

# **CCQM-K6: Key Comparison on the Determination of Cholesterol In Serum\***

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### **INTRODUCTION**

The accuracy and traceability of routine measurements performed to assess health status is a subject of considerable interest. The European Union has addressed the efficacy of such tests through issuance of an In-vitro Diagnostic (IVD) Directive that places requirements on any products to be sold in the EU. Among the requirements are that all IVD devices sold for quantitative purposes must be traceable to higher order standards, if such standards exist. Other parts of the world are likely to follow the EU in requiring greater traceability for IVD products. Consequently, it is important that internationally recognized reference systems be established and maintained for health status markers.

Cholesterol is one of the most frequently measured substances in human blood. Large epidemiological studies have demonstrated a strong correlation between the concentration of cholesterol in blood and the risk of cardiovascular disease. The U.S. National Cholesterol Education Panel established guidelines for treatment based upon the cholesterol levels in blood. These guidelines are that individuals having cholesterol concentrations greater than 2.4 g/L are candidates for cholesterol-reducing drugs, while those having concentrations between 2.0 g/L and 2.4 g/L should reduce the fat and cholesterol in their diets and increase physical activity. Those with cholesterol concentrations below 2.0 g/L and lacking other heart disease risk factors are considered to be at low risk of developing cardiovascular disease. Because treatment decisions require accurate determinations of blood cholesterol, it is important that routine cholesterol measurements be traceable to higher order reference methods and materials. A Key Comparison for the determination of serum cholesterol has been recommended as a means of establishing an internationally recognized reference systems maintained by various NMIs.

At the CCQM Organic Working Group meetings in 1999, a Key Comparison on the determination of total cholesterol in human serum was approved and outlined, based upon a successful 1999 Pilot Study (CCQM-P6, initially named CCQM-7) in this area. In January 2000, the designated pilot laboratory, NIST, sent serum samples to participating laboratories for determination of total serum cholesterol. The six NMI laboratories that submitted results in the pilot study also participated in this Key Comparison study. In addition, one laboratory (NARL) that did not participate in the Pilot Study elected to participate in the Key Comparison. By prior agreement of the CCQM, only results from those laboratories that participated in the pilot study would be eligible to be included in calculation of the Key Comparison Reference Values.

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The participants in CCQM-K6 were:

National Institute of Standards and Technology (NIST) [USA] *Pilot Laboratory*  
Laboratory of the Government Chemist (LGC) [UK]  
National Analytical Reference Laboratory (NARL) [Australia]  
National Metrology Institute of Japan (NMIJ) [Japan]  
National Research Center for Certified Reference Materials (NRCCRM) [China]  
NMI Van Swinden Laboratorium (NMI) [Netherlands]  
Physikalisch-Technische Bundesanstalt (PTB) [Germany]

## **PILOT STUDY SUMMARY**

A pilot study (CCQM-P6, initially named CCQM-7) for the determination of total serum cholesterol was organized by NIST in 1999. Samples from two frozen human serum pools were sent to seven institutes who agreed to participate. These pools were unknown to the participants, except for the pilot laboratory, and were NIST SRM 965, Glucose in Frozen Human Serum, certified for glucose, but not cholesterol (Material A<sub>p</sub>), and SRM 1951a, Lipids in Frozen Human Serum, Level 2, (Material B<sub>p</sub>) certified for cholesterol, using the published isotope dilution/gas chromatography/mass spectrometry (ID/GC/MS) “definitive” method for serum cholesterol<sup>1</sup> described briefly below. Each participant also received a sample of SRM 911b, Cholesterol, to use for calibration. The participants were free to use whatever methods they chose. Results were received from six participants, all of whom used ID/GC/MS-based methods. The results are shown in Table 1. The scatter in the data was acceptable for this pilot exercise, but was greater than would be expected for experienced laboratories, probably because most of the participants had no previous experience with cholesterol measurements. The two most experienced laboratories (NIST and Deutsche Gesellschaft für Klinische Chemie E.V. (DGKC)) reported the highest values, suggesting that the results of other laboratories likely were biased low, perhaps due to incomplete hydrolysis of esters of cholesterol. DGKC did not participate in the Key Comparison study since they are not an NMI or an official representative of an NMI at this time. Participants in this pilot study met at the 1999 meeting of the Organic Working Group of the CCQM to discuss the results and possible sources of bias and how such biases might be overcome. With the expectation that further exercises would show improvement as laboratories became more familiar with the methods they used for the determination of cholesterol, the CCQM approved the Organic Working Group’s proposal to proceed with a Key Comparison.

## **KEY COMPARISON**

The CCQM-K8 Key Comparison study utilized samples from two pools of human serum with significantly different concentrations of cholesterol for the participants to analyze using a method of their choice based upon isotope dilution/gas chromatography/mass spectrometry. Two unfortified frozen serum materials, with concentrations unknown, except by the Pilot Laboratory, were sent on dry ice. For each material there were four vials included, one as a test sample to determine how much internal standard was needed and three vials for measurements of two aliquots per vial. Material A was a material prepared at NIST by thoroughly mixing sera from two volunteers known to have high blood cholesterol levels. Material B was SRM 965, Glucose in Frozen Human Serum. This SRM is not value assigned for cholesterol, but has a cholesterol level in the normal range for humans.

As all of the laboratories used isotope dilution/gas chromatography/mass spectrometry-based methods for the pilot study, only this technique was permitted for the Key Comparison. All of the laboratories followed the published ID/MS “definitive” method for serum cholesterol.<sup>1</sup> This method involves adding a known mass of a cholesterol material with a stable isotope label to a known mass of serum. After an equilibration period, the serum is treated with a strong base to hydrolyze all cholesteryl esters to free cholesterol. After neutralization of the solution, the cholesterol is extracted with a non-polar solvent. The cholesterol is converted to a trimethylsilyl derivative to improve its gas chromatographic and mass spectrometric properties. The derivatized material is injected into a gas chromatograph/mass spectrometer system to carefully measure the ratio between the native cholesterol and the isotope labeled material that was added. By comparing this ratio with that of known mixtures of the same labeled cholesterol and unlabeled cholesterol of known purity (calibration material), it is possible to accurately and precisely determine the cholesterol content of the serum.

The measurement equation used to calculate the cholesterol concentration in mg/g of serum is dependent upon how the calibration is performed. If bracketing is used as in the published NIST method<sup>1</sup>, the equation is as follows:

$$C = \frac{[(I_{\text{Sam}} - I_{\text{Lo}}) \times (W_{\text{Hi}} - W_{\text{Lo}}) + W_{\text{Lo}}] M_{\text{Lab}}}{(I_{\text{Hi}} - I_{\text{Lo}}) \times M_{\text{Ser}}}$$

Where:

- C is the concentration of cholesterol in the serum sample;
- I<sub>Sam</sub> is the unlabeled/labeled ion intensity ratio measured for serum sample;
- I<sub>Lo</sub> is the unlabeled/labeled ion intensity ratio for the lower ratio calibration standard;
- W<sub>Hi</sub> is the unlabeled/labeled mass ratio for the higher ratio calibration standard;
- W<sub>Lo</sub> is the unlabeled/labeled mass ratio for the lower ratio calibration standard;
- M<sub>Lab</sub> is the mass of the labeled cholesterol added to the serum sample;
- I<sub>Hi</sub> is the unlabeled/labeled ion intensity ratio for the higher ratio calibration standard;
- M<sub>Ser</sub> is the mass of serum sample

In addition to the ion intensity measurements, the other critical measurement is determining the mass of the reference compound used to prepare the calibration standards. This measurement requires careful weighing of a material that has a known purity and associated uncertainty. In contrast to the pilot study, no calibration material was supplied for the Key Comparison by the organizers. Thus, each participant was responsible for selecting a calibration material that had a stated purity and uncertainty, which were to be incorporated in calculations of concentration and uncertainty. Five of the laboratories (LGC, NARL, NIST, NMI, and PTB) used SRM 911b from NIST with a certified purity of 99.8 mass % ± 0.1 mass %. NRCCRM used GBW 09203b with a specified purity of 99.7 ± 0.1 mass %, while NMIJ used a material that was characterized at NMIJ to be 99.90 ± 0.032 mass % by differential scanning calorimetry.

The laboratories were instructed to prepare samples for measurement on three separate days, with one vial of each level sampled in a day. The measurements were also to be done on separate days. The laboratories were further instructed to provide a detailed description of their uncertainty budget and were encouraged to attend the April 2000 meeting of the CCQM to

discuss their uncertainties. All of the participants followed the prescribed measurement protocol. Cholesterol is quite stable in frozen serum, thus the actual dates of when the analyses were performed is immaterial.

## RESULTS

Results were received from all seven participants in the Key Comparison. The composite results including means and expanded uncertainties for all of the participants are shown in the Tables 2A and 2B. Six of the participants submitted detailed uncertainty budgets which are shown in Table 3. In a few cases, information needed to calculate the matrices of equivalence were either missing or were not self consistent. To address these problems, the Pilot Laboratory either made estimates of the number of degrees of freedom when not reported, or computed  $k$  from the reported degrees of freedom to replace  $k=2$ , resulting in small differences in the expanded uncertainties between Tables 2 and 3. The results for Materials A and B are graphically depicted in Figures 1a and 1b, respectively.

Key Comparison Reference Values (KCRV) and associated uncertainties: Based on guidelines established by the CCQM Organic Working Group, Key Comparison Reference Values are to be established based on results from study participants that had their method(s) validated through participation in the preceding Pilot Study. As NARL did not participate in the relevant pilot, their results were not eligible to be included in the KCRV calculation. For Material A, it is recommended that the KCRV be assigned as the mean  $\pm$  U of the eligible results, excluding the one statistical outlier. That calculation yields a KCRV of  $2.200 \pm 0.019$  mg/g corresponding to a 95% confidence interval of 2.181 to 2.219 mg/g. For material B, it is recommended that the KCRV be assigned as the mean  $\pm$  U of all of the eligible results. The KCRV would be  $1.726 \pm 0.013$  mg/g, corresponding to a 95% confidence interval of 1.713 mg/g to 1.739 mg/g. It is likely that systematic biases contribute significantly to the total biases of some of the participants' results. Because all of the eligible results, excluding statistical outliers, are included in calculating the KCRV, systematic biases in individual results would bias the KCRV. Therefore the KCRV may not be the best estimate of the true mass fraction of cholesterol in the materials. However, even with results with systematic biases included in the calculations, the true mass fractions should fall within the 95% confidence intervals. The Tables of Equivalence, which enumerates the relationships among the results of the participants in this Key Comparison, are shown in Appendix 1 and the graphs of equivalence are in Appendix 2.

## DISCUSSION

Material A was prepared only for use in the Key Comparison and thus had not been previously measured. Results from five of the six participants were in good agreement, but the other participant provided a result that was low relative to the others. This result is statistically an outlier by Dixon's Test<sup>2</sup>. When this result is excluded from the calculation, the mean increases by 0.5% and the expanded uncertainty decreases by 42%.

Although unknown to the participants, CCQM-K6 Material B was one of the materials that had been used in the pilot study (Material A<sub>p</sub>), approximately one year prior to the Key Comparison. Consequently, it is possible to compare the performance of the laboratories on the same material

over time. This comparison is shown in Figure 2. It is clear from this figure that interlaboratory precision was much better for the Key Comparison than it was for the pilot study on this material. For the pilot study, most of the participants had little experience with cholesterol measurements. From discussions after the pilot study, it appeared that these laboratories may not have used conditions that assured complete hydrolysis of cholesteryl esters, resulting in a low bias. For the Key Comparison, these laboratories had more experience with the cholesterol method and therefore, used more rigorous hydrolysis conditions, resulting in much better interlaboratory precision for the Key Comparison.

Uncertainty calculations involved both Type A and Type B components for all of the participants. All of the laboratories used the repeatability of their measurements as the type A component. Repeatability of measurements was the largest source of uncertainty for most of the laboratories and contributed significantly to the overall uncertainty for the others. The type B component consisted of uncertainty in the purity of the reference compounds that they used plus other factors that could introduce a systematic bias. The complete uncertainty budgets used for the Key Comparison measurements are shown in Table 3. From the NIST perspective, based upon many years experience with using an ID/MS-based method for determining cholesterol<sup>1</sup>, two of the most significant contributors to the type B uncertainty are the completeness of the hydrolysis of the cholesteryl esters and the stability of free cholesterol in the basic solution used for the hydrolysis. Incomplete hydrolysis results in a low bias, but it is not possible to prove complete hydrolysis. Model systems can show that the most common esters are completely hydrolyzed, but it is possible that small quantities of some less abundant species may resist hydrolysis. Cholesterol is known to be unstable in basic solution over long periods. If some degradation occurs before equilibration is achieved, a bias will occur. Recovery studies have shown that very little cholesterol is lost in the entire sample preparation procedure<sup>1</sup>, but some losses in the hydrolysis step cannot be ruled out. Therefore, degradation of cholesterol may contribute significantly to the uncertainty for methods with high precision measurements such as those used in this study. Some of the other laboratories in this study explicitly used these factors and one laboratory included a component for unknown systematic effects that would encompass effects such as these. Several laboratories included components related to the uncertainties in weighing. Uncertainties from weighings should be very small and, in most cases, will contribute to the type A measurement uncertainty. However, non-linearity in balance responses would be a systematic effect that could make a small contribution to the type B uncertainty. For all of the laboratories that used this approach, the uncertainties attributable to weighing steps contributed minimally to the overall uncertainty.

## CONCLUSIONS

This Key Comparison study demonstrated that the participating NMIs could successfully measure serum cholesterol for normal and elevated levels, using ID/MS-based methods, with interlaboratory expanded uncertainties of less than 1%. Comparison of results from the Key Comparison and the earlier pilot study demonstrated that laboratories previously inexperienced in the determination of serum cholesterol showed dramatic improvement as they gained experience and better understood potential sources of bias, such as incomplete hydrolysis.

Ideally, an internationally recognized reference system should be established for all important health status markers, but that is not possible in any reasonable time frame. Every serum analyte of interest as a health status marker provides a unique set of challenges. To provide a more

comprehensive measure of the capabilities of NMIs for measuring well-defined serum analytes, the CCQM also has conducted pilot studies for the determinations of serum glucose and creatinine. These two analytes were chosen because they present very different challenges than does cholesterol, thus providing a more complete picture of the capabilities of participating NMIs. Glucose is highly water-soluble and also associates strongly with proteins. Creatinine is very polar, present at much lower levels than cholesterol, and its determination requires considerable care to assure separation from creatine, without interconversion between creatinine and creatine. The CV among the results from the participating laboratories was less than 1 % for glucose and less than 1.5 % for the creatinine both at clinically significant levels. Key Comparisons for both of these additional measurands are underway. The combination of these three Key Comparisons may provide a basis for the evaluation of measurement capabilities of participating NMIs for other well-defined organic analytes present in serum at  $\mu\text{g/g}$  levels or higher, without having to actually conduct a Key Comparison for all such analytes.

## REFERENCES

1. Ellerbe, P., Meiselman, S., Sniegowski, L. T., Welch, M. J., and White V, E., Determination of Serum Cholesterol by a Modification of the Isotope Dilution Mass Spectrometric Definitive Method, *Anal. Chem.*, **614**, 1718-1723 (1989).
2. Dixon, W.J., "Processing Data for Outliers", *Biometrics*, IX, 74-89 (1953).

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**Table 1. Results of CCQM-P6 Pilot Study – Cholesterol (mg/g) in Serum**

<b>Material A<sub>p</sub></b>	(mg/g)	
<u>Lab</u>	<u>Mean</u>	<u>U</u>
DGKC	1.731	0.024
LGC	1.663	0.029
NIST	1.7408	0.0077
NMi	1.663	0.053
NMIJ	1.7106	0.0090
NRCCRM	1.6815	0.0062
PTB	1.707	0.016
Mean	1.700	
CV%	1.84	

<b>Material B<sub>p</sub></b>	(mg/g)	
<u>Lab</u>	<u>Mean</u>	<u>U</u>
DGKC	2.691	0.038
LGC	2.607	0.016
NIST	2.7038	0.0054
NMi	2.637	0.070
NMIJ	2.692	0.016
NRCCRM	2.686	0.011
PTB	2.701	0.024
Mean	2.674	
CV%	1.39	

\*Material B<sub>p</sub> is SRM 1951a, Lipids in Frozen Human Serum, Level II issued in 1997. The NIST result listed is the certified value and uncertainty from extensive NIST certification measurements of this material.

**Table 2A. CCQM-K6 Cholesterol in Serum: Material A**  
(Values used to calculate the matrices of equivalence)

Laboratory	Mean Result mg/g	Std. Uncertainty mg/g	degrees of freedom	k	Exp. Uncertainty mg/g
LGC <sup>1</sup>	2.214	0.0096	60	2.000	0.019
NARL <sup>2</sup>	2.250	0.0131	10.95	2.228	0.029
NIST	2.215	0.0043	12.3	2.179	0.009
NMI <sup>1</sup>	2.137	0.0068	60	2.000	0.014
NMIJ	2.195	0.0050	27	2.052	0.010
NRCCRM	2.197	0.0062	11.9	2.201	0.014
PTB	2.179	0.0114	62	1.999	0.023

<sup>1</sup>Degrees of freedom were not reported by the participant, but were estimated by the Pilot Laboratory to be large ( $\geq 60$ ), and therefore  $k=2$  is appropriate.

<sup>2</sup>The reported  $k$  value ( $k=2$ ) did not agree with the reported degrees of freedom, therefore the  $k$  value was calculated from the reported degrees of freedom.

Summary of results with NMI value (as outlier) (and NARL value\*) omitted from calculations:

Mean	2.200	mg/g
Std Dev of mean	0.0067	mg/g
% RSD	0.303	%
Degrees of Freedom	4	
k-factor	2.776	
Expanded Uncertainty, U	0.019	mg/g (0.84% relative)

Summary of all results\*:

Mean	2.189	mg/g
Std Dev of mean	0.0118	mg/g
RSD	0.540	%
Degrees of Freedom	5	
k-factor	2.571	
Expanded Uncertainty, U	0.030	mg/g (1.4% relative)

\*As NARL did not participate in the pilot study for cholesterol in serum, its results were not eligible to be included in the calculation of the Key Comparison means and uncertainties.

**Table 2B. CCQM-K6 Cholesterol in Serum: Material B**  
(Values used to calculate the matrices of equivalence)

Laboratory	Mean Result mg/g	Std. Uncertainty mg/g	degrees of freedom	k	Exp. Uncertainty mg/g
LGC <sup>1</sup>	1.732	0.0066	60	2.000	0.013
NARL <sup>2</sup>	1.777	0.0170	11.3	2.201	0.037
NIST	1.735	0.0033	13.5	2.160	0.007
NMI <sup>1</sup>	1.729	0.0045	60	2.000	0.009
NMIJ <sup>2</sup>	1.718	0.0039	27	2.052	0.008
NRCCRM	1.736	0.0062	7.4	2.365	0.015
PTB	1.705	0.0086	314	1.968	0.017

<sup>1</sup>Degrees of freedom were not reported by the participant, but were estimated by the Pilot Laboratory to be large ( $\geq 60$ ), and therefore  $k=2$  is appropriate.

<sup>2</sup>The reported  $k$  value ( $k=2$ ) did not agree with the reported degrees of freedom, therefore the  $k$  value was calculated from the reported degrees of freedom.

Summary of all results\*:

Mean	1.726	mg/g
Std Dev of mean	0.0049	mg/g
RSD	0.287	%
Degrees of Freedom	5	
k-factor	2.571	
Expanded Uncertainty, U	0.013	mg/g (0.73% relative)

\*As NARL did not participate in the pilot study for cholesterol in serum, its results were not eligible to be included in the calculation of the Key Comparison means and uncertainties.

**Table 3. Participant Uncertainty Budgets for CCQM-K6****LGC Uncertainty Budget CCQM-K6: Material A**

<b>Parameter</b>	<b>Uncertainty Type</b>	<b>Relative Uncertainty (%)</b>	<b>Degrees of Freedom</b>
Method precision	A	0.2177	5
Instrument repeatability	A	0.1676	2
Calibration solution concentration	B	0.0501	Large
Balance linearity, calibration solution	B	0.0020	Large
Balance linearity, sample spike	B	0.0111	Large
Balance linearity, calibration spike	B	0.0016	Large
Balance linearity, sample mass	B	0.0524	Large
Method bias	B	0.3300	Large
Combined relative standard uncertainty		0.0067	
Combined standard uncertainty		0.0096 mg g <sup>-1</sup>	
Coverage factor	2		
Combined expanded uncertainty		0.019 mg g <sup>-1</sup>	
Mean value of result		2.2138 mg g <sup>-1</sup>	

**LGC Uncertainty Budget CCQM-K6: Material B**

<b>Parameter</b>	<b>Uncertainty Type</b>	<b>Relative Uncertainty (%)</b>	<b>Degrees of Freedom</b>
Method precision	A	0.1386	5
Instrument repeatability	A	0.1143	2
Calibration solution concentration	B	0.0501	Large
Balance linearity, calibration solution	B	0.0020	Large
Balance linearity, sample spike	B	0.0090	Large
Balance linearity, calibration spike	B	0.0016	Large
Balance linearity, sample mass	B	0.0285	Large
Method bias	B	0.3300	Large
Combined relative standard uncertainty		0.3802	
Combined standard uncertainty		0.0066 mg g <sup>-1</sup>	
Coverage factor	2		
Combined expanded uncertainty		0.013 mg g <sup>-1</sup>	
Mean value of result		1.732 mg g <sup>-1</sup>	

### NARL uncertainty budget for CCQM-K6:

#### Material A

Steps in process	Uncertainty type		Relative Uncert (%)	Degrees of Freedom
	A	B		
Initial mass sample solution		X	0.008	Inf
Final mass sample solution after dilution		X	0.0004	Inf
Mass sample solution for blend		X	0.008	Inf
Mass spike solution for blend		X	0.014	Inf
Mass standard for calibration blend		X	0.008	Inf
Mass spike for calibration blend		X	0.014	Inf
Concentration of calibration solution		X	0.06	Inf
Repeatability of cholesterol/ <sup>13</sup> C <sub>3</sub> -cholesterol ratio for sample blends	X		0.426	5
Repeatability of cholesterol/ <sup>13</sup> C <sub>3</sub> -cholesterol ratio for standard blends	X		0.397	6
Combined rel std uncertainty (%)				0.58
Calculated degrees of freedom				10.95
k-factor				2.228
Relative expanded uncertainty (%)				1.29
Mean value of NARL results				2.250 mg/g
Absolute expanded uncertainty, U				0.029 mg/g

#### Material B

Steps in process	Uncertainty type		Relative Uncert (%)	Degrees of Freedom
	A	B		
Initial mass sample solution		X	0.008	Inf
Final mass sample solution after dilution		X	0.0004	Inf
Mass sample solution for blend		X	0.008	Inf
Mass spike solution for blend		X	0.009	Inf
Mass standard for calibration blend		X	0.007	Inf
Mass spike for calibration blend		X	0.009	Inf
Concentration of calibration solution		X	0.06	Inf
Repeatability of cholesterol/ <sup>13</sup> C <sub>3</sub> -cholesterol ratio for sample blends	X		0.764	6
Repeatability of cholesterol/ <sup>13</sup> C <sub>3</sub> -cholesterol ratio for standard blends	X		0.554	7
Combined rel std uncertainty (%)				0.944
Calculated degrees of freedom				11.30
k-factor				2.201
Relative expanded uncertainty (%)				2.077
Mean value of NARL results				1.777 mg/g
Absolute expanded uncertainty, U				0.037 mg/g

### NIST uncertainty budget for CCQM-K6:

Material A

<u>Steps in Process</u>	<u>Uncertainty type</u>		<u>Relative Uncert (%)</u>	<u>degrees of freedom</u>
	<u>A</u>	<u>B</u>		
Repeatability of GC/MS measurements	X		0.123	2
Purity of reference standard		X	0.050	inf
Hydrolysis of cholesteryl esters		X	0.100	inf
Stability of cholesterol in base		X	0.100	inf
Combined rel std uncertainty (%)			0.194	
Calculated degrees of freedom			12.3	
k-factor			2.179	
Relative expanded uncertainty (%)			0.423	
Mean value of NIST results			2.215	mg/g
Absolute expanded uncertainty, U			0.00938	mg/g

Material B

<u>Steps in Process</u>	<u>Uncertainty type</u>		<u>Relative Uncert (%)</u>	<u>d.f.</u>
	<u>A</u>	<u>B</u>		
Purity of reference standard		X	0.050	inf
Hydrolysis of cholesteryl esters and Equil.		X	0.100	inf
Stability of cholesterol in base		X	0.100	inf
GC/MS measurements	X		0.119	2
combined rel std uncertainty (%)			0.191	
Calculated degrees of freedom			13.5	
k-factor			2.160	
Relative expanded uncertainty (%)			0.413	
Mean value			1.735	mg/g
Abs. expanded uncertainty			0.00717	mg/g

## NMi Uncertainty Budget for CCQM-K6:

### Material A

<i>Steps in process:</i>	<i>Uncertainty type:</i>	<i>Rel. uncertainty (%)</i>
Repeatability of GC/MS measurements	A	0.241
Purity SRM 911b	B	0.05
Hydrolysis	B	0.2
Combined relative uncertainty:		0.317
k-factor:		2
Rel. expanded uncertainty:		0.634
Mean value of NMi results:		2.137
Absolute expanded uncertainty:		0.014

### Material B

<i>Steps in process:</i>	<i>Uncertainty type:</i>	<i>Rel. uncertainty (%)</i>
Repeatability of GC/MS measurements	A	0.181
Purity SRM 911b	B	0.05
Hydrolysis	B	0.2
Combined relative uncertainty:		0.274
k-factor:		2
Rel. expanded uncertainty:		0.549
Mean value of NMi results:		1.729
Absolute expanded uncertainty:		0.009

## NMIJ Uncertainty Budget for CCQM-K6

### Material A

<u>Parameter</u>	<u>Uncertainty type</u>		<u>Relative Uncert (%)</u>	<u>degrees of freedom</u>
	<u>A</u>	<u>B</u>		
Method Precision	X		0.200	16
Purity of reference standard	X		0.016	3
Preparation of calibration solution		X	0.100	Large
Balance linearity, calibration solution		X	0.005	Large
Balance linearity, sample spike		X	0.005	Large
Balance linearity, calibration spike		X	0.005	Large
Balance linearity, sample mass		X	0.005	Large
Combined rel std uncertainty			0.228	
Calculated degrees of freedom			27.0	
k-factor			2.052	
Relative expanded uncertainty (%)			0.468	
Mean value of NIST results			2.195	mg/g
Absolute expanded uncertainty, U			0.010	mg/g

### Material B

<u>Parameter</u>	<u>Uncertainty type</u>		<u>Relative Uncert (%)</u>	<u>d.f.</u>
	<u>A</u>	<u>B</u>		
Method Precision	X		0.200	16
Purity of reference standard	X		0.016	3
Preparation of calibration solution		X	0.100	Large
Balance linearity, calibration solution		X	0.005	Large
Balance linearity, sample spike		X	0.005	Large
Balance linearity, calibration spike		X	0.005	Large
Balance linearity, sample mass		X	0.005	Large
Combined rel std uncertainty			0.228	
Calculated degrees of freedom			27.0	
k-factor			2.052	
Relative expanded uncertainty (%)			0.468	
Mean value of NIST results			1.718	mg/g
Absolute expanded uncertainty, U			0.008	mg/g

**NRCCRM uncertainty budget for CCQM-K6:**

Material A

<u>Steps in Process</u>	<i>Uncertainty type</i>		<i>Relative Uncert (%)</i>	<i>degrees of freedom</i>
	<i>A</i>	<i>B</i>		
Repeatability of GC/MS measurements	X		0.236	6
Purity of reference standard		X	0.050	inf
Hydrolysis of cholesteryl esters		X	0.100	inf
Stability of cholesterol in base		X	0.100	inf
Combined rel std uncertainty			0.280	
Calculated degrees of freedom			11.9	
k-factor			2.18	
Relative expanded uncertainty (%)			0.610	
Mean value of NRCCRM results			2.197	mg/g
Absolute expanded uncertainty, U			0.014	mg/g

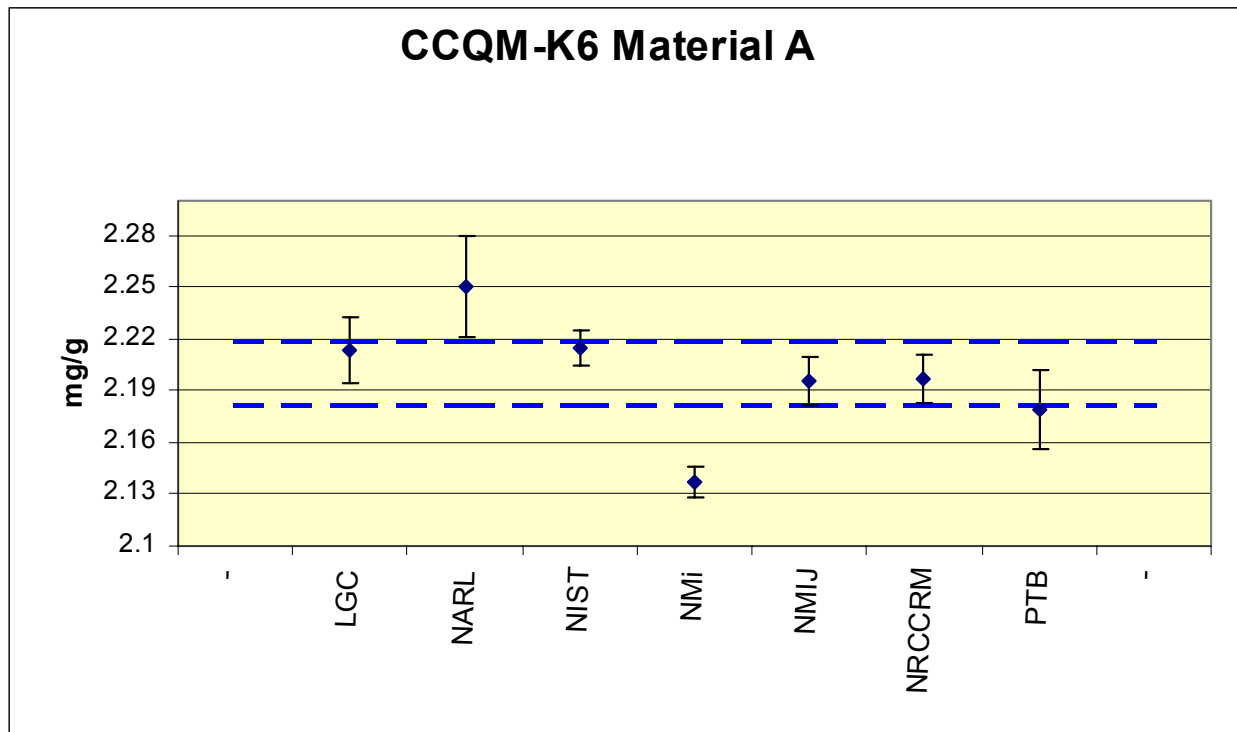
Material B

<u>Steps in Process</u>	<i>Uncertainty type</i>		<i>Relative Uncert (%)</i>	<i>d.f.</i>
	<i>A</i>	<i>B</i>		
Purity of reference standard		X	0.050	inf
Hydrolysis of cholesteryl esters and Equil.		X	0.100	inf
Stability of cholesterol in base		X	0.100	inf
GC/MS measurements	X		0.322	5
combined rel std uncertainty			0.355	
Calculated degrees of freedom			7.4	
k-factor			2.33	
Relative expanded uncertainty (%)			0.827	
Mean value			1.736	mg/g
Abs. expanded uncertainty			0.015	mg/g

**PTB uncertainty budget for CCQM-K6:**

<u>Material A</u>			<i>Relative</i>	<i>degrees</i>		
	<i>Uncertainty type</i>		<i>Uncert (%)</i>	<i>of freedom</i>		
<u>Steps in Process</u>	<u>A</u>	<u>B</u>				
Repeatability of GC/MS measurements	X		0.280	5		
Purity of reference standard		X	0.058	inf		
Sample preparation (prior to and excluding GC/MS)		X	0.440	inf		
Combined rel std uncertainty			0.525			
Calculated degrees of freedom			61.7			
k-factor			2.000			
Relative expanded uncertainty (%)			1.049			
Mean value of PTB results			2.179	mg/g		
Absolute expanded uncertainty, U			0.0229	mg/g		
<u>Material B</u>			<i>Relative</i>	<i>degrees</i>		
	<i>Uncertainty type</i>		<i>Uncert (%)</i>	<i>of freedom</i>		
<u>Steps in Process</u>	<u>A</u>	<u>B</u>				
Repeatability of GC/MS measurements	X		0.180	5		
Purity of reference standard		X	0.058	inf		
Sample preparation (prior to and excluding GC/MS)		X	0.470	inf		
Combined rel std uncertainty			0.507			
Calculated degrees of freedom			313.7			
k-factor			1.968			
Relative expanded uncertainty (%)			0.997			
Mean value of PTB results			1.705	mg/g		
Absolute expanded uncertainty, U			0.0170	mg/g		

**Figure 1a. Key Comparison Results for Cholesterol in Serum - Material A**  
 The area between the dashed lines represents the 95% C.I. of the KCRV.



**Figure 1b. Key Comparison Results for Cholesterol in Serum - Material B**  
 The area between the dashed lines represents the 95% C.I. of the KCRV

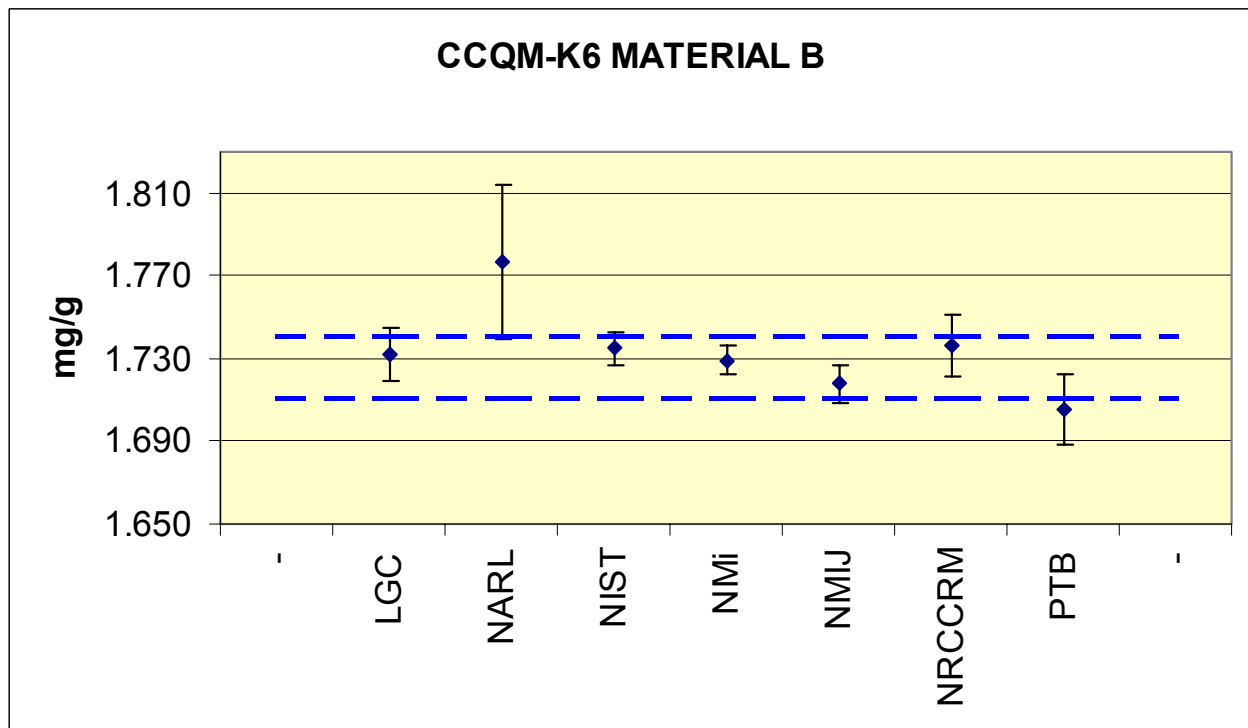
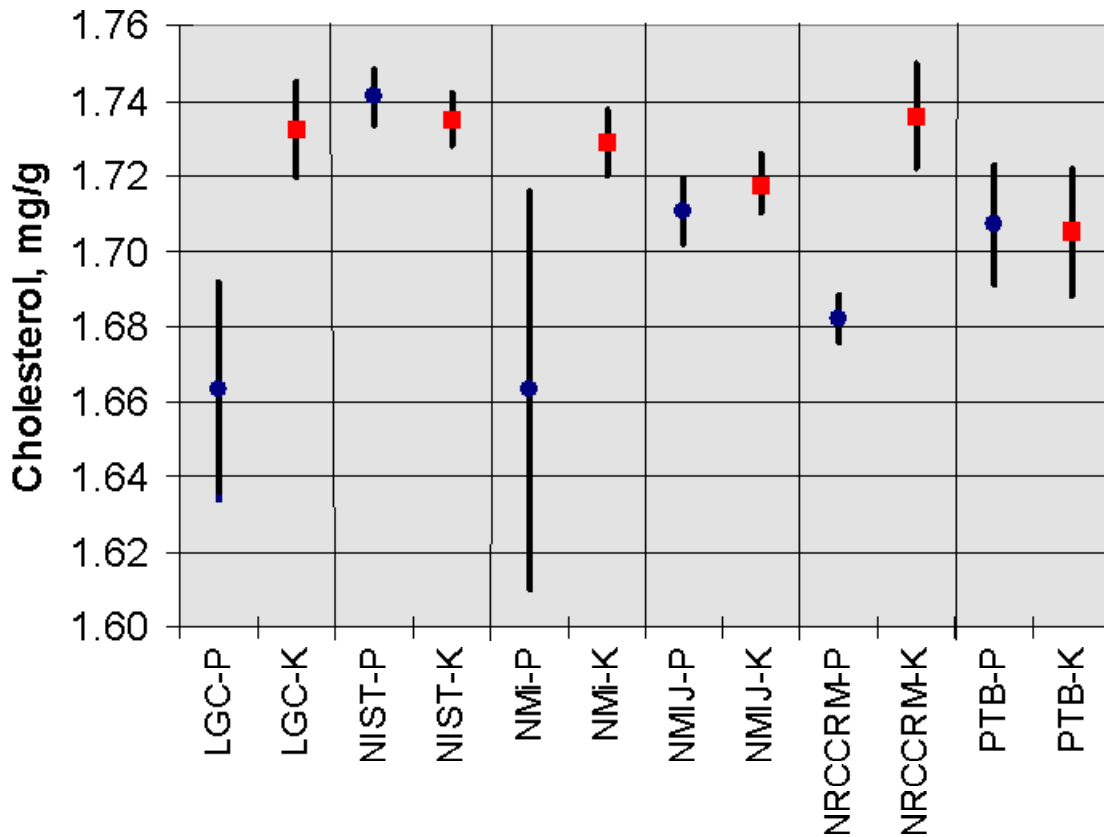


Figure 2. Comparison of Results from the Key Comparison and the Pilot Study on the Same Material

**CCQM – Comparison of Results for Cholesterol in Serum  
in 1999 Pilot Study ● and in 2000 Key Comparison ■ (CCQM-K6)**

mean ± U: 1.700 ± 0.029 mg/g (Pilot: CCQM-P6 Matl A<sub>p</sub>)  
1.726 ± 0.013 mg/g (Key: CCQM-K6 Matl B)



**Appendix 1. Tables of Matrices of Equivalence for CCQM-K6**

MATERIAL A		KCRV		LGC		NARL		NIST		NMI		NMIJ		NRCCRM		PTB	
	D <sub>i</sub>	U <sub>i</sub>	D <sub>ij</sub>	U <sub>ij</sub>	D <sub>ij</sub>	U <sub>ij</sub>	D <sub>ij</sub>	U <sub>ij</sub>	D <sub>ij</sub>	U <sub>ij</sub>	D <sub>ij</sub>	U <sub>ij</sub>	D <sub>ij</sub>	U <sub>ij</sub>	D <sub>ij</sub>	U <sub>ij</sub>	
	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	
LGC	0.014	0.024			-0.036	0.034			-0.001	0.021	0.077	0.023	0.019	0.022	0.017	0.023	
NARL	0.050	0.032							0.035	0.030	0.113	0.031	0.055	0.030	0.053	0.031	
NIST	0.015	0.019			-0.035	0.030					0.078	0.016	0.020	0.013	0.018	0.016	
NMI	-0.063	0.020			-0.113	0.031			-0.078	0.016			-0.058	0.017	-0.060	0.019	
NMIJ	-0.005	0.019			-0.055	0.030			-0.020	0.013	0.058	0.017			-0.002	0.016	
NRCCRM	-0.003	0.020			-0.053	0.031			-0.018	0.016	0.060	0.019	0.002	0.016			
PTB	-0.021	0.027			-0.071	0.035			-0.036	0.024	0.042	0.026	-0.016	0.025	-0.018	0.026	
MATERIAL B		KCRV		LGC		NARL		NIST		NMI		NMIJ		NRCCRM		PTB	
	D <sub>i</sub>	U <sub>i</sub>	D <sub>ij</sub>	U <sub>ij</sub>	D <sub>ij</sub>	U <sub>ij</sub>	D <sub>ij</sub>	U <sub>ij</sub>	D <sub>ij</sub>	U <sub>ij</sub>	D <sub>ij</sub>	U <sub>ij</sub>	D <sub>ij</sub>	U <sub>ij</sub>	D <sub>ij</sub>	U <sub>ij</sub>	
	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g	
LGC	0.006	0.017			-0.045	0.039			-0.003	0.015	0.003	0.016	0.014	0.015	-0.004	0.019	
NARL	0.051	0.038							0.042	0.038	0.048	0.038	0.059	0.041	0.039	0.040	
NIST	0.009	0.013			-0.042	0.038					0.006	0.011	0.017	0.010	-0.001	0.015	
NMI	0.003	0.014			-0.048	0.038			-0.006	0.011			0.011	0.012	-0.007	0.016	
NMIJ	-0.008	0.014			-0.059	0.038			-0.017	0.010	-0.011	0.012			-0.018	0.016	
NRCCRM	0.010	0.017			-0.041	0.039			0.001	0.015	0.007	0.016	0.018	0.016			
PTB	-0.021	0.020			-0.072	0.040			-0.030	0.018	-0.024	0.019	-0.013	0.019	-0.031	0.021	

## Appendix 2 Degrees of Equivalence Graphs

