Registration Form, the Reporting Form, and the Core Competency Table Form.

MEASURANDS

The study required the determination of mass fraction for the two measurands, 25(OH)D_3 and 25(OH)D_2, needed for estimating the 25(OH)D concentration in human serum. While participants were required to report mass fractions for 25(OH)D_3 and 25(OH)D_2 in both serum pools, no KCRV for 25(OH)D_2 in Serum Pool I is assigned because of its very low mass fraction in this material. Participants were requested to report mass fractions for 3-*epi*-25(OH)D_3 to demonstrate that their analytical method is capable of resolving the 3-epimer from the 25(OH)D_3 (see Figure 1). However, no KCRV is assigned for the 3-*epi*-25(OH)D_3 measurements.

STUDY MATERIALS

Two human serum pools with normal and high levels of 25(OH)D_2 and 25(OH)D_3 were used as study materials. Serum Pool I was (then candidate) SRM 2973 Vitamin D Metabolites in Human Serum (High Level) [9]. For the majority of the U.S. population, serum concentrations of 25(OH)D typically range from 16 ng/g to 30 ng/g (40 nmol/L to 75 nmol/L) [10]. About 10 % of the population has 25(OH)D concentrations from 30 ng/g to 50 ng/g (75 nmol/L to 125 nmol/L) [10]. SRM 2973 was prepared specifically to provide a serum material with a 25(OH)D concentration near 40 ng/g (100 nmol/L), which complements the lower normal levels available in other SRMs with values assigned for 25(OH)D. The study protocol indicated that the target mass fractions of the vitamin D metabolites were: 25(OH)D_3 = 30 ng/g to 50 ng/g; 25(OH)D_2 = 50× lower than 25(OH)D_3; and 3-*epi*-25(OH)D_3 ≈10 % of 25(OH)D_3 level. SRM 2973 Vitamin D Metabolites in Human Serum (High Level) was issued 4-Feb-2016 with a certified value of (38.6 ± 0.8) ng/g for the mass fraction of 25(OH)D_3 [7].

Serum Pool II represented a normal level of 25(OH)D_3 with a higher than normal level of 25(OH)D_2. The study protocol indicated that the target mass fractions of the vitamin D metabolites in Serum Pool II were: 25(OH)D_3 = 15 ng/g to 30 ng/g; 25(OH)D_2 = 1 ng/g to 10 ng/g; and 3-*epi*-25(OH)D_3 ≈10 % of 25(OH)D_3 level. Serum Pool II was distributed as an unknown sample in two exercises of the Vitamin D Metabolites Quality Assurance Program (VitDQAP) [11] coordinated by NIST. Serum Pool II was used (labeled VitDQAP-III) in the Winter 2014 [12] and Winter 2015 [13] VitDQAP exercises.

Both Serum Pool I and Serum Pool II were provided as 1.0 mL samples in cryovials. The samples were shipped frozen (on dry ice) to the participants. Participants were instructed that the serum materials required storage at between -20 ºC and -80 ºC.

Homogeneity Assessment of Study Material

The homogeneity of the two serum pools was evaluated at NIST using the NIST ID LC-MS/MS RMP for the determination of 25(OH)D_3 and 25(OH)D_2 [2].

For Serum Pool I, 15 samples (1 g each) were selected from across the total production lot of the pool. For the determination of 25(OH)D_2, 12 samples (2 g each from combined contents of two vials) were analyzed. The percent relative standard deviation (coefficient of variation, CV) for 25(OH)D_3 and 25(OH)D_2 were 0.7 % and 3.4 %. Table 2 details the 25(OH)D_3 and 25(OH)D_2 measurements in Serum Pool I.

For Serum Pool II, 10 samples (1 g each) were analyzed for the determination of 25(OH)D_3 and 25(OH)D_2. The CVs for 25(OH)D_3 and 25(OH)D_2, were 0.3 % and 0.6 %. Table 3 details the 25(OH)D_3 and 25(OH)D_2 measurements in Serum Pool II.

Table 2: Homogeneity Assessment of Serum Pool I (SRM 2973)

25(OH)D_3

Set	Box	Vial	Index	ng/g
1	30	1	21	38.5
1	1	1	22	21.2[a]
1	82	34	23	38.4
1	16	7	24	38.4
1	35	15	31	38.8
2	66	43	32	38.8
2	43	25	33	39.0
2	73	10	34	38.8
2	1	2	35	38.6
2	1	3	36	38.1
3	8	10	43	38.5
3	23	15	44	38.7
3	50	3	45	38.4
3	57	20	46	38.5
3	2	25	47	38.1
			Mean	38.54
			Standard deviation	0.26
			CV (%)	0.68

25(OH)D_2

Set	Box	Vials	Index	ng/g
4	57	6,7	55	0.66
4	1	4,5	56	0.64
4	43	11,14	57	0.61
4	30	20,21	58	0.62
4	82	32,33	59	0.66
4	16	1,2	60	0.62
5	66	46,47	68	0.67
5	23	30,31	69	0.68
5	8	17,18	70	0.63
5	73	21,22	71	0.62
5	35	27,28	72	0.63
5	50	10,11	73	0.64
			Mean	0.640
			Standard deviation	0.023
			CV (%)	3.5

a) First vial. Given its very low value, the second- and third-filled vials were added to the design and tested as part of Set 2. Since their results agree well with those of the later-filled vials, the result for this vial is considered a production-related technical outlier and is excluded from the summary statistics.

Table 3: Homogeneity Assessment of Serum Pool II

25(OH)D_3

Set	Box	Vial	Index	ng/g
6	9	5	85	25.8
6	5	49	86	25.6
6	17	33	87	25.8
6	11	21	88	25.8
6	1	1	89	25.7
7	7	20	96	25.7
7	15	8	97	25.8
7	1	44	98	25.6
7	2	15	99	25.8
7	4	39	100	25.7
			Mean	25.73
			Standard deviation	0.08
			CV (%)	0.32

25(OH)D_2

Set	Box	Vials	Index	ng/g
8	15	1	107	6.35
8	4	36	108	6.32
8	11	31	109	6.33
8	7	29	110	6.35
8	2	14	111	6.45
9	1	2	118	6.32
9	10	26	119	6.34
9	11	40	120	6.38
9	16	47	121	6.37
9	8	11	122	6.34
			Mean	6.355
			Standard deviation	0.039
			CV (%)	0.61

Stability Assessment of Study Materials

Serum Pools I and II were originally analyzed in September 2013 and July 2014, respectively. Formal stability studies have not been performed since these original measurements. However, Serum Pool II was distributed as an unknown in the VitDQAP in early 2015, and the results indicated no instability in the material [13]. Serum pools prepared in the same manner and issued as SRM 972 and SRM 972a Vitamin D Metabolites in Human Serum have been shown to be stable for over 3 years when stored at temperatures between -20 ºC and -80 ºC.

PARTICIPANTS AND INSTRUCTIONS

The call for participation was distributed in May 2015 and samples were distributed in June to seven NMIs and one DI as shown in Table 4.

Table 4: Institutions Receiving CCQM-K132 Sample Materials

NMI or DI	Code	Contact
Health Sciences Authority, Singapore	HSA	Liu Qinde
Korea Research Institute of Standards and Technology	KRISS	Dukjin Kan
National Institute of Metrology, China	NIM	Can Quan
National Institute of Metrology, Thailand	NIMT	Jintana Nammoonnoy
National Institute of Standards and Technology, USA	NIST	Susan Tai
National Measurement Institute Australia	NMIA	Veronica Vamathevan
National Metrology Institute of Turkey	UME	Ahmet Ceyhan Gören
University of Ghent, Belgium	Ugent	Katleen Van Uyfanghe

Each participant received six vials of each of the two serum pools, each vial containing 1.0 mL of frozen serum. Three vials were intended for analyses and the remaining three were available for practice, screening analysis, or for combining contents if required by the laboratory method. If more than six vials of each sample were required, the participants were instructed to request additional vials. Participants were instructed to store the samples in the dark frozen at between -20 ºC and -80 ºC and to avoid exposure of the samples to strong UV light or sunlight.

For Serum Pool I and Serum Pool II, participants were requested to report a single estimate of the mass fraction (ng/g) for $25(OH)D_2$ and $25(OH)D_3$ based on analysis of three subsamples from multiple vials (i.e., three independent replicates). The suggested minimum sample size for analysis for this study is 1 mL of serum. Participants were also requested to report a single estimate of the mass fraction (ng/g) for 3-*epi*-$25(OH)D_3$.

Participants were instructed to use their preferred laboratory procedures; however, only methods based on an isotope dilution quantification approach are eligible for use in the determination of the KCRV. It was recommended that laboratories analyze a serum-matrix CRM in conjunction with the study for quality control and report the results of these analyses. The reporting form also requested descriptions of methods used, reference compounds used as calibrants with purity corrections, the method used to calculate results, and a description of uncertainty calculations.

RESULTS

Methods Used by Participants

CCQM-K132 results were received from seven of the eight institutions that received samples: the University of Ghent did not provide results. All of the participants used ID LC-MS/MS for the measurement of the vitamin D metabolites. Brief descriptions of the analytical methods used by the participants, including sample preparation, analytical technique, calibrants, and quantification approach are summarized in Appendix F. The participants' approaches to estimating uncertainty are provided in Appendix G. The participants' results as reported are provided in Appendix H.

Informative results

After the initial discussion of results in October 2015, NIM requested and received an additional set of Pool I and Pool II samples to evaluate their 25(OH)D_2 measurement procedure. In April 2016 NIM reported the results of their analysis of these samples for 25(OH)D_3 in both Pools and 25(OH)D_2 in Pool II. These unofficial, informative results are listed and displayed with the identifier "NIM/2016".

Participant Results for 25(OH)D_3

The results for CCQM-K132 for the determination of 25(OH)D_3 are detailed in Table 5 and presented graphically in Figure 2.

Table 5: Reported Results for 25(OH)D_3 in CCQM-K132 Pools I and II, ng/g

	25(OH)D_3 - Pool I				25(OH)D_3 - Pool II			
NMI	***x***	***u*(*x*)**	***k***	***U*(*x*)**	***x***	***u*(*x*)**	***k***	***U*(*x*)**
HSA	38.83	0.744	2	1.49	25.82	0.52	2	1.04
KRISS	37.0	0.792	3.18	2.52	24.3	0.526	2.78	1.46
NIM	37.8	1.6	2	3.2	24.4	1.0	2	2.0
NIM/2016	37.37[a]	0.69[a]	2	1.4	26.11[a]	0.72[a]	2	1.5
NIMT	37.87	2.37	1.99	4.72	26.10	1.64	1.97	3.23
NIST	38.6	0.42	2	0.84	25.7	0.28	2	0.55
NMIA	37.0	0.75	1.98	1.5	25.0	0.51	1.98	1.0
UME	37.823	1.355	2.0	2.710	25.842	0.921	2.0	1.842
n	7				7			
$\bar{x}$	37.85				25.31			
$s; \bar{u}$	0.70	1.30			0.74	0.88		

n = number of results included in summary statistics; $\bar{x}$ = mean; s = standard deviation;

$\bar{u} = \sqrt{\sum_i^n u^2(x_i)/n}$, the "average" reported uncertainty

a result of post-study analysis of additional samples, not included in the summary statistics

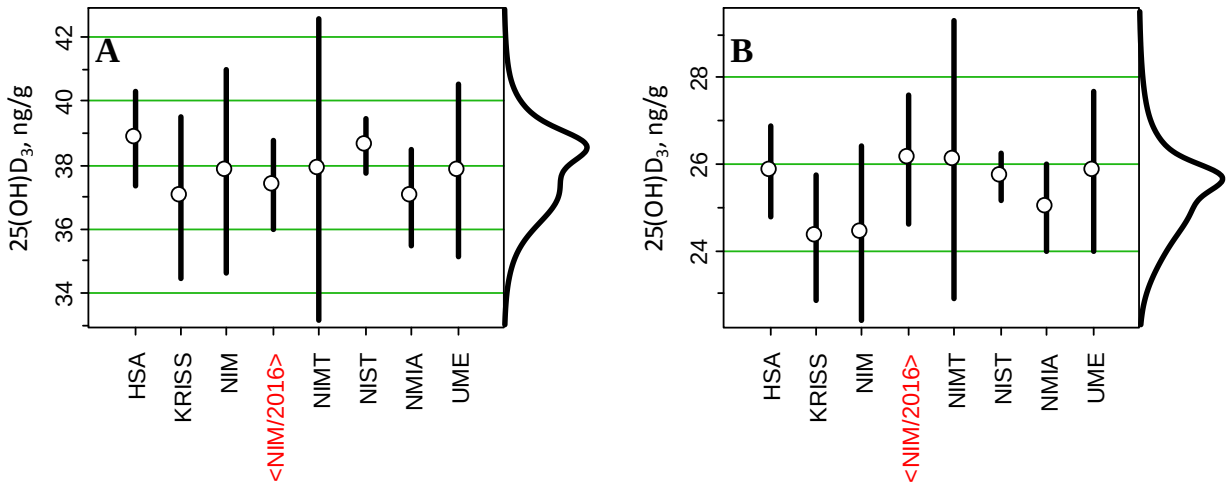

Figure 2: Dot-and-Bar Display of 25(OH)D_3 in CCQM-K132 Pools I and II, ng/g

Panel A displays reported results for 25(OH)D_3 in Serum Pool I; panel B displays results for 25(OH)D_3 in Serum Pool II. Dots represent the reported mean values, x; bars their 95 % expanded uncertainties, $U(x)$. The curve to the right side of each panel is the empirical probability density function (PDF) for the ensemble of reported results. The participant labels in <red> designate results received April 2016; they are not included in the PDFs.

Participant Results for 25(OH)D_2

The results for CCQM-K132 for the determination of 25(OH)D_2 are detailed in Table 6 and presented graphically in Figure 3.

Table 6: Reported Values of 25(OH)D_2 in CCQM-K132, ng/g

	25(OH)D_2 - Pool I				25(OH)D_2 - Pool II			
NMI	***x***	***u*(*x*)**	***k***	***U*(*x*)**	***x***	***u*(*x*)**	***k***	***U*(*x*)**
HSA	0.579	0.0285	2	0.057	6.05	0.205	2	0.41
KRISS	*a*				6.07	0.155	2.57	0.398
NIM	0.79[b]	0.08[b]	2	0.16	8.4[b]	0.50[b]	2	1.0
NIM/2016	*c*				6.13[d]	0.10	2	0.2
NIMT	*c*				7.09[b]	0.45	2.10	0.95
NIST	0.66	0.01	2	0.03	6.33	0.12	2	0.23
NMIA	0.61	*c*			6.41	0.15	1.98	0.31
UME	0.503	0.015	2	0.030	6.050	0.180	2.0	0.361
n	4				5			
$\bar{x}$	0.59				6.18			
$s; \bar{u}$	0.07	0.02			0.17	0.22		

n = number of results included in summary statistics; $\bar{x}$ = mean; s = standard deviation;

$\bar{u} = \sqrt{\sum_i^n u^2(x_i)/n}$, the "average" reported uncertainty

a result reported as "lower than the limit of quantification"

b result declared technically invalid by participant, not included in the summary statistics

c not reported

d result of post-study analysis of additional samples, not included in the summary statistics

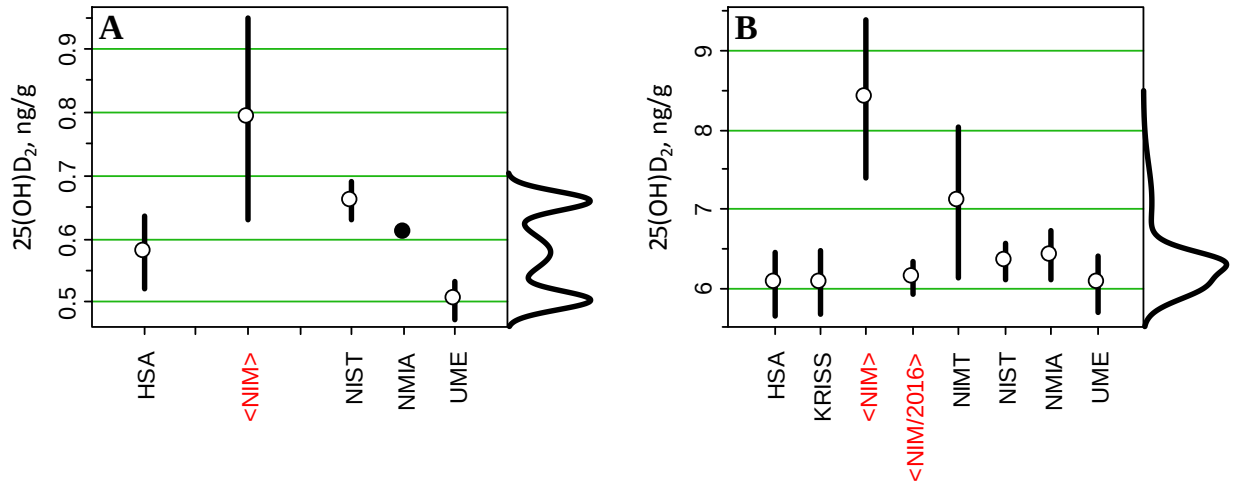

Figure 3: Dot-and-Bar Display of 25(OH)D_2 in CCQM-K132 Pools I and II, ng/g

Panel A displays reported results for 25(OH)D_2 in Serum Pool I; panel B displays results for 25(OH)D_2 in Serum Pool II. Dots represent the reported mean values, x; bars their 95 % expanded uncertainties, $U(x)$. The curve to the right side of each panel is the empirical probability density function (PDF) for the ensemble of reported results. The participant labels in <red> indicate that the value was declared technically invalid by the submitting NMI or that the result was received April 2016; they are not included in the PDFs. The solid dot indicates that no uncertainty was reported; this result is not included in the PDF. Note: the study protocol specified that no KCRV was to be estimated for the 25(OH)D_2 in the Pool I material; these results were requested solely for exploratory purposes.

Participant Results for 3-*epi*-25(OH)D_3

The results for CCQM-K132 for the determination of 3-*epi*-25(OH)D_3 are detailed in Table 7 and presented graphically in Figure 4.

Table 7: Reported Values of 3-*epi*-25(OH)D_3 in CCQM-K132, ng/g

	3-*epi*-25(OH)D_3 - Pool I				3-*epi*-25(OH)D_3 - Pool II			
NMI	***x***	***u*(*x*)**	***k***	***U*(*x*)**	***x***	***u*(*x*)**	***k***	***U*(*x*)**
HSA	3.14	0.16	2	0.32	1.67	0.21	2	0.42
KRISS	1.55	0.0547	2.57	0.141	1.13	0.0332	2.78	0.0921
NIM	3.75	0.14	2	0.28	2.68	0.16	2	0.32
NIMT	2.00	0.17	1.99	0.33	2.73	0.23	1.97	0.45
NIST	2.04	0.04	2	0.08	1.56	0.03	2	0.06
NMIA	2.0	*a*			1.52	*a*		
UME	2.020	0.064	2	0.127	1.483	0.047	2	0.093
n	7				7			
$\bar{x}$	2.36				1.82			
$s; \bar{u}$	0.78	0.12			0.62	0.15		

n = number of results included in summary statistics; $\bar{x}$ = mean; s = standard deviation;

$\bar{u} = \sqrt{\sum_i^n u^2(x_i)/n}$, the "average" reported uncertainty

a not reported

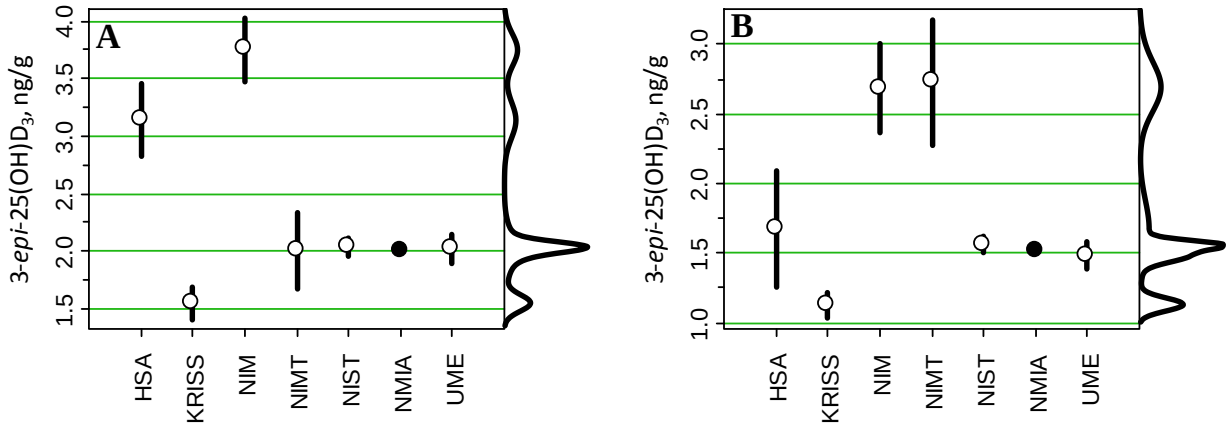

Figure 4: Dot-and-Bar Display of 3-*epi*-25(OH)D_3 in CCQM-K132 Pools I and II, ng/g

Panel A displays reported results for 3-*epi*-25(OH)D_3 in Serum Pool I; panel B displays results for 3-*epi*-25(OH)D_3 in Serum Pool II. Dots represent the reported mean values, x; bars their 95 % expanded uncertainties, $U(x)$. The curve to the right side of each panel is the empirical probability density function (PDF) for the ensemble of reported results. The solid dots indicate that no uncertainty was reported; these results are not included in the PDFs. Note: the study protocol specified that no KCRV was to be estimated 3-*epi*-25(OH)D_3 in either the Pool I or Pool II materials; these results were requested solely for exploratory purposes.

Within- and Between-Measurand Comparisons

Comparison of measurement results for the same measurand in two samples or for two measurands in the same sample can help differentiate systematic measurement system biases from sample-specific issues or measurement imprecision. When systematic biases are dominant, the correlation between pair sets of results should be strongly positive. The square of the correlation coefficient, R^2, directly estimates the fraction of the between-participant covariance that is attributable to the systematic biases. Note, however, that R^2 values for small numbers of results must be interpreted with great caution.

Figure 5 presents Youden-style [14] between-Pool / within-measurand comparisons for the three measurands, plotting standardized ("z-score") values rather than the reported x results to facilitate visual interpretation. The z-scores are defined: $z_i \pm u(z_i) = (x_i - \bar{x})/s \pm u(x_i)/s$, where x_i is a given result, $u(x_i)$ is its reported standard uncertainty, $\bar{x}$ is the mean of all results in a given set that can be paired with a quantitative result in the other set, and s is their standard deviation. Large R^2 are often attributable to systematic biases related to calibration.

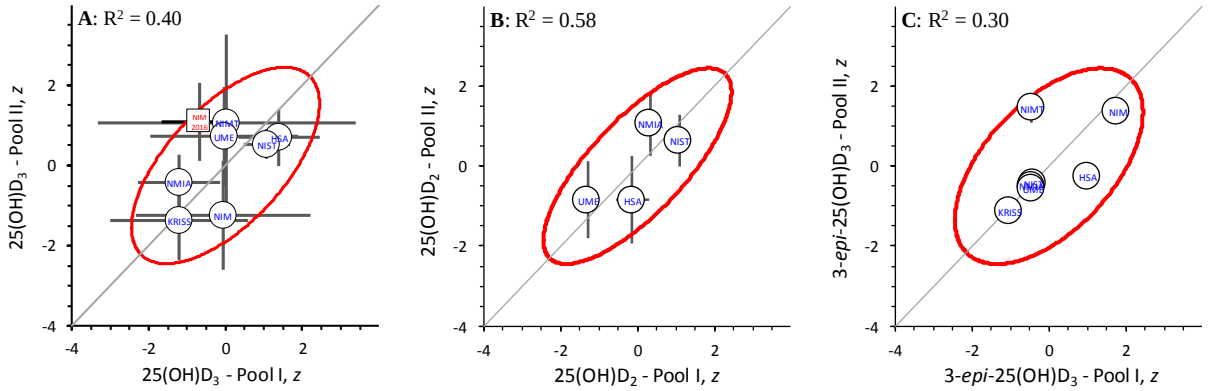

Figure 5: Youden-Style Within-Measurand Comparison of Pool I and II Results

Each panel displays the bivariate distribution of the reported results for one measurand in Serum Pool I (horizontal axis) and Pool II (vertical axis). Panel A displays results for 25(OH)D_3, B for 25(OH)D_2, and C for 3-*epi*-25(OH)D_3. Open circles each represent a {Pool I, Pool II} pair of z-scored original results for one measurand; bars span $z_i \pm u(z_i)$; the open square in Panel A represents the NIM/2016 results. The ellipses bound approximate 95 % bivariate distributions. The diagonal line marks where the pairs would be expected to lie if the measurements in the two Pools were perfectly correlated; it is provided for visual guidance.

Figure 6 presents Youden-style comparisons for the three between-measurand / within-Pool II comparisons. A large R^2 (and associated narrow ellipse) suggests that for a subset of participants the measurement processes for the two measurands are not independent, e.g., use of the same internal standard or external calibrant for both measurands, coelution with incomplete detection specificity, or a shared sample-specific chemical interference.

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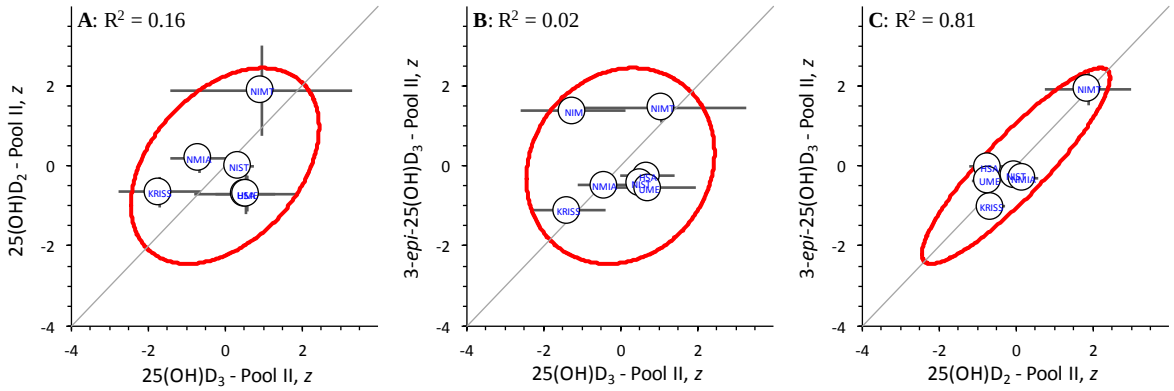

Figure 6: Youden-Style Between-Measurand Comparison of Pool II Results

Each panel displays the bivariate distribution of the reported Serum Pool II results for two measurands. Panel A displays results for 25(OH)D_3 (horizontal axis) and 25(OH)D_2 (vertical axis), B for 25(OH)D_3 (horizontal axis) and 3-*epi*-25(OH)D_3 (vertical axis), and C for 25(OH)D_2 (horizontal axis) and 3-*epi*-25(OH)D_3 (vertical axis). Open circles each represent a {Measurand-X, Measurand-Y} pair of z-scored results in Serum Pool II; bars span $z_i \pm u(z_i)$. The ellipses bound approximate 95 % bivariate distributions. The diagonal line marks where the pairs would be expected to lie if the measurements in the two Pools were perfectly correlated; it is provided for visual guidance.

KEY COMPARISON REFERENCE VALUE (KCRV)

Key Comparison Reference Values, KCRV, for CCQM-K132 were determined for 25(OH)D_3 in both serum pools and for 25(OH)D_2 in Serum Pool II. While participants were requested to provide values for 25(OH)D_2 in Serum Pool I and 3-*epi*-25(OH)D_3 in both serum pools, these values were for information purposes only; no KCRV is estimated for these measurements.

25(OH)D_3 in Serum Pools I and II

Given the good agreement among the 25(OH)D_3 results in both Serum Pools I and II, all values reported for each Pool can be considered as random draws from a Gaussian distribution. For such data, use of robust estimators of location and dispersion such as the median and adjusted median absolute deviation from the median (MAD_E) yields unrepresentatively wide uncertainty intervals [15]. The choice of appropriate estimators for the KCRV therefore depends upon whether or not the reported measurement uncertainties are deemed credible (for the purpose of establishing the KCRV): 1) if not all are credible, the simplest appropriate estimator is the equally-weighted arithmetic mean and its standard deviation-based uncertainty and 2) if all are credible, a variance-weighted mean and its uncertainty [15]. In April 2016, the OAWG decided to define the KCRVs as the arithmetic mean and the standard uncertainty of the KCRV, u(KCRV), as the standard deviation of the mean (the standard deviation, s, divided by the square root of the number of valid results, n). The 95 % expanded uncertainty of the KCRV, U_{95}(KCRV), is estimated as: $U_{95}(\text{KCRV}) = t_s \cdot u(\text{KCRV})$, where t_s is the Student's t two-tailed expansion factor for n-1 degrees of freedom and 95 % coverage.

Table 8 lists the summary statistics for the reported results. The KCRV values and their associated 95 % expanded uncertainties are displayed relative to the reported results in Figure 7.

Table 8: KCRVs for 25(OH)D_3 in Serum Pool I and Serum Pool II

Parameter	Pool I	Pool II
Number of results, n	7	7
Arithmetic mean, $\bar{x}$	37.85 ng/g	25.31 ng/g
Standard deviation, s	0.70 ng/g	0.74 ng/g
Coefficient of variation, CV	1.9 %	2.9 %
Standard uncertainty, u	0.27 ng/g	0.28 ng/g
Coverage factor, t_s	2.45	2.45
95 % uncertainty, U_{95}	0.65 ng/g	0.68 ng/g
KCRV ± U_{95}(KCRV)	(37.85 ±0.65) ng/g	(25.31 ±0.68) ng/g

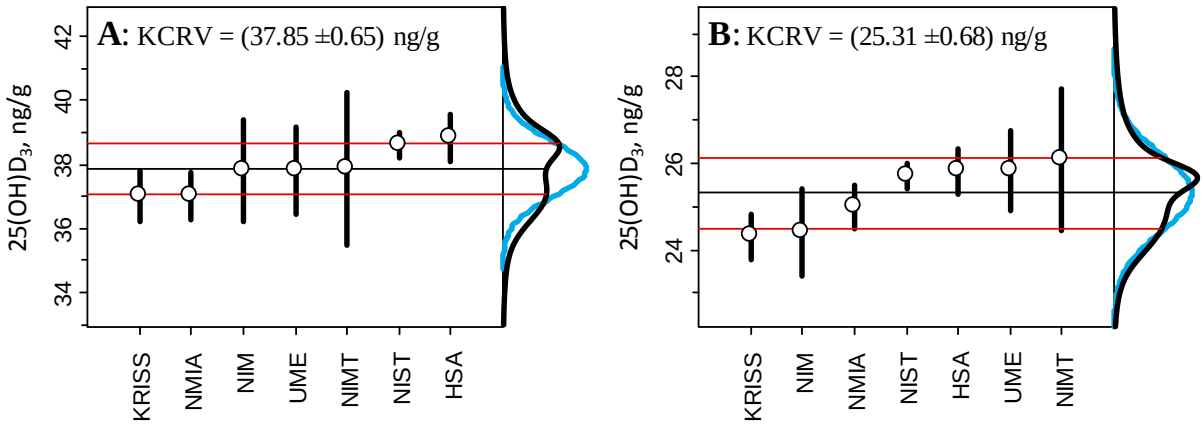

Figure 7: Dot-and-Bar Display of KCRVs for $25(OH)D_3$

Panel A displays the KCRV± KCRU for $25(OH)D_3$ in Serum Pool I; Panel B displays the KCRV± KCRU for $25(OH)D_3$ in Serum Pool II. Dots represent the reported mean values, x; bars their standard uncertainties, $u(x)$. The black horizontal lines represent the KCRVs; the bracketing red horizontal lines the KCRV± KCRU. The black and blue curves to the right side of each panel are the empirical and Gaussian probability density functions (PDFs) for the ensemble of reported results.

$25(OH)D_2$ in Serum Pool II

The results reported by NIM and NIMT for $25(OH)D_2$ in Serum Pool II are not in good accord with those reported by the other participants. After receiving and evaluating additional samples, NIM indicated that their result should not be used in estimating the KCRV for this measurand. NIMT elected to withdraw their result after reviewing their method. The remaining five results can be considered as draws from a single Gaussian distribution. In April 2016, the OAWG decided to base the KCRV and U_{95}(KCRV) for this measurand on the arithmetic mean and the standard deviation of the mean, as described above for $25(OH)D_3$ in Serum Pools I and II.

Table 9 lists the summary statistics for the five reported results remaining after excluding the results declared technically invalid by their submitting NMIs. The KCRV value and its associated 95 % expanded uncertainty are displayed relative to the reported results in Figure 8.

Table 9: KCRV for $25(OH)D_2$ in Serum Pool II

Parameter	Pool II
Number of results, n	5
Arithmetic mean, $\bar{x}$	6.18 ng/g
Standard deviation, s	0.17 ng/g
Coefficient of variation, CV	2.8 %
Standard uncertainty, u	0.08 ng/g
Coverage factor, t_s	2.78
95 % uncertainty, U_{95}	0.22 ng/g
KCRV ± U_{95}(KCRV)	(6.18 ±0.22) ng/g

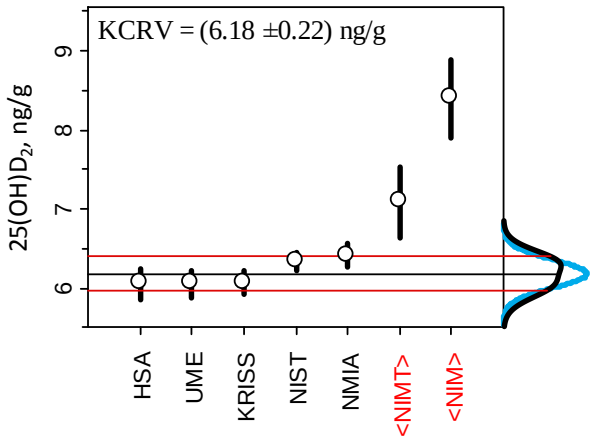

Figure 8: Dot-and-Bar Display of KCRV for 25(OH)D_2 in CCQM-K132 Pool II

This panel displays the KCRV± KCRU derived using the arithmetic mean and standard deviation. Dots represent the reported mean values, x; bars their standard uncertainties, $u(x)$. The black horizontal lines represent the KCRVs; the bracketing red horizontal lines the KCRV± KCRU. The black and blue curves to the right side of the panel are the empirical and Gaussian probability density functions (PDFs) for the ensemble of reported results. The participant labels in <red> designate results that were withdrawn from use in KCRV estimation.

DEGREES OF EQUIVALENCE (DoE)

Regardless of how a KCRV is established, the degree of equivalence (DoE), d_i, for a given result, x_i, is: $d_i = x_i - \text{KCRV}$. When the KCRV is defined from the arithmetic mean, the standard uncertainty of the degree of equivalence, $u(d_i)$, for any x_i not used to define the mean is: $u(d_i) = \sqrt{u^2(x_i) + u^2(\text{KCRV})}$. However, estimation of $u(d_i)$, for x_i used to define the mean depends upon whether the $u(x_i)$ are deemed credible and if the $u(x_i)$ fully account for the observed standard deviation, s.

For the x_i used to define the mean, if the $u(x_i)$ are not deemed credible (for the purpose of estimating DoE) then the $u(d_i)$ are the same for all d_i:
$u(d_i) = s \cdot \sqrt{(1 - 1/n)}$, where n = number of results used to estimate the mean [15]. If the $u(x_i)$ are deemed credible (for the purpose of estimating DoE) and the $u(x_i)$ are mostly smaller than s, then to account for the excess variance the $u(d_i)$ are estimated as:
$u(d_i) = \sqrt{(1 - 2/n) \cdot u^2(x_i) + u^2(\text{KCRV})}$ [15]. In April 2016, the OAWG decided to use the excess-variance definition for the $u(d_i)$.

By convention, the expanded uncertainty for all d_i is estimated using a coverage factor, k, of 2: $U_{k=2}(d_i) = 2 \cdot u(d_i)$.

Figure 9 presents the DoE estimates for the reported 25(OH)D_3 results in Serum Pools I and II. Figure 10 presents the DoE estimates for the reported 25(OH)D_2 results in Serum Pool II.

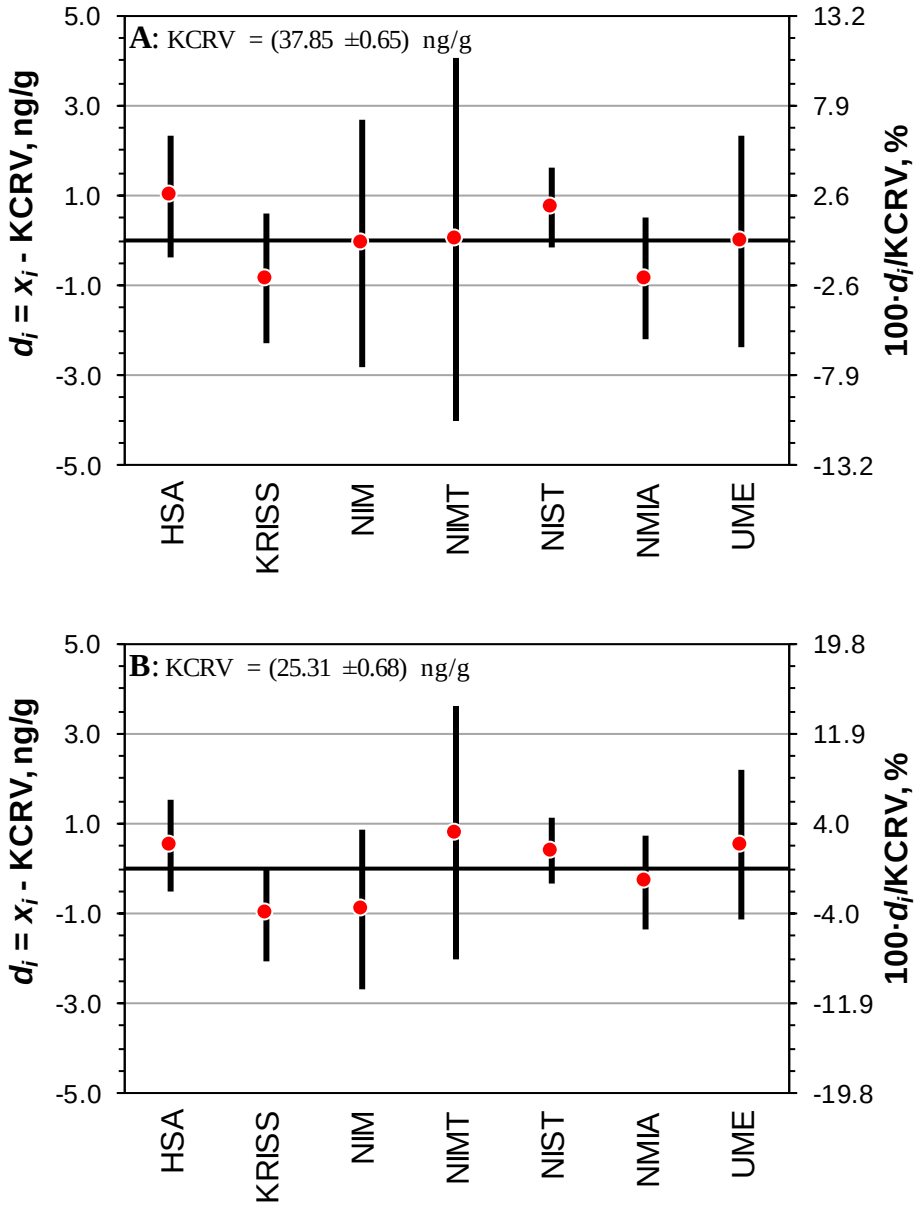

Figure 9: Degrees of Equivalence for 25(OH)D_3 in CCQM-K132 Pools I and II

Panel A displays the $d_i \pm U_{k=2}(d_i)$ for 25(OH)D_3 in Serum Pool I; Panel B displays the $d_i \pm U_{k=2}(d_i)$ for 25(OH)D_3 in Serum Pool II. The dots denote the d_i; the bars denote their k=2 expanded uncertainties. The black horizontal line marks complete agreement with the KCRV. The thin grey lines are intended to facilitate visual comparison. The left-hand axis scale reports in ng/g; the right-hand axis reports in the equivalent %.

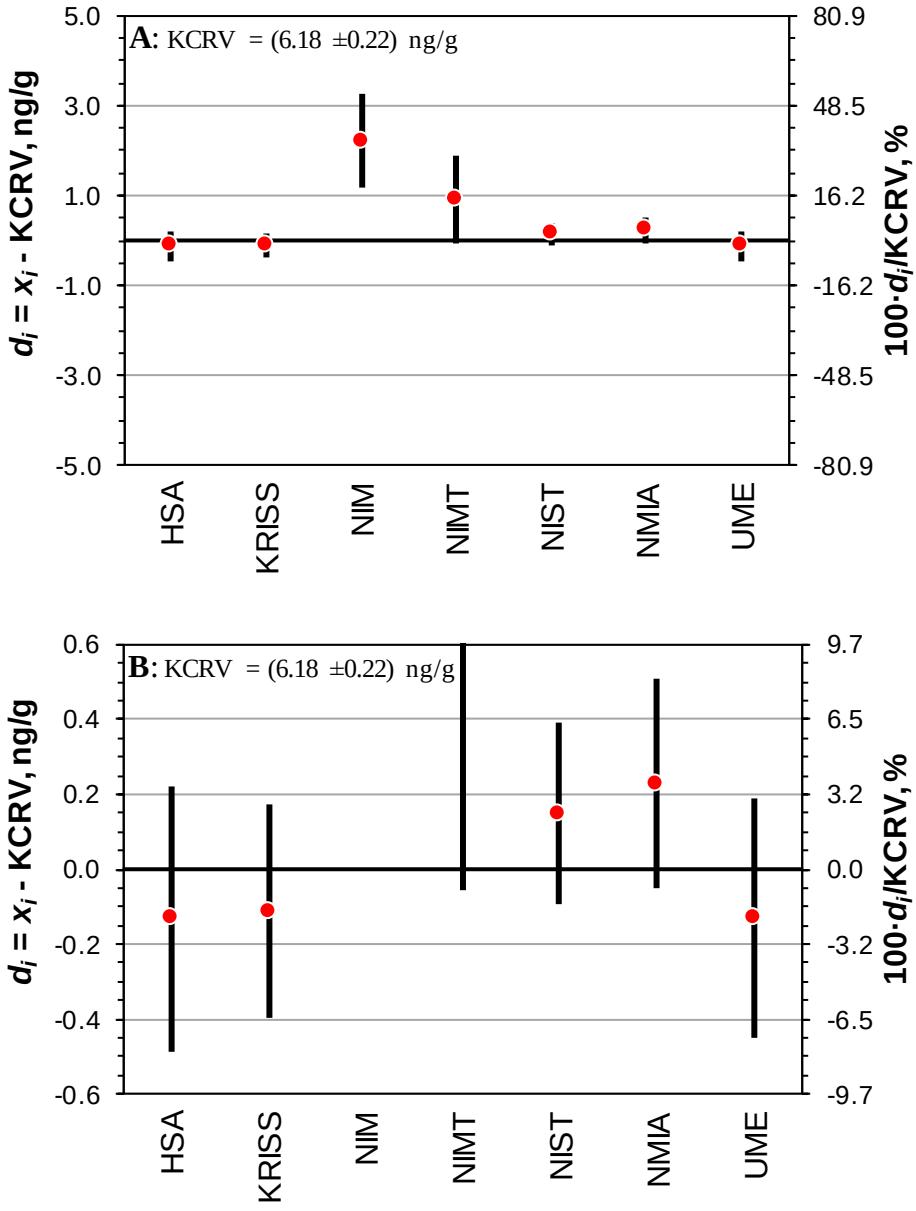

Figure 10: Degrees of Equivalence for 25(OH)D_2 in CCQM-K132 Pool II

Panel A displays the $d_i \pm U_{k=2}(d_i)$ for 25(OH)D_2 in Serum Pool II for all participants; B displays the same data at higher graphical resolution. The dots denote the d_i; the bars denote their k=2 expanded uncertainties. The black horizontal line marks complete agreement with the KCRV. The thin grey lines are intended to facilitate visual comparison. The left-hand axis scale reports in ng/g; the right-hand axis reports in the equivalent %.

USE OF CCQM-K132 IN SUPPORT OF CALIBRATION AND MEASUREMENT CAPABILITY (CMC) CLAIMS

How Far the Light Shines

Successful participation in CCQM-K132 demonstrates capabilities in analysis of low molecular mass (100 g/mol to 500 g/mol) and low-polarity (nonpolar, $pK_{ow} < -2$) analytes at the 1 ng/g to 500 ng/g mass fraction range in complex biological matrixes with core competencies for sample preparation and analysis using ID LC-MS/MS. This study extends the mass fraction capability range to 10^5 to 10^6 times lower than that previously demonstrated in previous CCQM Key Comparisons for cholesterol in serum, another nonpolar clinical analyte.

Core Competency Statements

Tables 10a to 10g list the Core Competencies claimed by the participants in CCQM-K132. The information in these Tables is as provided by the participants; however, the presentation of some entries has been condensed and standardized. Details of the analytical methods used by each participant in this study are provided in Appendix F.

CCQM is considering the application of "broader-scope" Calibration and Measurement Capabilities (CMCs). Appendix I presents a prototype "broader-scope" CMC that could be claimed on the basis of successful participation in CCQM-K132 and relevant previous CCQM Key Comparisons.

Table 10a: Core Competencies Demonstrated in CCQM-K132 by HSA

CCQM-K132	***HSA***	**Low-Polarity Analytes in a Biological Matrix: Vitamin D Metabolites in Human Serum**
Scope of Measurement: This Key Comparison will demonstrate capabilities for low molecular mass (100 g/mol to 500 g/mol) and low polarity (nonpolar, $pK_{ow} < -2$) analytes at the 1 ng/g to 500 ng/g mass fraction range in a biological matrix (human serum or blood) with core competencies for sample preparation and analysis using isotope dilution LC-MS, LC-MS/MS, or GC-MS. This study will complement capabilities demonstrated by previous key comparisons for cholesterol in serum (another nonpolar clinical analyte) by extending the mass fraction levels to 10^5 to 10^6 lower analyte levels then demonstrated with cholesterol. This Key Comparison is also appropriate to support claims for steroid hormone; e.g. cortisol, progesterone, or testosterone.		
Competency	**✓, ×, or N/A**	**Specific Information as Provided by *HSA***
Competencies for Value-Assignment of Calibrant		
Calibrant: Did you use a "highly-pure substance" or calibration solution?		25-Hydroxyvitamin D Calibration Solutions (SRM 2972a) from NIST was used as the calibration standards.
Identity verification of analyte(s) in calibration material	✓	LC-MS/MS was used to verify the $[M+H]^+$ ion and the corresponding daughter ions.
For calibrants which are a highly-pure substance: Value-Assignment / Purity Assessment method(s)	N/A	CRM calibration solutions were used.
For calibrants which are a calibration solution: Value-assignment method(s)	N/A	The mass fractions of the calibration solutions were certified by NIST.
Sample Analysis Competencies		
Identification of analyte(s) in sample	✓	The analytes in the samples were identified against CRM calibration solutions (SRM 2972a) by comparing their MRM transitions and retention times on the LC-MS/MS.
Extraction of analyte(s) of interest from matrix	✓	Liquid/liquid extraction was used. After spiking the isotope labeled internal standards, the sample was added carbonate buffer solution (200 µL per mL of liquid, pH 9.8±0.2). The mixture was then vortexed vigorously, and was added 5 mL of hexane/ethyl acetate solvent mixture (50:50 volume ratio) for extraction. The mixture was further shaken vigorously for 10 min using an orbital shaker, and was centrifuged at 4200 rpm for 5 min. The upper organic phase was transferred to a 15 mL centrifuge tube. The aqueous phase was extracted two more times following the same procedure with shortened shaking time (3 min), and the organic phase was combined.
Cleanup - separation of analyte(s) of interest from other interfering matrix components (if used)	✓	The combined organic phase from extraction was dried under nitrogen at 40 °C, and the residue was reconstituted with 90 µL of methanol. The solution was vortexed and centrifuged at 4200 rpm for 5 min. The supernatant was transferred to a sampling vial with a 150 µL insert for LC-MS/MS measurement.

Table 10a: Core Competencies Demonstrated in CCQM-K132 by HSA (continued)

Sample Analysis Competencies (continued)		
Transformation - conversion of analyte(s) of interest to detectable/measurable form (if used)	N/A	No conversion of analytes was performed.
Analytical system	✓	AB Sciex Qtrap® 5500 MS/MS instrument coupled with Shimadzu Prominence UFLC XR LC system.
Calibration approach for value-assignment of analyte(s) in matrix	✓	Four-point calibration curve IDMS method was used for the quantification of 25(OH)D_2 and 25(OH)D_3. Four calibration blends with isotope ratios being close to 0.7, 0.85, 1.15, and 1.3 were prepared, and the isotope ratio in the sample blends were controlled to be close to 1.0.
Verification method(s) for value-assignment of analyte(s) in sample	✓	Serum CRMs from NIST [SRM 972a (Levels 1, 2 and 3)] were used as the quality control materials, which were measured together with the comparison samples. The obtained values of 25(OH)D_2 were 0.552 ng/g, 0.755 ng/g, and 12.8 ng/g for Levels 1, 2, and 3, respectively. These values were within the uncertainties of the reference values of 25(OH)D_2 (0.52 ± 0.06 ng/g for Level 1, 0.80 ± 0.06 ng/g for Level 2, and 13.0 ± 0.3 ng/g for Level 3). The obtained values of 25(OH)D_3 were 28.7 ng/g, 17.9 ng/g, and 19.2 ng/g for Levels 1, 2, and 3, respectively. These values were within the uncertainties of the reference values of 25(OH)D_3 (28.1 ± 1.1 ng/g for Level 1, 17.7 ± 0.4 ng/g for Level 2, and 19.4 ± 0.4 ng/g for Level 3).
Other		

Table 10b: Core Competencies Demonstrated in CCQM-K132 by KRISS

CCQM-K132	***KRISS***	**Low-Polarity Analytes in a Biological Matrix: Vitamin D Metabolites in Human Serum**
Scope of Measurement: This Key Comparison will demonstrate capabilities for low molecular mass (100 g/mol to 500 g/mol) and low polarity (nonpolar, $pK_{ow} < -2$) analytes at the 1 ng/g to 500 ng/g mass fraction range in a biological matrix (human serum or blood) with core competencies for sample preparation and analysis using isotope dilution LC-MS, LC-MS/MS, or GC-MS. This study will complement capabilities demonstrated by previous key comparisons for cholesterol in serum (another nonpolar clinical analyte) by extending the mass fraction levels to 10^5 to 10^6 lower analyte levels then demonstrated with cholesterol. This Key Comparison is also appropriate to support claims for steroid hormone; e.g. cortisol, progesterone, or testosterone.		
Competency	**✓,×, or N/A**	**Specific Information as Provided by *KRISS***
Competencies for Value-Assignment of Calibrant		
Calibrant: Did you use a "highly-pure substance" or calibration solution?		Calibration solution (SRM 2972a) obtained from NIST was used
Identity verification of analyte(s) in calibration material	✓	LC-ESI-MS
For calibrants which are a highly-pure substance: Value-Assignment / Purity Assessment method(s)	✓	Both value-assignment and purity assessment of calibrants were used the given values by NIST SRM 2972a
For calibrants which are a calibration solution: Value-assignment method(s)	✓	The same as above
Sample Analysis Competencies		
Identification of analyte(s) in sample	✓	The identification of each analyte in the sample was carried out by comparison of both retention time in UPLC and MS/MS analysis of each analyte between that of calibration solution (NIST SRM 2972a)
Extraction of analyte(s) of interest from matrix	✓	Liquid/liquid Extraction (LLE) with hexane/ethyl acetate (50/50, v/v)
Cleanup - separation of analyte(s) of interest from other interfering matrix components (if used)	✓	SPE cartridge packed with C18-resins
Transformation - conversion of analyte(s) of interest to detectable/measurable form (if used)	N/A	
Analytical system	✓	For MRM analyses of each analyte, LC-ESI-MS/MS was used in positive ion mode
Calibration approach for value-assignment of analyte(s) in matrix	✓	IDMS with exact matching single-point calibration
Verification method(s) for value-assignment of analyte(s) in sample	N/A	
Other	N/A	

Table 10c: Core Competencies Demonstrated in CCQM-K132 by NIM
Information supplied with results received April-2016

CCQM-K132	***NIM***	**Low-Polarity Analytes in a Biological Matrix: Vitamin D Metabolites in Human Serum**

Scope of Measurement: This Key Comparison will demonstrate capabilities for low molecular mass (100 g/mol to 500 g/mol) and low polarity (nonpolar, $pK_{ow} < -2$) analytes at the 1 ng/g to 500 ng/g mass fraction range in a biological matrix (human serum or blood) with core competencies for sample preparation and analysis using isotope dilution LC-MS, LC-MS/MS, or GC-MS. This study will complement capabilities demonstrated by previous key comparisons for cholesterol in serum (another nonpolar clinical analyte) by extending the mass fraction levels to 10^5 to 10^6 lower analyte levels then demonstrated with cholesterol. This Key Comparison is also appropriate to support claims for steroid hormone; e.g. cortisol, progesterone, or testosterone.

Competency	**✓, ×, or N/A**	**Specific Information as Provided by *NIM***
Competencies for Value-Assignment of Calibrant		
Calibrant: Did you use a "highly-pure substance" or calibration solution?		SRM 2972a was obtained from NIST
Identity verification of analyte(s) in calibration material	✓	LC-ESI-MS
For calibrants which are a highly-pure substance: Value-Assignment / Purity Assessment method(s)	✓	Both value-assignment and purity assessment of calibrants used NIST SRM values
For calibrants which are a calibration solution: Value-assignment method(s)	✓	The same as above
Sample Analysis Competencies		
Identification of analyte(s) in sample	✓	The analytes in the samples were identified against CRM calibration solutions (SRM 2972a) by comparing their MRM transitions and retention times on the LCMS/MS.
Extraction of analyte(s) of interest from matrix	×	Liquid/liquid extraction was used. After spiking the isotope labeled internal standards, the sample was added carbonate buffer solution (200 µL per mL of liquid, pH 9.8±0.2). The mixture was then vortexed vigorously, and was added 4.3 mL of hexane/ethyl acetate solvent mixture (50:50 volume ratio) for extraction. The mixture was further shaken vigorously for 60 min using an orbital shaker, and was centrifuged at 8000 rpm for 6 min. The upper organic phase was transferred to a 5 mL centrifuge tube. The aqueous phase was extracted two more times following the same procedure with shortened shaking time (30 min), and the organic phase was combined.
Cleanup - separation of analyte(s) of interest from other interfering matrix components (if used)	✓	The combined organic phase from extraction was dried under nitrogen at 400 °C, and the residue was reconstituted with 200 µL of methanol and filtered by 0.2 µm for LC-MS/MS measurement.
Transformation - conversion of analyte(s) of interest to detectable/measurable form	N/A	No conversion of analytes was performed.

Table 10c: Core Competencies Demonstrated in CCQM-K132 by NIM (continued)

Sample Analysis Competencies (continued)		
Analytical system	✓	Waters UPLC- Xevo TQS MS system
Calibration approach for value-assignment of analyte(s) in matrix	✓	Six-point calibration curve IDMS method was used for the quantification of 25(OH)D_2 and 25(OH)D_3. Four calibration blends with isotope ratios being close to 0.7, 0.9, 1.0,1.1,1.3 and 1.5 were prepared, and the isotope ratio in the sample blends were controlled to be close to 1.0.
Verification method(s) for value-assignment of analyte(s) in sample	N/A	
Other	N/A	

The NIM result for 25(OH)D_2 in Serum Pool II result is not consistent with the KCRV for that measurand and yields a DoE that does not cross zero. After further method development and the analysis of additional samples, NIM identified that their method required extraction of larger sample volumes (800 µL to 1000 µL rather than 100 µL to 400 µL) to accurately quantify the low concentration of analyte in this material.

Table 10d: Core Competencies Demonstrated in CCQM-K132 by NIMT

CCQM-K132	***NIMT***	**Low-Polarity Analytes in a Biological Matrix: Vitamin D Metabolites in Human Serum**
Scope of Measurement: This Key Comparison will demonstrate capabilities for low molecular mass (100 g/mol to 500 g/mol) and low polarity (nonpolar, $pK_{ow} < -2$) analytes at the 1 ng/g to 500 ng/g mass fraction range in a biological matrix (human serum or blood) with core competencies for sample preparation and analysis using isotope dilution LC-MS, LC-MS/MS, or GC-MS. This study will complement capabilities demonstrated by previous key comparisons for cholesterol in serum (another nonpolar clinical analyte) by extending the mass fraction levels to 10^5 to 10^6 lower analyte levels then demonstrated with cholesterol. This Key Comparison is also appropriate to support claims for steroid hormone; e.g. cortisol, progesterone, or testosterone.		
Competency	**✓,×, or N/A**	**Specific Information as Provided by *NIMT***
Competencies for Value-Assignment of Calibrant		
Calibrant: Did you use a "highly-pure substance" or calibration solution?		SRM 2972a was obtained from NIST
Identity verification of analyte(s) in calibration material	✓	LC-MS/MS
For calibrants which are a highly-pure substance: Value-Assignment / Purity Assessment method(s)	N/A	
For calibrants which are a calibration solution: Value-assignment method(s)	✓	Gravimetric
Sample Analysis Competencies		
Identification of analyte(s) in sample	✓	LC-MS/MS
Extraction of analyte(s) of interest from matrix	✓	Liquid/liquid extraction using 1:1 (v/v) hexane:ethyl acetate
Cleanup - separation of analyte(s) of interest from other interfering matrix components (if used)	✓	Protein precipitation using 80:20 MeOH:Iso propyl alcohol
Transformation - conversion of analyte(s) of interest to detectable/measurable form (if used)	N/A	
Analytical system	✓	LC-MS/MS
Calibration approach for value-assignment of analyte(s) in matrix	✓	a) IDMS b) 6-point calibration curve
Verification method(s) for value-assignment of analyte(s) in sample	N/A	
Other	N/A	

Table 10e: Core Competencies Demonstrated in CCQM-K132 by NIST

CCQM-K132	***NIST***	**Low-Polarity Analytes in a Biological Matrix: Vitamin D Metabolites in Human Serum**
Scope of Measurement: This Key Comparison will demonstrate capabilities for low molecular mass (100 g/mol to 500 g/mol) and low polarity (nonpolar, $pK_{ow} < -2$) analytes at the 1 ng/g to 500 ng/g mass fraction range in a biological matrix (human serum or blood) with core competencies for sample preparation and analysis using isotope dilution LC-MS, LC-MS/MS, or GC-MS. This study will complement capabilities demonstrated by previous key comparisons for cholesterol in serum (another nonpolar clinical analyte) by extending the mass fraction levels to 10^5 to 10^6 lower analyte levels then demonstrated with cholesterol. This Key Comparison is also appropriate to support claims for steroid hormone; e.g. cortisol, progesterone, or testosterone.		
Competency	**✓, ×, or N/A**	**Specific Information as Provided by *NIST***
Competencies for Value-Assignment of Calibrant		
Calibrant: Did you use a "highly-pure substance" or calibration solution?		SRM 2972a for 25(OH)D_2
Identity verification of analyte(s) in calibration material	✓	qNMR and LC-UV for 25(OH)D_3 and 3-*epi*-25(OH)D_3
For calibrants which are a highly-pure substance: Value-Assignment / Purity Assessment method(s)	✓	25(OH)D_3: qNMR and mass balance (LC-UV, TGA, Karl Fischer titration) 3-*epi*-25(OH)D_3: qNMR and mass balance (LC-UV, TGA)
For calibrants which are a calibration solution: Value-assignment method(s)	N/A	
Sample Analysis Competencies		
Identification of analyte(s) in sample	✓	LC retention time and MRM transitions
Extraction of analyte(s) of interest from matrix	✓	Liquid/liquid extraction
Cleanup - separation of analyte(s) of interest from other interfering matrix components (if used)	✓	Liquid/liquid extraction
Transformation - conversion of analyte(s) of interest to detectable/measurable form (if used)	N/A	
Analytical system	✓	LC-MS/MS [RMPs for 25(OH)D_3 and 25(OH)D_2]
Calibration approach for value-assignment of analyte(s) in matrix	✓	a) Quantification mode used: IDMS b) Calibration mode used :6-point calibration curve
Verification method(s) for value-assignment of analyte(s) in sample	N/A	
Other	N/A	

Table 10f: Core Competencies Demonstrated in CCQM-K132 by NMIA

CCQM-K132	***NMIA***	**Low-Polarity Analytes in a Biological Matrix: Vitamin D Metabolites in Human Serum**
Scope of Measurement: This Key Comparison will demonstrate capabilities for low molecular mass (100 g/mol to 500 g/mol) and low polarity (nonpolar, $pK_{ow} < -2$) analytes at the 1 ng/g to 500 ng/g mass fraction range in a biological matrix (human serum or blood) with core competencies for sample preparation and analysis using isotope dilution LC-MS, LC-MS/MS, or GC-MS. This study will complement capabilities demonstrated by previous key comparisons for cholesterol in serum (another nonpolar clinical analyte) by extending the mass fraction levels to 10^5 to 10^6 lower analyte levels then demonstrated with cholesterol. This Key Comparison is also appropriate to support claims for steroid hormone; e.g. cortisol, progesterone, or testosterone.		
Competency	**✓, ×, or N/A**	**Specific Information as Provided by *NMIA***
Competencies for Value-Assignment of Calibrant		
Calibrant: Did you use a "highly-pure substance" or calibration solution?		NIST SRM2972a for 25-hydroxyvitamin D_3, 25-hydroxyvitamin D_2 and 3-*epi*-25-hydroxyvitamin D_3
Identity verification of analyte(s) in calibration material.	✓	Chromatographic retention time LC-MS/MS – a minimum of 2-3 SRM transitions GC-MS/MS – a minimum of 3 SRM transitions
For highly-pure substances…	N/A	
For calibration solution…	N/A	
Sample Analysis Competencies		
Identification of analyte(s) in sample	✓	Chromatographic retention time LC-MS/MS – a minimum of 2-3 SRM transitions GC-MS/MS – a minimum of 3 SRM transitions
Extraction of analyte(s) of interest from matrix	✓	Liquid-liquid extraction with ethyl acetate/hexane (1:1) (5 mL extraction solvent per gram of serum)
Cleanup - separation of analyte(s) of interest from other interfering matrix components (if used)	✓	High-performance liquid chromatography (HPLC) with ultraviolet (UV) absorbance detection and fraction collection
Transformation - conversion of analyte(s) of interest to detectable/measurable form (if used)	✓	Derivatisation with Amplifex reagent for LC-MS/MS Derivatisation with heptafluorobutyric acid anhydride (HFBA) reagent for GC-MS/MS analysis
Analytical system	✓	LC-MS/MS (ESI-positive, underivatised analytes) LC-MS/MS (ESI, positive, derivatised analytes) GC-MS/MS (CI, negative, derivatised analytes)
Calibration approach for value-assignment of analyte(s) in matrix	✓	Exact-matching (single-point calibration) double isotope dilution mass spectrometry with bracketing
Verification method(s) for value-assignment of analyte(s) in sample	✓	LC-MS/MS (ESI) with HPLC; clean-up and sample derivatisation with Amplifex reagent GC-MS/MS (CI) analysis with HPLC; clean-up and sample derivatisation with HFBA reagent
Other	✓	Analyte/internal standard equilibration for 1 h at room temperature with gentle sample mixing prior to solvent extraction. Equilibration time of 1 h selected based on a time profile study conducted over 24 h which showed that an equilibration time of 15 min or more was suitable.

Table 10g: Core Competencies Demonstrated in CCQM-K132 by UME

CCQM-K132	***UME***	**Low-Polarity Analytes in a Biological Matrix: Vitamin D Metabolites in Human Serum**
Scope of Measurement: This Key Comparison will demonstrate capabilities for low molecular mass (100 g/mol to 500 g/mol) and low polarity (nonpolar, $pK_{ow} < -2$) analytes at the 1 ng/g to 500 ng/g mass fraction range in a biological matrix (human serum or blood) with core competencies for sample preparation and analysis using isotope dilution LC-MS, LC-MS/MS, or GC-MS. This study will complement capabilities demonstrated by previous key comparisons for cholesterol in serum (another nonpolar clinical analyte) by extending the mass fraction levels to 10^5 to 10^6 lower analyte levels then demonstrated with cholesterol. This Key Comparison is also appropriate to support claims for steroid hormone; e.g. cortisol, progesterone, or testosterone.		
Competency	**✓,×, or N/A**	**Specific Information as Provided by *UME***
Competencies for Value-Assignment of Calibrant		
Calibrant: Did you use a "highly-pure substance" or calibration solution?		Matrix CRMa: UME CRM 1308 (25-Hydroxy Vitamin D_2 and 25-Hyroxy Vitamin D_3 in Lyophilized Serum) and NIST SRM2972a
Identity verification of analyte(s) in calibration material	✓	LC-MS/MS HPLC-UV
For calibrants which are a highly-pure substance: Value-Assignment / Purity Assessment method(s)	N/A	-
For calibrants which are a calibration solution: Value-assignment method(s)	✓	IDMS LCMS/MS
Sample Analysis Competencies		
Identification of analyte(s) in sample	✓	Retention time, mass spec ion ratios
Extraction of analyte(s) of interest from matrix	✓	Liquid/liquid extraction
Cleanup - separation of analyte(s) of interest from other interfering matrix components (if used)	N/A	-
Transformation - conversion of analyte(s) of interest to detectable/measurable form (if used)	N/A	-
Analytical system	✓	LC-MS/MS
Calibration approach for value-assignment of analyte(s) in matrix	✓	a) Internal standard b) IDMS, Single-point calibration
Verification method(s) for value-assignment of analyte(s) in sample	N/A	
Other	N/A	

CONCLUSIONS

All participants demonstrated the capability of determining 25(OH)D_3 in a complex biological matrix at levels 25 ng/g and above with a CV of 3% or better. Five of the seven participants also demonstrated the capability of determining 25(OH)D_2 at levels 6 ng/g and above with a CV of 3% or better, indicating that these participants can determine 25(OH)D at levels 6 ng/g and above with a CV of 3% or better. The four results for 25(OH)D_2 in Serum Pool I that were considered technically valid by their submitters agreed within 12% at a level of 0.6 ng/g. The spread of the results for 3-*epi*-25(OH)D_3 indicates that some of the measurement systems employed for this analyte were insufficiently sensitive and/or selective for this analyte.

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APPENDIX A: Call for Participation

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Armishaw, Paul <Paul.Armishaw@measurement.gov.au>
Subject: CCQM-K132 Vitamin D in serum - key comparison call for participation

Dear OAWG members

As we discussed at our recent meeting in April we have a key comparison for Vitamin D in serum which NIST will be coordinating in 2015. Steve Wise has updated the protocol in line with the discussion at our last meeting and thus this is a formal call for participation.
Please contact Steve on stephen.wise@nist.gov to register by 22 May 2015. If you have any questions about this don't hesitate to email Steve or myself.

Best regards

Lindsey

APPENDIX B: Protocol

K132 Low-Polarity Analytes in a Biological Matrix: Vitamin D Metabolites in Human Serum

Key Comparison
Track C

Coordinating Laboratory: NIST
Study Protocol
May 8, 2015

Introduction

Vitamin D is a fat-soluble vitamin that occurs primarily in two forms, vitamin D_2 and vitamin D_3. Vitamin D_3 is produced naturally when skin is exposed to UV radiation and is also naturally-occurring in foods (generally of animal origin) and is also fortified in foods and dietary supplements. Vitamin D_2 occurs in food (generally plant sources) and is the vitamin form in many dietary supplements. Vitamin D is metabolized in the body to produce several closely related, hydroxylated species (metabolites), with 25-hydroxyvitamin D_3 [$25(OH)D_3$] and 25-hydroxyvitamin D_2 [$25(OH)D_2$] as the most common metabolites measured in human serum. Vitamin D levels in serum are typically measured as the total 25-hydroxyvitamin D [25(OH)D] metabolites of vitamin D_3 and vitamin D_2. Total vitamin D [$25(OH)D_2$ + $25(OH)D_3$] concentrations in human serum are typically in the 5 nmol/L to 150 nmol/L (2 ng/g to 60 ng/g) range, with the $25(OH)D_3$ typically accounting for more than 90 % of the total. An epimer of the $25(OH)D_3$, 3-*epi*-$25(OH)D_3$, can be present at levels up to 10 % of $25(OH)D_3$ concentration. 3-epi-$25(OH)D_2$ has been reported, however, it would be expected to be less than 10 % of the $25(OH)D_2$, and it is not currently measured in serum samples. 3-*epi*-$25(OH)D_3$ needs to be chromatographically separated from $25(OH)D_3$, for proper quantification.

Measurement of vitamin D in serum is used in the investigation of bone health as well as a variety of emerging non-skeletal conditions (i.e., immunity, cancer). Vitamin D deficiency (total vitamin D < 50 nmol/L) can lead to bone pain and weakness and compromised immunity.

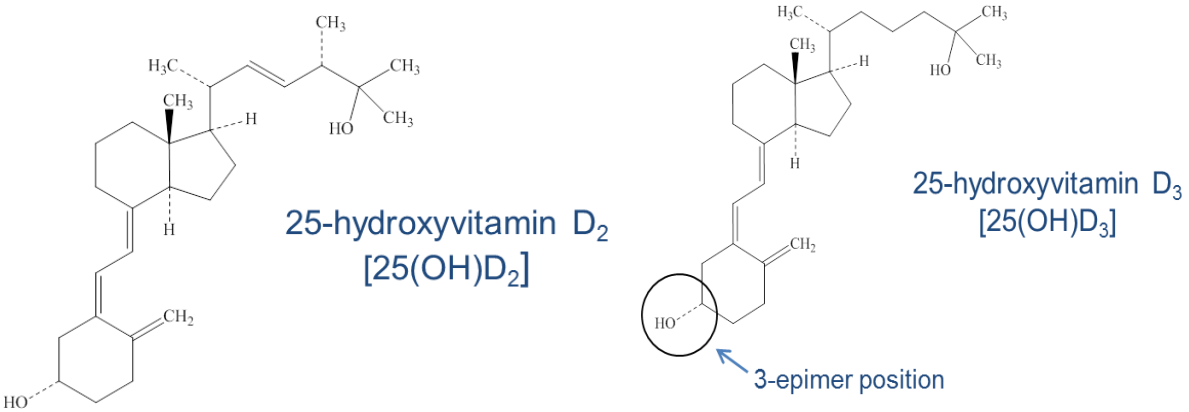


Figure 1. Structures of 25-hydroxyvitamin D_2 and 25-hydroxyvitamin D_3

Why Does the OAWG Need this Key Comparison?

$25(OH)D_2$ and $25(OH)D_3$ are low polarity analytes (molar mass of 412 g/mol and 400 g/mol, respectively) in the same pK_{ow} range as cholesterol (molar mass of 386 g/mol); however, the concentrations in serum of $25(OH)D_2$ and $25(OH)D_3$ are significantly lower than cholesterol levels (factor of 10^5 to 10^6 lower). The OAWG has coordinated a series of KCs for cholesterol but has not carried out any KCs for this type of analyte at this concentration range. At least three National Metrology Institutes (NMIs) and one designated institute are currently providing measurement services in the determination of vitamin D metabolites.

Study Materials

NIST has several activities on-going related to the measurement of vitamin D metabolites in human serum, and as a result has potential comparison materials available. The study would consist of two human serum pools. Serum Pool I contains a high level of $25(OH)D_3$. Target concentrations of the vitamin D metabolites are: $25(OH)D_3$ = 30 ng/g to 50 ng/g; $25(OH)D_2$ = 50x lower than $25(OH)D_3$; and 3-*epi*-$25(OH)D_3$ = typically ~10 % of $25(OH)D_3$ level. Serum Pool II would be a normal level of $25(OH)D_3$ with higher than normal level of $25(OH)D_2$. Target concentrations of the vitamin D metabolites in Serum Pool II are: $25(OH)D_3$ = 15 ng/g to 30 ng/g; $25(OH)D_2$ = 1 ng/g to 10 ng/g; and 3-*epi*-$25(OH)D_3$ = typically ≈10 % of $25(OH)D_3$ level. These two serum materials would be provided as 0.5 mL samples in vials, and would be shipped frozen (on dry ice) to the participants. The materials require storage at between -20 ºC and -80 ºC. Both serum pools have been analyzed and homogeneity assessed.

Measurands

The study requires the determination of two measurands: the mass fractions of 25-hydroxyvitamin D_3 [$25(OH)D_3$] and 25-hydroxyvitamin D_2 [$25(OH)D_2$] in human serum. These two analytes are required to provide the total vitamin D concentration typically reported clinically. A third measurand, 3-*epi*-$25(OH)D_3$ mass fraction, should also be determined (and reported) to demonstrate that the analytical method is capable of resolving the 3-epimer from the $25(OH)D_3$ (see Figure 1). However, no KCRV will be assigned for the 3-*epi*-$25(OH)D_3$. For the determination of mass fraction of $25(OH)D_2$, we ask that you report a value for this measurand in both serum pools; however, because of the low level in Serum Pool I, a KCRV will not be determined for this analyte in Serum Pool I.

Methods

Currently two methods for determination of $25(OH)D_3$ and $25(OH)D_2$ based on isotope dilution liquid chromatography with tandem mass spectrometry (ID LC-MS/MS) have been published [1,2] and recognized as reference measurement procedures (RMP) by the Joint Committee for Traceability in Laboratory Medicine (JCTLM). There are no RMP for the determination of 3-*epi*-$25(OH)D_3$. It is anticipated that participants would use an ID LC-MS/MS method; however, isotope dilution LC-MS methods and isotope dilution gas chromatography-mass spectrometry (ID GC-MS) methods have been reported and would be acceptable.

Homogeneity Assessment and Stability of Serum Pools

The homogeneity of the two serum pools was evaluated at NIST using the NIST RMP based on LC-MS/MS for determination of $25(OH)D_3$, $25(OH)D_2$, and 3-*epi*-$25(OH)D_3$. For Serum Pool I 15 samples (1 g each) were selected from across the total production lot of the serum pool and

analyzed by the RMP for determination of 25(OH)D_3. For the determination 25(OH)D_2 and 3-*epi*-25(OH)D_3 using the RMP, 12 samples (2 g each from combined contents of two vials) were analyzed. For the determination of 25(OH)D_3, 25(OH)D_2, and 3-*epi*-25(OH)D_3, the CVs were 0.7 %, 3.4 %, and 1.2 % for Serum Pool I. For Serum Pool II, 10 samples (1 g each) were analyzed for the determination of 25(OH)D_3 and 25(OH)D_2. For determination of 3-*epi*-25(OH)D_3, four samples (2 g each from combined contents of two vials). For the analysis of Serum Pool II, the CVs for 25(OH)D_3, 25(OH)D_2, and 3-*epi*-25(OH)D_3 were 0.3 %, 0.6 %, and 1.0 %.

These serum pools were analyzed during the previous 18 months (Serum Pool I in Sept. 2011 and Serum Pool II in July 2014), and no formal stability study has been performed since these original measurements. Serum pools prepared in the same manner and issued as SRM 972 and SRM 972a Vitamin D Metabolites in Human Serum have been shown to be stable for over 3 years when stored at temperatures between -20 ºC and -80 ºC. Therefore, we anticipate that the vitamin D metabolites would be stable in the serum material during the period of the study.

Reference Standards Available

Reference standards for 25(OH)D_2, 25(OH)D_3, and 3-epi-25(OH)D_3 are commercially available from several sources including:

USP (USA) www.usp.org
LCG Standards www.lgcstandards.com
IsoSciences (USA) www.isosciences.com
Cerilliant (USA) www.cerilliant.com
Toronto Research Chemicals Inc. (Canada) www.trc-canada.com

Isotopically-labeled vitamin D metabolites for use as internal standards (^{13}C-labeled and ^{2}H-labeled) are available from:

IsoSciences (USA) www.isosciences.com
Cerilliant (USA) www.cerilliant.com
Toronto Research Chemicals Inc. (Canada) www.trc-canada.com

CRMs Available

A calibration solution CRM is available from NIST for the three vitamin D metabolites in ethanol, SRM 2972a 25-Hydroxyvitamin D Calibration Solutions. SRM 2972a consists of four separate ethanolic solutions of vitamin D metabolites as indicated below:

25-Hydroxyvitamin D_3 Calibrant in Ethanol Level 1 (nominally 400 ng/g)
25-Hydroxyvitamin D_3 Calibrant in Ethanol Level 2 (nominally 800 ng/g)
25-Hydroxyvitamin D_2 Calibrant in Ethanol (nominally 300 ng/g)
3-Epi-25-hydroxyvitamin D_3 Calibrant in Ethanol (nominally 300 ng/g)

Several serum-matrix CRMs with certified values for the three vitamin D metabolites of interest are available from NIST. These CRMs include: SRM 972a Vitamin D Metabolites in Frozen Human Serum (four levels); SRM 968e Fat-Soluble Vitamins, Carotenoids, and Cholesterol in Human Serum (three levels for 25(OH)D_3 only); and SRM 1950 Metabolites in Human Plasma (one level, 25(OH)D_2 and 25(OH)D_3 only).

Study Guidelines
Each participant will receive six vials of each of the two serum pools, each vial containing 1.0 mL of frozen serum. Three vials are intended for analyses and the remaining are available for practice, screening analysis, or for combining contents of more than one vial if required by the laboratory method. If more than six vials of each sample are required, please contact the study coordinator. Samples must be stored in the dark frozen at between -20 ºC and -80 ºC. Avoid exposure to strong UV light or sunlight.

For Serum Pool I and Serum Pool II, participants are requested to report a single estimate of the mass fraction (ng/g) for 25(OH)D_2 and 25(OH)D_3 based on analysis of three subsamples from multiple vials (i.e., three independent replicates). The minimum sample size for analysis for this study is 1 mL of serum. Participants should also report a single estimate of the mass fraction (ng/g) for 3-*epi*-25(OH)D_3; however, there will be no KCRV established for this measurand. Participants may use their preferred laboratory procedures; however, methods should be based on an isotope dilution quantification approach to be considered for use in the determination of the KCRV. It is recommended that laboratories analyze a serum-matrix CRM in conjunction with the study for quality control and report the results of these analyses.

Submission of Results
Each participant must provide results using a reporting sheet (provided with the samples) and sent to the study coordinator (stephen.wise@nist.gov) via email with the core competency table before the submission deadline. Submitted results are considered final and no corrections or adjustments of analytical data will be accepted unless approved by the OAWG. The results must include: (1) the mass fraction (ng/g) of each of the three metabolites of interest, and (2) the standard and expanded uncertainties with detailed description of the full uncertainty budget. A description of the analytical procedure (extraction, sample clean-up, LC-MS/MS, LC-MS, or GC-MS column and conditions, and quantification approach) must be provided in the reporting forms. Details must also be provided concerning the calibration and internal standards used with appropriate purity statement and/or laboratory assessment.

How Far Does the Light Shine?
This Key Comparison will demonstrate capabilities for low molecular mass (100 g/mol to 500 g/mol) and low polarity (nonpolar, $pK_{ow} < -2$) analytes at the 1 ng/g to 500 ng/g mass fraction range in a biological matrix (human serum or blood) with core competencies for sample preparation and analysis using isotope dilution LC-MS, LC-MS/MS, or GC-MS. This study will complement capabilities demonstrated by previous key comparisons for cholesterol in serum (another nonpolar clinical analyte) by extending the mass fraction levels by 10^5 to 10^6 times lower than previously demonstrated capabilities for cholesterol. This Key Comparison is also appropriate to support claims for steroid hormones (e.g. cortisol, progesterone, testosterone).

Time Schedule
Call for participants in CCQM-K132 will be in May 2015. Samples will be distributed in late May or early June 2015. The deadline for submission of results will be September 15, 2015. The first discussion of the results will be during the OAWG meeting at NIST in October in Gaithersburg, MD.

References

1. Tai SS, Bedner MM, Phinney KW, Development of a candidate reference measurement procedure for the determination of 25-hydroxyvitamin D_3 and 25-hydroxyvitamin D_2 in human serum using isotope-dilution liquid chromatography-tandem mass spectrometry, *Anal. Chem*. 82:1942-1948 (2010)
2. Stepman HC, Vanderroost A, Van Uytfanghe K, Thienpont LM, Candidate reference measurement procedures for serum 25-hydroxyvitamin D_3 and 25-hydroxyvitamin D_2 by using isotope-dilution liquid chromatography-tandem mass spectrometry, *Clin. Chem*. 57:441-448 (2011)

APPENDIX C: Registration Form

Registration Form

CCQM K132 - Low-Polarity Analytes in a Biological Matrix: Vitamin D Metabolites in Human Serum

ORGANIZATION / DEPARTMENT / LABORATORY

FULL ADDRESS FOR SHIPMENT OF SAMPLES (no PO box)

CONTACT PERSON

E-MAIL AND TELEPHONE

Date _____

Please complete the form and send it back to stephen.wise@nist.gov **before May 22, 2015**.

APPENDIX D: Reporting Form

The original form was distributed as an Excel workbook. The following are pictures of the relevant portions of the workbook's three worksheets.

"Participant Details" worksheet

CCQM-K132

Low Polarity Analytes in Biological Matrix: Vitamin D Metabolites in Human Serum

Data Submission Form

Please complete all pages of the reporting form and submit it by email before September 15, 2015 to:
stephen.wise@nist.gov

Reporting Date	
Institute	
Submitted by (name)	
E-mail address	

"Results" Worksheet

CCQM-K132 RESULTS				
Serum Pool I				
Measurand	Mass Fraction (ng/g)	Combined Standard Uncertainty (ng/g)	Coverage Factor (k)	Expanded Uncertainty (ng/g)
25(OH)D2				
25(OH)D3				
3-epi-25(OH)D3				
Serum Pool II				
Measurand	Mass Fraction (ng/g)	Combined Standard Uncertainty (ng/g)	Coverage Factor (k)	Expanded Uncertainty (ng/g)
25(OH)D2				
25(OH)D3				
3-epi-25(OH)D3				
Note: We request that you report results for all three analytes in both serum pools; however, the results for 3-epi-25(OH)D3 in both serum pools and 25(OH)D2 in Serum Pool I will **not** be used for determination of a KCRV				

“Analytical Information” Worksheet, Page 1

Information about the analytical procedure	
Sample amount used for analysis	g
Sample pre-treatment (if applicable)	
Extraction method/conditions (e.g., liquid/liquid extraction, solvent)	
Clean-up procedure (e.g., SPE, LC)	
Analytical instrumentation used (e.g.,LC-MS, LC-MS/MS, GC-MS)	
MS settings	
Chromatographic Column (i.e., specify tpe and manufacturer)	
Chromatographic Conditions (e.g., GC temperature program, LC mobile phase and gradient)	

“Analytical Information” Worksheet, Page 2

Calibration type / details (e.g., single-point, bracketing / external calibration, internal standard calibration, IDMS)	
Calibration standards (e.g., source, purity, and traceability of standards)	
Internal standards used (Please specify the compounds, sourse, and at which stage of the analysis were the internal standards	
Purity assessment of the calibrant (if applicable) (e.g. methods used for value assignment/verification)	
Estimation of impurities (if applicable) (e.g. type of impurity, mass fraction, uncertainty)	
Indicate ion/MRM monitored in Mass Spectrometer	
Measurement equation and uncertainty budget (please include breakdown of the budget, describing individual uncertainty contributions and how they were combine	

“Analytical Information” Worksheet, Page 3

Additional Comments or Observations		

APPENDIX E: Core Competency Table Form

CCQM OAWG: Competency Template for Analyte(s) in Matrix

CCQM-K132	*NMI/DI*	Low-Polarity Analytes in a Biological Matrix: Vitamin D Metabolites in Human Serum

Scope of Measurement: This Key Comparison will demonstrate capabilities for low molecular mass (100 g/mol to 500 g/mol) and low polarity (nonpolar, $pK_{ow} < -2$) analytes at the 1 ng/g to 500 ng/g mass fraction range in a biological matrix (human serum or blood) with core competencies for sample preparation and analysis using isotope dilution LC-MS, LC-MS/MS, or GC-MS. This study will complement capabilities demonstrated by previous key comparisons for cholesterol in serum (another nonpolar clinical analyte) by extending the mass fraction levels to 10^5 to 10^6 lower analyte levels then demonstrated with cholesterol. This Key Comparison is also appropriate to support claims for steroid hormone; e.g. cortisol, progesterone, or testosterone.

Competency	✓, ×, or N/A"	Specific Information as Provided by *NMI/DI*
Competencies for Value-Assignment of Calibrant		
Calibrant: Did you use a "highly-pure substance" or calibration solution?		*Indicate if you used a "pure material" or a calibration solution. Indicate its source and ID, eg CRM identifier*
Identity verification of analyte(s) in calibration material		*Indicate method(s) you used to identify analyte(s)*
For calibrants which are a highly-pure substance: Value-Assignment / Purity Assessment method(s)		*Indicate how you established analyte mass fraction/purity (i.e., mass balance (list techniques used), qNMR, other)*
For calibrants which are a calibration solution: Value-assignment method(s)		*Indicate how you established analyte mass fraction in calibration solution*
Sample Analysis Competencies		
Identification of analyte(s) in sample		*Indicate method(s) you used to identify analyte(s) in the sample (i.e., Retention time, mass spec ion ratios, other)*
Extraction of analyte(s) of interest from matrix		*Indicate extraction technique(s) used, if any, (i.e. Liquid/liquid, Soxhlet, ASE, other)*
Cleanup - separation of analyte(s) of interest from other interfering matrix components (if used)		*Indicate cleanup technique(s) used, if any (i.e., SPE, LC fractionation, other)*
Transformation - conversion of analyte(s) of interest to detectable/measurable form (if used)		*Indicate chemical transformation method(s), if any, (i.e., hydrolysis, derivatization, other)*
Analytical system		*Indicate analytical system (i.e., LC-MS/MS, GC-HRMS, GC-ECD, other)*
Calibration approach for value-assignment of analyte(s) in matrix		*a) Indicate quantification mode used (i.e., IDMS, internal standard, external standard, other)* *b) Indicate calibration mode used (i.e., single-point calibration, bracketing, x-point calibration curve, other)*
Verification method(s) for value-assignment of analyte(s) in sample (if used)		*Indicate any confirmative method(s) used, if any.*
Other		*Indicate any other competencies demonstrated.*

Instructions:

- In the middle column place a tick, cross or say the entry is not applicable for each of the competencies listed (the first row does not require a response)
- Fill in the right hand column with the information requested in blue in each row
- Enter the details of the calibrant in the top row, then for materials which would not meet the CIPM traceability requirements the three rows with a # require entries.

APPENDIX F: Summary of Participants' Analytical Information

The following Tables summarize the analytical information provided by the participants in the "Analytical Information" worksheet of the "CCQM-K132 Reporting Form" Excel workbook.

The summary is provided as three Tables:

Table F-1: CCQM-K132 Sample Size, Extraction, and Cleanup,
Table F-2: CCQM-K132 Analytical Techniques, and
Table F-3: CCQM-K132 Calibrants and Standards.

Disclaimer

Certain commercial equipment, instruments, or materials are identified in these Tables to specify adequately experimental conditions or reported results. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology or other participant in this Key Comparison, nor does it imply that the equipment, instruments, or materials identified are necessarily the best available for the purpose.

Table F-1: Summary of Sample Size, Extraction, and Cleanup for CCQM-K132

Institute	Sample Size (g)	Extraction	Cleanup
HSA	1.8 g (I) 0.9 g (II)	Carbonate buffer added, vortex mixing; liquid/liquid extraction with hexane:ethyl acetate (50:50 v/v); shaken and centrifugation (4200 rpm for 5 min); repeat 3x	Combined organic phase dried under N_2 @ 40 ºC; residue reconstituted with MeOH; vortex and centrifugation (4200 rpm, 5 min); supernatant removed
KRISS	1.0 g	Adjust pH to 10 with NH_4OH; vortex mixing with hexane:ethyl acetate (1:1,v/v) for 2 h; remove organic layer and freeze-dry; reconstitute residue in MeOH	Apply to HLB cartridge and elute with 60 % MeOH then 100 % MeOH to isolate vitamin D metabolites
NIM	0.1 g to 0.4 g	0.9 % NaCl solution added; liquid/liquid extraction with hexane for 20 min	Centrifugation (8000 rpm for 5 min @ 4 ºC); repeat; remove supernatant; dry under N_2 at 45 ºC; reconstitute in MeOH
NIMT	0.5 g	Liquid/liquid extraction 3 x 1 mL hexane:ethyl acetate (1:1, v/v)	Protein precipitation using MeOH with IPA
NIST	1 g (I) 2 g (II)	Add H_2O + IS, equilibrate 1 h; adjust pH to 9.8 with carbonate buffer; liquid/liquid extraction with hexane:ethyl acetate (50:50) 10 min with mechanical shaker; remove supernatant; repeat extraction	Combined extracts dried under N_2 at 45 ºC; residue reconstitute with MeOH
NMIA (I)	2 g	H_2O added + IS, mixed 1 h; extract 2x hexane:ethyl acetate (1:1); extract evaporated to dryness under N_2 (RT); reconstitute with MeOH	Without derivatization: LC fraction collected using Biphenyl Allure (4.6 x 250 mm, 5 µm particles) (Restek) with MeOH:H_2O @ 40 ºC; single fraction for all metabolites; fraction dried, reconstitute with MeOH
NMIA (II)	2g	Same as above	With derivatization: same LC fractionation as above with fractions collected for each metabolite; fraction derivatized with ABSciex Ampliflex diene reagent 45 min @ 45 ºC
UME	0.4 g	Add 0.6 g 50 % ammonium sulfate solution; Vortex and centrifugation for 15 min; upper layer removed and H_2O added; vortex	

Table F-2: Summary of Analytical Techniques for CCQM-K132

Institute	Analytical Technique	Chromatographic Column	Chromatographic and Mass Spectrometry Conditions
HSA	LC-MS/MS	Kinetex PFP; 4.6 x 150 mm, 2.6 µm particles (Phenomenex)	Mobile phase: 75:25 MeOH:H_2O @ 0.8 mL/min Ions monitored: 413.3/395.4 *m/z* for 25(OH)D_2; 401.4/383.4 *m/z* for 25(OH)D_3 and *epi*-25(OH)D_3; 419.5/401.3 *m/z* for 25(OH)D_2-d_6; 407.3/389.5 *m/z* for 25(OH)D_3-d_6
KRISS	UPLC-MS/MS	HSS PFP, 2.1 x 100 mm, 1.8 µm particles (Waters)	Mobile phase: 70:30 MeOH:H_2O with 0.1 % formic acid @ 0.15 mL/min @ 30 ºC Ions monitored: 25(OH)D_2 395.4/377.4 *m/z*; 25(OH)D_3 and *epi*-25(OH)D_3 383.4/365.4 *m/z*; IS 398.4/380.4 *m/z* for 25(OH)D_2-d_3 and 386.4/368.4 *m/z* for 25(OH)D_3-d_3 and *epi*-25(OH)D_3-d_3
NIM	LC-MS/MS Positive APCI	Kinetex PFP; 2.1 x 100 mm, 2.6 µm particles (Phenomenex)	Mobile phase: 50:50 MeOH:H2O with 0.1 % formic acid @ 0.4 mL/min Ions monitored: 25(OH)D_2 395.4/119.2 *m/z*; 25(OH)D_3 and *epi*-25(OH)D_3 383.8/365.3 *m/z*; IS 401.4/119.0 *m/z* for 25(OH)D_2-d_6 and 386.4/368.3 *m/z* for 25(OH)D_3-d_3
NIMT	LC-MS/MS Positive APCI	Kinetex PFP; 2.1 x 150 mm, 2.6 µm particles (Phenomenex)	Mobile phase: 70:30 MeOH:H_2O @ 0.15 mL/min; @ 40 ºC Ions monitored: 25(OH)D_2 413.4/395.3 *m/z*; 25(OH)D_3 and epi-25(OH)D_3 401.4/383.4 *m/z*; IS 419.5/401.4 *m/z* for 25(OH)D_2-d_6 and 407.5/389.6 *m/z* for 25(OH)D_3-d_6
NIST	LC-MS/MS APCI	25(OH)D_3: Zorbax SB CN, 4.6 x 150 mm, 3.5 µm particles (Agilent) 25(OH)D_2 and 3-*epi*-25(OH)D_3: Ascentis Express F5, 4.6 x 150 mm, 2.7 µm particles	Mobile phase: 25(OH)D_3 - 67:33 (v:v) MeOH:H_2O @ 30 ºC @ 0.75 mL/min Ions monitored: 401/383 *m/z* and 407/389 *m/z* for 25(OH)D_3 and 25(OH)D_3-d_6 25(OH)D_2 and 3-*epi*-25(OH)D_3 - 73:27 (v:v) MeOH:H_2O @ 30 ºC @ 0.75 mL/min Ions monitored: 401/383 *m/z*, 407/389 *m/z*, 413/395 *m/z*, and 416/398 *m/z* for 3-*epi*-25(OH)D_3, 3-*epi*-25(OH)D_3-d_6, 25(OH)D_2, and 25(OH)D_2-d_3, respectively

Table F-2: Summary of Analytical Techniques for CCQM-K132 (Continued)

Institute	Analytical Technique	Chromatographic Column	Chromatographic and Mass Spectrometry Conditions
NMIA (I)	2D LC-MS/MS	1st Dimension: Restek PFPP, 2.1 x 100 mm, 1.9 µm particles 2nd Dimension: Waters CSH Fluoro-Phenyl, 2.1 x 100 mm, 1.7 µm particles	Mobile phase: 1st D gradient MeOH:H_2O at 50 ºC; 2nd D gradient MeOH:H_2O with 0.2 % formic acid at 50 ºC MRM Monitored: 383/257, 383/365, 388/362, 388/370 *m/z* for 25(OH)D_3/25(OH)D_3-$^{13}C_5$, 3-*epi*-25(OH)D_3/3-*epi*-25(OH)D_3-$^{13}C_5$; 395/209, 395/269, 398/209, and 398/269 *m/z* for 25(OH)D_2/25(OH)D_2-$^{13}C_3$
NMIA (II)	2D LC-MS/MS	1st Dimension: Phenomenex Kinetex F5, 2.1 mm x 100 mm, 2.6 µm particles 2nd Dimension: Phenomenex Kinetex XB-C18, 2.1 mm x 100 mm, 2.6 µm particles	Mobile phase: 1st D gradient ACN:H_2O with 2 % formic acid @ 50 ºC @ 0.3 mL/min; 2nd D gradient MeOH:H_2O with 2 % formic acid @ 50 ºC @ 0.3 mL/min MRM Monitored: 732/217, 732/673, 737/217, 737/678 *m/z* for 25(OH)D_3/25(OH)D_3-$^{13}C_5$ and 3-*epi*-25(OH)D_3/3-*epi*-25(OH)D_3-$^{13}C_5$; 744/217, 744/685, 747/217, and 747/676 *m/z* for 25(OH)D_2/25(OH)D_2-$^{13}C_3$
UME	LC-MS/MS APCI	Reprosil Fluosil PFP, 2.0 mm x 150 mm, 3 µm particles	Mobile phase: isocratic @ 78 % MeOH 22 % H_2O with 0.1 % formic acid; flow @ 0.3mL/min. Ions monitored: 395.3/269.3 *m/z* for 25(OH)D_2; 383.3/257.3 *m/z* for 25(OH)D_3 and epi-25(OH)D_3; IS 389.3/263.3 *m/z*

Table F-3: Summary of Calibrants and Standards for CCQM-K132

Institute	Calibrants	Type of Calibration	Internal Standards	Matrix CRM
HSA	SRM 2972a (NIST)	IDMS 4-point calibration 25(OH)D_2 and 25(OH)D_3 IS 4-point calibration for epi-25(OH)D_3	25(OH)D_3-d_6, epimer (Medical Isotopes) 25(OH)D_2-d_6 (Medical Isotopes)	SRM 972a (NIST)
KRISS	SRM 2972a (NIST)	Single point exact matching double IDMS	25(OH)D_3-d_3 *epi*-25(OH)D_3-d_3 25(OH)D_2-d_3	SRM 972a (NIST)
NIM	SRM 2972a (NIST)	IDMS Bracketing	25(OH)D_3-d_3 (CIL) *epi*-25(OH)D_3-d_3 (CIL) 25(OH)D_2-d_6 (TRC)	SRM 972a (NIST)
NIMT	SRM 2972a (NIST)	IDMS 6-point calibration	25(OH)D_3-d_6 *epi*-25(OH)D_3-d_6 25(OH)D_2-d_6 (TRC)	SRM 972a (NIST)
NIST	25(OH)D_2: SRM 2972a (NIST) 25(OH)D_3 (USP) 3-*epi*-25(OH)D_3 (IsoSciences) purity by LC-UV, TGA, KF, and qNMR	IDMS 6-point calibration	25(OH)D_3-d_6 (Cerilliant) 25(OH)D_2-d_3 (IsoSciences) *epi*-25(OH)D_3-d_6 (TRC)	SRM 972a (NIST)
NMIA (I)	SRM 2972a (NIST)	Exact matching double IDMS with bracketing	25(OH)D_3-$^{13}C_5$ 25(OH)D_2-$^{13}C_3$ *epi*-25(OH)D_3-$^{13}C_5$ (IsoSciences)	SRM 972a (NIST)
NMIA (II)	SRM 2972a (NIST)	Exact matching double IDMS with bracketing	25(OH)D_3-$^{13}C_5$ 25(OH)D_2-$^{13}C_3$ *epi*-25(OH)D_3-$^{13}C_5$ (IsoSciences)	SRM 972a (NIST)
UME	SRM 2972a (NIST)	IDMS single point calibration	25(OH)D_3-d_6 (Sigma-Aldrich)	CRM 1308 (UME, Serum)

APPENDIX G: Summary of Participants' Uncertainty Estimation Approaches

The following are pictures of the uncertainty-related information provided by the participants in the "Analytical Information" worksheet of the "Reporting Form" Excel workbook. Information is grouped by participant and presented in alphabetized acronym order.

Uncertainty Information from HSA

The mass fraction of the analyte [25(OH)D2, 25(OH)D3 or 3-epi-25(OH)D3)] in serum was calculated based on the equation as follows:

$$C_X = (mR_B + b) \times \frac{W_Y}{M_X} = (mR_B + b) \times \frac{M_Y C_Y}{M_X} \qquad (1)$$

where

C_X = mass fraction of measurand in the serum sample

M_X = mass of serum sample (determined by weighing)

M_Y = mass of isotope standard solution (determined by weighing)

W_Y = mass of the isotope labeled standard spiked into the serum sample (equals to MY × CY)

R_B = peak area ratio of sample blend (determined by LC-MS/MS measurements)

C_Y = concentration of isotope labeled standard solution (determined by weighing and from purity of the isotope labeled standard)

m = gradient of the slope of linear regression plot (determined by the linear fit of the isotope mass ratio and the peak area ratio of the calibration blends)

b = intercept on y axis of the linear regression plot (determined by the linear fit of the isotope mass ratio and the peak area ratio of the calibration blends)

For the estimation of uncertainty, considering $R_M = mR_B + b$, and let $R_M = R_M' C_Y / C_Z$, Equation (1) is converted to:

$$C_X = R_M' \times \frac{M_Y C_Z}{M_X} \qquad (2)$$

where

R_M = isotope mass ratio in sample blend

C_Z = concentration of measurant in the calibration standard solution

A standard uncertainty was estimated for all components of the measurement in Equation (2), which were then combined using respective derived sensitivity coefficients to estimate a combined standard uncertainty in the reported result of the measurands in serum samples. A coverage factor k with a value of 2 is used to expand the combined standard uncertainty at a 95 % confidence interval.The factor of method precision (F_P) was accounted for in the final uncertainty budget with the use of the measurement equation:

$$C_X = F_P \times R_M' \times \frac{M_Y C_Z}{M_X} \qquad (3)$$

The sensitivity coefficients of each component can be expressed as follows:

$$\frac{\partial C_X}{\partial R_M'} = \frac{C_X}{R_M'} \qquad \frac{\partial C_X}{\partial M_Y} = \frac{C_X}{M_Y} \qquad \frac{\partial C_X}{\partial M_X} = -\frac{C_X}{M_X} \qquad \frac{\partial C_X}{\partial C_Z} = \frac{C_X}{C_Z} \qquad \frac{\partial C_X}{\partial F_P} = \frac{C_X}{F_P}$$

The standard uncertainty of each component was calculated as follows:

(1) M_Y and M_X: The standard uncertainty was calculated based on the calibration report using the standard weights calibrated by the National Metrology Centre, A*STAR.

(2) F_P: The standard deviation of the mean of the results was used as the the standard uncertainty of method precision.

(3) C_Z: The certified purity and uncertainty of NIST SRM 914a in combination with the uncertainty of weighing for preparation of the calibration standard solution.

(4) R_M': Consider $R_M = R_M' \times C_Z / C_Y$, the conversion of equation $R_M = mR_B + b$ leads to:

$R_B = (C_Z \times R_M') / (C_Y \times m) - b/m$

Let $m' = C_Z / (C_Y \times m)$ and $b' = -b/m$, we have:

$R_B = m' R_M' + b'$

The standard uncertainty of R_M' was calculated using the following equation:

$$\mu_{R_M} = \frac{1}{m'} \times s_{y/x} \times \sqrt{\frac{1}{N} + \frac{1}{n} + \frac{\left(R_B - \overline{R_{Bc}}\right)^2}{m'^2 \sum_{i=1}^{n} (R_{Mc} - \overline{R_{Mc}})^2}} \qquad (4)$$

where

$s_{y/x}$ = standard deviation of the regression

R_B = peak area ratio of sample blend

$R_{Bc}(bar)$ = average peak area ratio of calibration blends

n = number of calibration blends used for the linear regression plot

N = injection time for each sample

R_{Mc} = isotope mass ratio in calibration blends

$R_{Mc}(bar)$ = average isotope mass ratio in calibration blends

The combined standard uncertainty was calculated using the equation below:

$$u = \sqrt{\sum_i c_i^2 u_{xi}^2} \qquad (5)$$

where

u = combined standard uncertainty

c_i = sensitivity coefficient of each component

u_{xi} = standard uncertainty of each component

The expanded uncertainty (U) was calculated by mutiplying the combined standand uncertainty (u) with a coveragy factor (k = 2) for 95% confidence level.

Table 1 Uncertainty budget of 25(OH)D2 in Serum Pool I.

Factor	Value x	Uncertainty u(x)	Relative Uncertainty u(x)/(x)	Sensitivity Coefficient (c) δCx/δx	c2 . u(x)2	% contribution
M_X (g)	1.7366	0.000085	0.0049%	0.000333	7.993E-16	0.00010%
M_Y (g)	0.5062	0.000085	0.0168%	0.00114	9.409E-15	0.00116%
C_Z (µg/g)	0.0017517	0.0000271	1.550%	0.3303	8.042E-11	9.89%
R_M'	0.11185	0.00091	0.815%	0.00517	2.223E-11	2.74%
F_P (µg/g)	0.000579	0.0000266	4.605%	1.00	7.101E-10	87.37%

Table 2 Uncertainty budget of 25(OH)D3 in Serum Pool I.

Factor	Value x	Uncertainty u(x)	Relative Uncertainty u(x)/(x)	Sensitivity Coefficient (c) δCx/δx	c2 . u(x)2	% contribution
M_X (g)	1.7366	0.000085	0.0049%	0.02236	3.600E-12	0.00065%
M_Y (g)	0.5129	0.000085	0.0165%	0.07571	4.127E-11	0.00745%
C_Z (µg/g)	0.009546	0.0001716	1.798%	4.0680	4.876E-07	88.05%
R_M'	1.101	0.00184	0.167%	0.03527	4.212E-09	0.76%
F_P (µg/g)	0.03883	0.0002488	0.641%	1.00	6.191E-08	11.18%

Table 3 Uncertainty budget of 3-epi-25(OH)D3 in Serum Pool I.

Factor	Value x	Uncertainty u(x)	Relative Uncertainty u(x)/(x)	Sensitivity Coefficient (c) δCx/δx	c2 . u(x)2	% contribution
M_X (g)	1.8469	0.000078	0.0042%	0.00170	1.751E-14	0.00007%
M_Y (g)	0.5053	0.000085	0.0168%	0.00622	2.785E-13	0.00111%
C_Z (µg/g)	0.00167	0.0000383	2.301%	1.8865	5.227E-09	20.75%
R_M'	2.854	0.08439	2.957%	0.00110	8.635E-09	34.27%
F_P (µg/g)	0.00314	0.0001065	3.388%	1.00	1.133E-08	44.98%

Table 4 Uncertainty budget of 25(OH)D2 in Serum Pool II.

Factor	Value x	Uncertainty u(x)	Relative Uncertainty u(x)/(x)	Sensitivity Coefficient (c) δCx/δx	c2 . u(x)2	% contribution
M_X (g)	0.9149	0.000085	0.0093%	0.006610	3.146E-13	0.00075%
M_Y (g)	0.4949	0.000085	0.0171%	0.01222	1.075E-12	0.00255%
C_Z (µg/g)	0.003132	0.0000485	1.550%	1.9308	8.785E-09	20.87%
R_M'	0.3490	0.00274	0.784%	0.01733	2.247E-09	5.34%
F_P (µg/g)	0.00605	0.0001763	2.914%	1.00	3.106E-08	73.79%

Table 5 Uncertainty budget of 25(OH)D3 in Serum Pool II.

Factor	Value x	Uncertainty u(x)	Relative Uncertainty u(x)/(x)	Sensitivity Coefficient (c) δCx/δx	c2 . u(x)2	% contribution
M_X (g)	0.9096	0.000085	0.0093%	0.028391	5.804E-12	0.00215%
M_Y (g)	0.4920	0.000085	0.0172%	0.05249	1.984E-11	0.00736%
C_Z (μg/g)	0.01194	0.0002170	1.817%	2.1626	2.202E-07	81.73%
R_M'	0.3853	0.00112	0.290%	0.06703	5.595E-09	2.08%
F_P (μg/g)	0.02582	0.0002088	0.808%	1.00	4.359E-08	16.18%

Table 6 Uncertainty budget of 3-epi-25(OH)D3 in Serum Pool II.

Factor	Value x	Uncertainty u(x)	Relative Uncertainty u(x)/(x)	Sensitivity Coefficient (c) δCx/δx	c2 . u(x)2	% contribution
M_X (g)	0.9075	0.000085	0.0094%	0.001843	2.446E-14	0.00006%
M_Y (g)	0.4815	0.000085	0.0176%	0.00347	8.690E-14	0.00020%
C_Z (μg/g)	0.00146	0.0000335	2.301%	1.1480	1.481E-09	3.39%
R_M'	0.3074	0.01496	4.865%	0.00544	6.621E-09	15.17%
F_P (μg/g)	0.00167	0.0001886	11.274%	1.00	3.556E-08	81.44%

(1) The mass fractions of 3-epi-25(OH)D3 were not determined using IDMS method, but using the internal standard method. The d_6-25(OH)D3 was used as the internal standard for both 3-epi-25(OH)D3 and 25(OH)D3. As it was necessary to control the mass ratio of 25(OH)D3 over d_6-25(OH)D3 to about 1, the mass ratio of 3-epi-25(OH)D3 over d_6-25(OH)D3 was about 0.1. In addition, the mass fractions of 3-epi-25(OH)D3 in both serum samples were very low. All these factors affected the accuracy and precision of the measurement of 3-epi-25(OH)D3. However, it was demonstrated that 3-epi-25(OH)D3 was baseline-separated from 25(OH)D3 using our LC conditions (see the typical chromatogram attached below).

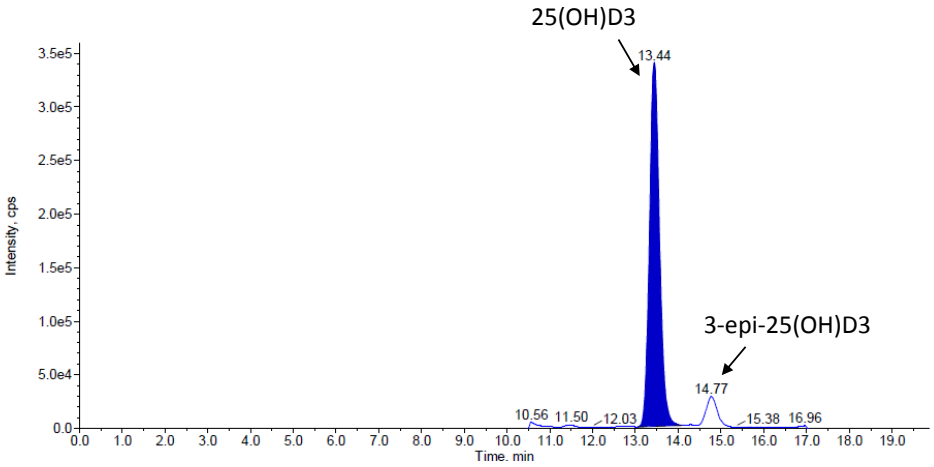
(2) The mass fraction of 25(OH)D2 in Serum Pool I was very low. We combined the sera in two bottles to obtain a larger sampling size (about 1.8 g). However, the peak intensity of 25(OH)D2 for Serum Pool I was still very low with signal-to-noise of around 20, which was only slightly higher than the Limit of Quantification (LOD) with a signal-to-noise of 10. This significantly affected the precision and accuracy of the measurement of 25(OH)D2 in Serum Pool I.

(3) Serum CRMs from NIST [SRM 972a (Levels 1, 2 and 3)] were used as the quality control materials, which were measured together with the comparison samples. The obtained values of 25(OH)D2 were 0.552 ng/g, 0.755 ng/g, and 12.8 ng/g for Levels 1, 2, and 3, respectively. These values were within the uncertainties of the reference values of 25(OH)D2 (0.52 ± 0.06 ng/g for Level 1, 0.80 ± 0.06 ng/g for Level 2, and 13.0 ± 0.3 ng/g for Level 3). The obtained values of 25(OH)D3 were 28.7 ng/g, 17.9 ng/g, and 19.2 ng/g for Levels 1, 2, and 3, respectively. These values were within the uncertainties of the reference values of 25(OH)D3 (28.1 ± 1.1 ng/g for Level 1, 17.7 ± 0.4 ng/g for Level 2, and 19.4 ± 0.4 ng/g for Level 3). The obtained values of 3-epi-25(OH)D3 were found to be all out of the uncertainties of the reference values, which may be attributed to the reasons stated above.

(4) One of the sample vials of Serum Pool II was found to be empty without the comparison material.

Sample Name: "S1-1" Sample ID: "" File: "150827 HL VitD KC Pool II.wiff"
Peak Name: "401.4 / 383.4" Mass(es): "401.4/383.4 Da"
Comment: "" Annotation: ""

Sample Index:	9
Sample Type:	Unknown
Concentration:	N/A
Calculated Conc:	0.00 ng/mL
Acq. Date:	8/28/2015
Acq. Time:	4:25:48 AM
Modified:	No
Proc. Algorithm:	Analyst Classic
Bunching Factor:	1
Noise Threshold:	59.99 cps
Area Threshold:	299.97 cps
,Num. Smooths:	3
Sep. Width:	0.20
Sep. Height:	0.01
Exp. Peak Ratio:	5.00
Exp. Adj. Ratio:	4.00
Exp. Val. Ratio:	3.00
RT Window:	30.0 sec
Expected RT:	13.5 min
Use Relative RT:	No
Int. Type:	Base To Base
Retention Time:	13.4 min
Area:	5471522 counts
Height:	3.40e+005 cps
Start Time:	13.1 min
End Time:	14.1 min



Uncertainty Information from KRISS

Measurement equation

$$C_{\text{sample}} = \frac{M_{\text{is-sol,spiked}} \cdot AR_{\text{sample}} \cdot M_{\text{s-sol,std. mix.}} \cdot C_{\text{s-sol}}}{M_{\text{sample}} \cdot AR_{\text{std. mix.}} \cdot M_{\text{is-sol,std.mix.}}}$$

C_{sample}: is the concentration of analytes in the sample;
$C_{s\text{-}sol}$: is the concentration of the analytes standard solution;
M_{sample}: is the mass of the sample taken for analysis;
$M_{is\text{-}sol,\ spiked}$: is the mass of the isotope standard solution added to the sample
$M_{is\text{-}sol,\ std.\ mix.}$: is the mass of the isotope standard solution added to the
$M_{s\text{-}sol,\ std.\ mix.}$: is the mass of the standard solution added to the isotope ratio
AR_{sample}: is the area ratio of analyte/isotope for sample extract, observed by
$AR_{std.\ mix.}$: is the area ratio of analyte/isotope for the isotope ratio standard

Random : Standard deviations of multiple measurement results from five subsamplings
Combined standard uncertainties were obtained by combining systematic uncertainties and random

$$u(C_{\text{mean}}) = \sqrt{u^2_{\text{s.p.,systematic}} + \frac{s^2}{n}}$$

Pool 1

25(OH)vitaminD$_3$

Parameter	Input	Unit	Standard uncertainty	Degree of freedom	Type of uncertainty	Uncertainty contribution (%)
$M_{is\text{-}sol,spiked}$	0.1329	g	0.0001	∞	Type B	0.06%
AR_{sample}	2.1751		0.0247	3	Type A	0.57%
W_s	0.9693	g	0.0001	∞	Type B	0.01%
AR_{std}	2.3768		0.0399	3	Type A	0.84%
				Average (ng/g)		36.958
				Combined uncertainty (%)		2.14%
				Effective degree of freedom		3
				k (95% level of confidence)		3.18
				Expanded uncertainty		6.826%

3-epi-25(OH)vitaminD$_3$

Parameter	Input	Unit	Standard uncertainty	Degree of freedom	Type of uncertainty	Uncertainty contribution (%)
$M_{is\text{-}sol,spiked}$	0.0363	g	0.0001	∞	Type B	0.23%
AR_{sample}	0.9516		0.0717	3	Type A	4.27%
W_s	0.9693	g	0.0001	∞	Type B	0.01%
AR_{std}	2.1698		0.0432	3	Type A	1.00%
				Average (ng/g)		1.547
				Combined uncertainty (%)		3.54%
				Effective degree of freedom		5
				k (95% level of confidence)		2.57
				Expanded uncertainty		9.095%

Uncertainty Information from KRISS (Continued)

Pool 2

25(OH)vitaminD$_3$

Parameter	Input	Unit	Standard uncertainty	Degree of freedom	Type of uncertainty	Uncertainty contribution (%)
$M_{is\text{-}sol,spiked}$	0.0738	g	0.0001	∞	Type B	0.12%
AR_{sample}	2.9833		0.0368	3	Type A	0.65%
W_s	1.0709	g	0.0001	∞	Type B	0.01%
AR_{std}	2.3768		0.0399	3	Type A	0.84%
				Average (ng/g)		24.259
				Combined uncertainty (%)		2.17%
				Effective degree of freedom		4
				k (95% level of confidence)		2.78
				Expanded uncertainty		6.023%

3-epi-25(OH)vitaminD$_3$

Parameter	Input	Unit	Standard uncertainty	Degree of freedom	Type of uncertainty	Uncertainty contribution (%)
$M_{is\text{-}sol,spiked}$	0.0208	g	0.0001	∞	Type B	0.41%
AR_{sample}	2.9833		0.0413	3	Type A	1.71%
W_s	1.0709	g	0.0001	∞	Type B	0.01%
AR_{std}	2.1698		0.0432	3	Type A	1.00%
				Average (ng/g)		1.132
				Combined uncertainty (%)		2.93%
				Effective degree of freedom		4
				k (95% level of confidence)		2.78
				Expanded uncertainty		8.135%

25(OH)vitaminD$_2$

Parameter	Input	Unit	Standard uncertainty	Degree of freedom	Type of uncertainty	Uncertainty contribution (%)
$M_{is\text{-}sol,spiked}$	0.0758	g	0.0001	∞	Type B	0.11%
AR_{sample}	2.9833		0.1226	3	Type A	2.05%
W_s	1.0709	g	0.0001	∞	Type B	0.01%
AR_{std}	2.4357		0.0720	3	Type A	1.48%
				Average (ng/g)		6.074
				Combined uncertainty (%)		2.55%
				Effective degree of freedom		5
				k (95% level of confidence)		2.57
				Expanded uncertainty		6.556%

Uncertainty Information from NIM

Information supplied for results of 31-Mar-2016

$R_M = aR_A + b$,

where:

R_M : isotope mass ratio in sample blend

R_A : isotope ratio in sample blend measured by LC-MS/MS

a: slope of the linear regression plot based on the 6 calibration blends

b :interception on y axis for the linear regression plot

C_S : mass fraction of D-glucose in serum sample

M_S : mass of serum sample

M_L : mass of $25(OH)D_2$, $25(OH)D_3$ ID-labled internal standard solution added to the serum sample

C_L : concentration of $25(OH)D_2$, $25(OH)D_3$ ID-labled internal standard solution added to the serum sample

The measurement equations can be converted to

where

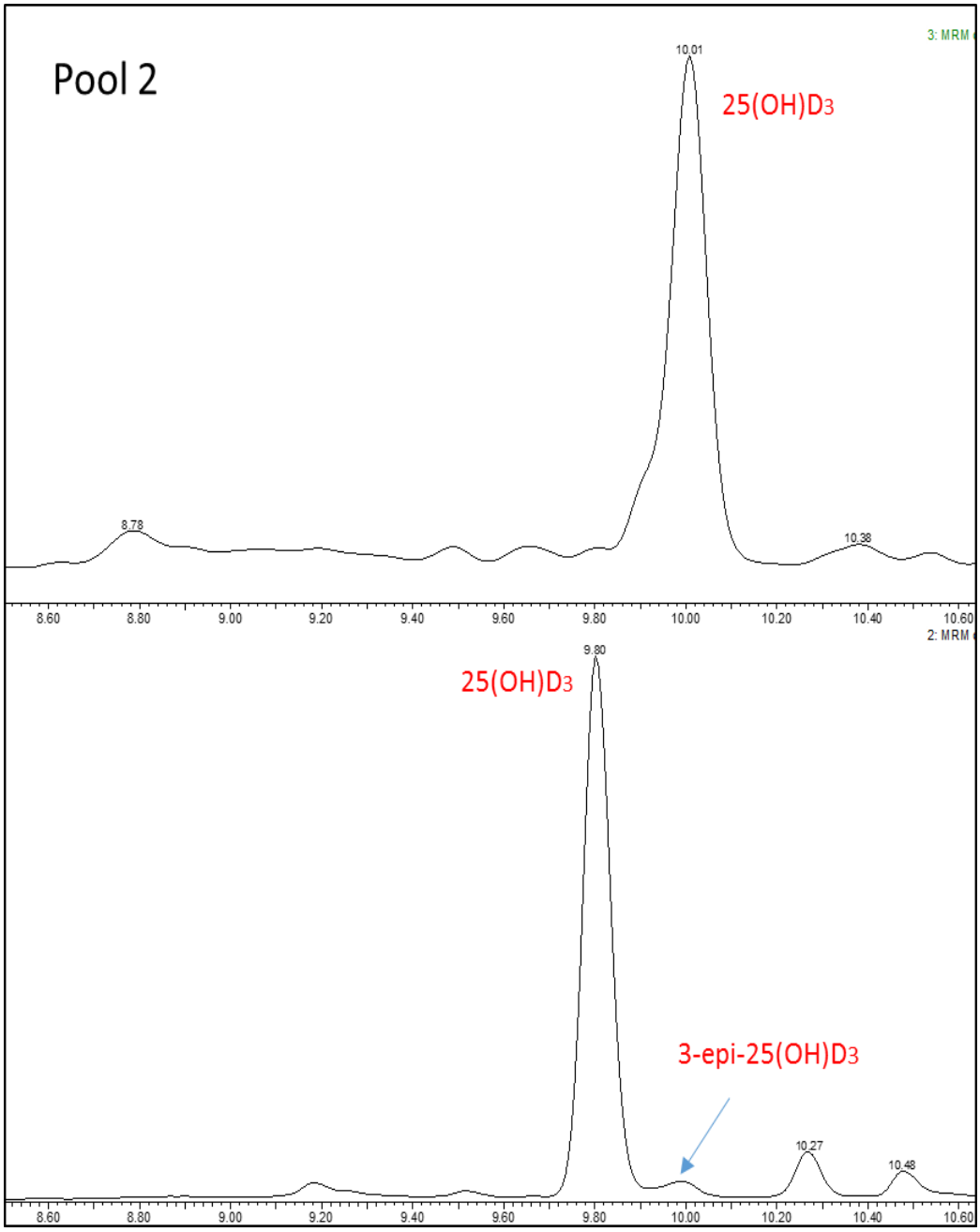
M_C : mass of calibrator

M_{PC} : mass of primary calibration solution

P_C : calibrator purity

R_M ' : parameter only relevant to isotope ratio of calibration solution blends measured and weighing of calibration solution blends

	Serum Pool I		**Serum Pool II**		
Component	**$25(OH)D_3$**	**$25(OH)D_2$**	**$25(OH)D_3$**	**$25(OH)D_2$**	Source
u_I	0.27%	0.23%	0.27%	0.23%	Linear regression of calibration curve
u_A	1.86%	1.78%	2.13%	1.11%	RSD of mean
u_{Ms}	0.01%	0.01%	0.01%	0.01%	Serum sample weighing
u_{Mc}	0.02%	0.02%	0.02%	0.02%	Calibrator weighing
u_{Ml}	0.03%	0.03%	0.03%	0.03%	weighing of Internal standard solution added to sample
u_{Pc}	1.80%	1.55%	1.80%	1.55%	Calibrator purity
u_c	2.60%	2.37%	2.80%	1.92%	Combined standard uncertainty
U_{rel}	5.2%	4.7%	5.6%	3.8%	Expanded uncertainty (k =2)
Obtained value (ng/g)	37.8	0.79	26.11	6.13	Mean of three aliquots measurement value (ng/g)
U(ng/g)	3.2	0.04	1.5	0.2	Expanded uncertainty(ng/g,k =2)


Pool 2
3: MRM
10.01
25(OH)D3
8.78
10.38
8.60
8.80
9.00
9.20
9.40
9.60
9.80
10.00
10.20
10.40
10.60
2: MRM
9.80
25(OH)D3
3-epi-25(OH)D3
10.27
10.48

Uncertainty Information from NIMT

$$w_x = F_P . F(w_{z(c)}) . w_0 . w_{y(x)} . \frac{m_{y(x)}}{m_x}$$

F_P = Method precision factor

w_x = Mass fraction of measurand in serum sample (ng/g)

$F(w_{zc})$ = Mass fraction of the calibration standard factor estimated from the purity and weighings

w_o = Mass fraction ratio (between unlabeled/labeled) obtained from the calibration curve (ng/ng)

$w_{y(x)}$ = Mass fraction of internal standard, ng/g

$m_{y(x)}$ = Mass of internal standard spiked into sample (g)

m_x = Mass of serum sample (g)

$$\frac{u(w_x)}{w_x} = \sqrt{\left(\frac{u(F_P)}{F_P}\right)^2 + \left(\frac{u(F(w_{z(c)}))}{F(w_{z(c)})}\right)^2 + \left(\frac{u(w_0)}{w_0}\right)^2 + \left(\frac{u(w_{y(x)})}{w_{y(x)}}\right)^2 + \left(\frac{u(m_{y(x)})}{m_{y(x)}}\right)^2 + \left(\frac{u(m_x)}{m_x}\right)^2}$$

$u(w_x)$ = Standard uncertainty of measurand in sample

$u(w_0)$ = Standard uncertainty of mass fraction ratio of unlabeled/label analogue estimated from standard error of the calibration curvet

$u(w_{y(x)})$ = Standard uncertainty of mass fraction of labeled standard

$u(m_{y(x)})$ = Standard uncertainty of mass of labeled internal standard

$u(m_x)$ = Standard uncertainty of mass of serum sample

$u(F_P)$ = Standard uncertainty of method precision

u(F(wzc) =Standard uncertainty of calibration standard factor estimated from the purity and weighings

25-OH-D2 (II) Red cap

Factor	Values	Uncertainties	
	x	u(x)	u(x)/(x)
FP	1.00000	0.03657	0.03657
F wzc	1.00000	0.01002	0.01002
w0	18.75551	0.10222	0.00545
wy(x)	7.45780	0.38164	0.05117
my(x)	0.02526	0.00005	0.00197
mx	0.49311	0.00005	0.00010

Uncertainty Analysis Results

Cx =	7.0896	ng/g
u(x) =	0.4534	ng/g
u(x)/x =	0.0640	
Veff(total) =	18.0144	
k=	2.1009	(@ 95% level)
U(x) =	0.95257	
%U(x) =	13.44%	

25-OH-D3 (I) White cap

Factor	Values	Uncertainties	
	x	u(x)	u(x)/(x)
Fp	1.00000	0.02267	0.02267
F(wzc)	1.00000	0.02058	0.02058
w0	1.76328	0.03148	0.01785
wy(x)	102.11692	5.67734	0.05560
my(x)	0.07654	0.00004	0.00051
mx	0.49705	0.00004	0.00008

Uncertainty Analysis Results

Cx =	37.8711	ng/g
u(x) =	2.3723	ng/g
u(x)/x =	0.0626	
Veff(total) =	85.3950	
k=	1.9883	(@ 95% level)
U(x) =	4.7167	
%U(x) =	0.1245	

Uncertainty Information from NIMT (Continued)

25-OH-D3 (II) Red cap

Factor	Values	Uncertainties	
	x	u(x)	u(x)/(x)
FP	1.00000	0.01379	0.01379
F(wzc)	1.00000	0.02058	0.02058
w0	1.76328	0.04682	0.02655
wy(x)	102.11692	5.63322	0.05516
my(x)	0.07513	0.00004	0.00052
mx	0.49311	0.00004	0.00008

Uncertainty Analysis Results

Cx =	26.0975	ng/g
u(x) =	1.6378	ng/g
u(x)/x =	0.0628	
Veff(total) =	231.9584	
k=	1.9703	(@ 95% level)
U(x) =	3.2270	
%U(x) =	0.1237	

3-epi-25-OH-D3 (I) White cap

Factor	Values	Uncertainties	
	x	u(x)	u(x)/(x)
FP	1.00000	0.02907	0.02907
F(wzc)	1.00000	0.02509	0.02509
w0	1.69449	0.03792	0.02238
wy(x)	10.04288	0.74959	0.07464
my(x)	0.07498	0.00004	0.00052
mx	0.49640	0.00004	0.00008

Uncertainty Analysis Results

Cx =	1.9990	ng/g
u(x) =	0.1663	ng/g
u(x)/x =	0.0832	
Veff(total) =	93.2093	
k=	1.9858	(@ 95% level)
U(x) =	0.3302	
%U(x) =	0.1652	

3-epi-25-OH-D3 (II) Red cap

Factor	Values	Uncertainties	
	x	u(x)	u(x)/(x)
FP	1.00000	0.01103	0.01103
F(wzc)	1.00000	0.02509	0.02509
w0	1.69449	0.03806	0.02246
wy(x)	9.63711	0.73352	0.07611
my(x)	0.07661	0.00004	0.00051
mx	0.49311	0.00004	0.00008

Uncertainty Analysis Results

Cx =	2.7297	ng/g
u(x) =	0.2292	ng/g
u(x)/x =	0.0840	
Veff(total) =	281.5641	
k=	1.9684	(@ 95% level)
U(x) =	0.4511	
%U(x) =	0.1653	

Uncertainty Information from NIST

Measurement protocol: Linear regression fit of calibration data;
The standard uncertainty of the measurement was attributable to two types of uncertainties, Type A and Type B. For the Type A component of measurement uncertainty, the standard deviation (SD) of the mean was calculated by dividing the standard deviation by the square root of n, where n =3. The type B uncertainties include uncertainties of the purity of reference standards and an estimate of unidentified systematic errors (1 %). Type A and type B uncertainty components were combined quadratically to determine the standard uncertainty, uc, which was multiplied by a coverage factor, k =2, to calculate the expanded uncertainty, U.

Uncertainty Information from NMIA

The measurement equation used to calculate the mass fraction of each analyte is as follows:

$$\omega_X = \omega_Z \bullet \frac{M_Y}{M_X} \bullet \frac{M_{Zc}}{M_{Yc}} \bullet \frac{R_Y - R_B}{R_B - R_X} \bullet \frac{R_{Bc} - R_Z}{R_Y - R_{Bc}} \bullet F_{MP} \bullet F_{MT}$$

where;
ωx = mass fraction of analyte in sample
ωz = mass fraction of analyte in the calibration standard solution used to prepare calibration blend
My = mass of internal standard solution added to sample blend
Myc = mass of internal standard solution added to calibration blend
Mx = mass of sample added to sample blend
Mzc = mass of calibration standard solution added to calibration blend
Rb = ratio of measured peak areas corresponding to the analyte and internal standard in the sample blend
Rbc = ratio of measured peak areas corresponding to the analyte and internal standard in the calibration blend
Rx = ratio of measured peak areas corresponding to the analyte and internal standard in the sample
Rz = ratio of measured peak areas corresponding to the analyte and internal standard in the calibration standard
Ry = ratio of measured peak areas corresponding to the analyte and internal standard in the internal standard
F(MP) = method precision term
F(MT) = method trueness factor

All masses used to calculate ωx were determined using calibrated balances
with metrological traceability to the SI unit of the kilogram through Australian national standards for mass.
Peak areas were determined from chromatographic traces generated for characteristic ions corresponding to analytes and
internal standards. 14 sub-samples of Serum Pool I and 9 Sub-samples of Serum Pool II were analysed in
6 batches between June - September 2015. Certified matrix reference materials, SRM 1950 and SRM 972a were analysed in these batches for quality control.
The results obtained for the matrix CRMs are summarised in the Excel worksheet named, "Matrix SRMs Data".
Tables showing the measurement uncertainty budgets for 25OHD3 in Serum Pool I, 25OHD3 in Serum Pool II and 25OHD2 in Serum Pool II are provided on the Tabs named,
"25OHD3 Pool I MUB", "25OHD3 Pool II MUB", and "25OHD2 Pool II MUB".

A standard uncertainty was estimated for all components in the measurement equation. These were combined using
derived sensitivity coefficients to estimate a combined standard uncertainty in the reported result for each analyte
in the study sample. The total effective degrees of freedom was determined using the Welch-Satterthwaite
equation to calculate the appropriate coverage (k) factor to expand the combined standard uncertainty to a 95% confidence
interval for reporting. The method precision term was calculated as the standard deviation of the mean of all the measurements used in the
calculation of the reference value.

To ensure that all likely sources of bias would be accounted for in the final uncertainty budget a trueness factor was also included.
This factor was assigned a nominal value of one with an uncertainty representing the potential magnitude of undetected bias due to
factors affecting the measured peak area ratios such as matrix interferences and matrix effects estimated from the average differences in analyte mass fractions
determined from the use of alternative ion pairs for quantification and alternative methods of sample analysis (underivatised versus
Amplifex derivatised samples, LC-MS/MS versus GC-MS/MS analysis).

25-Hydroxyvitamin D3

	Average mass fraction (ng/g)	Relative standard deviation (%)	Number of analyses performed
SRM972a Level 1	27.5	2.2%	8
SRM972a Level 2	17.0	1.9%	3
SRM972a Level 3	18.5	1.4%	4
SRM972a Level 4	27.3	3.2%	5
SRM1950	23.0	2.8%	7

25-Hydroxyvitamin D2

	Average mass fraction (ng/g)	Relative standard deviation (%)	Number of analyses performed
SRM972a Level 1	-	-	-
SRM972a Level 2	0.8	-	1
SRM972a Level 3	13.0	3.6%	5
SRM972a Level 4	-	-	-
SRM1950	0.3	-	1

3-epi-25-Hydroxyvitamin D3

	Average mass fraction (ng/g)	Relative standard deviation (%)	Number of analyses performed
SRM972a Level 1	1.7	-	1
SRM972a Level 2	1.2		1
SRM972a Level 3	-	-	-
SRM972a Level 4	26.0	2.8%	4
SRM1950	-	-	-

Details	
Project	CCQM K132
Sample Name	Serum Pool I
Analyte	25OHD3
Matrix	Human Serum
Measurand	Mass Fraction
Measurand Symbol	Wx
Reporting units	ng/g

Summary of Contributions to Total Combined Measurement Uncertainty

Number	*Name of Component*	*Symbol*	*Units*	*Value*	*Standard Uncertainty*	*Relative Standard Uncertainty*	*Degrees of Freedom*
i	Xi			xi	u(xi)	u(xi)/xi (%)	vi
1	Method Precision	F(MP)	dimensionless	1.0000	0.0033	0.33%	16.0
2	Method Trueness	F(MT)	dimensionless	1.0000	0.014	1.4%	30.0
3	Standard	Wz	ng/g	410	5.5	1.3%	100.0
4	Moisture Content	MC	n/a	1.00	0.00	0.00%	1.0
5	Gravimetry	Mx	g	1.83584	0.00026	0.014%	100.0
6	Gravimetry	My(SB)	g	0.09734	0.00026	0.27%	100.0
7	Gravimetry	Mz	g	0.14518	0.00026	0.18%	100.0
8	Gravimetry	My(CB)	g	0.13189	0.00026	0.20%	100.0
9	Isotope Amount Ratio	Rx,Rz	mol/mol	3.8E+05	2.98E+05	78%	4.0
10	Isotope Amount Ratio	Ry	mol/mol	0.000049	0.000018	36%	4.0
11	Blend Isotope Amount Ratio	R(SB)	mol/mol	1.1405			1.0
12	Blend Isotope Amount Ratio	R(CB)	mol/mol	1.0851			1.0

Details	
Project	CCQM K132
Sample Name	Serum Pool II
Analyte	25OHD3
Matrix	Human Serum
Measurand	Mass Fraction
Measurand Symbol	Wx
Reporting units	ng/g

Summary of Contributions to Total Combined Measurement Uncertainty

Number	*Name of Component*	*Symbol*	*Units*	*Value*	*ndard Uncerta ive Standard Uncert*		*Degrees of Freedom*
i	Xi			xi	u(xi)	u(xi)/xi (%)	vi
1	Method Precision	F(MP)	dimensionless	1.0000	0.0039	0.39%	13.0
2	Method Trueness	F(MT)	dimensionless	1.0000	0.014	1.4%	30.0
3	Standard	Wz	ng/g	410	5.5	1.3%	100.0
4	Moisture Content	MC	n/a	1.00	0.00	0.00%	1.0
5	Gravimetry	Mx	g	1.99466	0.00026	0.013%	100.0
6	Gravimetry	My(SB)	g	0.11460	0.00026	0.23%	100.0
7	Gravimetry	Mz	g	0.11927	0.00026	0.22%	100.0
8	Gravimetry	My(CB)	g	0.14835	0.00026	0.18%	100.0
9	Isotope Amount Ratio	Rx,Rz	mol/mol	3.8E+05	2.98E+05	78%	4.0
10	Isotope Amount Ratio	Ry	mol/mol	0.000049	0.000018	36%	4.0
11	Blend Isotope Amount Ratio	R(SB)	mol/mol	1.1162			1.0
12	Blend Isotope Amount Ratio	R(CB)	mol/mol	1.0695			1.0

Details	
Project	**CCQM K132**
Sample Name	**Serum Pool II**
Analyte	**25OHD2**
Matrix	**Human Serum**
Measurand	**Mass Fraction**
Measurand Symbol	**Wx**
Reporting units	**ng/g**

Summary of Contributions to Total Combined Measurement Uncertainty

Number	*Name of Component*	*Symbol*	*Units*	*Value*	*Standard Uncertainty*	*Relative Standard Uncertainty*	*Degrees of Freedom*
i	Xi			xi	u(xi)	u(xi)/xi (%)	vi
1	Method Precision	F(MP)	dimensionless	1.0000	0.0082	0.82%	4.0
2	Method Trueness	F(MT)	dimensionless	1.0000	0.015	1.5%	30.0
3	Standard	Wz	ng/g	294	4.6	1.5%	100.0
4	Moisture Content	MC	n/a	1.00	0.00	0.00%	1.0
5	Gravimetry	Mx	g	2.00388	0.00026	0.013%	100.0
6	Gravimetry	My(SB)	g	0.06026	0.00026	0.43%	100.0
7	Gravimetry	Mz	g	0.05678	0.00026	0.46%	100.0
8	Gravimetry	My(CB)	g	0.07636	0.00026	0.34%	100.0
9	Isotope Amount Ratio	Rx,Rz	mol/mol	1.5E+03	7.11E+01	5%	4.0
10	Isotope Amount Ratio	Ry	mol/mol	0.000254	0.000031	12%	4.0
11	Blend Isotope Amount Ratio	R(SB)	mol/mol	1.0394			1.0
12	Blend Isotope Amount Ratio	R(CB)	mol/mol	1.0703			1.0

Uncertainty of Mass of Sample and IS

$$u(W_{SM}) = \sqrt{\left(u_{sample}\right)^2 + (u_{IS})^2 + (u_{tare})^2}$$

Uncertainty of stock solution of IS

$$\frac{u\left(C_{Analyte}\right)}{C_{Analyte}} = \frac{u(W_{IS})}{W_{IS}}$$

Uncertainty of stock solution of IS

$$u(R_m) = R_m\sqrt{\left(\frac{u(\overline{C_{obs}})}{\overline{C_{obs}}}\right)^2 + \left(\frac{u(C_{cert})}{C_{cert}}\right)^2} \;;\; R_m = \frac{\overline{C_{obs}}}{C_{cert}}$$

Uncertainty of Repeatability

$$u(r) = \frac{RSD}{\sqrt{n}}$$

Combined Standard Measurement Uncertainty

$$\frac{u_c(A)}{C_A} = \sqrt{\left(\frac{u(W_{SM})}{W_{SM}}\right)^2 + \left(\frac{u(W_{IS})}{W_{IS}}\right)^2 + \left(\frac{u(C_{LS})}{C_{LS}}\right)^2 + u(R_m)^2 + u(r)^2}$$

APPENDIX H: Participants' Quantitative Results as Reported

The following are pictures of the quantitative reults as provided by the participants in the "Results" worksheet of the "Reporting Form" Excel workbook. Information is grouped by participant and presented in alphabetized acronym order.

Quantitative Results from HSA

Serum Pool I				
Measurand	Mass Fraction (ng/g)	Combined Standard Uncertainty (ng/g)	Coverage Factor (k)	Expanded Uncertainty (ng/g)
25(OH)D2	0.579	0.0285	2	0.057
25(OH)D3	38.83	0.744	2	1.49
3-epi-25(OH)D3	3.14	0.16	2	0.32

Serum Pool II				
Measurand	Mass Fraction (ng/g)	Combined Standard Uncertainty (ng/g)	Coverage Factor (k)	Expanded Uncertainty (ng/g)
25(OH)D2	6.05	0.205	2	0.41
25(OH)D3	25.82	0.52	2	1.04
3-epi-25(OH)D3	1.67	0.21	2	0.42

Quantitative Results from KRISS

Serum Pool I				
Measurand	Mass Fraction (ng/g)	Combined Standard Uncertainty (ng/g)	Coverage Factor (k)	Expanded Uncertainty (ng/g)
25(OH)D2	Lower than LOQ	-	-	-
25(OH)D3	37.0	0.792	3.18	2.52
3-epi-25(OH)D3	1.55	0.0547	2.57	0.141

Serum Pool II				
Measurand	Mass Fraction (ng/g)	Combined Standard Uncertainty (ng/g)	Coverage Factor (k)	Expanded Uncertainty (ng/g)
25(OH)D2	6.07	0.155	2.57	0.398
25(OH)D3	24.3	0.526	2.78	1.46
3-epi-25(OH)D3	1.13	0.0332	2.78	0.0921

Quantitative Results from NIM (Official Results, 18-Oct-2015)

Serum Pool I				
Measurand	Mass Fraction (ng/g)	Combined Standard Uncertainty (ng/g)	Coverage Factor (k)	Expanded Uncertainty (ng/g)
25(OH)D2	0.79	0.08	2	0.16
25(OH)D3	37.8	1.6	2	3.2
3-epi-25(OH)D3	3.75	0.14	2	0.28

Serum Pool II				
Measurand	Mass Fraction (ng/g)	Combined Standard Uncertainty (ng/g)	Coverage Factor (k)	Expanded Uncertainty (ng/g)
25(OH)D2	8.4	0.50	2	1.0
25(OH)D3	24.4	1.0	2	2.0
3-epi-25(OH)D3	2.68	0.16	2	0.32

Quantitative Results from NIM (Follow-up Results, 31-Mar-2015)

Serum Pool I				
Measurand	Mass Fraction (ng/g)	Combined Standard Uncertainty (ng/g)	Coverage Factor (k)	Expanded Uncertainty (ng/g)
25(OH)D2				
25(OH)D3	37.37	0.69	2	1.4
3-epi-25(OH)D3				

Serum Pool II				
Measurand	Mass Fraction (ng/g)	Combined Standard Uncertainty (ng/g)	Coverage Factor (k)	Expanded Uncertainty (ng/g)
25(OH)D2	6.13	0.10	2	0.2
25(OH)D3	26.11	0.72	2	1.5
3-epi-25(OH)D3				

Quantitative Results from NMIT

Serum Pool I (White cap)				
Measurand	Mass Fraction (ng/g)	Combined Standard Uncertainty (ng/g)	Coverage Factor (k)	Expanded Uncertainty (ng/g)
25(OH)D2	ND	-	-	-
25(OH)D3	37.87	2.37	1.99	4.72
3-epi-25(OH)D3	2.00	0.17	1.99	0.33

Serum Pool II (Red cap)				
Measurand	Mass Fraction (ng/g)	Combined Standard Uncertainty (ng/g)	Coverage Factor (k)	Expanded Uncertainty (ng/g)
25(OH)D2	7.09	0.45	2.10	0.95
25(OH)D3	26.10	1.64	1.97	3.23
3-epi-25(OH)D3	2.73	0.23	1.97	0.45

Quantitative Results from NIST

Serum Pool I				
Measurand	Mass Fraction (ng/g)	Combined Standard Uncertainty (ng/g)	Coverage Factor (k)	Expanded Uncertainty (ng/g)
25(OH)D2	0.66	0.01	2	0.03
25(OH)D3	38.6	0.42	2	0.84
3-epi-25(OH)D3	2.04	0.04	2	0.08

Serum Pool II				
Measurand	Mass Fraction (ng/g)	Combined Standard Uncertainty (ng/g)	Coverage Factor (k)	Expanded Uncertainty (ng/g)
25(OH)D2	6.33	0.12	2	0.23
25(OH)D3	25.7	0.28	2	0.55
3-epi-25(OH)D3	1.56	0.03	2	0.06

Quantitative Results from NMIA

Serum Pool I				
Measurand	Mass Fraction (ng/g)	Combined Standard Uncertainty (ng/g)	Coverage Factor (k)	Expanded Uncertainty (ng/g)
25(OH)D2	0.61	-	-	-
25(OH)D3	37.0	0.75	1.98	1.5
3-epi-25(OH)D3	2.00	-	-	-

Serum Pool II				
Measurand	Mass Fraction (ng/g)	Combined Standard Uncertainty (ng/g)	Coverage Factor (k)	Expanded Uncertainty (ng/g)
25(OH)D2	6.41	0.15	1.98	0.31
25(OH)D3	25.0	0.51	1.98	1.0
3-epi-25(OH)D3	1.52	-	-	-

Quantitative Results from UME

Serum Pool I (white cap)				
Measurand	Mass Fraction (ng/g)	Combined Standard Uncertainty (ng/g)	Coverage Factor (k)	Expanded Uncertainty (ng/g)
25(OH)D2	0.503	0.015	2	0.030
25(OH)D3	37,823	1,355	2	2,710
3-epi-25(OH)D3	2,020	0.064	2	0.127

Serum Pool II (red cap)				
Measurand	Mass Fraction (ng/g)	Combined Standard Uncertainty (ng/g)	Coverage Factor (k)	Expanded Uncertainty (ng/g)
25(OH)D2	6,050	0.180	2	0.361
25(OH)D3	25,842	0.921	2	1,842
3-epi-25(OH)D3	1,483	0.047	2	0.093

APPENDIX I: Prototype Broader-Scope Core Competency Claim

Table I-1: Prototype Broader-Scope Category 10 Claim
from successful participation in CCQM-K132

Measurement service	Category 10. Biological fluids and materials
Measurement service sub-category	Blood serum (10.1)
Matrix	Human serum, plasma, whole blood(?)
Measurand from the HFTLS Statement	Analyte or Component: low-polarity ($pK_{ow} < -2$) organic analyte of low molecular mass (100 g/mol to 500 g/mol) Quantity: Mass fraction
Dissemination range of measurement capability	From 1 to 500 Unit: ng/g
Range of expanded uncertainties as disseminated	From 2 to 6 Unit: % Coverage factor: 2 or Student's $t_{1-0.95,n-1}$ Level of confidence: 95% Expanded uncertainty is a relative one: Yes
Example measurands within this scope	Consider more specific subclasses within the low-polarity/low molecular mass sector. A primary level of claims might be: steroidal vitamin metabolites, steroidal hormones, other steroid-based metabolites A secondary level of claims might be to extend further for other subclasses such as: organochlorine pesticides, PCBs, triglycerides, fat-soluble vitamins, flame retardants. This would be possible where a much broader range of evidence existed
Supporting Evidence	Successfully participated in CCQM-K132 and other potentially related KCs: CCQM-K6/-K6.2, CCQM-K69, and CCQM-K95