CCQM-K6.2 Determination of Total Cholesterol in Human Serum

Final Report
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SUMMARY

Cholesterol is one of the most frequently measured substances in human blood/serum to assist in assessing the health status of individuals. Because of its clinical significance, CCQM-K6 Determination of Cholesterol in Serum was completed in 2000 as one of the first Key Comparison (KC) studies performed within the Organic Analysis Working Group (OAWG). The first Subsequent KC for cholesterol, CCQM-K6.1, was completed in 2001. Measurements for this second Subsequent, CCQM-K6.2, were completed in 2012. These Subsequent comparisons were conducted to enable CCQM members that had not participated in earlier studies to demonstrate their capabilities to measure a nonpolar ($pK_{ow} < -2$), low molecular mass (100 g/mol to 500 g/mol) metabolite in human serum at relatively high concentrations (1 mg/g to 3 mg/g) found in normal populations. Successful participation in CCQM-K6.2 demonstrated capabilities in analysis of complex biological matrices including sample preparation (extraction, derivatization), LC or GC separation, and quantification using an isotope dilution mass spectrometry approach.

Normally in a subsequent KC, no Key Comparison Reference Value (KCRV) would be established and assessment of performance would be via the deviation of participants' results to the anchor institute's results, adjusted to account for the anchor's performance in the original comparison versus its KCRV. Due to the very long-time period since the original key comparison, the OAWG decided that this did not represent the best approach to assess performance in what is a relatively complex measurement. Given the excellent agreement between the anchor institute's results and robust consensus summary of the participants' values, the Reference Value for this study was taken as the anchor institute's result and treated as a "KCRV". Seven of the nine participants demonstrated agreement with the reference value.

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ACRONYMS

CCQM	Consultative Committee for Amount of Substance: Metrology in Chemistry and Biology
CDC	Centers for Disease Control and Prevention, USA
CENAM	Centro Nacional de Metrologia, México
CMC	calibration and measurement capabilities
CRM	certified reference material
DI	designated institute
GC	gas chromatography
GC-MS	gas chromatography separation with mass spectrometry detection
HSA	Health Sciences Authority, Singapore
ID	isotope dilution
INMETRO	Instituto Nacional de Metrologia, Qualidade e Tecnologia, Brazil
INTI	Instituto Nacional de Tecnologia Industrial, Argentina
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	liquid chromatography
LC-MS	liquid chromatography with mass spectrometry detection
LC-MS/MS	liquid chromatography with tandem mass spectrometry detection
LNE	Laboratoire National de Métrologie et d'Essais, France
MADe	median absolute deviation from the median (MAD)-based estimate of s: MADe = 1.4826·MAD, where MAD = median($\|x_i$-median(x_i)$\|$)
NIMT	National Institute of Metrology (Thailand), Thailand
NIST	National Institute of Standards and Technology, USA
NMI	national metrology institute
OAWG	Organic Analysis Working Group
pK_{ow}	logarithm of the octanol-water partition coefficient
PTB	Physikalisch-Technische Bundesanstalt, Germany
SRM	Standard Reference Material, a NIST CRM
UME	National Metrology Institute of Turkey, Turkey
VNIIM	D.I. Mendeleyev Institute for Metrology, Russian Federation

SYMBOLS

d_i	degree of equivalence: x_i - KCRV
$\%d_i$	percent relative degree of equivalence: $100 \cdot d_i$/KCRV
k	coverage factor: $U(x) = k \cdot u(x)$
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: $\bar{u}(x) = \sqrt{\sum_{i=1}^{n} u^2(x_i)/n}$
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: $\bar{x} = \sum_{i=1}^{n} x_i/n$
$U_{95}(x)$	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 $U_{k=2}(x) = 2 \cdot u(x)$

INTRODUCTION

Cholesterol, glucose, and creatinine are three of the most frequently measured substances in human blood/serum to assist in assessing the health status of individuals. Because of their clinical significance, measurements of cholesterol, glucose, and creatinine were three of the first Key Comparison (KC) studies performed within the Consultative Committee for Amount of Substance: Metrology in Chemistry and Biology (CCQM) Organic Analysis Working Group (OAWG). These studies were performed in 2000 and 2001 with the National Institute of Standards and Technology (NIST) as the coordinating laboratory (see Table 1) and were published in Metrologia [1,2,3]. Subsequent Key Comparisons were conducted for each analyte in 2005 with the Korea Research Institute of Standards and Science (KRISS) as the coordinating laboratory for CCQM-K11.1 Glucose and CCQM-12.1 Creatinine and NIST as the coordinating laboratory for CCQM-K6.1 Cholesterol.

Table 1: Previous CCQM Comparisons for Cholesterol, Glucose, and Creatinine

Comparison	Name of Comparison Study	Date	Coordinating Laboratory	Number of Participants
CCQM-P6	Cholesterol in Human Serum	1999	NIST	7
CCQM-K6	Cholesterol in Human Serum	2000	NIST	7
CCQM-K6.1	Cholesterol in Human Serum	2001	NIST	2
CCQM-P8	Glucose in Human Serum	2000	NIST	4
CCQM-K11	Glucose in Human Serum	2001	NIST	3
CCQM-K11.1	Glucose in Human Serum	2005	KRISS	3
CCQM-P9	Creatinine in Human Serum	2000	NIST	4
CCQM-K12	Creatinine in Human Serum	2001	NIST	5
CCQM-K12.1	Creatinine in Human Serum	2005	KRISS	3

Since these earlier studies were conducted, additional national metrology institutes (NMIs) or their designated institutes (DIs) are now providing measurement services for one or more of these clinical analytes. At the April 2012 OAWG meeting a proposal was accepted to conduct Subsequent Key Comparison studies for the three analytes with NIST as the coordinating laboratory. These three studies were conducted in parallel as CCQM-K6.2, -K11.2, and K-12.2.

The three studies were designed as Subsequent Key Comparisons, with NIST designated as both the coordinating and anchor laboratory. Therefore, participant results were to be compared with NIST measurements. Due to discordant results in CCQM-K11.2 and CCQM-K12.2 between NIST and the participating laboratories, KRISS and the Physikalisch-Technische Bundesanstalt (PTB) – laboratories that had successfully participated in the original studies – were requested by the OAWG to provide measurements for both glucose and creatinine. At the April 2015 OAWG meeting, the decision was made to treat CCQM-K11.2 and CCQM-K12.2 as modified Track C Key Comparisons rather than as Subsequent Key Comparisons. This report describes only CCQM-K6.2. CCQM-K11.2 and CCQM-K12.2 are described in a separate report.

The timeline for CCQM-K6.2 study "Determination of Total Cholesterol in Human Serum" is summarized in Table 2.

Table 2: CCQM-K6.2 Timeline

Date	Action
April 2012	OAWG authorized CCQM-K6.2, -K11.2, and -K12.2 subsequent studies and approved protocols
Nov. 2012	Call for Participation to OAWG members
Dec. 2012	Samples shipped to participants
April 2013	Preliminary results presented to OAWG at Paris meeting. Results for CCQM-K6.2 in good agreement; KRISS and PTB asked to provide reference measurements for CCQM-K11.2 and CCQM-K12.2
Nov. 2013	Reference results for CCQM-K11.2 and CCQM-K12.2 from PTB and KRISS received and discussed at CCQM meeting in South Africa
April 2014	Further discussion of how to assign KCRV for CCQM-K6.2, CCQM-K11.2 and CCQM-K12.2; decision to treat CCQM-K6.2 results as true Subsequent Key Comparison with NIST results as anchor; decision to assign KCRV for CCQM-K11.2 and CCQM-K12.2 from participant and reference laboratory results
April 2015	Draft A Report discussed; decision to prepare two Draft A Reports, one for CCQM-K6.2 and a second for CCQM-K11.2 and CCQM-K12.2, which are to be treated as Track C Key comparisons rather than Subsequent Key Comparisons
Oct. 2015	Draft A Report distributed to OAWG
Nov. 2015	Draft B report distributed to OAWG
June 2016	Draft Final report delivered to OAWG Chair
Sep. 2016	Review by CCQM WG chairs
June 2017	Revised Draft Final report delivered to OAWG Chair
April 2018	Final report delivered to OAWG Chair

MEASURAND

The measurands for the three clinical analyte studies were cholesterol, glucose, and creatinine as previously defined in the original studies (CCQM-K6, CCQM-K11, and CCQM-K12). These three clinical health status markers were selected in the original Key Comparison studies to be representative of measurement challenges associated with well-defined and low molar mass organic substances in blood. For CCQM-K6.2 the measurand was the mass fraction of total cholesterol in human serum.

Cholesterol (molar mass 365 g/mol) is a low polarity (nonpolar) analyte that is present in human serum at relatively high concentrations (1 mg/g to 3 mg/g). Cholesterol is predominantly esterified with fatty acids in the blood.

STUDY MATERIAL

The study material for CCQM-K6.2 was NIST candidate Standard Reference Material (SRM) 1951c Lipids in Frozen Human Serum (Level 2) [4], prepared as a replacement for SRM 1951b Lipids in Frozen Human Serum and issued in June 2013 after the CCQM-K6.2 results were reported to the OAWG. Participants were provided with three vials of serum for the determination of cholesterol. Each vial contained 1 mL of human serum. Samples were shipped frozen (on dry ice), and participants were instructed that a -20 ºC freezer was adequate for storage up to one week; however, if longer storage time was anticipated, the material should be stored at temperatures of -60 ºC or below.

Homogeneity Assessment of Study Material

Based on nearly two decades of experience with frozen serum samples prepared as SRMs for the determination of cholesterol, there were limited concerns regarding the homogeneity or the stability of the study material. No formal stability study was conducted for the study material. However, studies to assess homogeneity were conducted. For the material used in CCQM-K6.2, cholesterol homogeneity was assessed as part of the certification measurements. A total of 15 vials were selected for analysis based on a stratified sampling plan designed to test for homogeneity across the lot of vials. The 15 vials were analyzed in three sets of five vials per set with duplicate GC-MS injections. The results of the homogeneity assessment are shown in Table 3, where $\bar{x}$ designates a mean value, s a standard deviation, and $100 \cdot s/\bar{x}$, the percent relative standard deviation. There was no trend apparent in the data when plotted against the sequence in which the vials were prepared [4].

Table 3: Determination of Cholesterol in SRM 1951c Level 2

				Set Statistics		
Set	**Tray**	**Sample**	**Sample $\bar{x}$**	**$\bar{x}$**	**s**	**$100 \cdot s/\bar{x}$**
1	83	7	241.31	241.19	0.71	0.29 %
1	32	8	241.50			
1	14	9	240.10			
1	112	10	242.00			
1	40	11	241.02			
2	2	19	240.24	240.70	0.54	0.23 %
2	31	20	240.57			
2	65	21	240.72			
2	102	22	240.37			
2	81	23	241.62			
3	114	31	238.54	240.84	1.89	0.79 %
3	24	32	239.57			
3	73	33	243.38			
3	119	34	240.89			
3	27	35	241.82			

PARTICIPANTS AND INSTRUCTIONS

Participants were requested to analyze two vials of material for cholesterol; the number of subsamples from each vial was left up to the laboratories. Participants were encouraged to use an appropriate serum-matrix CRM as a control material. Participants were to report the mass of cholesterol per mass of serum (mg/g) in the reporting form provided. The reporting form also included descriptions of methods used, number and order of measurements, reference compounds used as calibrants with purity corrections, control materials used, and method of calculating results. A complete description of their uncertainty calculations was also requested in the reporting form.

The National Metrology Institutes or Designated Institutes that participated in CCQM-K6.2 are listed in Table 4. NIST was designated as the anchor laboratory.

Table 4: Participants and Anchor in CCQM-K6.2 Cholesterol in Human Serum

NMI/DI	CCQM-K6.2 Cholesterol
CENAM	Participant
HSA	Participant
INMETRO	Participant
INTI	Participant
KRISS	Participant
LNE	Participant
NIMT	Participant
UME	Participant
VNIIM	Participant
NIST	Anchor

METHODS

Methods Used by Participants

For CCQM-K6.2 Cholesterol in Human Serum, results were received from nine participants. The participants used either isotope dilution gas chromatography-mass spectrometry (ID GC-MS) (six labs) or isotope dilution liquid chromatography-tandem mass spectrometry (ID LC-MS/MS) (three labs). The analytical methods used by the participants, including sample preparation, analytical technique, and quantification approach, are summarized in Tables A1 to A3 of Appendix A.

Methods Used by Anchor Laboratory

The anchor laboratory used the ID GC-MS procedure published as a definitive method in 1989 [5] and now recognized by the Joint Committee for Traceability in Laboratory Medicine (JCTLM) as a reference measurement procedure. The method as used by the anchor laboratory is summarized in Tables A1 to A3.

RESULTS

Participant Results: Reported

The results for K6.2 as received from the participants for measurements on each of two vials (as requested) are summarized in Table 5.

Table 5: Results for CCQM-K6.2 Cholesterol in Human Serum as Received

		Mass Fraction, mg/g			Coverage
NMI/DI	**Vial**	x	$u(x)$	$U_{95}(x)$	Factor (k)
CENAM	1	2.443	0.042	0.091	2.16
	2	2.486	0.021	0.044	2.16
HSA	1	2.357	0.0136	0.027	2
	2	2.353	0.0135	0.027	2
INMETRO	1	2.35	0.016	0.04	2.262
	2	2.35	0.020	0.05	2.306
INTI	1	1.72	0.12	0.25	2
	2	1.71	0.12	0.25	2
KRISS	1	2.333	0.017	0.037	2.2
	2	2.340	0.017	0.037	2.2
LNE	1	2.350	0.026	0.053	2
	2	2.353	0.028	0.056	2
NIMT	1	2.39	0.039	0.079	2.05
	2	2.38	0.045	0.093	2.06
UME	1	2.265	0.031	0.062	2
	2	2.310	0.033	0.066	2
VNIIM	1	2.316	0.029	0.058	2
	2	2.309	0.029	0.058	2

Participant Results: Combined

Due to an oversight in the study's design, the report form did *not* request participants to combine their results for the two vials into a single overall result for the study material. Rather than retrospectively requesting that the participants supply this additional information, the coordinating laboratory calculated the combined results for all participants from their reported results. These combined results are summarized in Table 6 and displayed in Figure 1.

The combined value, $\bar{x}$, was estimated as the mean of the two reported results, x_1 and x_2. The standard uncertainty on this mean, $u(\bar{x})$, combined the standard deviation, s, of x_1 and x_2 and the pooled value of their associated uncertainties, $\bar{u}(x) = \sqrt{[(U_{95}(x_1)/2)^2 + (U_{95}(x_2)/2)^2]/2}$, yeilding the combined standard uncertainty: $u(\bar{x}) = \left(\sqrt{s^2 + \bar{u}^2(x)}\right)/\sqrt{2}$. The expanded uncertainty on the combined value, $U_{k=2}(\bar{x})$, was estimated using the usual k=2 coverage factor: $U_{k=2}(\bar{x}) = 2 \cdot u(\bar{x})$. Note that $\bar{u}(x)$ is estimated from the reported expanded uncertainties divided by 2, $U_{95}(x)/2$, to ensure that $U_{k=2}(\bar{x})$ reflects the participant's uncertainty expansion policy.

Table 6: Participant Results for CCQM-K6.2 Cholesterol in Human Serum as Combined

	Mass Fraction, mg/g				
NMI/DI	$\bar{x}$	s	$\bar{u}(x)$	$u(\bar{x})$	$U_{k=2}(\bar{x})$
CENAM	2.465	0.030	0.071	0.033	0.066
HSA	2.355	0.004	0.027	0.010	0.020
INMETRO	2.350	0.000	0.045	0.016	0.032
INTI	1.72	0.01	0.25	0.09	0.18
KRISS	2.337	0.005	0.037	0.014	0.027
LNE	2.352	0.002	0.055	0.019	0.039
NIMT	2.383	0.0045	0.086	0.031	0.061
UME	2.288	0.032	0.064	0.032	0.064
VNIIM	2.313	0.005	0.058	0.021	0.042

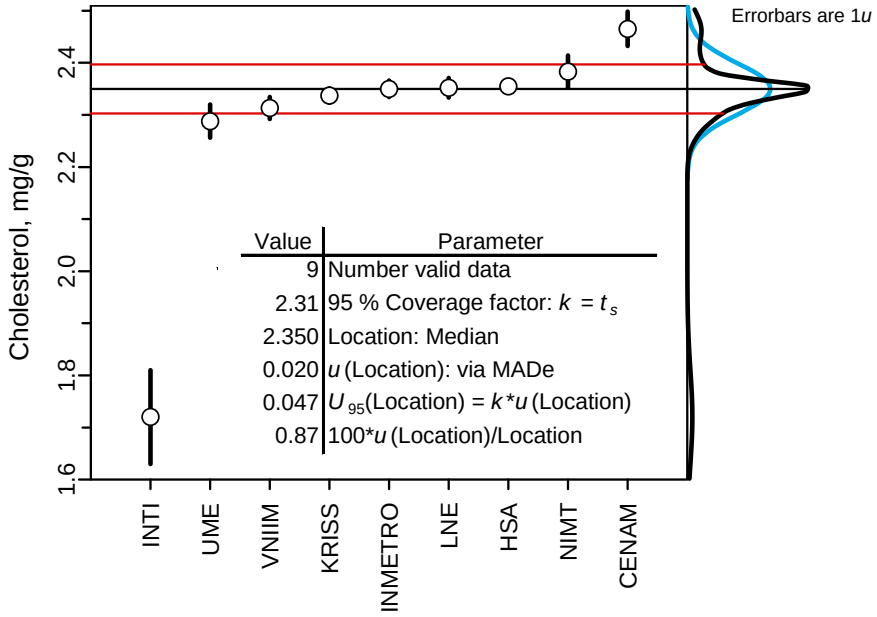

Figure 1: Combined results and robust consensus estimates of location and dispersion

Dots represent the combined values; the vertical bars on the dots span the $k = 2$ expanded uncertainties. The black horizontal line represents the median. The red horizontal lines bracket a robust estimate of the 95 % coverage interval about the median, U_{95}. This interval is estimated as the product of the: standard uncertainty, u, estimated as the median absolute deviation from the median scaled to have the same coverage of a normal distribution as provided by the standard deviation (MADe) [6]; a factor of 1.25 reflecting the efficiency of the median as an estimator of the location for normally distributed data; and the 2.31 expansion factor of the Student's t_s distribution for 8 degrees of freedom. The black curve to the right edge is the empirical probability density for the reported results; the blue curve to the right is the Gaussian distribution parameterized with the robust consensus estimates.

Anchor Laboratory Results

The serum sample used for CCQM-K6.2 was NIST candidate SRM 1951c Level 2. The anchor laboratory's certification measurements for this material, (240.91 ± 2.8) mg cholesterol/dL and a density of (102.521 ± 0.016) g/dL serum, were completed in June 2011 [5]. The certified value for this material, (241.41 ± 2.8) mg/dL, combines measurements made at NIST and at the U.S. Centers for Disease Control and Prevention (CDC). The date of issue for SRM 1951c was 27-June-2013, shortly after the CCQM-K6.2 results were revealed to participants. All uncertainties are here stated at an approximate 95 % level of confidence.

KEY COMPARISON REFERENCE VALUE (KCRV)

The certified value for SRM 1951c-2 and the anchor laboratory's result, both transformed to mass fraction, are shown in Table 7 along with the robust consensus summary of the CCQM-K6.2 participant's results.

Normally in a subsequent KC no KCRV would be established and assessment of performance would be via the deviation of participants' results to the anchor lab's results, adjusted to account for the anchor lab's performance in the original comparison versus its KCRV. Due to the very long-time period since the original key comparison it was decided that this did not represent the best approach to assess performance in what is a relatively complex measurement.

Considering the excellent agreement between the anchor laboratory's result and the consensus value, the OAWG at the April 2014 meeting agreed to use the anchor value and its U_{95} expanded uncertainty a "KCRV" for this comparison.

The participant results, both as reported and as combined, are displayed as $x \pm U_{95}(x)$ in Figure 2 with KCRV$\pm U_{95}$(KCRV) reference lines. The combined results are displayed as $x \pm u(x)$ in Figure 3 with KCRV$\pm u$(KCRV) reference lines.

Table 7: Key Comparison Reference Value for CCQM-K6.2

Source	Value	u(Value)	U_{95}(Value)	Units
SRM 1951c-2 Certified Value	2.355	0.014	0.027	mg/g
Anchor Laboratory Result	2.350	0.019	0.038	mg/g
Robust Consensus	2.350	0.024	0.047	mg/g
KCRV	2.350	0.019	0.038	mg/g

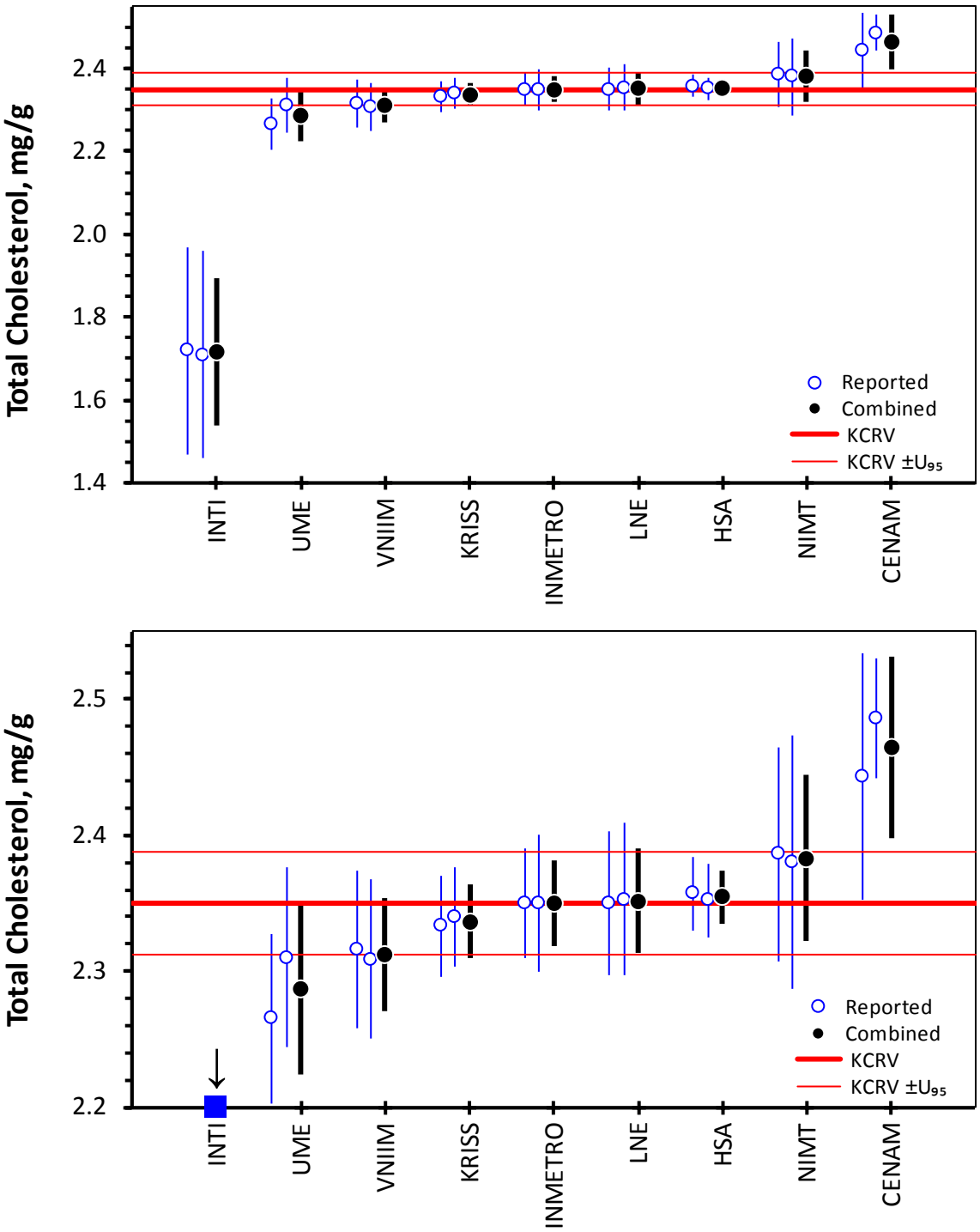

Figure 2: Participant results for CCQM-K6.2 relative to the KCRV.

The blue symbols and vertical bars represent the results as reported; the black symbols and bars represent the results as combined by the coordinating laboratory. The bars are approximate 95 % expanded uncertainties. The horizontal lines represent the KCRV and the KCRV ± U_{95}(KCRV) interval. The lower panel is identical to the upper, but displayed at higher vertical resolution.

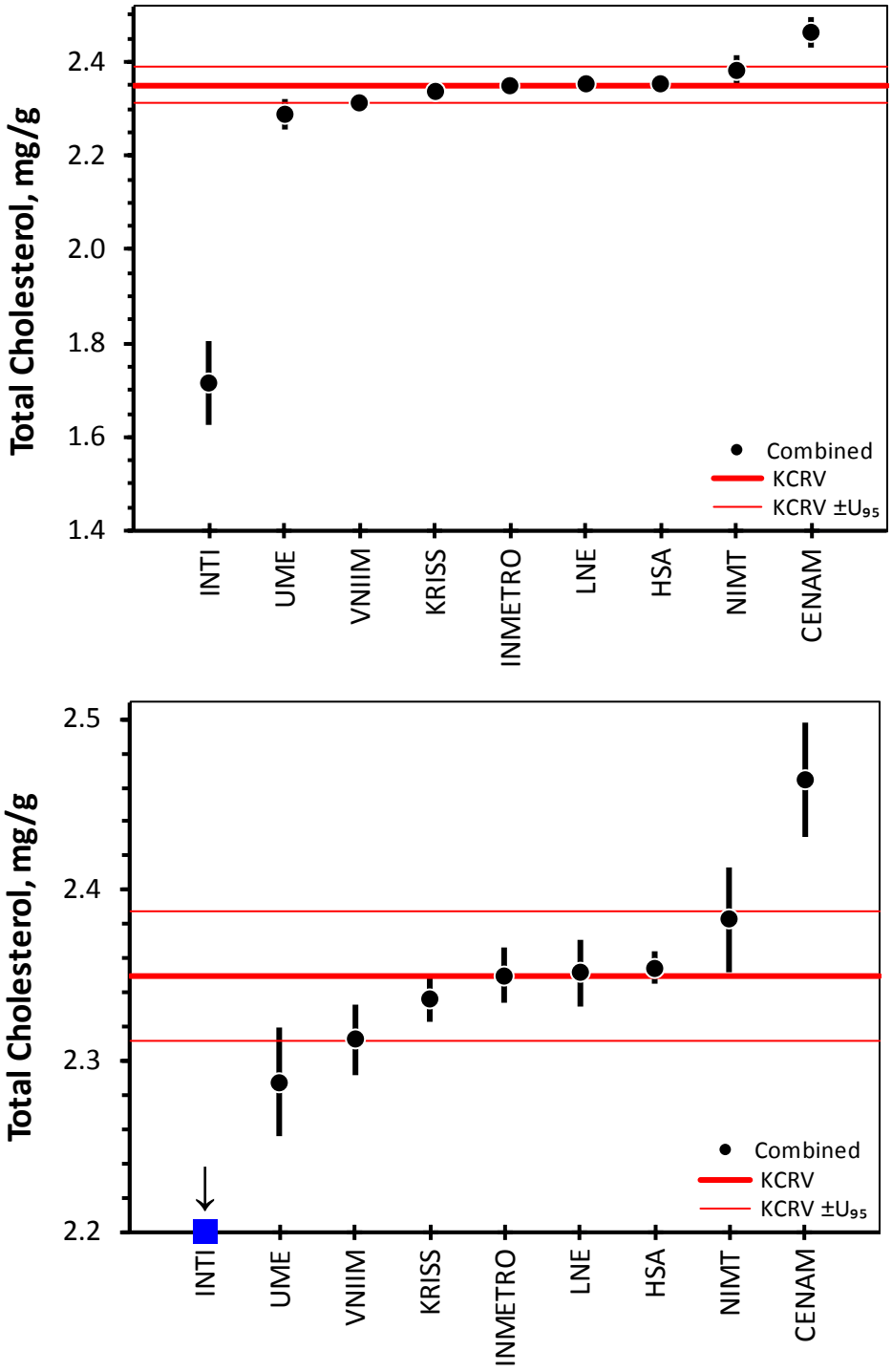

Figure 3: Combined Participant results for CCQM-K6.2 relative to the KCRV.

The black symbols and bars represent the results as combined by the coordinating laboratory. The bars are standard uncertainties. The horizontal lines represent the KCRV and the KCRV ± *u*(KCRV) interval. The lower panel is identical to the upper, but displayed at higher vertical resolution.

DEGREES OF EQUIVALENCE

The absolute degrees of equivalence for the participants in CCQM-K6.2 are estimated as the signed difference between the combined value and the KCRV: $d_i = x_i$ – KCRV. Since the KCRV is not estimated from the participant values, the 95 % expanded uncertainty on the d_i, $U_{95}(d_i)$, is estimated as the square root of the sum of the squares of the expanded uncertainties of the two components: $U_{95}(d_i) = \sqrt{U_{k=2}^2(x_i) + U_{95}^2(\mathrm{KCRV})}$.

To enable comparison with the degrees of equivalence estimates from CCQM-K6 and –K6.1, it is convenient to express the d_i and $U_{95}(d_i)$ as percentages relative to the KCRV: $\%d_i = 100 \cdot d_i/\mathrm{KCRV}$ and $U_{95}(\%d_i) = 100 \cdot U_{95}(d_i)/\mathrm{KCRV}$. Table 7 lists the numeric values of d_i, $U_{95}(d_i)$, d_i, and $U_{95}(d_i)$ for all participants in CCQM-K6.2. Figure 4 displays the absolute $d_i \pm U_{95}(d_i)$ for CCQM-K6.2; Figure 5 displays the relative $\%d_i \pm U_{95}(\%d_i)$ for CCQM-K6, CCQM-K6.1, and CCQM-K6.2.

Table 8: Degrees of Equivalence for CCQM-K6.2 Cholesterol in Human Serum

	mg/g		**%**	
NMI/DI	d_i	$U_{k=2}(d_i)$	$\%d_i$	$U_{k=2}(\%d_i)$
CENAM	0.115	0.076	4.9	3.3
HSA	0.004	0.043	0.2	1.8
INMETRO	0.000	0.050	0.0	2.1
INTI	-0.635	0.181	-27.0	7.7
KRISS	-0.014	0.047	-0.6	2.0
LNE	0.002	0.054	0.1	2.3
NIMT	0.033	0.072	1.4	3.1
UME	-0.063	0.074	-2.7	3.2
VNIIM	-0.038	0.056	-1.6	2.4

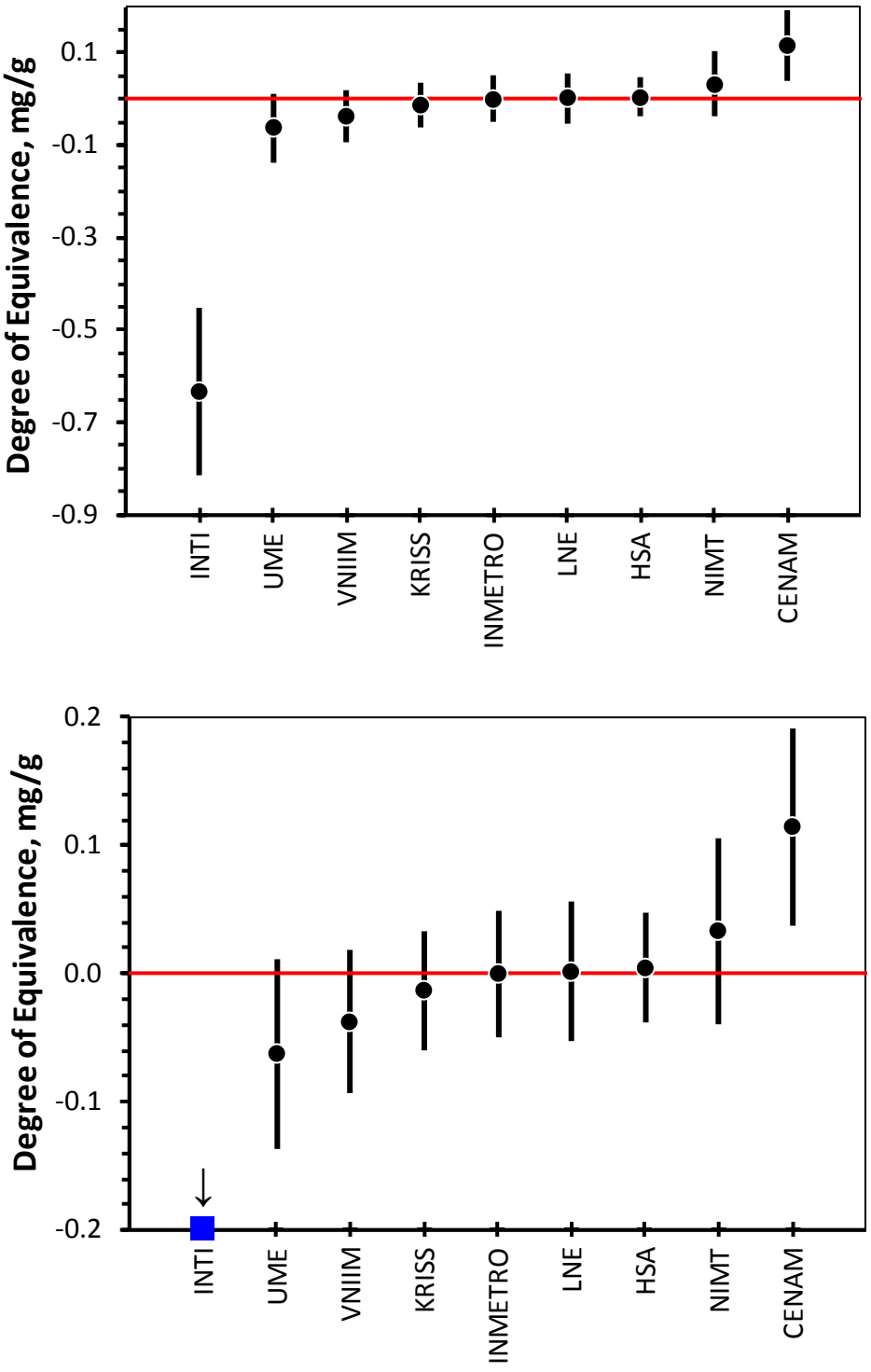

Figure 4: Absolute degrees of equivalence for CCQM-K6.2

The black symbols and vertical bars represent the $d_i \pm U_{95}(d_i)$. The horizontal line marks the ideal zero deviation from the KCRV. The lower panel is identical to the upper, but displayed at higher vertical resolution.

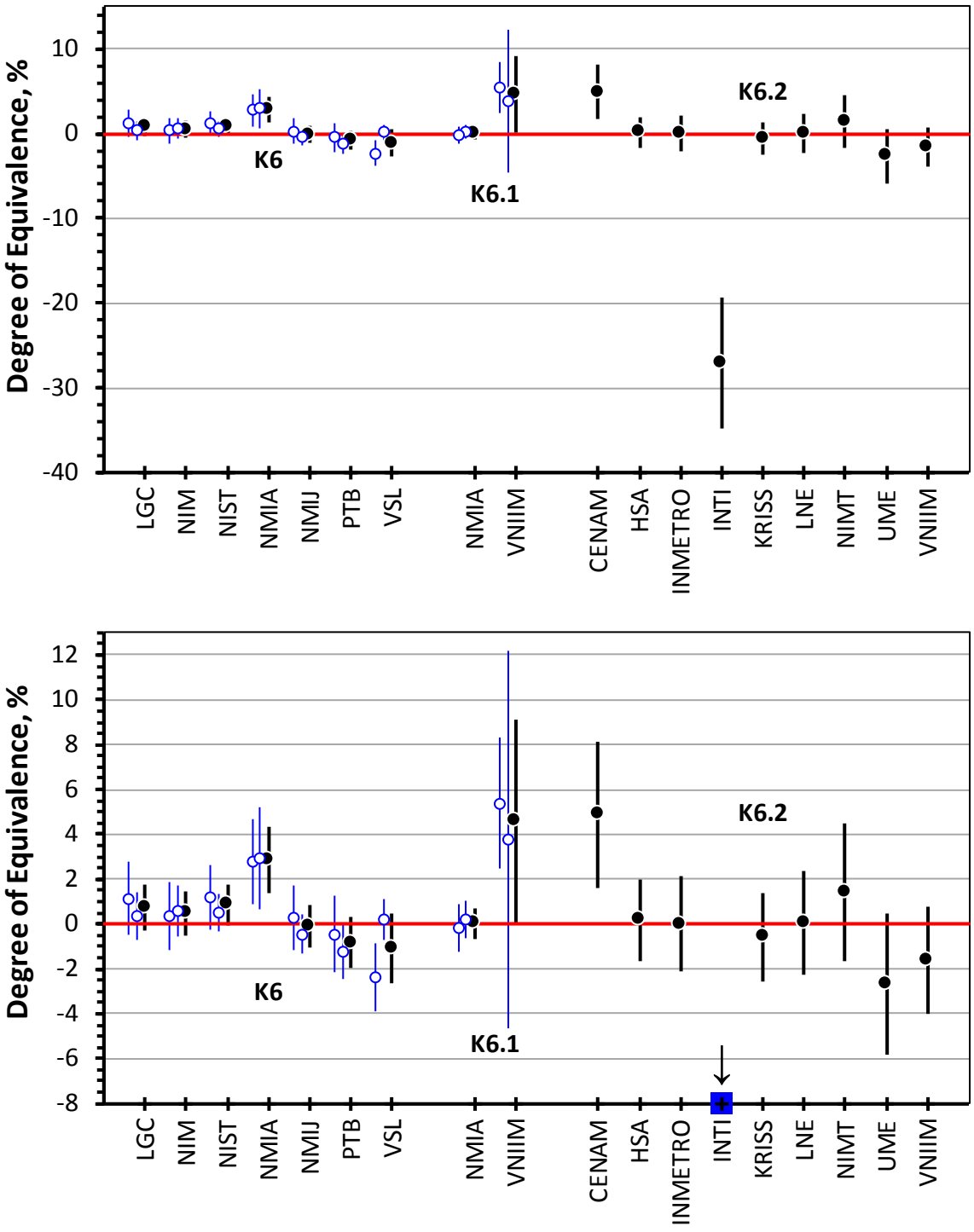

Figure 5: Relative degrees of equivalence for CCQM-K6, -K6.1 and -K6.2

The blue symbols and bars represent $\%d_i \pm U_{95}(\%d_i)$ for individual vials distributed in CCQM-K6 and -K6.1; the black symbols and vertical bars represent their combined $\%d_i \pm U_{95}(\%d_i)$. The red horizontal line marks the ideal zero deviation from the KCRV; the light grey lines are for visual guidance. The lower panel is identical to the upper, but displayed at higher vertical resolution.

USE OF CCQM-K6.2 IN SUPPORT OF CMCs

CCQM-K6.2 Cholesterol in Human Serum was designed as a Subsequent Key Comparison for NMIs and DIs that had not participated in earlier studies for determination of cholesterol. The study demonstrates a laboratory's capabilities to measure a nonpolar ($pK_{ow} < -2$), low molecular mass (100 g/mol to 500 g/mol) metabolite in human serum at relatively high concentrations (1 mg/g to 3 mg/g) found in normal populations. At the time of this study, the OAWG had not formalized the reporting of "core competencies". However, participation in this study demonstrates calibration and measurement capabilities (CMCs) in analysis of complex biological matrices including sample preparation (extraction, derivatization), LC or GC separation, and quantification using an isotope dilution mass spectrometry approach.

CONCLUSIONS

Intended as a Subsequent Key Comparison, CCQM-K6.2 met the expectations for such a study in that seven of the nine participants demonstrated agreement with the KCRV.

ACKNOWLEDGEMENTS

The study coordinators thank all the participating laboratories for providing the requested information during the course of these studies.

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APPENDIX A: CCQM-K6.2 Summary of Analytical Information

The following Tables summarize the analytical information provided by the participants in the "Analytical Information" worksheet of the "CCQM-K6.2 Reporting Form" Excel workbook.

The summary is provided as three Tables:

Table A-1: CCQM-K6.2 Sample Size, Extraction, and Cleanup,
Table A-2: CCQM-K6.2 Analytical Techniques, and
Table A-3: CCQM-K6.2 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 A-1: CCQM-K6.2 Sample Size, Extraction, and Cleanup

NMI/DI	Sample Size (g)	Extraction Method	Post Extraction Cleanup
Anchor Laboratory (NIST)	0.1	Basic hydrolysis (KOH) at 37 ºC for 3 h; liquid/liquid extraction with hexane.	Hexane extract evaporated to dryness and derivatized with N,O-bis(trimethyl)acetamide (BSA) at 65 ºC for 30 min.
CENAM	0.5	Basic hydrolysis and liquid/liquid extraction using cyclohexane for 30 min.	Free cholesterol in alcoholic medium was derivatized with N-methyl-N-(trimethylsilyl) trifluoroacetamide in cyclohexane; heat at 60 ºC for 1 h; after cooling to room temperature adding 0.5 mL pyridine.
HSA	0.1	Basic hydrolysis at 50 ºC for 3 h followed by liquid/liquid extraction using cyclohexane; extract evaporated to dryness and reconstituted in ethanol.	GC-MS: Ethanolic solution derivatized using N,O-bis(trimethylsilyl)acetamide (BSA). LC-MS: Ethanolic solution diluted with MeOH/water for injection to LC-MS.
INTI	0.2	Basic hydrolysis for 3 h followed by liquid/liquid extraction using hexane for 1 min	Hexane extract derivatized using N,O-bis(trimethylsilyl)acetamide
INMETRO	0.035	Basic (KOH) hydrolysis for 1 h followed by liquid/liquid extraction using hexane; extract evaporated to dryness	Extract residue derivatized using MSTFA at 60 ºC for 15 min
KRISS	0.075	Basic (KOH) hydrolysis for 3 h followed by liquid/liquid extraction using hexane; extract evaporated to dryness and reconstituted with ethanol	No further cleanup
LNE	0.08	Basic (aqueous KOH and ethanol) hydrolysis for 2 h followed by liquid/liquid extraction using hexane	Hexane extract derivatized using MSTFA/pyridine.
NIMT	0.1	Basic (KOH) hydrolysis at 50 ºC for 3 h followed by liquid/liquid extraction using hexane for 3 h. Hexane extract evaporated to dryness and reconstituted in MeOH	

Table A-1: CCQM-K6.2 Sample Size, Extraction, and Cleanup (Continued)

NMI/DI	Sample Size (g)	Extraction Method	Post Extraction Cleanup
UME	0.2	Basic (aqueous KOH/ethanol) hydrolysis at 50 ºC for 4 h followed by liquid/liquid extraction using cyclohexane (5 min)	Cyclohexane extract filtered through 0.2 µm membrane filter
VNIIM	0.1	Alkaline (NaOH) hydrolysis followed by liquid/liquid extraction using hexane	Derivatization with BSTFA + 10 % TMCS

Table A-2: CCQM-K6.2 Analytical Techniques

NMI/DI	Analytical Method	Chromatographic Column	Chromatographic and Mass Spectrometry Conditions
Anchor Laboratory (NIST)	GC-MS	DB5-MS 30 m capillary column	Split mode injection (8:1); 200 ºC 0.5 min hold, 20 ºC/min to 300 ºC 5 min hold. MSD quadrupole at 200 ºC, source at 230 ºC Ions monitored: *m/z* 458 cholesterol trimethylsilyl ether and *m/z* 461 labeled cholesterol trimethylsilyl ether
CENAM	GC-MS	HP-1MS capillary column, 30 m × 0.32 id, 0.25 µm film thickness	Split mode injection: 190 ºC 1 min, 30 ºC/min to 280 ºC hold 10 min; He carrier gas at 0.9 mL/min constant flow. MSD: transfer line at 270 ºC, quadrupole at 150 ºC, source at 250 ºC Ions monitored: *m/z* 458 cholesterol trimethylsilyl ether and *m/z* 464 deuterated cholesterol trimethylsilyl ether
HSA	GC-MS; LC-MS	GC-MS: DB5-MS, 15 m × 0.25 mm id × 0.25 µm film thickness LC-MS: Hypersil GOLD Phenyl, 100 mm × 2.1 mm, 3 µm particles	GC-MS: Inlet at 280 ºC, 70 ºC to 300 ºC at 50 ºC/min then hold 6 min; Flow at 1.0 mL/min; transfer line at 270 ºC Ions monitored: *m/z* 458 and *m/z* 460 (IS) (quantifying ions) and *m/z* 368 and *m/z* 370 (IS) (confirmatory ions) LC-MS: 93 % methanol/7 % 10 nmol/L ammonium formate at 0.5 mL/min Ions Monitored: *m/z* 369 and *m/z* 371 (IS) (quantifying ions)
INMETRO	GC-MS	VF1ms, 10 m × 110 mm id × 0.1 µm film thickness	170 ºC for 1 min, 30 ºC/min to 280 ºC and hold 10 min. Split mode injection; helium carrier gas Mass Selective Detector: Ions monitored: *m/z* 458.4 cholesterol trimethylsilyl ether and *m/z* 464.4 deuterated cholesterol trimethylsilyl ether
INTI	GC-MS	HP5-MS capillary column, 30 m × 0.25 id, 0.25 µm film thickness	Inlet at 280 ºC, 180 ºC to 250 ºC at 40 ºC/min then to 320 ºC (hold 2 min) at 20 ºC/min. Flow at 0.5 mL/min; split injection (1:100). Ions monitored: m/z 129, 329, 368, and 458 (quantifying ions) and m/z 131, 333, 374, and 464 (IS) (quantifying ions)
KRISS	LC-MS/MS	Hypersil ODS 100 mm × 2.1 mm, 3 µm particles	Mobile phase: (A) 1 % acetic acid in water, (B) 0.05 % acetic acid in MeOH; 1 % A and 99 % B isocratic
LNE	GC-MS	DB5-MS capillary column, 30 m × 250 µm id, 0.25 µm film thickness	Initial 100 ºC, then 20 ºC/min to 280 ºC and hold 8 min; split injection (20:1) at 270 ºC; Mass Selective Detector at 230 ºC Ions monitored: *m/z* 458 and *m/z* 460

Table A-2: CCQM-K6.2 Analytical Techniques (Continued)

NMI/DI	Analytical Method	Chromatographic Column	Chromatographic and Mass Spectrometry Conditions
NIMT	LC-MS/MS	Haisil C18, 100 mm × 0.3 mm id, 5 µm particles	Isocratic mobile phase 20 % isopropanol with 0.1 % formic acid and 80 % methanol with 0.1 % formic acid at 0.8 mL/min
UME	LC-MS/MS	Kintex C18 100 mm × 2.1 mm, 2.6 µm particles	Mobile phase: isocratic at 80 % acetonitrile and 20 % methanol at 0.25 mL/min Ions Monitored: *m/z* 369.0 and 161.0 and *m/z* 372.3 and 161.0 (IS)
VNIIM	GC-MS	Rtx-5MS 20 m × 0.18 mm × 0.18 µm film thickness	Initial 70 ºC, then 15 ºC/min to 270 ºC and hold 10 min Ions monitored: *m/z* 368 and *m/z* 370

Table A-3: CCQM-K6.2 Calibrants and Standards

NMI/DI	Quantification Method	Type of Calibration	Internal Standard	Source of Traceability
Anchor Laboratory (NIST)	IDMS	bracketing	Cholesterol-25,26,27-$^{13}C_3$ (99 atom % ^{13}C, 99 % CP) (Isotec, Miamisburg, OH)	NIST SRM 911c
CENAM	IDMS	bracketing	Labeled cholesterol of 99 % purity added before hydrolysis	Purity assessed at CENAM using HPLC, DSC, and Karl Fischer
HSA	IDMS	6-point calibration	$^{13}C_2$-cholesterol (Cambridge Isotopes) of 99.6 % purity added during gravimetric preparation of sample and calibrants	NIST SRM 911c
INMETRO	IDMS	One standard point-to-point	Deuterium-labeled (6) cholesterol (Cambridge Isotopes) of 99.8 % purity, isotopic enrichment 98.3 %	NIST SRM 911c
INTI	IDMS	bracketing	Deuterium-labeled (6) cholesterol (Cambridge Isotopes)	NIST SRM 911c
KRISS	IDMS/MS	bracketing	Deuterium-labeled (4) cholesterol (CDN Isotopes)	SRM 911c (NIST)
LNE	IDMS	5-point calibration	$^{13}C_2$-cholesterol (Cambridge Isotopes) of 99 % purity added prior to hydrolysis	NIST SRM 911c
NIMT	IDMS/MS	Exact matching IDMS with single-point calibration	$^{13}C_2$-cholesterol (Cambridge Isotopes)	Calibration blend prepared from matrix matched NIST SRM 1951b
UME	IDMS/MS	2-point calibration	$^{13}C_3$-cholesterol added prior to hydrolysis	NIST SRM 1951b NIST SRM 968e
VNIIM	IDMS	Single-point	$^{13}C_2$-cholesterol (Cambridge Isotopes)	NIST SRM 911c

APPENDIX B: CCQM-K6.2 Summary of Uncertainty Estimation Methods

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 CENAM

$$w_x = \left(\frac{\left(\frac{m_2}{m_{I2}}\right) \cdot w_2 \cdot (\overline{R_x} - \overline{R_1}) - \left(\frac{m_1}{m_{I1}}\right) \cdot w_1 \cdot (\overline{R_x} - \overline{R_2})}{\overline{R_2} - \overline{R_1}} \right) \cdot \frac{m_{Ix}}{m_x}$$

w_1	Mass fraction of the solution calibration standard (low level) (mg/g)
$w2$	Mass fraction of the solution calibration standard (high level) (mg/g)
$R1$	Response relationship of low level solution
$R2$	Response relationship of high level solution
$mi\ (1)$	Mass of the isotope solution added to the low level solution calibration standard (g).
m_1	Mass of the analyte standard solution of low level calibration (g)
$mi\ (2)$	Mass of the isotope solution added to the high level solution calibration standard (g).
$m2$	Mass of the analyte standard solution of high level calibration (g)
m_x	Mass of sample to be measured (g).
m_{Ix}	Mass isotope of the solution added to the sample (g).
R_x	Instrument response relationship (GC or LC) between the analyte in the sample and its isotope added (dimensionless).

Symbol	Value	Units	Uncertainty source	Type of distribution	Standard uncertainty	Units	Relative uncertainty ui(y)
w_1	**2.3512**	mg/g	experimental	normal type A	0.0036	mg/g	0.1515%
WNA (w2)	**2.7385**	mg/g	experimental	normal type A	0.0034	mg/g	0.1255%
R1	**1.6887**		experimental	normal type A	0.006570		0.3891%
R2	**2.0109**		experimental	normal type A	0.004795		0.2385%
mi (1)	**0.49931**	g	experimental	normal type B	0.00003	g	0.0061%
m_1	**0.5004**	g	experimental	normal type B	0.00003	g	0.0054%
mi (2)	**0.5009**	g	experimental	normal type B	0.00002	g	0.0050%
m2	**0.5024**	g	experimental	normal type B	0.00003	g	0.0051%
m_x	**0.4950**	g	experimental	normal type B	0.000051	g	0.0103%
m_{Ix}	**0.4946**	g	experimental	normal type B	0.000053	g	0.0107%
R_x	**1.7792**		experimental	normal type A	0.0089		0.4995%
mathematical model uncertainty	**0.0172**		**0.7%**				
Repeatibility between subsamples	0.0383						
Combined Uncertainty	0.0420						
Expanded Uncertainty	**0.0908**		**5.1%**				
k(95%)	2.16						

Uncertainty Information from HSA

The mass fraction of total cholesterol in serum was calculated based on the IDMS calibration curve as follows:

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

where
C_X = mass fraction of total cholesterol 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 $M_Y \times C_Y$)
R_B = peak area ratio of sample blend (determined by GC-MS or LC-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 $R_M = R_M' \times C_Y/C_Z$, Equation (1) is converted to:

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

where
R_M = isotope mass ratio in sample blend
C_Z = concentration of cholesterol 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 total cholesterol in the serum samples. A coverage factor *k* with a value of 2 was used to expand the combined standard uncertainty at a 95 % confidence interval. Possible sources of biases [method precision (F_P), choice of different ion pair (F_I), and other factors during sample extraction (F_{C1}) and derivatisation (F_{C2})] are accounted for in the final uncertainty budget with the use of the measurement equation:

$$C_X = F_P \times F_I \times F_{C1} \times F_{C2} \times R_M' \times \frac{M_Y C_Z}{M_X} \quad (3)$$

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

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

$$\frac{\partial C_X}{\partial F_P} = \frac{C_X}{F_P} \qquad \frac{\partial C_X}{\partial F_I} = \frac{C_X}{F_I} \qquad \frac{\partial C_X}{\partial F_{C1}} = \frac{C_X}{F_{C1}} \qquad \frac{\partial C_X}{\partial F_{C2}} = \frac{C_X}{F_{C2}}$$

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 pooled standard deviation of the mean of the GC-MS and LC-MS results for each sample was used as the standard uncertainty of method precision.
(3) F_I: The standard deviation of the difference of the results using two ion pairs divided by the square root of the number of samples (for insignificant difference using t-test) or the average of the difference of the results using two ion pairs divided by 2 (for significant difference using t-test).
(4) F_{C1} and F_{C2}: A relatively standard uncertainty of 0.2 % was employed for each of these two factors.
(5) C_Z: The certified purity and uncertainty of NIST SRM 911c in combination with the uncertainty of weighing for the preparation of the calibration standard solution.
(6) 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:

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

where

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

R_B = peak area ratio of sample blend

$\overline{R_{Bc}}$ = average peak area ratio of calibration blends

m' = number of calibration blends used for the linear regression plot

N = injection time for each sample

R_{Mc} = isotope mass ratio in calibration blends

$\overline{R_{Mc}}$ = average isotope mass ratio in calibration blends

The combined standard uncertainty was calculated using the equation below:

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

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 a confidence level of 95 %.

Table 1. Uncertainty Budget for Sample 1

Factor	Value x	Uncertainty u(x)	Relative Uncertainty u(x)/(x)	Sensitivity Coefficient (c) δCx/δx	$c^2 . u(x)^2$	Contribution %
M_X (g)	0.0960	0.000099	0.103%	24545.16	5.9042	3.2%
M_Y (g)	0.6538	0.000099	0.015%	3605.11	0.1274	0.1%
C_Z (µg/g)	1519.3	5.1353	0.338%	1.55	63.4705	34.5%
R_M'	1.1505	0.0034	0.299%	2048.71	49.6015	27.0%
F_P (µg/g)	2357	2.2651	0.096%	1.00	5.1308	2.8%
F_I (µg/g)	2357	3.9038	0.166%	1.00	15.2398	8.3%
F_{C1} (µg/g)	2357	4.7140	0.200%	1.00	22.2220	12.1%
F_{C2} (µg/g)	2357	4.7140	0.200%	1.00	22.2220	12.1%

Table 2. Uncertainty Budget for Sample 2

Factor	Value x	Uncertainty u(x)	Relative Uncertainty u(x)/(x)	Sensitivity Coefficient (c) δCx/δx	$c^2 . u(x)^2$	Contribution %
M_X (g)	0.0992	0.000099	0.100%	23708.42	5.5085	3.0%
M_Y (g)	0.6565	0.000099	0.015%	3582.97	0.1258	0.1%
C_Z (µg/g)	1519.3	5.1353	0.338%	1.55	63.2069	34.6%
R_M'	1.1505	0.0034	0.299%	2044.45	49.3955	27.0%
F_P (µg/g)	2352	2.2604	0.096%	1.00	5.1095	2.8%
F_I (µg/g)	2352	3.8957	0.166%	1.00	15.1765	8.3%
F_{C1} (µg/g)	2352	4.7042	0.200%	1.00	22.1297	12.1%
F_{C2} (µg/g)	2352	4.7042	0.200%	1.00	22.1297	12.1%

Uncertainty Information from INMETRO

All factors from the measurement equation were considered in the uncertainty estimation.
All of the evaluated uncertainties were of type B except for the R'B and R'Bc repeatabilities.
Hence their standard uncertainties were obtained by dividing the expanded uncertainties by the coverage factors encountered in the certificates.
For the repeatabilities, standard uncertainties were obtained by the standard errors of the means
The standard uncertainties were multiplied by their sensitivity coefficients using the GUM methodology and then combined using the square root of the squared sum of the components.
Effective degrees of freedom were calculated and the coverage factors for 95 % probability were taken for the expanded uncertainties.

The full uncertainty budget is presented below as for sample 1:

Factor	% contribution
m_{final}	0,00001
m_{solute}	2,14496
P	8,70135
m_z	0,13319
m_{yc}	0,03935
m_y	0,04002
m_x	0,15553
R'B	37,89210
R'Bc	50,89350
Total	**100**

Method was validated by the preparation by two different analysts of the CRM from NIST 909c.
These results showed that both analysts were capable of generating results equivalent to the certified property values for the CRM by comparison of the Δm (absolute difference between the mean measured value and the certified value) and the UΔ (expanded uncertainty of the difference between the measurement result and the certified value), obtaining $\Delta m < U\Delta$ which means the measured value and the certified value have no significant differences according to ERM Application Note 1.

These experiments demonstrated repeatability, intermediate precision and trueness (bias) evaluations of the method.

Uncertainty Information from INTI

$$w_x = \frac{m_{Ix} \cdot R_x \cdot m_0}{m_x \cdot R_0 \cdot m_{I0}} \cdot w_0$$

w0 Fracción de masa de la disolución estándar de calibración. (concentracion STD)
R0 Relación de respuesta del instrumento (CG o CL) entre el analito en el patrón de calibración y el isótopo adicionado (adimensional).
mI0 Masa de disolución de isótopo adicionado a las disoluciones patrón de calibración (g). (masa STDi al STD)
m0 Masa de disolución de analito patrón de calibración (g) (masa STD agregada a los viales)
mx Masa de muestra problema a medir (g).
mIx Masa de la disolución de isótopo adicionado a la muestra (g).
Rx Relación de respuesta del instrumento (CG o CL) entre el analito en la muestra y su isótopo adicionado (adimensional).

Parámetro (simbolo)	Descripción	Valor	Unidades	Origen de la incertidumbre	Tipo de distribution	Incetidumbre estandar	Units	Incertidumbre relativa ui(y)
w_0	Fracción de masa de la disolución estándar de calibración. (concentracion STD)	**1.602**	mg/g	certificado/experimental	normal tipo A	0.016825	mg/g	1.05%
R_0	Relación de respuesta del instrumento (CG o CL) entre el analito en el patrón de calibración y el isótopo adicionado (adimensional).	**1.576**	1	experimental	normal tipo A	0.001795		0.11%
m_{I0}	Masa de disolución de isótopo adicionado a las disoluciones patrón de calibración (g). (masa STDi al STD)	**0.156**	g	certificado/experimental	normal tipo B	0.000035	g	0.02%
m_0	Masa de disolución de analito patrón de calibración (g) (masa STD agregada a los viales)	**0.315**	g	certificado/experimental	normal tipo A	0.000038	g	0.01%
m_x	Masa de muestra problema a medir (g).	**0.1487**	g	experimental	normal tipo A	0.000122	g	0.08%
m_{Ix}	Masa de la disolución de isótopo adicionado a la muestra (g).	**0.1453**	g	experimental	normal tipo A	0.000088	g	0.06%
R_x	Relación de respuesta del instrumento (CG o CL) entre el analito en la muestra y su isótopo adicionado (adimensional).	**1.631**		experimental	normal tipo A	0.0039		0.24%
	Bias measuring SRM 909c	**1.398**		experimental	normal tipo A	0.1		7.15%

u(Cmtra)	**Inc. combinada**	0.1241	**0.072**
U exp	**Inc expandida**	0.2482	
k(95%)	**2**		

Uncertainty Information from KRISS

$$C=(M_{(is-sol,\,spiked)}\cdot C_{(s-sol)})/W_s \cdot[(([\![AR]\!]_{sample}-[\![AR]\!]_1)/([\![AR]\!]_2-[\![AR]\!]_1))\cdot([\![MR]\!]_{(mix,\,2)}-[\![MR]\!]_{(mix,\,1)})+[\![MR]\!]_{(mix,\,1)}]$$

Here, $M_{is\text{-}sol,spiked}$ is the weight of the cholesterol-d_4 solution spiked in the sample, $C_{s\text{-}sol}$ is the concentration of the cholesterol standard solution (mg/kg), and W_s is the weight of the sample, . AR_{sample} is the observed area ratio of cholesterol/cholesterol-d_4 of the sample from the LC/MS/MS measurement, AR_i is the observed area ratio of cholesterol/cholesterol-d_4 of the calibration standard mixture i (i=1,2) from the LC/MS/MS measurement, and $MR_{mix\,i}$ is the weight ratio of the cholesterol solution/cholesterol-d_4 solution in the calibration standard mixture i (i=1,2) from the LC/MS/MS measurement.

Measurement protocol: each subsample was separately measured by LC/MS/MS in comparison with Isotope ratio standard

Uncertainty	CCQM sample2	CCQM sample3
1		2.328
2	2.352	2.370
3	2.320	2.329
4	2.328	2.331
Avg	2.333	2.340
Stdev	0.017	0.020
Rel. stdev (%)	0.7	0.9
Standard unc. (%)	0.41	0.44
STD solution unc. (prep, %)	0.4	0.4
Purity unc. (%)	0.4	0.4
STD Mix (prep)	included in std sol unc	
unc of LC/MS/MS meas. For sample	included in rel stdev	
unc of LC/MS/MS meas. For STD Mix	included in rel stdev	
Combined standard unc. (%)	0.72	0.73
DOE	10	12
k	2.2	2.2
Urel%	1.6	1.6
Uexp (mg/kg)	0.037	0.037

Uncertainty Information from LNE

$$C = (aR_{458/460} + b) \times ((m_{Lab} C_{Lab}) / m_{ser})$$

C = mass fraction of cholesterol in the serum sample (mg/g)
m_{Lab} = mass of labeled cholesterol solution (g)
C_{Lab} = concentration of labeled cholesterol solution (mg/g)
a = gradient of the slope for linear regression plot
b = intercept on y axis for the linear regression plot
$R_{458/460}$ = unlabeled/labeled ion peak area ratio of serum sample
m_{ser} = mass of serum sample (g)

sample1

Component	Type (A or B)	relative Uncertainty (%)
Purity of primary standard	B	2.45%
preparationof sample blends (weighings)	B	6.66%
Calibration model	B	5.63%
Preparation of calibration blend (weighings)	B	1.79%
Precision	B	83.47%

sample2

Component	Type (A or B)	relative Uncertainty (%)
Purity of primary standard	B	2.06%
preparationof sample blends (weighings)	B	5.88%
Calibration model	B	5.63%
Preparation of calibration blend (weighings)	B	1.51%
Precision	B	84.92%

Uncertainty Information from NIMT

Expanded measurement equation:

$$w_x = F_P . F_E . F_I . w_{z,c} \cdot \frac{m_y \cdot m_{zc}}{m_x \cdot m_{yc}} \cdot \frac{R'_b}{R'_{bc}}$$

$w_{z,c}$	is the mass fraction of analyte in the calibration solution used to prepare the calibration blend
m_y	is the mass of spike solution added to sample blend
$m_{y,c}$	is the mass of spike solution added to calibration blend
m_x	is the mass of sample added to sample blend
$m_{z,c}$	is the mass of standard solution added to calibration blend
R'_B and $R'_{B,C}$	are the observed isotope amount ratios in the sample blend and the calibration blend, respectively
F_E	is the extraction efficiency factor
F_P	is the method precision factor
F_I	is the interference effect factor

$$\frac{u(w_x)}{w_x} = \sqrt{\left(\frac{u(w_{Z,C})}{w_{Z,C}}\right)^2 + \left(\frac{u(m_Y)}{m_Y}\right)^2 + \left(\frac{u(m_{Y,C})}{m_{Y,C}}\right)^2 + \left(\frac{u(m_X)}{m_X}\right)^2 + \left(\frac{u(m_{Z,C})}{m_{Z,C}}\right)^2 + \left(\frac{u(F_P)}{F_P}\right)^2 + \left(\frac{u(F_E)}{F_E}\right)^2 + \left(\frac{u(F_I)}{F_I}\right)^2}$$

$u(w_{z,c})$ is the standard uncertainty of the mass fraction of analyte in the calibration solution used to prepare the calibration blend. The value was estimated from the certified mass fraction value of matrix-matched calibration standard, masses weighed for preparation of calibration standard and uncertainty using different standards (standard comparison).

$u(m_y)$, $u(m_{y,c})$, $u(m_x)$ and $u(m_{z,c})$ are standard uncertainties of the masses. These values were estimated from the bias and precison effect of the balance.

$u(F_P)$ is the standard uncertainty of the precision factor. This value was estimated from standard deviation of the multiple IDMS results.

$u(F_E)$ is the standard uncertainty of the extraction efficiency factor which was estimated from the extraction and protein precipitaion

$u(F_I)$ is the standard uncertainty of the interference effect. This value was estimated from potential bias between primary ion pair and secondary ion pair of the MRM program.

Note: For the uncertainty contributing to the R'_B and $R'_{B,C}$,the precision in measuring the isotope amount ratios of the analyte and the internal standard in the sample and calibration blends was assumed to be incorporated in the overall method precision.The effect of any biases on these ratios was assumed to be negligible as any systematic biases should cancel out since the calibration blends and sample blends were exact-matched for analyte concentration and isotope ratio. Other biases that may arise from extractions are captured in other factors.

Uncertainty budget of Cholesterol (sample I)			
Factor	**Values**	**Uncertainties**	
	x	**u(x)**	**u(x)/(x)**
Parameter (unit)			
Method Precision, F_P(1)	1.0000	0.01004	1.004%
$m_{z,c}$ (g)	0.08372	0.000049	0.0591%
m_y (g)	0.07478	0.000049	0.0662%
$m_{y,c}$ (g)	0.07489	0.000049	0.0661%
m_x (g)	0.10035	0.000049	0.0493%
$w_{z,c}$ (ug/g)	0.2295	0.0016	0.6944%
Additional Factors			
Extraction effects, F_E (1)	1.000	0.0100	1.000%
Interference from two different ion pairs, F_I (1)	1.000	0.0028	0.283%

Uncertainty Analysis Results		
wx=	**2.386**	ug/g
u(x) =	0.038	ug/g
u(x)/x =	1.61%	
Veff(total) =	27.151	
k=	2.05	(@ 95% level)
U(x) =	**0.079**	ug/g
%U(x) =	**3.30%**	

Uncertainty budget of Cholesterol (sample II)			
Factor	**Values**	**Uncertainties**	
	x	**u(x)**	**u(x)/(x)**
Parameter (unit)			
Method Precision, F_P(1)	1.0000	0.01407	1.407%
$m_{z,c}$ (g)	0.08372	0.000049	0.0591%
m_y (g)	0.07478	0.000049	0.0662%
$m_{y,c}$ (g)	0.07489	0.000049	0.0661%
m_x (g)	0.10035	0.000049	0.0493%
$w_{z,c}$ (ug/g)	0.2295	0.0015	0.6617%
Additional Factors			
Extraction effects, F_E (1)	1.000	0.0100	1.000%
Interference from two different ion pairs, F_I (1)	1.000	0.0036	0.357%

Uncertainty Analysis Results		
wx=	**2.380**	ug/g
u(x) =	0.045	ug/g
u(x)/x =	1.89%	
Veff(total) =	25.181	
k=	2.06	(@ 95% level)
U(x) =	**0.092**	ug/g
%U(x) =	**3.89%**	

Uncertainty Information from UME

$$RF = \frac{A_{ABx}\ xC_{ISx}}{A_{ISx}\ xC_{ABx}}$$

RF : Response factor
CABx : Concentration of native compound (mg/g)
AABx : Peak area of native compound
AISx : Peak area of labelled compound
CISx : Concentration of labelled compound (mg/g)

1-Mass of sample

	Value	Standard Uncertainty
Mass of compound	$m_{Compound}$	
Calibration		$uCm_{Compound}$
Mass of Tare	m_{tare}	
Calibration		uCm_{tare}

$$u(m_{Compound}) = \sqrt{u_{CmCompound}^{\ 2} + u_{CmTare}^{\ 2}}$$

2-Mass of Labelled STD

	Value	Standard Uncertainty
Mass of labelled compound	$m_{Compound}$	
Calibration		$uCm_{Compound}$
Mass of Tare	m_{tare}	
Calibration		uCm_{tare}

$$u(m_{Compound\ \ ^{13}C3}) = \sqrt{u_{CmCompound\ \ ^{13}C3}^{\ 2} + u_{CmTare}^{\ 2}}$$

3-Labelled Compounds Stock Solution

	Value	Standard Uncertainty
Mass of Compound $^{13}C3$	m_{13C3}	
Calibration		uCm_{C13C3}
Mass of Tare	m_{tare}	
Calibration		uCm_{tare}
Mass of Solvent	$m_{solvent}$	
Calibration		$uCm_{solvent}$

$$u(m_{stock\ ^{13}C3}) = \sqrt{u_{stock\ ^{13}C3}^{\ 2} + u_{CmSolvent}^{\ 2} + u_{CmTare}^{\ 2}}$$

4- Uncertainty of calibration standard

	Value	Standard Uncertainty
Mass of calib	$m_{Compound}$	
Calibration		$uCm_{Compound}$
Mass of Tare	m_{tare}	
Calibration		uCm_{tare}

$$u(m_{Calib}) = \sqrt{u_{CmCalib}^{\ 2} + u_{CmTare}^{\ 2}}$$

5-Method Precision

where,
$u(rep)$: Uncertainty of repeatability
SD: Standard deviation
n: Number of sample

$$u(rep) = \frac{SD}{\sqrt{n}}$$

6-Instrument Repeatability

where,
$u(rep)$: Uncertainty of repeatability
SD: Standard deviation
n: Number of sample

$$u(rep) = \frac{SD}{\sqrt{n}}$$

7-Calibration Graph

$$u(c_0) = \frac{S}{B_1}\sqrt{\frac{1}{p} + \frac{1}{n} + \frac{(c_0 - \bar{c})^2}{S_{xx}}} \qquad S_{XX} = \sum_{i=1}^{n}(c_i - \bar{c})^2$$

S: Residual standard deviation
B_1: Slope
p: number of measurement to determine c_0
n: number of measurement for the calibration
c_0: determined concentration
$\bar{c}$: mean value of the different calibration standards
i : index for the number of calibration standards

Uncertainty Information from UME (Continued)

CCQM SAMPLE 1			
Parameter	***Value(X)***	**u(x)**	**u(x)/X**
Mass of sample (mg)	2.046E+02	2.860E-05	1.398E-07
Mass of labelled std(mg)	*7.566E+01*	3.910E-06	5.168E-08
Labelled stock solution (mg/kg)	*4.000E+03*	8.303E-03	2.076E-06
Uncertainty of calibration standard level 2 (mg)	*2.070E+02*	2.927E-05	1.414E-07
Uncertainty of calibration standard level 3 (mg)	*2.063E+02*	2.907E-05	1.409E-07
Method Precision	*1.000E+02*	1.887E-01	1.887E-03
Instrument repeatability	1.000E+02	1.770E-01	1.770E-03
Calibration curve	2.265E+00	3.036E-02	1.340E-02
Relative Combined Uncertainty			1.365E-02
Result (mg/g)	2.265E+00		
Combined Standard Measurement Uncertainty		3.092E-02	
Expanded Uncertainty (k=2)		6.185E-02	
Relative Uncertainty		2.730E+00	

CCQM SAMPLE 3			
Parameter	***Value(X)***	**u(x)**	**u(x)/X**
Mass of sample (mg)	2.053E+02	2.878E-05	1.402E-07
Mass of isotopic standard (mg)	*7.651E+01*	3.999E-06	5.227E-08
Labelled stock solution (mg/kg)	*4.000E+03*	8.303E-03	2.076E-06
Uncertainty of calibration standard level 2 (mg)	*2.070E+02*	2.927E-05	1.414E-07
Uncertainty of calibration standard level 3 (mg)	*2.063E+02*	2.907E-05	1.409E-07
Method Precision	*1.000E+02*	1.887E-01	1.887E-03
Instrument repeatability	1.000E+02	1.556E-01	1.556E-03
Calibration curve (mg/g)	2.310E+00	3.248E-02	1.406E-02
Relative Combined Uncertainty			1.428E-02
Result (mg/g)	2.310E+00		
Combined Standard Measurement Uncertainty		3.297E-02	
Expanded Uncertainty (k=2)		6.594E-02	
Relative Uncertainty		2.855E+00	

Uncertainty Information from VNIIM

$W=(San*m_{is})/(Sis*m*F)$

W - mass fraction of the creatinine in the sample, mg/g;

mis - mass of internal standard added to sample before sample preparation, mg;

m - mass of sample, g;

F - response factor; $F=(San_{cal}*Cis)/(Sis_{cal}*Can)$

Can_{cal}- concentration of creatinine in calibration solution;

Cis - concentration of internal standard in calibration solution

San_{cal} - peak area for the creatinine; Sis - peak area for the internal standard

Source of uncertainty	u, %
mass of sample (m)	0.29
mass of internal standard added to sample before extraction (mIS)	0.58
response factor (F)	0.85
purity of referense standard	*0.12*
preparation of calibration solution	*0.82*
RSD of F determination	*0.19*
RSD of results, %	0.47

comb.std uncertainty	1.16
expanded uncertainty (k=2)	2.32