EURAMET.QM-K12

EURAMET Key Comparison on the Determination of the Mass Fraction of Creatinine in Human Serum

Final report

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Introduction

Creatinine is a well known marker for the evaluation of renal function. It is a small product of protein metabolism being formed by the spontaneous, non-enzymatic cyclization of creatine, a key component involved in muscle contractions. Creatinine concentration in human serum is measured to estimate the glomerular filtration rate (eGFR). High creatinine concentration is a diagnostic marker for chronic kidney disease, which can result in kidney failure. Currently, creatinine concentration is routinely measured using methods that involve the Jaffe reaction, where creatinine reacts with alkaline picrate to form a red complex, which is often measured with a spectrophotometer. It has been shown that the picrate reagent cross-reacts with a wide variety of blood substances like proteins, glucose, and bilirubine, thus potentially generating biased results. For this reason, various approaches are used to eliminate or correct for known interferences. In recent years, enzymatic assays have become the methods of choice for routine measurements of creatinine in serum. Therefore, reference methods and materials are needed to evaluate the accuracy of routine measurements for creatinine in serum.

To address the need to assess the equivalence of NMIs or DI providing higher order standards for clinical laboratory measurements that are traceable to the SI, the Organic Analysis Working Group of the Consultative Committee on the Amount of Substance (CCQM) has previously conducted two key comparisons to assess the capabilities of NMIs to characterise the mass fraction of creatinine in human serum. NIST first organized a pilot study for the determination of serum creatinine in 2000 (CCQM-P9) and a follow on key comparison in 2003 (CCQM-K12). The results from the key comparison suggested that there was excellent agreement between the NMIs with all reported values agreeing with the KCRV within their reported uncertainties. A more recent study (CCQM-K80) which compared a wide number of certified reference materials from a number of NMIs and DIs showed an excellent degree of comparability over a broad range of concentrations.

The purpose of this study was to enable NMIs, who missed the previous studies, to demonstrate their capability for characterising serum materials from 1 to 100 µg/g of creatinine in serum by participating in this Regional Metrology organisation (RMO) Key Comparison. Newly designated NMIs in EURAMET, or those assessing new approaches for dissemination of services were encouraged to participate in the parallel pilot study, results for which are included in this report.

Summary of CCQM-K12 study

The initial Key comparison on creatinine in serum was co-ordinated by NIST in 2002. The study had five participants but the results from four of these were used to calculate the KCRV and UCRV. Further details of the study can be found on the BIPM website. The conclusion from this study was that participating NMIs could successfully measure creatinine at normal and

elevated levels with an interlaboratory expanded uncertainty of less than 0.8%. The results of CCQM-K12 are presented in Tables 1 and 2.

Participant	Mean (µg/g)	Stand Uncert. (µg/g)	K	Expanded Uncert. (µg/g)
IRMM	8.360	0.1060	2	0.212
KRISS	8.186	0.0796	1.995	0.159
LGC	8.193	0.0080	2	0.016
NIST	8.277	0.0319	3.182	0.102
PTB	8.211	0.0289	2	0.058

Table 1 A: Summary of individual participants results for CCQM-K12 Creatinine in Human Serum for Material I

Mean	8.217
Range (%)	2.12
Std dev of mean	0.0208
Degrees of freedom	3
K Factor	3.182
U	0.0663
U (rel) %	0.807
KCRV	8.217 ± 0.066 µg/g

Table 1 B: Summary of results for CCQM-K12 Creatinine in Human Serum for Material I

Participant	Mean (µg/g)	Stand Uncert. (µg/g)	K	Expanded Uncert. (µg/g)
IRMM	18.720	0.2396	2	0.479
KRISS	18.539	0.1627	1.965	0.320
LGC	18.614	0.0316	2	0.063
NIST	18.708	0.0722	3.182	0.230
PTB	18.718	0.0650	2.032	0.132

Table 2 A: Summary of individual participants results for CCQM-K12 Creatinine in Human Serum for Material II

Mean	18.645
Range (%)	0.97
Std dev of mean	0.0423
Degrees of freedom	3
K Factor	3.182
U	0.135
U (rel) %	0.723
KCRV	18.645 ± 0.135 µg/g

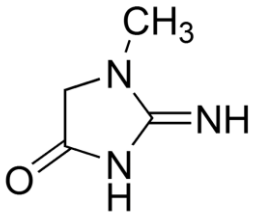
Table 2 B: Summary of results for CCQM-K12 Creatinine in Human Serum for Material II

Summary of CCQM-K12.1 subsequent study of CCQM-K12

The subsequent study, CCQM-K12.1, was co-ordinated by KRISS and had three participants. A detailed draft-A report is available on the BIPM OAWG private web pages but as it is only Draft A, it will not be discussed further here.

EURAMET.QM-K12 Study outline

The study measurand was the mass fraction of creatinine (2-amino-3-methyl-4H-imidazol-5-one) in lyophilised human serum.



Chemical Formula: $C_4H_7N_3O$
Relative molecular mass: 113.12 g / mol
CAS Number: 82016-55-5

Instructions for Study participants

All participants were requested to sign up with the co-ordinator and request samples direct from the RELA organisers at http://www.dgkl-rfb.de:81
Samples were stored lyophilised at 4°C until required for analysis.

Reporting

Two replicate measurements, for creatinine in the reconstituted serum were to be reported for each of three vials of the material received at each of the two levels. A single estimate for each material based on six replicates was reported by each laboratory. A data reporting sheet was emailed directly to participants on requesting samples and was used for the submission of results.

Participants were requested that all results returned should include,

- The mass fraction of creatinine in reconstituted serum as µg/g
- A full uncertainty budget
- The source and details of all primary standards used
- The source and details of any labelled materials used
- An outline of the methodology, a full measurement equation and a breakdown of the uncertainty estimation submitted

Participants

NMI/DI	Country	RMO
EXHM (General State Chemical Laboratory/Hellenic Metrology Institute)	**Greece**	**EURAMET**
Health Sciences Authority	**Singapore**	**APMP**
LGC	**UK**	**EURAMET**
Laboratoire National del Metrologie et d'Essais (LNE)	**France**	**EURAMET**
PTB	**Germany**	**EURAMET**

Table 3: Identity of participants of EURAMET.QM-K12.

Material

The study utilised two lyophilised serum materials provided as part of the 2010/2011 International Federation of Clinical Chemists (IFCC) external quality control for reference laboratories (RELA) ring trial for the determination of creatinine in lyophilised serum.

No approximate target value was provided for either material. Although the exact levels were not known by the co-ordinating lab, it was expected that one of the materials would have a creatinine level in the normal range for adults or children while the other would have a level representative of an elevated concentration in adults. Therefore the two materials covered a large range of concentration and were representative of both physiological and pathological creatinine concentrations.

An estimation of the homogeneity and stability of the material was not performed. However, the material was prepared for an IFCC RELA ring trial. Results from previous materials suggest that homogeneity and stability over the study period was not an issue and materials from this provider formed part of the recent CCQM-K80 comparison on creatinine materials.

Participants received samples directly from the IFCC RELA trial organizers. Both materials where shipped as unknowns to all of the participants, including the coordinating laboratory.

Although participants were free to use whatever methods and calibrants they chose, participants of the EURAMET key comparison study were encouraged to use isotope dilution-based methods: either gas chromatography mass spectrometry (GC-IDMS) or liquid chromatography/mass spectrometry (LC-IDMS) as these had been proven in CCQM-K12.

Timing of study

The call for participation was announced at the CCQM-OAWG meeting in November 2010 with a sign-up deadline of 25 Feb. 2011. Samples were shipped directly to participants, in the first week in March, with a results submission deadline of 1st April 2011. This gave participants less than four weeks to provide a result. With many laboratories claiming the provision of reference measurement services to customers this turnaround time was considered appropriate for such dissemination activities.

Summary of methods

A summary of the extraction, clean-up and instrumental parameters used by each participant is shown in Table 4. There was a split in technologies used by the key participants. Two participants opted to use a GC-MS approach whilst three preferred the more direct LC-MS or LC-MS/MS approach. The only pilot participant used an innovative isotope dilution methodology using surface enhanced Raman spectroscopy (SERS). The different separation and detection technologies required vastly different extraction and clean-up procedures but a similar range of approaches had been used in CCQM-K12.

NMI/DI	Sample size (g)	Extraction method	Solvent	Post extraction clean-up / manipulation	Analytical Instrument	Analytical separation	Method of quantification	Calibration regime
LGC	0.5	Protein crash	Cold ethanol	Centrifugation	LC-MS	Phenomenex Luna C18 (2); 150 x 2 mm, 3 µm	IDMS, Creatinine-d3	Single point exact matched
LNE	0.15	Cation exchange clean-up (AG50W-X2)		MSTFA derivitisation	GC-MS	Agilent HP5 : 5% phenyl:95% methylsiloxane (30m x 0.25 mm ; 0.25µm)	IDMS, Creatinine ^{13}C, $^{15}N2$	Calibration curve
HSA	0.15	Protein precipitation	Acetonitrile	Centrifugation	LC-MS/MS	Agilent Zorbax SB-Aq, 100 x 2.1 mm, 3.5 µm	IDMS, Creatinine-d3	Calibration curve
EXHM	Sample A: 0.6 Sample B 0.9	Protein crash	Cold ethanol	Filtration 0.45µm	LC-MS/MS	XTerra MS C-18, 150 x 2.1mm, 3.5 µm	IDMS, Creatinine d3	Single point exact match
PTB	1	Cation exchange clean-up (AG50W-X8) pH 4.8-5		MSTFA derivitisation	GC-MS	5% phenyl:95% methylpoly siloxane (30m x 0.25 mm; 0.25 µm)	IDMS, Creatinine ^{13}C, $^{15}N2$	Single point Exact match
PTB (SERS)		Centrifugation and cation exchange		lyophilisation & filtration	Raman Spectrometer		ID-SERS, Creatinine ^{13}C, $^{15}N2$	Calibration curve

Table 4: Brief summary of the approaches used in EURAMET.QM-K12 and the parallel pilot study

Primary calibrator

All participants used certified reference materials as calibration materials, whereby the results of the materials were directly traceable to the SI. Five of the participants, LGC, LNE, HSA, PTB and PTB (SERS) used NIST 914a which is a highly purified material characterised for the mass fraction of creatinine (99.7 ± 0.3%). One of the participants used a matrix CRM (NIST SRM 967a) as the primary calibrator. NIST has a CMC for the provision of services for such a material. Further confirmation of the traceability and comparability of NIST SRM 967a can be obtained from the recent CCQM-K80 study. Therefore all the returned results were traceable to the SI.

Summary of Participant results

	Material A (high level)			Material B (low level)		
NMI/DI	**Creatinine (µg/g)**	**Standard uncert. (µg/g) (u)**	**Expanded uncert. (µg/g) (U)**	**Creatinine (µg/g)**	**Standard uncert. (µg/g) (u)**	**Expanded uncert. (µg/g) (U)**
LGC	53.50	0.27	0.54	37.71	0.12	0.24
LNE	54.04	0.22	0.44	37.70	0.22	0.43
HSA	54.24	0.29	0.58	37.94	0.22	0.43
EXHM	54.509	0.697	1.394	38.438	0.4534	0.907
PTB	55.06	0.41	0.82	38.28	0.29	0.58
PTB (SERS)*	54.6*	1.2	2.4	38.0*	0.5	1.0

* participated as a pilot laboratory. These results was not used in the calculation of the CRV or UCRV.

Table 5: Results reported for the mass fraction of creatinine in human serum by participant laboratories for EURAMET.QM-K12 and parallel pilot study

Approaches to Uncertainty Estimation

A detailed description of the measurement equations and the participants associated measurement uncertainty budgets is provided in Appendix A. However, a summary of the major components used in the estimation of uncertainty are reported in Table 6.

	Relative Standard Uncertainty (%)		Contributions to the uncertainty budget
NMI/DI	**Material A**	**Material B**	
LGC	0.50	0.32	▪ Mass of calibration standard ▪ Mass of spike added to calibration blend ▪ Mass of sample ▪ Mass of spike added to the sample blend ▪ Uncertainty of mass fraction of the calibration standard ▪ Blend to blend variation (includes differences in re-suspension of sample material etc.)
LNE	0.41	0.58	▪ Purity of primary standard ▪ Preparation of calibrators ▪ Calibration curve ▪ Sample preparation ▪ Precision
HSA	0.54	0.57	▪ Mass of sample ▪ Mass of isotopic spike ▪ Mass fraction of primary calibration solution ▪ Linear regression of the calibration curve ▪ Method precision ▪ Method bias
EXHM	1.28	1.18	▪ Method precision ▪ Uncertainty from calibration standard ▪ Mass of isotopic spike ▪ Mass of sample ▪ Mass of calibration standard ▪ Mass of reconstitution
PTB	0.74	0.76	▪ Mass fraction of creatinine in serum ▪ Mass fraction of creatinine in serum per vial, mean of 3 single observations ▪ Purity of the reference material ▪ Uncertainty of weighing ▪ Estimated factor for unidentified systematic error
PTB (SERS)*	2.20	1.32	▪ Mass fraction of creatinine in reference solution ▪ Prediction of isotopologue abundance ratio in sample ▪ Prediction of isotopologue abundance ratio in reference ▪ Mass fraction of creatinine in serum per vial, mean of 3 single observations

* participated as a pilot laboratory. These results were not used in the calculation of the CRV or UCRV.

Table 6: Summary of the major contributors used to estimate the reported measurement uncertainty by each laboratory.

Comparison Reference value (CRV) calculation

A number of approaches for the calculation of CCQM key comparison reference values have been suggested over the past decade. These have included the use of arithmetic mean, median and weighted mean. Whilst all have their perceived merits and drawbacks, the approach used in this instance is somewhat reliant on the approach used in the original key comparison (CCQM-K12). The approach taken in CCQM-K12 was to use the mean of the eligible key participants. For uKCRV the standard deviation of the mean was used and this was expanded by the relevant K factor determined for the appropriate degrees of freedom from a t-distribution at the 95% confidence level. For ease of direct comparison between the old study and this RMO key a similar method of calculating the comparison reference value (CRV) and it associated uncertainty (uCRV) was adopted. The results for different approaches and the final agreed CRV and UCRV can be found in Table 7. A graph showing the individual participant results for material A and B are shown in Figures 1 and 2 respectively.

	Material A	**Material B**
Arithmetic Mean (µg/g)	54.27	38.01
Standard deviation (µg/g)	0.58	0.33
Data points used (n)	5	5
Standard deviation of the mean $(s.d/(n)^{0.5})$	0.26	0.15
K	2.78	2.78
Median	54.24	37.94
MADe	0.40	0.36

Table 7A: Results of the approaches used for the suggested comparison reference value for EURAMET.QM-K12

	Material A	**Material B**
CRV (µg/g)	**54.27**	**38.01**
uCRV (µg/g)	**0.26**	**0.15**
UCRV (µg/g)	**0.72**	**0.42**
UCRV (%)	**1.3**	**1.1**

Table 7 B: Agreed comparison reference value and its standard and expanded uncertainty for EURAMET.QM-K12

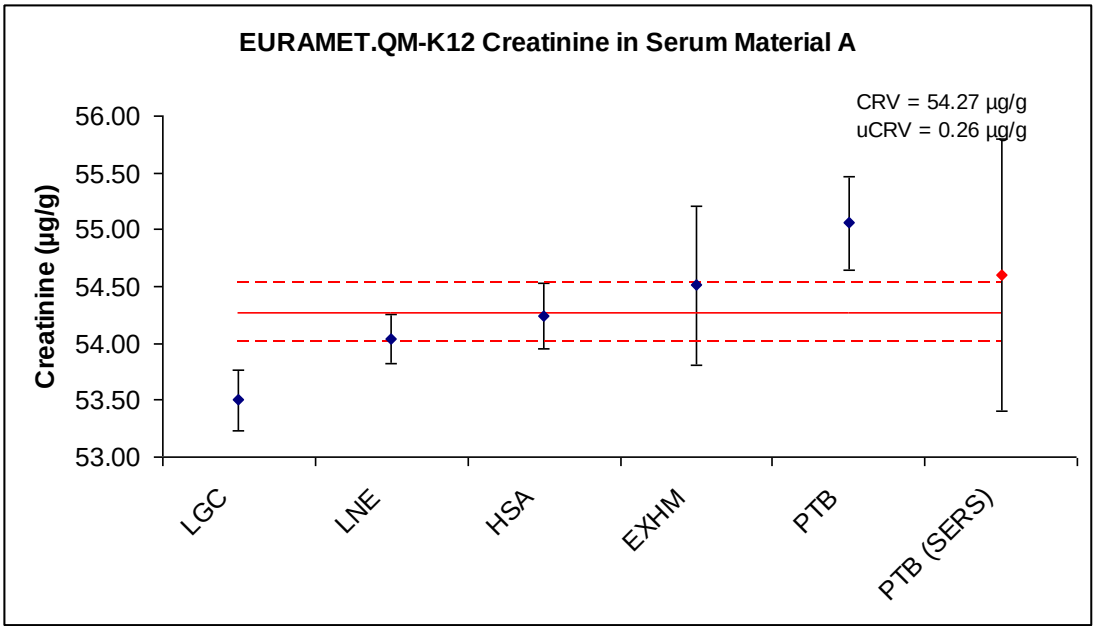


Figure 1: Individual participant results for key participants (blue diamonds) and pilot participants (red diamonds), with reported standard uncertainties, for EURAMET.QM-K12 Material A. The solid red line represents the agreed CRV with the dashed lines indicating uCRV.

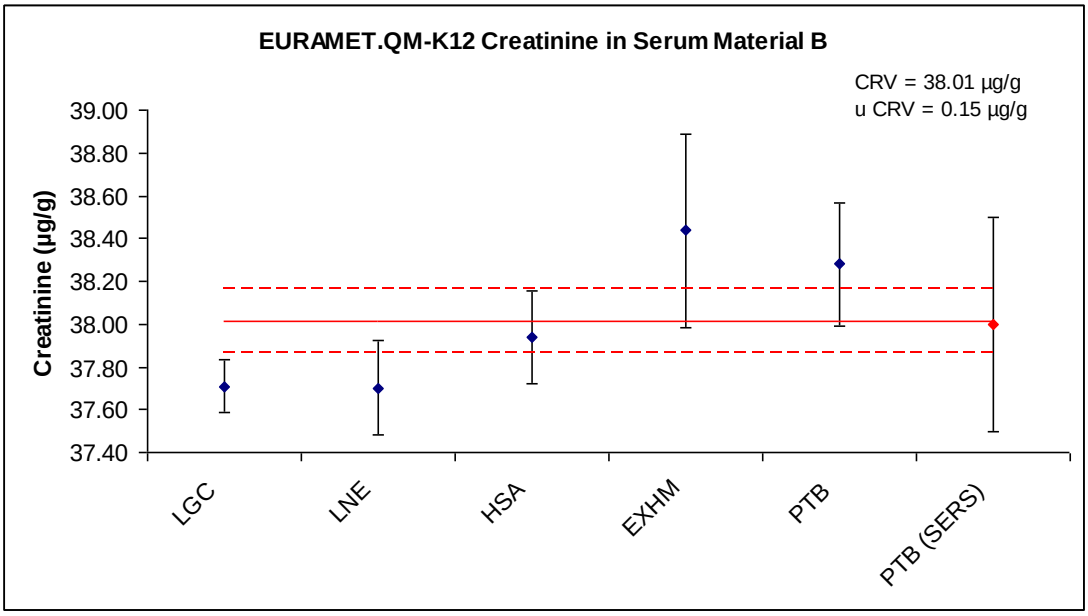


Figure 2: Individual participant results for key participants (blue diamonds) and pilot participants (red diamonds), with reported standard uncertainties, for EURAMET.QM-K12 Material B. The solid red line represents the agreed CRV with the dashed lines indicating uCRV.

Calculation of Degrees of Equivalence

The degrees of equivalence (DoE) and relative degrees of equivalence were calculated from the comparison reference value using the following standard approaches:

$$DoE = \left(x_i - x_{ref}\right)$$

Where Xi is the individual participants results and Xref is the comparison reference value.
The uncertainty associated with the DoE for each participant was estimated as:

$$uDoE = \sqrt{\left(u_{x_i}\right)^2 + \left(u_{x_{ref}}\right)^2}$$

Whilst the expanded uncertainty of DoE was calculated for 95% coverage by using a K factor of 2.

$$UDoE = k \cdot uDoE$$

The calculated Doe and UDoE for each Key participant are shown in Table 8 with graphical representation of the results shown in Figures 3 and 4.

	Material A				Material B			
	Di		U(Di)		Di		U(Di)	
	(µg/g)	(%)	(µg/g)	(%)	(µg/g)	(%)	(µg/g)	(%)
LGC	-0.77	-1.42	0.75	1.38	-0.30	-0.80	0.38	1.01
LNE	-0.23	-0.42	0.68	1.25	-0.31	-0.82	0.53	1.40
HSA	-0.03	-0.06	0.78	1.43	-0.07	-0.19	0.52	1.38
EXHM	0.24	0.44	1.49	2.74	0.42	1.12	0.95	2.51
PTB	0.79	1.46	0.97	1.78	0.27	0.70	0.65	1.72

Table 8: Degree of equivalence and relative degree of equivalence for key participants in EURAMET.QM-K12

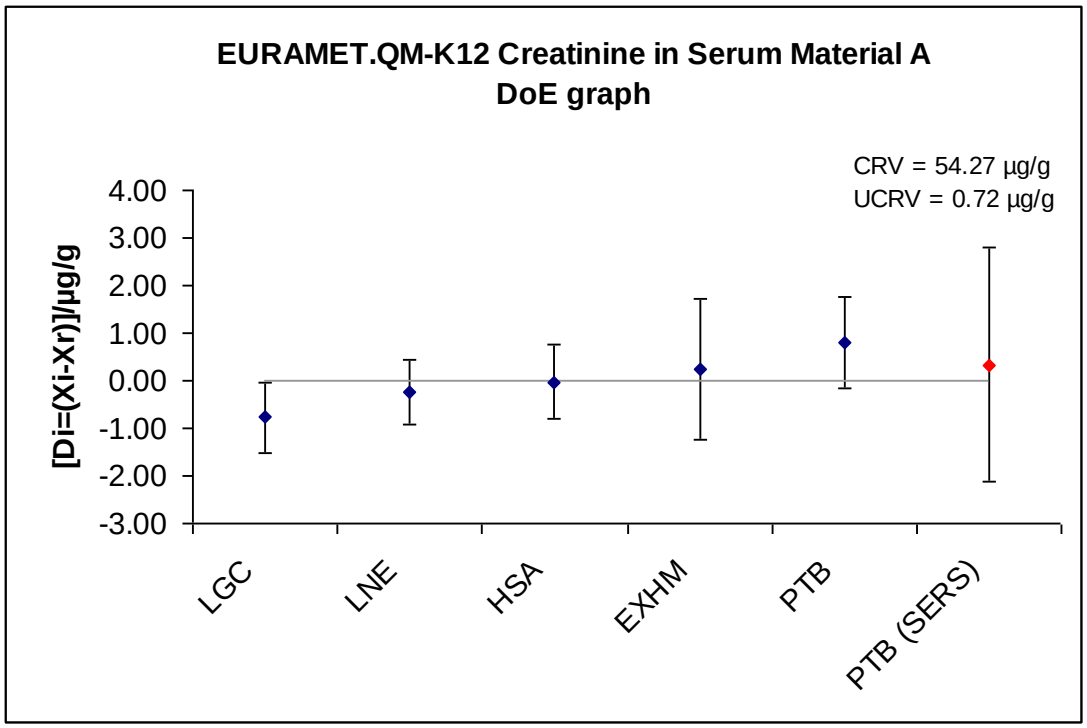


Figure 3A: Individual participant DoE for key participants (blue diamonds) and pilot participants (red diamonds), with expanded uncertainties, for EURAMET.QM-K12 Material A.

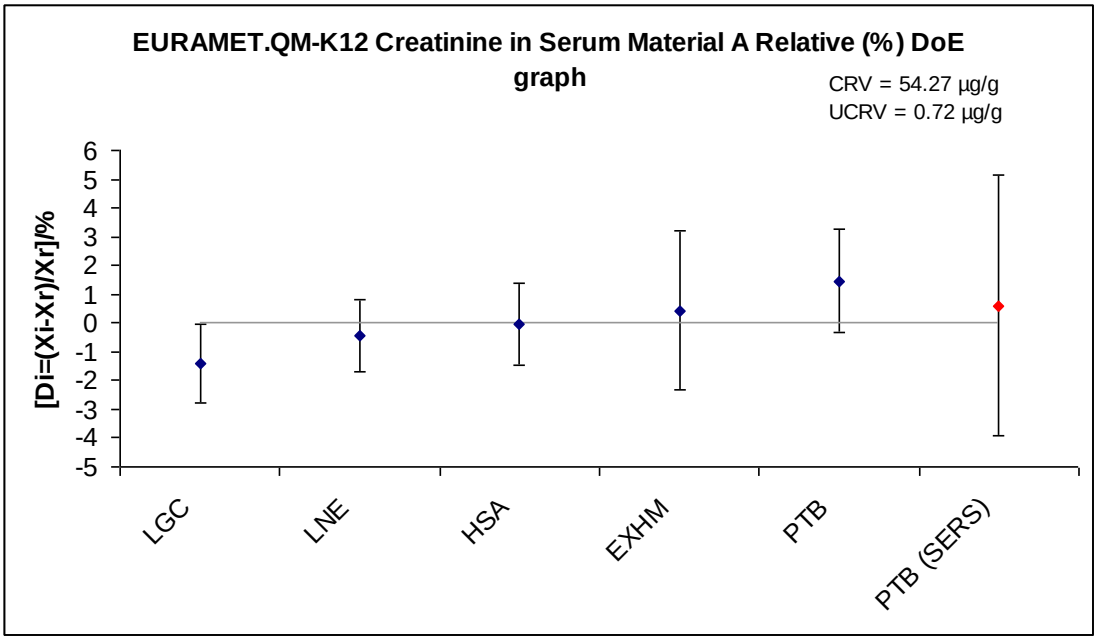


Figure 3A: Individual participant relative (%) DoE for key participants (blue diamonds) and pilot participants (red diamonds), with expanded uncertainties, for EURAMET.QM-K12 Material A

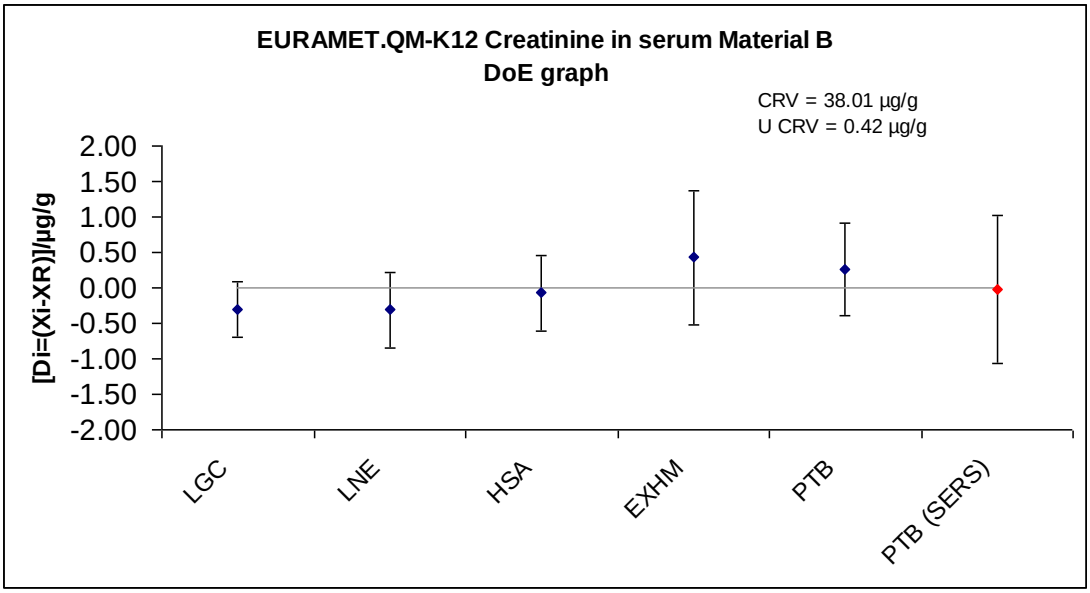


Figure 4A: Individual participant DoE for key participants (blue diamonds) and pilot participants (red diamonds), with expanded uncertainties, for EURAMET.QM-K12 Material B

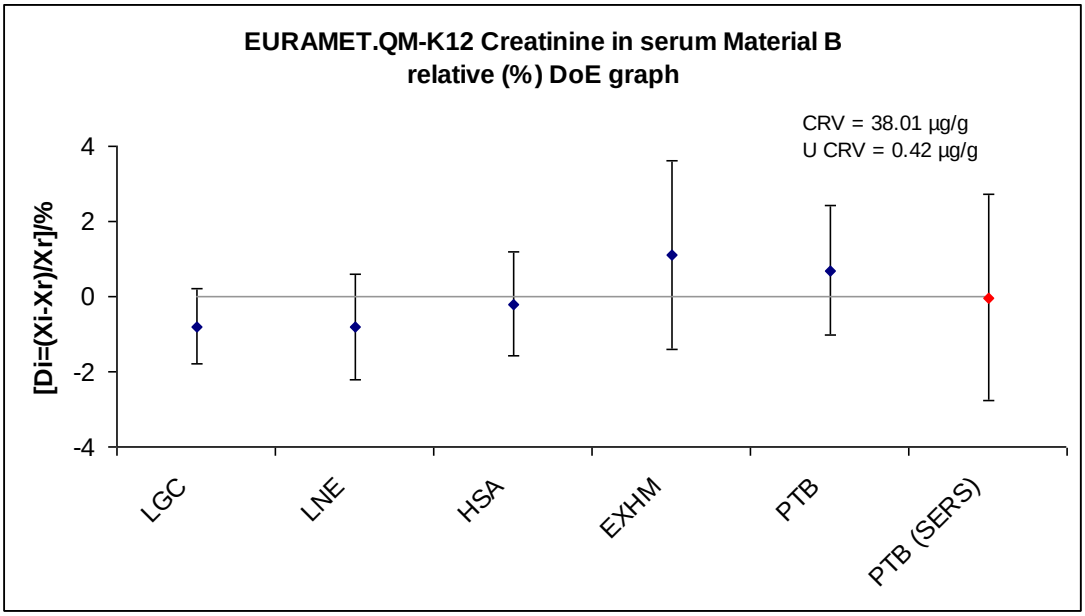


Figure 4B: Individual participant relative (%) DoE for key participants (blue diamonds) and pilot participants (red diamonds), with expanded uncertainties, for EURAMET.QM-K12 Material B

Linking EURAMET.OQ-K12 to CCQM-K12

For the original CCQM-K12 study the OAWG decision was to accept the mean as the KCRV and that UKCRV be expressed as the standard deviation of the mean multiplied by a K factor assigned by the number of observations used to calculate the mean. As the objective of this study is to link back to the original study it is somewhat incumbent that we used the same statistical methods. Failure to do so may result in the degrees of equivalence (DoE) and UDoE being very different.

Two participants of EURAMET.QM-K12 participated in CCQM-K12 (LGC and PTB). In this instance the mean of all participants is virtually identical to the average of the PTB/LGC results (38.01µg/g v's 37.99µg/g for material A, 54.27µg/g v's 54.28µg/g). This combined with the extra information provided by all participants in the RELA study seems to suggest the mean is a good estimate of the true value.

It is normal in RMO key studies to link the degrees of equivalence to the original study CC key comparison. When one "linking" lab is used the new study is assigned a reference value (RV) based on the linking labs performance in both studies, normally by adjusting the new study RV by the relative DoE of the linking lab in the previous study. This makes the assumption that the linking labs performance is identical in each study and the DoE is a static bias. In this study we have two possible linking labs. The evidence suggests that a key comparison is nothing but a "snap shot in time", whereby in this instance the relative DoE expressed for the new study would be different if you calculated it based on PTB or LGC. On this occasion we have decided to calculate the DoE of all labs based on the mean of the key participants (which is the same as the PTB/LGC average). The relative degrees of equivalence were then graphically represented against the relative DoE calculated from the data of Material II of CCQM-K12. Material II was used in both instances as this was closer in mass fraction to both materials in EURAMET.QM-K12. As the relative DoE were missing from the original CCQM-K12 these were calculated as described above and are shown in Table 9. The graphical representation of the DoE from both CCQM-K12 and EURAMET.QM-K12 are shown in Figures 5 and 6.

	Material I				Material II			
	Di		U(Di)		Di		U(Di)	
	(µg/g)	(%)	(µg/g)	(%)	(µg/g)	(%)	(µg/g)	(%)
IRMM	0.143	1.7	0.216	2.6	0.075	0.40	0.487	2.6
KRISS	-0.031	-0.4	0.164	2.0	-0.106	-0.57	0.331	1.8
LGC	-0.024	-0.3	0.071	0.9	-0.031	-0.16	0.125	0.7
NIST	0.060	0.7	0.093	1.1	0.063	0.34	0.205	1.1
PTB	-0.006	-0.1	0.074	0.9	0.073	0.39	0.161	0.9

Table 9: Degree of equivalence and relative degree of equivalence for key participants in CCQM-K12

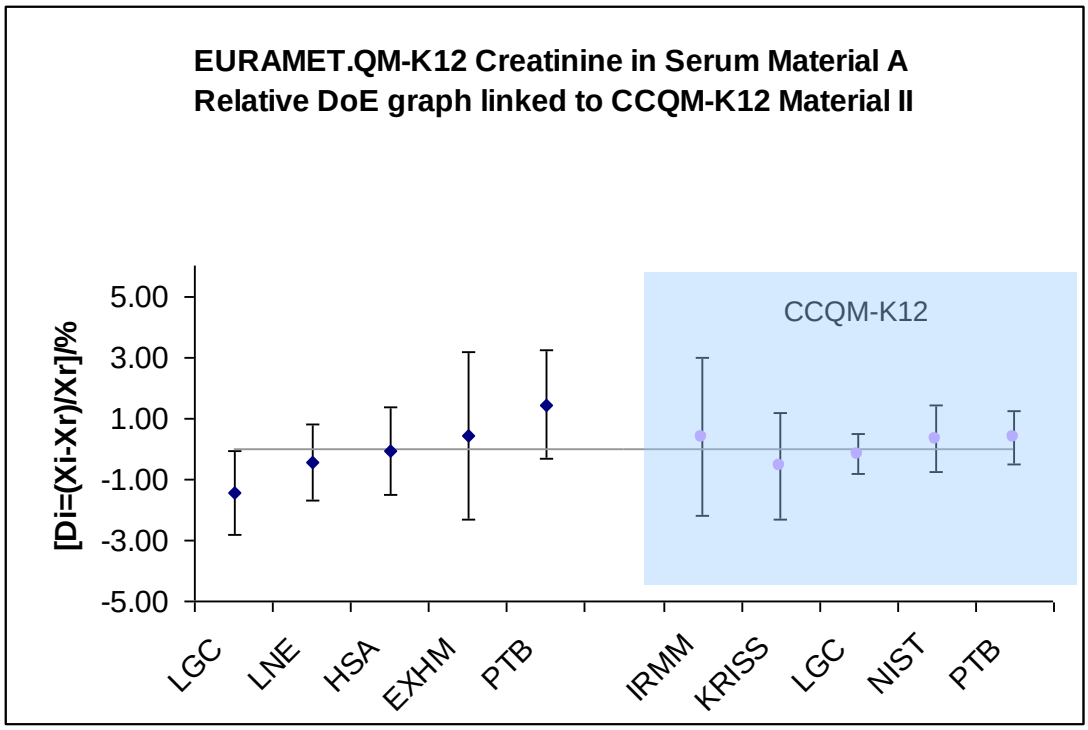


Figure 5: Individual participant relative (%) DoE for key participants in EURAMET.AM-K12 (blue diamonds) and CCQM-K12 (lilac diamonds), with expanded uncertainties. EURAMET.QM-K12 Material A and CCQM-K12 Material II.

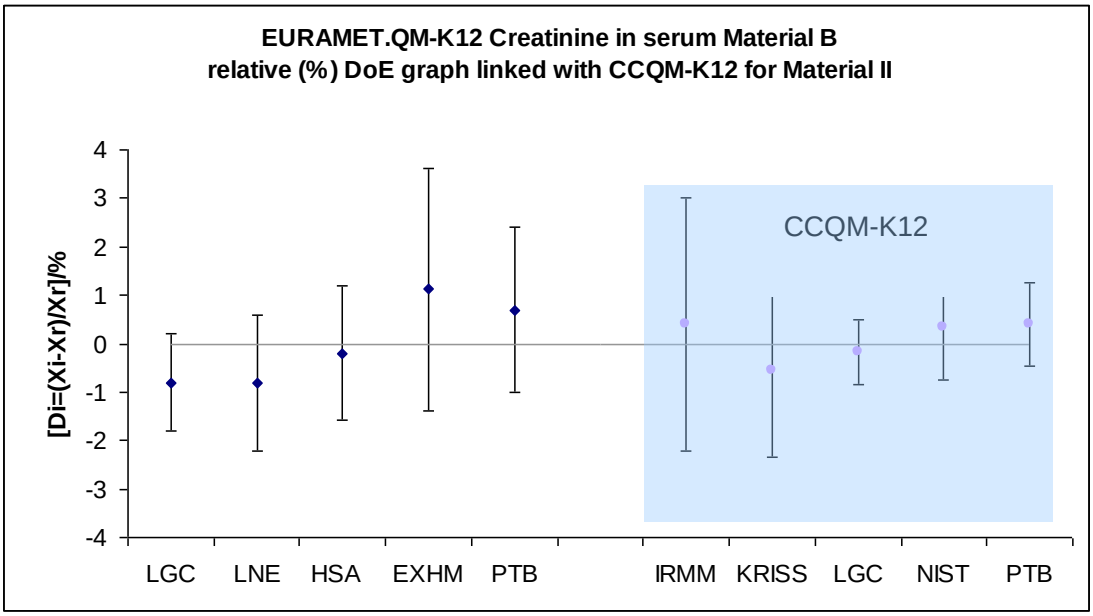


Figure 5: Individual participant relative (%) DoE for key participants in EURAMET.AM-K12 (blue diamonds) and CCQM-K12 (lilac diamonds in blue surround), with expanded uncertainties. EURAMET.QM-K12 Material B and CCQM-K12 Material II.

Conclusions

The range of participant's results in EURAMET.QM-K12 for both levels (2% and 3% respectively for materials A and B) is greater than the 1% achieved in CCQM-K12.

All participants DoE, as calculated using the same approach adopted in CCQM-K12, resulted in all participants UDoE's overlapping with zero. This suggests that all participants can at least provide services with a bias of less then 1.5%.

Current CMCs for creatinine have an expanded uncertainty range from 0.3% to 7% with three of the five institutes with current CMCs claiming a relative expanded uncertainty on their dissemination range of between 1 and 1.5%

This study is suitable to support CMCs for creatinine in serum in the range from 1 to 100 µg/g with uncertainties at the 1 to 1.5% level.

Appendix A: Measurement equation and Uncertainty calculation as described by Participants.

Participant: LGC

Method of Calculating results

Results were calculated using the double IDMS equation (equation 1).

Equation 1

$$W_x = W_z \cdot \frac{m_z}{m_{yc}} \cdot \frac{m_y}{m_x} \cdot \frac{R'_B}{R'_{BC}}$$

Where:

W_x= the concentration of creatinine in sample (mg/g);
W_z = the concentration of natural creatinine solution used to prepare the calibration blend (mg/g);
m_z = mass of the natural creatinine standard added to the calibration blend;
m_x = mass of the sample used
m_{yc} = mass of the labelled creatinine standard added to the calibration blend;
m_y = mass of the labelled creatinine standard added to the sample blend;
R'_B = measured ratio (peak area m/z 114 / peak area m/z 117) of the sample blend;
R'_{BC} = Average measured ratio (peak area m/z 114 / peak area m/z 117) of the calibration blend injected before and after the sample;

The uncertainty of an individual measurement was calculated by combining the relative standard uncertainties of the weighings, solution concentrations and ratio measurements of the calibration and sample blends as shown in equation 2. The combined standard uncertainty, as shown in Table 1. was calculated using equation 2.

Equation 2

$$u = w_x \sqrt{\left(\frac{u_{Cz}}{c_z}\right)^2 + \left(\frac{u_{p_m}}{p_m}\right)^2 + \left(\frac{um_x}{m_x}\right)^2 + \left(\frac{um_y}{m_y}\right)^2 + \left(\frac{um_z}{m_z}\right)^2 + \left(\frac{um_{yc}}{m_{yc}}\right)^2}$$

Where: $u_{pm} = s.d.$

and s.d = standard deviation of all results

Results

Are detailed in the summary sheets and the reporting sheets

Participant: LNE

Material A

Purity of primary standard	4.54%
Calibrators preparation	14.95%
Calibration curve	47.62%
Sample preparation	27.45%
Precision	5.42%

Material B

Purity of primary standard	2.33%
Calibrators preparation	7.68%
Calibration curve	25.42%
Sample preparation	15.71%
Precision	48.84%

Participant: HSA

Uncertainty Estimation:
Please give full details including equations where appropriate.

The mass fraction of creatinine in serum was calculated based on the IDMS Linear Regression Plot as follows:

$$C_X = (mR_B + b) \times \frac{W_Y}{M_X} = (mR_B + b) \times \frac{M_Y C_Y}{M_X} \qquad (3)$$

where C_X = mass fraction of creatinine in serum sample
M_X = mass of serum sample
M_Y = mass of isotope stadard solution
W_Y = mass of the isotope labeled standard spiked in serum sample
R_B = peak area ratio of sample blend
C_Y = concentration of isotope labeled standard solution
m = gradient of the slope for linear regression plot
b = intercept on y axis for the linear regression plot

Consider $R_M = mR_B + b$, Equation (3) is converted to:

$$C_X = R_M \times \frac{M_Y C_Y}{M_X} \qquad (4)$$

Where: R_M = isotope mass ratio in sample blend
C_Z = concentration of creatinine in calibration standard solution
Let $R_M' = R_M \times C_Y / C_Z$
Equation (4) can be coverted to:

$$C_X = R_M' \times \frac{M_Y C_Z}{M_X} \qquad (5)$$

A standard uncertainty was estimated for all components of the measurement Equation (5), which were then combined using respective derived sensitivity coefficients to estimate a combined standard uncertainty in the reported result of creatinine in serum samples. A coverage factor k of 2 is used to expand the combined standard uncertainty to a 95 % confidence interval. Possible sources of biases (Method Precision, F_P, and Choice of Different Column, F_C) are accounted for in the final uncertainty budget with the use of the measurement equation:

$$C_X = F_P \times F_C \times R_M' \times \frac{M_Y C_Z}{M_X} \qquad (6)$$

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

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

The standard uncertainty of each component was calculated as follow:
(1) M_Y and M_X: The standard uncertainty was calculated based on the calibration report using the standard weights calibrated by NMC.
(2) F_P: The standard deviation of mean of the six reported results for each sample was used as the the standard uncertainty of method precision.
(3) F_C: The average difference of all the results between using Agilent Zorbax SB-Aq column and Imtakt Unison UK-C8 column divided by 2.
(4) C_Z: The certified purity and uncertainty of NIST SRM 914a in combination with the uncertainty of balance weighing for preparation of the calibration standard solution

(5) 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:

$$\mu_{R_M} = \frac{1}{m'} \times s_{y/x} \times \sqrt{\frac{1}{N} + \frac{1}{n} + \frac{(R_B - \overline{R_{Bc}})^2}{m'^2 \sum_{i=1}^{n} (R_{Mc} - \overline{R_{Mc}})^2}} \qquad (6)$$

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

The uncertainty budgets for Samples A and B are listed in Table 1 and Table 2, respectively

Table 1. Uncertainty Budget for Sample A

Factor	Value x	Uncertainty u(x)	Relative Uncertainty u(x)/(x)	Sensitivity Coefficient (c) δCx/δx	$c^2 . u(x)^2$	% contribution
M_X	0.1490	0.000106	0.071%	364.01	0.00149	1.8%
M_Y	0.1370	0.000106	0.077%	395.84	0.00176	2.1%
C_Z	1457.86	4.169	0.286%	0.04	0.02406	28.6%
R_M'	3.0556	0.010795	0.353%	17.75	0.03672	43.6%
F_P	54.24	0.081168	0.150%	1.00	0.00659	7.8%
F_C	54.24	0.116828	0.215%	1.00	0.01365	16.2%
Combined Standard Uncertainty	0.29					
Coverage Factor (95%)	2					
Expanded Uncertainty	0.58					

Table 12. Uncertainty Budget for Sample B

Factor	Value x	Uncertainty u(x)	Relative Uncertainty u(x)/(x)	Sensitivity Coefficient (c) δCx/δx	$c^2 . u(x)^2$	% contribution
M_X	0.1483	0.000106	0.072%	255.83	0.00074	1.6%
M_Y	0.0949	0.000106	0.112%	399.63	0.00180	3.9%
C_Z	1457.86	4.169	0.286%	0.03	0.01178	25.4%
R_M'	3.0845	0.010880	0.353%	12.30	0.01791	38.6%
F_P	37.94	0.031837	0.084%	1.00	0.00101	2.2%
F_C	37.94	0.114712	0.302%	1.00	0.01316	28.4%
Combined Standard Uncertainty	0.22					
Coverage Factor (95%)	2					
Expanded Uncertainty	0.43					

Participant: EXHM

Sample A

Uncertainty component	relative unc	comb. std unc.	k=2	expanded unc
method precision (n=9)	0.0072	0.697		1.394
calibrant (NIST SRM 967a)	0.0106			
mass of spiked isotopic reagent	< 0,0001			
mass of reconstituted serum	0.0001			
mass of calibrant	< 0,0001			
mass of water for reconstitution	< 0,0001			

Sample B

Uncertainty component	relative unc	comb. std unc.	k=2	expanded unc
method precision (n=9)	0.0052	0.4534		0.9068
calibrant (NIST SRM 967a)	0.0106			
mass of spiked isotopic reagent	< 0,0001			
mass of reconstituted serum	0.0001			
mass of calibrant	< 0,0001			
mass of water for reconstitution	< 0,0001			

Participant: PTB

Model Equation:

$c_{sample}=w_{sample}*P_{creatinine}*K_W*Sys$

List of Quantities:

Quantity	Unit	Definition
c_{sample}	µg/g	Mass fraction of creatinin in serum
w_{sample}	µg/g	mass fraction of creatinin in serum per vial, mean of 3 single observations
$P_{creatinine}$		purity of the reference material
K_W		uncertainty of weighing
Sys		estimated factor for unidentified systematic error

c_{sample}:

Result

w_{sample}:

Type A
Method of observation: Direct
Number of observation: 3

No.	Observation
1	55.2624
2	55.0026
3	54.9162

Arithmetic Mean: 55.060 µg/g
Standard Deviation: 0.18 µg/g
Standard Uncertainty: 0.104 µg/g
Degrees of Freedom: 2

The observations (w) are the determined mass fractions of creatinine in serum (in µg/g).
3 vials, 2 aliquots per vial used. The inserted observations are the means per vial.

$P_{creatinine}$:

Type B rectangular distribution
Value: 1
Halfwidth of Limits: 0.003

Uncertainty purity of the reference compound creatinine in used NIST SRM 914a, according to certificate +/- 0,3%
The purity (99,7%) was already calculated in the excel sheet for the determination of w.
Therefore the value was set here to 1 .

K_W:
Type B normal distribution
Value: 1
Expanded Uncertainty: 0.00017
Coverage Factor: 2

Uncertainty of the microbalance MC 5 (Sartorius)
calibration certificate dated 14.07.2010
U = 0,0008 mg + 1,44 * 10 E- 0 5 * m (w);
= 0,017% (5 mg)

Sys:
Type B normal distribution
Value: 1
Expanded Uncertainty: 0.014
Coverage Factor: 2

unknown factor for systematic unidentified discrepancies including sample preparation and GC-MS interferences (estimated value = +/- 1,4 %).

Uncertainty Budget:

Quantity	Value	Standard Uncertainty	Degrees of Freedom	Sensitivity Coefficient	Uncertainty Contribution	Index
w_{sample}	55.060 µg/g	0.104 µg/g	2	1.0	0.10 µg/g	6.4 %
$P_{creatinine}$	1.00000	$1.73 \cdot 10^{-3}$	∞	55	0.095 µg/g	5.4 %
K_W	1.0000000	$85.0 \cdot 10^{-6}$	50	55	$4.7 \cdot 10^{-3}$ µg/g	0.0 %
Sys	1.00000	$7.00 \cdot 10^{-3}$	50	55	0.39 µg/g	88.2 %
c_{sample}	55.06 µg/g	0.410 µg/g	56			