CCQM-K78.a
Polar Analytes in Aqueous Solvent:
Multicomponent Amino Acids in Dilute HCl Solution
Track A Comparison
Final Report
November 2018

Steven Westwood[1], Ralf Josephs, Tiphaine Choteau, Adeline Daireaux and Robert Wielgosz
Bureau International des Poids et Mesures (BIPM),

With contributions from:

Jeremy Melanson, Marie-Pier Thibeault and Juris Meija
Measurement Science and Standards - National Research Council Canada (NRC)
Ottawa, Canada

Can Quan, Hongmei Li, Ting Huang, Wei Zhang and Dewei Song
National Institute of Metrology (China) (NIMC)
Beijing, China

Gustavo Martos
Laboratoire National de Métrologie et d'Essais (LNE)
Paris, France

Rüdiger Ohlendorf and André Henrion
Physikalisch-Technische Bundesanstalt (PTB)
Braunschweig, Germany

Elias Kakoulides, Panagiota Giannikopoulou and Charalampos Alexopoulos
Chemical Metrology Laboratory (EXHM/GCSL-EIM),
Athens, Greece

Kelly W. Y. Chan, W.H. Lam and W.H. Fung
Government Laboratory of Hong Kong SAR (GLHK)
Kowloon, Hong Kong SAR

Taichi Yamazaki
National Metrology Institute of Japan (NMIJ)
Tsukuba, Japan

Krylov A.I., Lopushanskaya E.M., Haritonov S.G., Belyakov M.V. and Sokolova O.B.
D.I. Mendeleyev Scientific and Research Institute for Metrology (VNIIM)
St Petersburg, Russia

Qinde Liu, Hong Liu, Sharon Yong
Health Sciences Authority (HSA)
Singapore

[1] Comparison coordinator: Steven Westwood (steven.westwood@bipm.org)

Désirée Prevoo-Franzsen and Maria Fernandes-Whaley
National Metrology Institute of South Africa (NMISA)
Pretoria, South Africa

Ji-Seon Jeong, Ha-Jeong Kwon, Inchul Yang and Byungjoo Kim
Korea Research Institute of Standards and Science (KRISS)
Daejeon, South Korea

Jintana Nammoonnoy
National Institute of Metrology (Thailand) (NIMT)
Bangkok, Thailand

Ahmet Ceyhan Gören, Simay Gündüz, Ilker Ün and Gökhan Bilsel
National Metrology Institute of Turkey (UME)
Gebze-Kocaeli, Turkey

Sabine Biesenbruch
LGC Limited (LGC)
Teddington, United Kingdom

Mark Lowenthal
National Institute of Standards and Technology
Gaithersburg, MD, USA

SUMMARY

The CCQM-K78.a comparison and parallel CCQM-P121.a pilot study was coordinated by the BIPM on behalf of the CCQM Organic Analysis Working Group (OAWG) for National Measurement Institutes (NMIs) and Designated Institutes (DIs) which provide measurement services in organic analysis under the CIPM MRA. Gravimetrically-prepared solutions having an assigned mass fraction of specified organic analytes are routinely used to calibrate measurement processes for the quantification of the same analytes in matrix samples. Appropriate assignments of the property value and associated uncertainty of the content of calibration solutions thus underpin the traceability of routine analysis and are critical for accurate measurements. Evidence of successful participation in relevant international comparisons is needed to document calibration and measurement capability claims (CMCs) made by national metrology institutes (NMIs) and designated institutes (DIs).

Fifteen National Metrology Institutes in addition to the BIPM participated in the Track A Key Comparison CCQM-K78.a [Multicomponent amino acids in dilute HCl solution]. Participants were requested to assign the mass fractions, expressed in μg/g, of phenylalanine (Phe), leucine (Leu), isoleucine (Ile) and proline (Pro) in a 0.01 N hydrochloric acid solution. The Key Comparison Reference Values (KCRVs) for each analyte were assigned as the Der Simonian-Laird combination of all participant values that agreed within their expanded uncertainty with the independent gravimetric values calculated by the coordinating laboratory from their preparation procedure.

Successful participation in CCQM-K78.a was intended to demonstrate measurement capabilities for assigning the mass fraction content of polar organic compounds ($pK_{ow} > -2$) present at a mass fraction range from 50 μg/g to 500 μg/g in an aqueous solution.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF APPENDICES

ACRONYMS

ACN	acetonitrile
CCQM	Consultative Committee for Amount of Substance: Metrology in Chemistry and Biology
CMC	Calibration and Measurement Capability
DI	Designated Institute
DoE	degree of equivalence
DSL	DerSimonian-Laird model for meta-analysis of data
GC-FLD	gas chromatography with fluorescence detection
GC-MS	gas chromatography with mass spectrometric detection
GC-MS/MS	gas chromatography with tandem mass spectrometric detection
GC-TOFMS	gas chromatography with time-of-flight mass spectrometric detection
Ile	(L)-Isoleucine
LC-DAD	liquid chromatography with diode array (UV) detection
LC-HRMS	liquid chromatography with high-resolution mass spectrometric detection
LC-FLD	liquid chromatography with fluorescence detection
LC-MS	liquid chromatography with mass spectrometric detection
LC-MS/MS	liquid chromatography with tandem mass spectrometric detection
Leu	(L)-Leucine
IDMS	isotope dilution mass spectrometry
KCRV	Key Comparison Reference Value
MRM	multiple reaction monitoring
NMI	National Metrology Institute
NMR	nuclear magnetic resonance spectroscopy
OAWG	Organic Analysis Working Group
pK_{ow}	negative log base 10 of the octanol-water partition coefficient
Phe	(L)-Phenylalanine
Pro	(L)-Proline
qNMR	quantitative nuclear magnetic resonance
SIM	selected ion monitoring

SYMBOLS

d_i	degree of equivalence: x_i - KCRV
$\%d_i$	percent relative degree of equivalence: $100 \cdot d_i/\text{KCRV}$
k	coverage factor: $U(x) = k \cdot u(x)$
n	number of quantity values in a series of quantity values
$u(x_i)$	standard uncertainty of quantity value x_i
$U(x_i)$	expanded uncertainty of quantity value x_i
$U_{95}(x_i)$	expanded uncertainty defined such that $x_i \pm U_{95}(x_i)$ is asserted to include the true value of the quantity with an approximate 95 % level of confidence
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: $\bar{x} = \sum_{i=1}^{n} x_i/n$
w_i	mass fraction content of organic analyte i in kg/kg or subunits thereof in a given matrix

INTRODUCTION

Gravimetrically-prepared solutions having an assigned mass fraction of specified organic analytes are essential for the calibration of many measurement processes for the quantification of the corresponding analytes in matrix samples. The ability to assign the property value and associated uncertainty of the analyte content of solutions for use in calibration is critical for the delivery of SI-traceable measurements in organic analysis and is thus a core competency for producers of reference materials as standard solutions and for providers of calibration and reference measurement services in organic analysis. Evidence of successful participation in formal, relevant international comparisons is needed to support calibration and measurement capability (CMC) claims for services in analytical organic chemistry made by national metrology institutes (NMIs) and designated institutes (DIs).

Key comparisons and pilot studies have been undertaken by the OAWG for the value assignment of the analyte content in standard solutions in organic solvent. These included the CCQM-P31 series in 2004,[1] the CCQM-K38 comparison on the determination of PAHs in solution in 2005 [2] and in 2015 the CCQM-K131 comparison on the determination of PAHs in acetonitrile.[3] There are numerous CMC claims listed in the KCDB Appendix C [4] for the provision of standard solutions of organic analytes. Figure 1 is a plot of NMI CMC claims (red data points) and relevant key comparison KCRVs (blue data points).

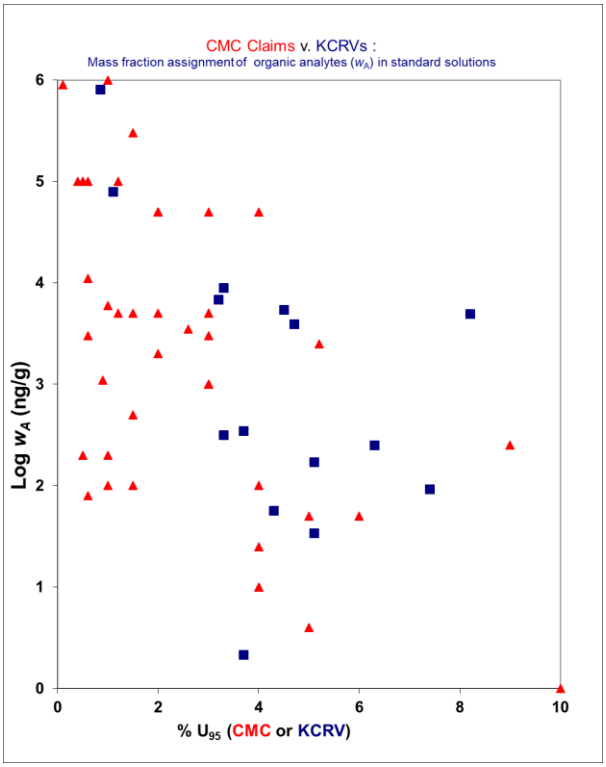


Figure 1: log [w_A] v. % U_{95} for CMC claims (in red) and KCRVs (in blue) for the preparation and assignment of standard solutions of organic analytes

As is clear from Figure 1, many current CMC claims for mass fraction assignment in standard solutions have a smaller expanded uncertainty than is supported solely by relevant KCRVs. It was also a concern that

the comparisons in this area have been undertaken only using solutions of organic analytes in non-polar organic solvent. As a number of NMIs currently provide or are developing reference measurement services for the assignment of polar analytes in aqueous biological and clinical samples, in particular for support of the quantification of amino acids in aqueous solution, it was considered desirable to undertake a key comparison investigating capabilities for standard solution assignment of organic analytes in an aqueous solution.

In April 2015 the Consultative Committee for Amount of Substance: Metrology in Chemistry and Biology (CCQM) approved the Key Comparison (KC) CCQM-K78.a, with the BIPM as the coordinating laboratory. CCQM-K78.a was designed to assess participants' capabilities for assignment of the mass fraction content of single or multi-component polar organic analytes in an aqueous standard solution. The target mass fraction content of the amino acids in the material to correspond to values in the range 4-5 on the *y*-axis with associated relative expanded measurement uncertainties of the participant results anticipated to correspond to values on the *x*-axis of less than 5% in Figure 1. All NMIs with ongoing programs in this area were strongly encouraged to participate in the comparison. Participation in CCQM-K78.a allowed NMIs and DIs to provide objective evidence that the procedures they use for the property value and associated measurement uncertainty assignment of aqueous standard solutions are suitable for their intended purpose. The purpose of a primary calibrator standard solution produced by an NMI could be either for provision to external users as a Certified Reference Material or for internal use to establish the calibration hierarchy of a reference measurement procedure.

The focus of this comparison was the demonstration of the capabilities of the participants to assign the analyte mass fraction content in a multicomponent amino acid solution. It was a deliberate decision not to require the participants to source or value assign their own primary calibrator materials, as this would introduce an additional source of uncertainty whose contribution might prove hard to resolve from that associated with the calibration strategy and analytical method. In addition the purity assignment capability had already been investigated through participation in the CCQM-K55 purity comparisons. Each participant was supplied by the coordinating laboratory with a set of value-assigned, pure substance calibrator materials which they were instructed to use as calibrants for methods requiring external calibration. They were however free to use to whatever analytical approach they chose to undertake the assignment. The participants were informed of the four amino acids present in the solution and were advised that the mass fraction content of the individual amino acids in the comparison material were in the range 50 μg/g - 500 μg/g (see Appendix C), but no additional information was provided.

The sections of this report document the timeline of CCQM-K78.a, target measurands, characterization of the study material, participants, results, and the measurement capability claims that the results of participation in CCQM-K78.a are intended to support. The Appendices reproduce the official communication materials and summaries, additional information about the characterization of the comparison material and details of the methods and approaches to the estimation of measurement uncertainty used by the participants.

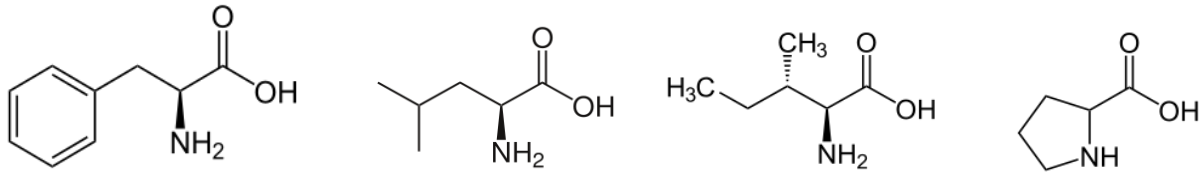
TIMELINE

Date	Action
April 2015	Proposed to CCQM
April 2016	Draft protocol presented to OAWG for potential Track A Key Comparison
April 2016	OAWG authorized CCQM-K78.a as a Track A Comparison; protocol approved
May 2016	Call for participation to OAWG members
October 2016 – January 2017	Study samples shipped to participants. The range in shipping times reflects delays experienced due to customs clearance issues.
March 2017	Results due to coordinating laboratory
April 2017	Result summary distributed to participants
April 2017	First discussion of participant results
September 2017	Second discussion of participant results, agreement on the HFTLS statement, the results for inclusion in the KCRV calculation and the approach its estimation
March 2018	Draft B report distributed to OAWG
July 2018	Final report approved by OAWG

MEASURANDS

BIPM prepared a standard solution in 0.01 N HCl containing phenylalanine (Phe), leucine (Leu), isoleucine (Ile) and proline (Pro). The approximate target levels of each analyte were as follows: Phe - 500 µg/mg, Leu & Ile - 200 µg/mg and Pro - 50 µg/mg. The study protocol identified the four amino acids present in the solution and provided a broad estimate of the mass fraction range at which they were present. These levels were intended to be representative of the range of mass fraction content of stable amino acids in a multicomponent standard solution provided as a primary calibrator for use in amino acid analysis. Phenylalanine, the only component containing a UV-chromophore, was present at a level that allowed for quantification by direct LC-UV methods. The isomeric amino acids Leu and Ile were selected to provide a challenge to achieve suitable analytical resolution. Proline was present at a lower level compared with that found in typical amino acid primary calibrant solutions.

The structures, nomenclature and *pK*ow values of each compound are shown below in Figure 2.



Name:	(L)-Phenylalanine	(L)-Leucine	(L)-Isoleucine	(L)-Proline
Symbol:	Phe	Leu	Ile	Pro
pK_{OW}	1.4	1.8	1.8	2.2

Figure 2: Amino acid components of the CCQM-K78.a solution

STUDY MATERIALS

Preparation of Candidate Material

To prepare the candidate comparison material high purity samples of phenylalanine (725 mg), leucine (298 mg), isoleucine (322 mg) and proline (70 mg), with each sample mass accurately determined using a calibrated Mettler MX5 balance reading to 0.001 mg, were placed in a large tared, acid-rinsed Ehrlenmeyer flask. The solid materials were taken up in 500 mL of 0.01 N HCl. The solvent was prepared immediately before use by dilution of analytical grade 6 N HCl with MilliQ-water.

The component mixture was stirred at room temperature for several hours to obtain a clear, homogenous solution and the total volume was made up to approximately 1.5 litre with additional 0.01 N HCl. The environmental temperature, pressure and relative humidity were noted. The gross mass of the flask containing the solution, and by difference the mass of the bulk solution, was determined using a Mettler XP1002 laboratory balance reading to 0.1 g.

Aliquots of the bulk solution (minimum volume 1.2 mL) were transferred into 2 mL amber ampoules and flame sealed under nitrogen. The integrity of the seal of each ampoule was tested under vacuum.

The resulting batch of 215 sealed ampoules, each containing a minimum of 1.2 mL aliquots of the amino acid solution, was stored at 4 °C.

The content of each amino acid component in the solution calculated from the purity of the source materials (see Table 4) and gravimetric operations used in the solution preparation are given in Table 1.

Table 1: Gravimetric mass fraction content of amino acids in the CCQM-K78.a solution

Amino acid	Mass fraction (w) [µg/g]	u(w) µg/g
Phe	487.4	0.5
Leu	199.5	0.4
Ile	215.0	0.5
Pro	46.9	0.05

The gravimetric values were consistent with the target concentration range for each analyte.

The assigned gravimetric value for leucine in the comparison solution was corrected for the contribution from leucine impurity in the isoleucine source material.

In each case the final uncertainty in the assigned value is dominated by the uncertainty in the purity assignment of the source material while the contribution from the combined uncertainty associated with gravimetric operations is negligible.

Homogeneity Assessment of Study Material

The homogeneity of the candidate material was investigated using high-performance liquid-chromatography coupled in sequence with a diode array detector (DAD) and charged aerosol detector (CAD). The CAD was able to detect and quantify each of the amino acid components and in addition the DAD was used to detect and quantify Phe (UV @ 260 nm). A Shiseido Capcell PAK MG (250 mm x 4.6 mm; 5µm) HPLC column with an isocratic elution using 89% mobile phase A (0.1 % aqueous formic acid) and 11% mobile phase B (0.1 % formic acid in methanol) was used to achieve baseline separation of the four amino acid components. Ten vials selected at a regular interval from the filling sequence were used for the homogeneity study. For quantification studies using the CAD detector, analysis of the Phe, Leu and Ile content was carried out in triplicate for each vial after 1:10 dilution of a 100 µl sample aliquot. For the analysis of the content of the component present at the lowest level, Pro, triplicate analysis of a separate 1:4 dilution of the same aliquot volume was used.

Individual data from the homogeneity study were normalised with respect to the gravimetric concentration to establish repeatability conditions. The data sets were tested for outliers by use of the Hampel, Grubbs and Nalimov-tests. No outliers were identified according to these criteria.

Plots of the normalised results following the vial filling sequence for each amino acid are presented in Appendix A. The results of the ANOVA of the homogeneity study data for each component are summarised in Table 1. No differences in the within- and between-sample variances was detected by F-test at the 95 % confidence level for each amino acid. The material was thus homogeneous by this criteria. An estimate of s_{bb} could not be calculated for isoleucine, leucine and proline because in each case the observed $MS_{between}$ was smaller than MS_{within}. Therefore an upper limit estimate for the potential relative uncertainty contribution due to inhomogeneity corresponding to the u^*_{bb} of 0.66 %, 0.99 %, and 0.54 % was assigned respectively for Ile, Leu and Pro. For phenylalanine the $MS_{between}$ was greater than MS_{within} and s_{bb} could be calculated directly as 0.46%.

Table 2. Results of the homogeneity assessment for CCQM-K78.a candidate material

ANOVA Estimate	Phe	Leu	Ile	Pro
Within-unit, $s_{wb\ (\%)}$:	1.10	2.02	3.05	1.64
Between-unit, s_{bb} (%):	0.47	–[1]	–[1]	–[1]
u^*_{bb} (%)	0.36	0.66	0.99	0.54
u_{bb} (%)/s_{bb} (%)[2]	**0.47**	**0.66**	**0.99**	**0.54**
F	1.549	0.659	0.657	0.484
F_{crit}	2.393	2.393	2.393	2.423

[1] Not calculable because $MS_{between} < MS_{within}$

[2] Higher value (u^*_{bb} or s_{bb}) was taken as uncertainty estimate for potential inhomogeneity

Stability Assessment of Study Material

An isochronous accelerated stability study of the amino acid content was performed using as a reference storage in the dark at -4 °C and test storage temperatures of 22 °C (dark), 22 °C (ambient light) and 40 °C (dark). Assigned sample units were transferred from the study temperatures to the reference storage every two weeks over an eight week period. Sample units were selected using a stratified sampling scheme from each quartile of the 215 units of candidate material. The study required two units stored throughout at the reference temperature to establish the reference stability values and twelve additional units for each of the study conditions.

An LC-DAD-CAD method was again used to quantify the amino acid content, with the individual amino acid content of each sample assigned using external calibration. Two solutions, corresponding to a 1:20 dilution, were gravimetrically prepared for each vial as follows: transfer of aliquots of approximately 50 mg of each solution into LC vials for accurate mass determination on a Mettler MX-5 ultrabalance. The volume was made up to approximately 1000 mL with ultrapure water and reweighed. The data for proline, isoleucine, leucine and phenylalanine obtained by analysis of each solution by LC-CAD were then used for the assessment of the stability of each analyte in the solution.

The mass fraction content data for each component were normalised with respect to the average mass fraction of the two reference samples stored at 4°C from week 0. The results were plotted according to increasing storage time for each condition and the slopes of each plotline were used to test the significance at a 95 % confidence level of the observed data for evidence of instability of the mass fraction of each amino acid in the solution under each storage condition.

No significant trends were observed in the stability of the mass fraction content of each amino acid component under the three test conditions. As no significant degradation was observed under the conditions applied it was concluded that no special precautions regarding temperature control during shipment and storage of the material in the course of the comparison were required. The uncertainty contribution due to the stability of each amino acid in solution was therefore assumed to be negligible.

For information, normalised plots of the results obtained for the stability of each amino acid component under storage for up to eight weeks at 40 °C are shown in Appendix B.

PARTICIPANTS, INSTRUCTIONS AND SAMPLE DISTRIBUTION

The call for participation was distributed in May 2016 with the intent to distribute samples in November 2016, for submission of results in March 2017, and an initial discussion of results at the April 2017 OAWG meeting. See the comparison Timeline above (page 3). Appendix C reproduces the combined Call for Participation and Protocol that was circulated to the OAWG membership.

Table 3 identifies the fifteen institutions registered to participate in CCQM-K78.a in addition to BIPM, the coordinating laboratory.

Table 3: Institutions Registered for CCQM-K78.a

NMI or DI	Acronym	Country	Contact
National Research Council of Canada	NRC	Canada	Jeremy Melanson jeremy.melanson@nrc-cnrc.gc.ca
National Institute of Metrology, China	NIMC	China	Can Quan quancan@nim.ac.cn
Laboratoire National de Métrologie et d'Essais	LNE	France	Vincent Delatour Vincent.Delatour@lne.fr
Physikalisch-Technische Bundesanstalt	PTB	Germany	Ruediger Ohlendorf Ruediger.Ohlendorf@ptb.de
National Chemical Metrology Laboratory	EXHM	Greece	Charalampos Alexopoulos x.alexopoulos@gcsl.gr
Government Laboratory of Hong Kong	GLHK	Hong Kong	Dr. Kelly WY Chan wychan2@govtlab.gov.hk
National Metrology Institute of Japan	NMIJ	Japan	Taichi Yamazaki t-yamazaki@aist.go.jp
D.I. Mendeleev Institute for Metrology	VNIIM	Russia	Anton Konopelko a.l.konopelko@vniim.ru
Health Sciences Authority	HSA	Singapore	Liu Qinde LIU_Qinde@hsa.gov.sg
National Metrology Institute of South Africa	NMISA	South Africa	Desiree Prevoo-Franzsen DPrevoo@nmisa.org
Korea Research Institute of Standards and Science	KRISS	South Korea	Byungjoo Kim byungjoo@kriss.re.kr
National Institute of Metrology (Thailand)	NIMT	Thailand	Jintana Nammoonnoy jintana@nimt.or.th
National Metrology Institute of Turkey	UME	Turkey	Ahmet Gören ahmetceyhan.goren@tubitak.gov.tr
LGC Limited	LGC	UK	John Warren John.Warren@lgcgroup.com
National Institute of Standards and Technology	NIST	USA	Karen Phinney karen.phinney@nist.gov

Four ampoules of the CCQM-K78.a comparison material were shipped by the coordinating laboratory to each participant. One ampoule was provided for method development purposes, and participants were requested to report a value derived by the combination of data obtained using at least one aliquot from each of the remaining three vials. Participants were requested to report a single estimate for the mass fraction of the amino acid components in units of μg/g

In addition to the quantitative results, participants were required to describe their analytical methods, their approach to uncertainty estimation, and the Core Competencies they felt were demonstrated in this study. Appendices E, F, and G reproduce the relevant report forms.

Calibration Materials

Each comparison participant was provided separately by the coordinating laboratory with a 500 mg sample of each of the four high purity amino acids used to prepare the CCQM-K78.a material and with the characterization data and property values assigned to each material by the BIPM. Participants were required to use these materials as their primary source material for the calibration of their analytical method(s) for the value assignment of the CCQM-K78.a material. They were also required to use the assigned property values in calculations and value assignments for their CCQM-K78.a result.

The pure substance amino acid source materials used in the preparation of the comparison solution were purchased from a commercial supplier. Each material was described as being of pharmaceutical grade and was not subject to further treatment. The mass fraction content of each amino acid was assigned by qNMR and the value obtained was checked for its consistency with supporting evidence (water content, elemental analysis, LC-MS/MS check for related structure impurities).

The mass fraction content assigned to the source materials by qNMR are given in Table 4.

Table 4: Mass fraction purity of amino acids used to prepare CCQM-K78.a

Amino acid	BIPM Ref	Mass fraction (mg/g, by qNMR)
Phe	OGO.087b	999 ± 1
Leu	OGO.084b	995 ± 2
Ile	OGO.089b	994 ± 2
Pro	OGO.083c	999 ± 1

The isoleucine source material contained small levels of leucine (1.5 mg/g) and valine (1.5 mg/g).

The leucine source material contained valine (1.5 mg/g) as the sole significant amino acid impurity.

RESULTS

Results for each of the four component amino acids were received from each of the sixteen institutions (fifteen registered participants plus the BIPM) that received samples.

In addition to a result for the CCQM-K78.a comparison, three participating institutes (LGC, NIMC and UME) reported a separate result, obtained using a method independent of that used to assign their key comparison result, in the parallel pilot study CCQM-P121.a.

The results reported by each participant for the determination of amino acid content of the comparison material are summarized in Tables 5 and 6 and presented graphically in Figures 3, 4, 5 and 6.

Table 5: Results for Phe and Ile

	Phe (µg/g)				*Ile (µg/g)*			
NMI	x	$u(x)$	$u_{rel}(x)$ (%)	$U(x)$	x	$u(x)$	$u_{rel}(x)$ (%)	$U(x)$
BIPM	486.3	7.2	1.49	14.5	212.8	3.6	1.70	7.2
EXHM	492.2	4.6	0.93	9.2	213.6	2.6	1.22	5.2
GLHK	489	11	2.25	21	215.6	3.2	1.48	6.3
HSA	483.6	5.8	1.21	11.7	214.7	2.2	1.02	4.4
KRISS	486.0	4.2	0.86	9.6	215.6	1.2	0.56	3.0
LGC	488.3	1.4	0.29	2.8	214.9	0.86	0.40	1.7
LNE	486.6	4.1	0.84	8.2	214.8	1.7	0.79	3.5
NIMC	488.53	2.5	0.50	4.9	215.72	1.8	0.85	3.7
NIMT	494.7	3.2	0.65	6.6	217.9	4.0	1.82	7.8
NIST	492.2	5.4	1.10	11.0	220.3	4.6	2.09	9.2
NMIJ	487.9	1.9	0.39	3.8	214.6	0.9	0.42	1.8
NMISA	488	7.2	1.48	14.0	208.0	5.3	2.55	11
NRC	489.1	1.8	0.37	3.6	215.3	0.6	0.26	1.1
PTB	487.8	2.5	0.51	5.0	215.20	1.10	0.51	2.3
UME	482.59	3.6	0.75	7.2	218.98	3.50	1.60	7.0
VNIIM	495	6.0	1.21	12.0	193.3	3.1	1.60	6.2
n	16				15			
$\bar{x}$	**488.6**				**215.2**			
s	3.5				2.8			
CV	0.72				1.3			
Gravimetric	**487.4**	**0.5**	**0.1**	**1.0**	**215**	**0.43**	**0.2**	**0.87**

n = number of results used for KCRV; $\bar{x}$ = mean; s = standard deviation; CV = $100 \cdot s/\bar{x}$;
Gravimetric = value from preparation
Values shown in red were not included for the calculation of the summary statistics

Table 6: Results for Leu and Pro

	Leu (µg/g)				*Pro (µg/g)*			
NMI	*x*	*u*(*x*)	$u_{rel}(x)$ (%)	*U*(*x*)	*x*	*u*(*x*)	$u_{rel}(x)$ (%)	*U*(*x*)
BIPM	199.4	3.6	1.80	7.2	47.4	0.83	1.75	1.7
EXHM	200.6	3.2	1.60	6.4	49.87	0.79	1.58	1.55
GLHK	199.5	3.1	1.55	6.1	46.6	1.2	2.58	2.3
HSA	198.1	2.1	1.07	4.4	46.7	0.44	0.94	0.88
KRISS	199.5	2.1	1.05	4.8	47.4	0.4	0.92	1.0
LGC	199.2	1.4	0.70	2.8	46.8	0.4	0.90	0.8
LNE	203.0	1.5	0.74	3.1	46.7	0.5	1.07	1.0
NIMC	199.9	1.56	0.78	3.1	47.0	0.2	0.51	0.5
NIMT	190.8	3.91	2.05	7.8	50.4	0.9	1.81	1.8
NIST	206.0	4.6	2.23	9.2	47.8	0.4	0.86	0.8
NMIJ	199.8	0.9	0.45	1.8	46.7	0.5	1.07	1.0
NMISA	187.5	4	2.13	8	47.2	1.4	2.97	2.9
NRC	199.6	0.62	0.31	1.2	46.6	0.1	0.20	0.2
PTB	199.7	1.1	0.55	2.1	47.1	0.26	0.55	0.54
UME	200.6	1.62	0.81	3.24	47.2	0.8	1.71	1.6
VNIIM	186.7	3.4	1.82	6.7	46.0	0.6	1.30	1.1
n	13				14			
$\bar{x}$	**200.4**				**46.94**			
s	2.03				0.45			
CV	1.01				0.96			
Gravimetric	**199.5**	**0.4**	**0.2**	**0.8**	**46.9**	**0.04**	**0.09**	**0.09**

n = number of results used for KCRV; $\bar{x}$ = mean; *s* = standard deviation; CV = $100 \cdot s/\bar{x}$;
Gravimetric = value from preparation;
Results shown in red font were not used for the calculation of the summary statistics

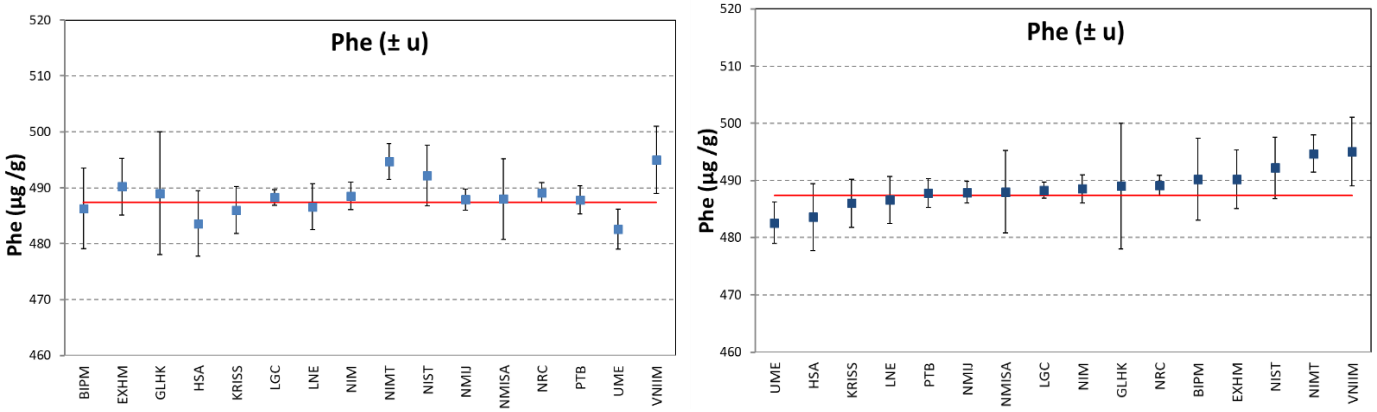


Figure 3: Reported Results for Phe

Reported results for Phe displayed sorted alphabetically by NMI acronym and by increasing reported value. Dots represent the reported value, *x*; the bars their associated standard uncertainty, *u*(*x*). The red reference line corresponds to the gravimetric value calculated for the amino acid content in the solution by the coordinating laboratory.

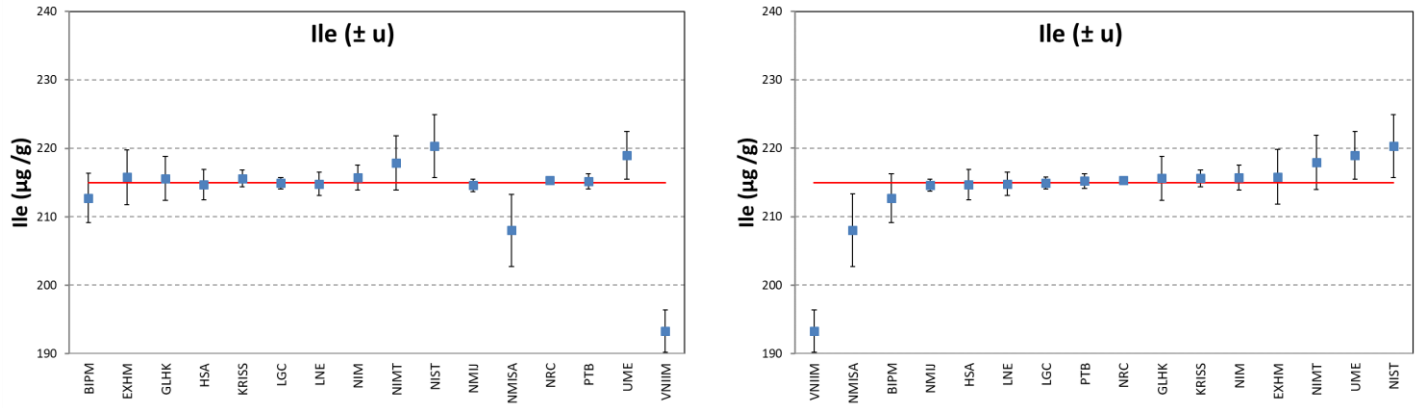


Figure 4: Reported Results for Ile

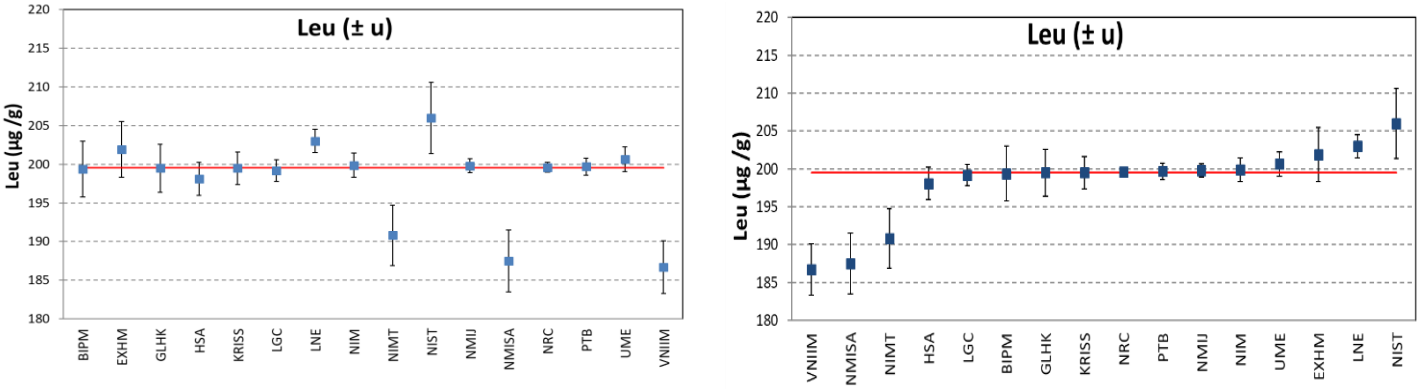


Figure 5: Reported Results for Leu

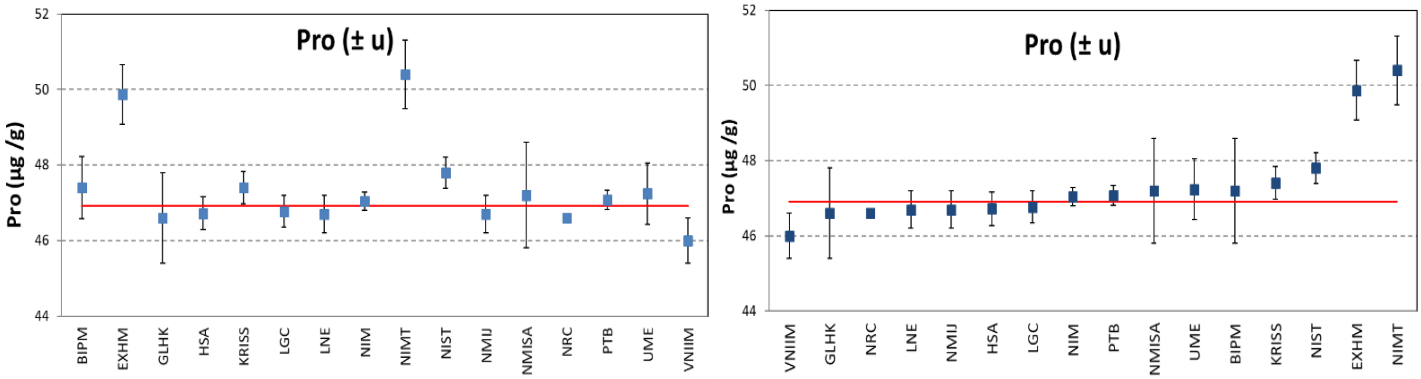


Figure 6: Reported Results for Pro

Panels display the reported results for Ile, Leu and Pro; sorted alphabetically by NMI acronym and by increasing reported value. Dots represent the reported value, x; bars their standard uncertainty, $u(x)$. The red reference line corresponds to the gravimetric value calculated for the amino acid content by the coordinating laboratory.

DISCUSSION

Methods

Appendix D provides a one page tabulated summary of the approaches used by all participants to assign the mass fraction content of each material. Fuller detail of the analytical methods used by the participants, including sample preparation, analytical technique, internal standards and quantification approach are given in Appendix E. An overview of the participants' reported approaches to estimating uncertainty are provided in Appendix F.

Thirteen of the sixteen participants used an IDMS-based method as the sole or as a contributing method to their result assignment. One participant, unable to obtain labelled standards in time for the comparison, used LC-MS/MS with L-Trp as internal standard in place of the corresponding labeled compound.

Two participants used LC-FLD and two used GC-IDMS based methods as the basis of their value assignment. One participant used LC-UV/FLD as a check method of their value assignment by LC-IDMS/MS. Three participants also used an LC-UV method as an additional method for quantification of the Phe content and one participant used an LC-UV method via derivatization with ninhydrin to quantify each of the amino acid components.

Participants were requested to report the mass fraction content in units of μg/g for each of the amino acids in the solution based on measurements for at least one subsample from each of three of the supplied ampoules of the CCQM-K78.a solution (i.e., at least three independent replicates). In addition to the quantitative results, participants were required to outline their analytical methods, their approach to uncertainty estimation, and to complete a form describing the Core Competencies claimed to have been demonstrated in this study.

The coordinating laboratory provided primary calibrator materials, each with an assigned mass fraction purity value, for each participant to use. As these materials provided a harmonized source of traceability for the comparison results, unlike the usual practice in other key comparisons, participants were not required to independently value-assign the materials or use their own source of traceable amino acid calibrator. Nevertheless several participants also reported value assignment of the comparison solution using an independent amino acid calibrator in addition to the material provided by the coordinating laboratory. The values assigned to the CCQM-K78.a material using separate amino acid calibration sources were all in good agreement with results obtained using the calibrators supplied by the coordinating laboratory.

The predominant quantification method used by the comparison participants involved variants on double IDMS-based approaches – either GC-IDMS, LC-IDMS or LC-IDMS/MS. Both selected ion monitoring (SIM) and multiple reaction monitoring (MRM) modes were used to generate the ion used for quantification. The participants using SIM quantification obtained results with a smaller uncertainty with no decrease in trueness relative to the KCRV value compared to the results reported by participants that were based on MRM quantification.

It was observed that every participant using double IDMS approaches involving a matched amino acid/labelled amino acid pair obtained results consistent with the KCRV and with the gravimetric values for amino acid content. However when the labelled standard and amino acid subject to quantification were not structurally identical, "IDMS" based methods did in some cases provide results that deviated significantly from the reference values.

Three participants used LC-FLD and three used an LC-UV method for the independent quantification of the Phe content only. Another used an LC-UV method with derivatization of amino acids with ninhydrin to quantify each amino acid component.

Further detail of analytical methods used by the participants, including sample preparation, analytical technique and quantification approach are summarized in Appendix E. The participant approaches to estimating the measurement uncertainty of their results are provided in Appendix F.

For phenylalanine all results were consistent with the gravimetric value within their associated expanded uncertainties. For the other analytes a small number of participants obtained results in each case which were not consistent with the gravimetric values within their stated expanded uncertainty. Every participant using an IDMS-based method for which they had available a labelled version of the amino acid subject to quantification obtained results that were consistent with the KCRV within their associated uncertainty.

KEY COMPARISON REFERENCE VALUE (KCRV)

The documents CCQM/11-18[5] and CCQM/13-22 *Guidance note: Estimation of a consensus KCRV and associated Degrees of Equivalence*,[6] describe recommended best practice for the choice of the appropriate estimators for a KCRV, depending on the range of participant results and their degree of consistency taking into account their associated measurement uncertainty.

After the discussion of the results at the OAWG meetings in April and September 2017 and with the agreement of the participants concerned, reported values for an amino acid component that did not agree with the gravimetric value within their expanded uncertainty were excluded from inclusion in the KCRV calculations. No results of the sixteen reported for Phe, three of sixteen for Leu, one of sixteen for Ile and two of sixteen for Pro were excluded from the KCRV calculation based on this requirement. Where results were excluded for an individual NMI, the identified cause of the lack of agreement with the KCRV is described in comments appended to the NMI's core competency claim in Table 10 below.

There was also a variation in the magnitude of the uncertainty reported by participants, to the extent that the results retained for use in each KCRV estimation, while consistent with the gravimetric values for each analyte, were not fully consistent with each other within their reported uncertainties. This is demonstrated graphically in Figure 7. As discussed in the Methods section above, this was ascribed in part to the choice by participants between the use of SIM- and MRM-based ion quantification when implementing double-IDMS based methods, the former approach generally providing results with smaller associated uncertainty.

It was proposed by the coordinating laboratory and agreed by the participants that, in compliance with the recommendations in Guidance note CCQM/13-22, it was appropriate to use the uncertainty information provided with the participant results in the estimation of the KCRV while making allowance for some

additional excess variance in the calculation. It was also agreed that this could be done using a method which assigned greater weight to results having smaller uncertainties without concern that the resulting KCRV would be unduly influenced toward these individual results.

In addition to the gravimetric value, the product of three result estimators - the mean, median and the DerSimonian-Laird variance-weighted mean (DSL-mean)[7,8] for each data set are shown in Table 7. All three estimators are in agreement with each other and with the gravimetric value: however only the DSL-mean takes into account the uncertainties of each participant result while introducing sufficient excess variance to allow for their observed dispersion. On the basis of complying pragmatically with best-practice recommendations of CCQM, the DSL-mean of the result set and its associated uncertainty was selected as respectively the KCRV and u(KCRV) for the mass fraction content of each analyte in CCQM-K78.a.

Table 7: Estimators for amino acid content in the CCQM-K78.a material

	Phe (µg/g)			**Leu (µg/g)**			**Ile (µg/g)**			**Pro (µg/g)**		
Estimator	X	$u(X)$	$U_{95}(X)$	X	$u(X)$	$U_{95}(X)$	X	$u(X)$	$U_{95}(X)$	X	$u(X)$	$U_{95}(X)$
Mean	488.6	0.87	1.9	200.4	0.56	1.22	215.2	0.72	1.55	46.9	0.12	0.26
Median	488.2	0.79	1.7	199.7	0.15	0.34	215.2	0.25	0.54	46.9	0.15	0.32
DSL-Mean	**488.5**	**0.49**	**1.1**	**199.9**	**0.26**	**0.57**	**215.1**	**0.15**	**0.31**	**46.9**	**0.13**	**0.28**
Gravimetric	487.4	0.5	1.0	199.5	0.4	0.8	215.0	0.43	0.87	46.9	0.04	0.08

$U_{95}(X) = t_s \cdot u(X)$, where t_s is the appropriate two-tailed Student's t critical value for 95 % coverage.

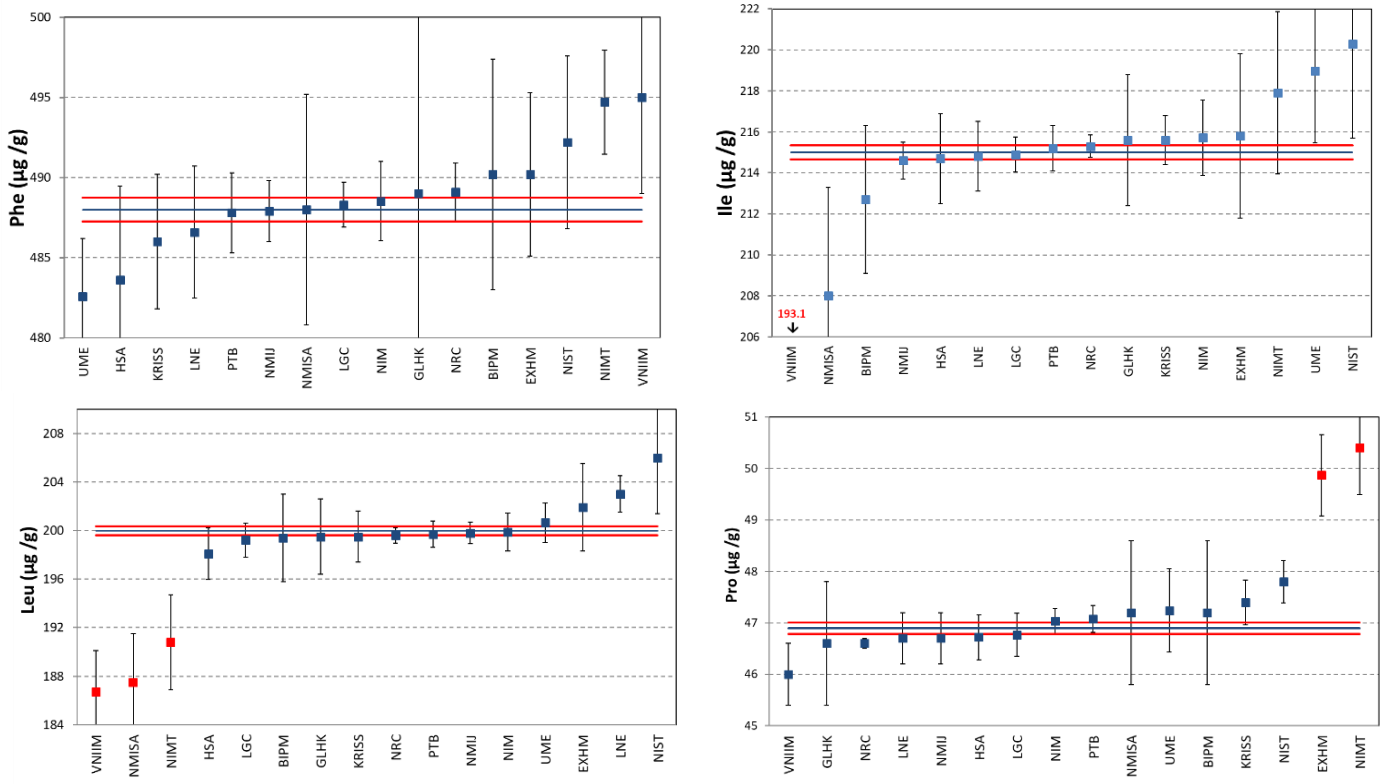


Figure 7: Participant results relative to KCRVs

Figure 7 displays plots of the DSL-Mean as the KCRV and the associated uncertainty of the assigned value relative to the reported participant results for each amino acid. The blue horizontal line denotes the KCRV. The bracketing red lines denote the KCRV plus/minus its standard uncertainty.
Results are sorted by increasing value. The dots represent individual participant reported values, x with the bars their associated standard uncertainties, $u(x)$. The results corresponding to the data points shown in red were not used for the KCRV calculation

DEGREES OF EQUIVALENCE (DoE)

The absolute degrees of equivalence of each result for amino acid content reported by the participants in CCQM-K78.a were estimated as the difference between the value and the KCRV: $d_i = x_i$ – KCRV.

The nominal $k = 2$ expanded uncertainty on the d_i, $U_{k=2}(d_i)$, was estimated as twice the square root of the sum of the squares of the standard uncertainties of the two components:

$$U_{k=2}(d_i) = 2\sqrt{u^2(x_i) + u^2(\text{KCRV})}.$$

The d_i and $U_{k=2}(d_i)$ were calculated as percentages relative to the KCRV:

$\%d_i = 100 \cdot d_i/\text{KCRV}$ and $U_{k=2}(\%d_i) = 100 \cdot U_{k=2}(d_i)/\text{KCRV}$.

Tables 9 and 10 list the numeric values of d_i, $U_{95}(d_i)$, $\%d_i$, and $U_{95}(\%d_i)$ for each amino acid for all participant results.

Figure 8 through 11 are plots of the absolute (primary y-axis) and relative (secondary y-axis) DoE for each participant result with the corresponding KCRV.

Table 8: Degrees of Equivalence with KCRV: Results for Phe and Ile

	Phe (µg/g)				Ile (µg/g)			
NMI	d	$U_{k=2}(d)$	$\%\ d$	$\%U_{k=2}(d)$	d	$U_{k=2}(d)$	$\%\ d$	$\%U_{k=2}(d)$
BIPM	-2.19	14.52	-0.45	2.97	-2.39	7.25	-1.11	3.37
EXHM	3.70	9.25	0.76	1.89	-1.53	5.21	-0.71	2.42
GLHK	0.50	22.02	0.10	4.51	0.47	6.41	0.22	2.98
HSA	-4.90	11.72	-1.00	2.40	-0.43	4.41	-0.20	2.05
KRISS	-2.50	8.46	-0.51	1.73	0.47	2.42	0.22	1.12
LGC	-0.20	2.96	-0.04	0.61	-0.23	1.74	-0.11	0.81
LNE	-1.90	8.26	-0.39	1.69	-0.33	3.41	-0.15	1.59
NIMC	0.03	5.01	0.01	1.03	0.59	3.69	0.27	1.72
NIMT	6.20	6.53	1.27	1.34	2.77	7.93	1.29	3.68
NIST	3.70	10.84	0.76	2.22	5.17	9.20	2.40	4.28
NMIJ	-0.60	3.92	-0.12	0.80	-0.53	1.82	-0.25	0.85
NMISA	-0.50	14.43	-0.10	2.95	-7.13	10.60	-3.31	4.93
NRC	0.60	3.73	0.12	0.76	0.17	1.14	0.08	0.53
PTB	-0.70	5.09	-0.14	1.04	0.07	2.22	0.03	1.03
UME	-5.91	7.27	-1.21	1.49	3.85	7.01	1.79	3.26
VNIIM	6.50	12.04	1.33	2.46	-21.83	6.21	-10.15	2.89

Table 9: Degrees of Equivalence with KCRV: Results for Leu and Pro

	Leu (µg/g)				Pro (µg/g)			
NMI	d	$U_{k=2}(d)$	% d	$\%U_{k=2}(d)$	d	$U_{k=2}(d)$	% d	$\%U_{k=2}(d)$
BIPM	-0.53	7.20	-0.26	3.61	0.49	1.68	1.05	3.58
EXHM	0.70	6.42	0.35	3.22	2.97	1.60	6.33	3.41
GLHK	-0.40	6.22	-0.20	3.12	-0.30	2.41	-0.64	5.15
HSA	-1.80	4.27	-0.90	2.14	-0.18	0.92	-0.38	1.95
KRISS	-0.40	4.23	-0.20	2.12	0.50	0.91	1.07	1.93
LGC	-0.70	2.85	-0.35	1.43	-0.13	0.88	-0.28	1.87
LNE	3.10	3.04	1.55	1.53	-0.20	1.03	-0.43	2.20
NIMC	-0.03	3.16	-0.01	1.59	0.14	0.55	0.30	1.16
NIMT	-9.10	7.84	-4.56	3.93	3.50	1.84	7.46	3.92
NIST	6.10	9.21	3.06	4.62	0.90	0.86	1.92	1.83
NMIJ	-0.10	1.87	-0.05	0.94	-0.20	1.03	-0.43	2.20
NMISA	-12.40	8.02	-6.21	4.02	0.30	2.81	0.64	5.99
NRC	-0.30	1.35	-0.15	0.67	-0.30	0.32	-0.64	0.68
PTB	-0.20	2.26	-0.10	1.13	0.18	0.58	0.38	1.24
UME	0.74	3.28	0.37	1.64	0.34	1.64	0.72	3.50
VNIIM	-13.20	6.82	-6.62	3.42	-0.90	1.23	-1.92	2.62

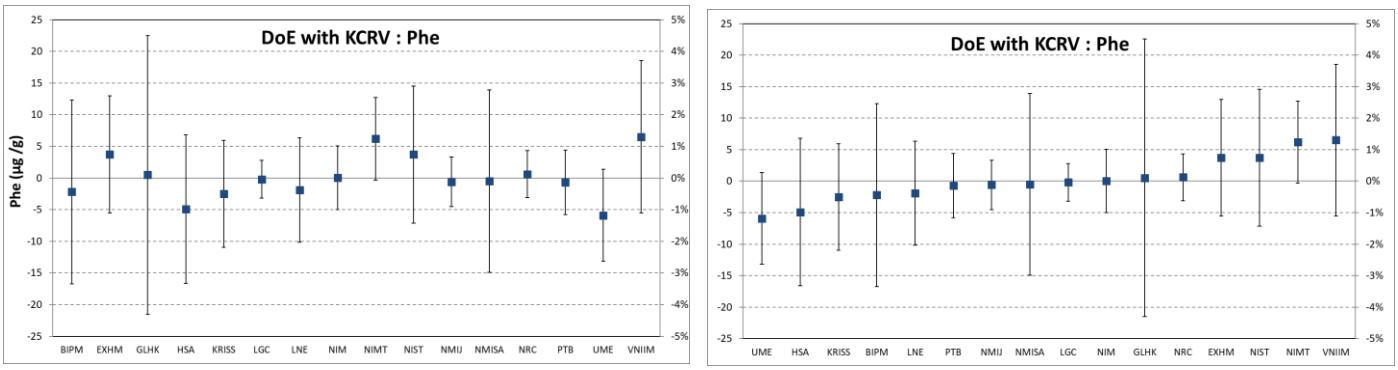


Figure 8: DoE plots for Phe in CCQM-K78.a

Figure 8: Plot of the DoE of the participants results with the KCRV for Phe. Results are listed alphabetically by NMI acronym and separately by increasing reported value. The left hand axis in both panels represents the absolute DoE in units of µg/g, the right-hand axis the DoE relative to the KCRV as percent. Individual data points represent the DoE (d) and the bars their 95% confidence expanded uncertainties $U(d)$.

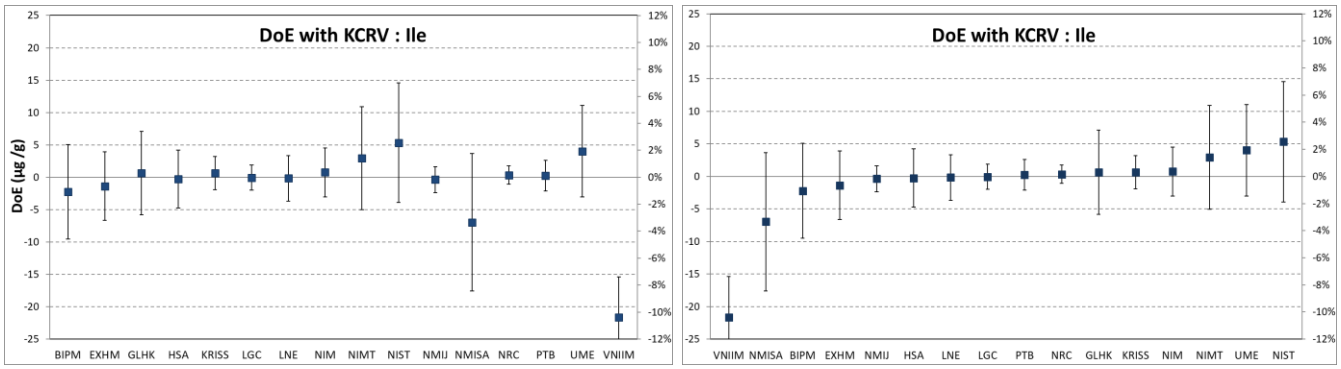


Figure 9: DoE plots for Ile in CCQM-K78.a

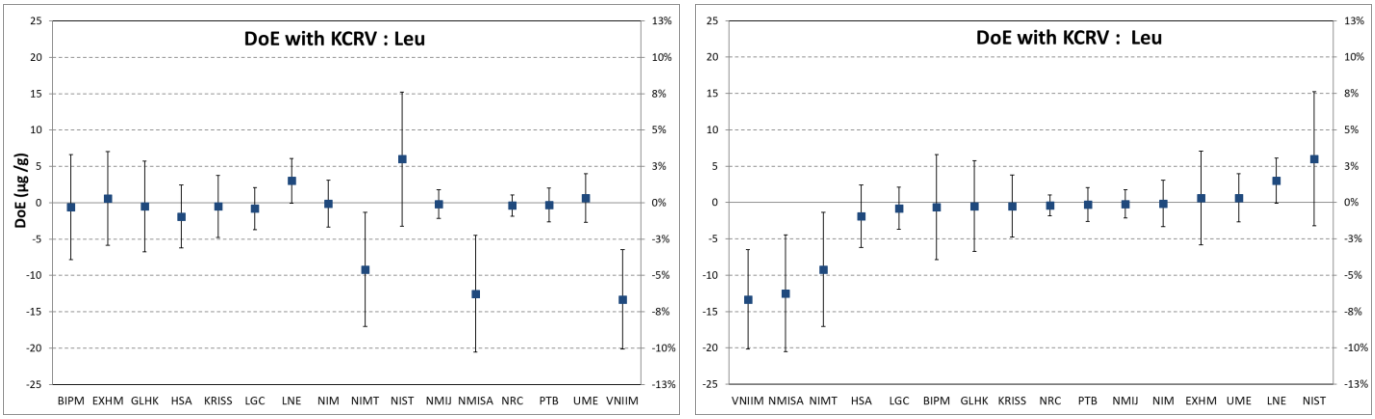


Figure 10: DoE plots for Leu in CCQM-K78.a

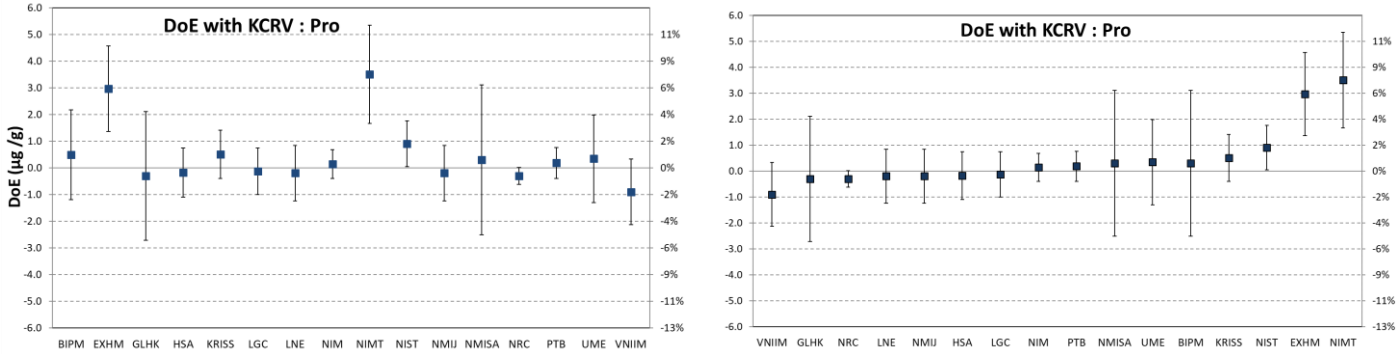


Figure 11: DoE plots for Pro in CCQM-K78.a

Figures 9, 10 and 11 plot the DoE of participant results with respectively the KCRVs for Ile, Leu and Pro. Results are listed alphabetically by NMI acronym and also by increasing reported value. The left hand axis in both panels represents the absolute DoE in units of µg/g, the right-hand axis the DoE relative to the KCRV plotted as percent. Individual data points represent the DoE (d) and the bars their 95% confidence expanded uncertainties $U(d)$.

USE OF CCQM-K78.a IN SUPPORT OF CALIBRATION AND MEASUREMENT CAPABILITY (CMC) CLAIMS

How Far the Light Shines

Successful participation in CCQM-K78.a demonstrates two measurement capabilities related to the assignment of the mass fraction of organic compounds present in the mass fraction range of 50 μg/g - 500 μg/g in a multicomponent aqueous calibration solution, where the molar mass of the analytes are in the range 75 g/mol* to 500 g/mol, the polarity (pK_{ow}) is in excess of -2, and the value assignment of the mass fraction content of the calibrators for each analyte are undertaken separately:

a. confirmation of a value assignment of a polar analyte in an aqueous calibration solution
b. separation and quantification of polar organic components, including those of similar structure and chromatographic behaviour

The HFTLS statement given in this report was modified from that provided in the original Protocol. The original HFTLS provided for a case where a participant only used an LC-UV method to quantify the Phe content of the material. However as all participants reported values for all components this was not relevant to the final HFTLS. In addition the limit of the polar analyte molar mass range was decreased to 75 g/mol from 100 g/mol so that CMCs including the amino acid glycine (molar mass 75.1 g/mol) would be included within the scope of the HFTLS.

It was noted in advance of the comparison that several of the methods for quantification of the amino acid content in solution would likely involve dilution of the comparison material prior to analysis and thus demonstrate the capability for confirmatory mass fraction assignment at levels significantly lower than that nominally covered by the HFTLS. It is recognized that where this is the case satisfactory performance in CCQM-K78.a can be used by an NMI to justify CMC claims for mass fraction assignment at lower levels than indicated by the HFTLS.

Core Competency Statements

Tables 10.a to 10.o list the Core Competencies claimed by the individual participants in CCQM-K78.a. The information in these Tables is as provided by the participants; however in some cases the presentation of some entries has been condensed and standardized. Details of the analytical methods used by each participant in this study are provided in Appendix E.

The agreement of a result with the KCRV was assessed by calculation of the absolute value of the ratio of the DoE (d) of a participant's result and the expanded uncertainty of the DoE [U(d)].

Where $\left|\frac{d}{U(d)}\right|$ exceeds 1 it is highlighted in red in the Core Competency tables. This indicates > 95% probability that the participant's result is not consistent with the KCRV within the stated uncertainties of each. In this case the identified cause of the inconsistency in the result is described in a comment appended to the table.

* The molar mass range covered by the HFTLS, as shown in the original Study Protocol and the Core Competency Claim Tables reproduced elsewhere in this report, was originally stated as 100 g/mol to 500 g/mol. During subsequent discussion of the comparison results within the OAWG it was agreed to decrease the lower limit to 75 g/mol so that standard solutions containing glycine would be included within the scope of HFTLS.

Core Competency Claims by Participant

CCQM-K78.a	*EXHM*	Polar analytes in aqueous solvent
Scope of measurement capabilities: Participation in this comparison demonstrates the laboratory's capabilities in determining the mass fraction in aqueous calibration solution of organics with molar mass of 100 g/mol to 500 g/mol having polarity pK_{ow} > -2 for one or more of the following: a. value assignment of a multi- component solution containing UV- and non-UV active organics at mass fractions of 50 µg/g to 500 µg/g (Phe, Leu, Ile and/or Pro reported) b. separation and quantification using chromatography (LC- or GC-systems)		
• Value assignment of Multicomponent Calibration solution		
Competency		**Specific Information**
Result for Phe content (µg/g)	$\left\|\frac{d}{U(d)}\right\|$ = 0.40	**492.2 ± 9.2**
Result for Leu content (µg/g)	$\left\|\frac{d}{U(d)}\right\|$ = 0.11	**200.6 ± 6.4**
Result for Ile content (µg/g)	$\left\|\frac{d}{U(d)}\right\|$ = 0.29	**213.6 ± 5.2**
Result for Pro content (µg/g)	$\left\|\frac{d}{U(d)}\right\|$ = 1.86	**49.87 ± 1.55***
Identification of analyte(s)	✓	*Retention time, ion ratios*
Extraction and clean up	**N/A**	*none*
Sample concentration adjustment	✓	*1:100 - 1:10000*
Derivatization of analyte(s)	**N/A**	*none*
Analytical system	✓	*LC-MS/MS, LC-UV, LC-FLD*
Gravimetric procedures for preparation of calibration solutions	✓	*(a) 0.02 % - rel. unc. of gravimetric operations in preparation of calibration solutions* *(b) 0.5 % Estimate of uncertainty due to gravimetric operations to the uncertainty of value assignment*
Calibration approach for value-assignment of analyte(s) in matrix	✓	*a) external standard and IDMS* *b) single-point calibration at exact matching* *c) 99.5% Estimate of contribution due to calibration to the uncertainty of the value assignment*
Verification method(s) for value-assignment of analyte(s) in sample	✓	*FL measurements were used to complement the UV values, NIST SRM 2389a was also used to assess the assigned values*

Table 10.a : Core competency claims for CCQM-K78.a - EXHM

* A transcription error occurred in the entry of the result for Pro into the Result Submission form forwarded to the study coordinator. The experimental value obtained by the laboratory was 46.87 ± 1.55 µg/g, consistent with the KCRV.

CCQM-K78.a	*GLHK*	Polar analytes in aqueous solvent
Scope of measurement capabilities: Participation in this comparison demonstrates the laboratory's capabilities in determining the mass fraction in aqueous calibration solution of organics with molar mass of 100 g/mol to 500 g/mol having polarity *pKow* > -2 for one or more of the following: a. value assignment of a multi- component solution containing UV- and non-UV active organics at mass fractions of 50 µg/g to 500 µg/g (Phe, Leu, Ile and/or Pro reported) b. separation and quantification using chromatography (LC- or GC-systems)		
• Value assignment of Multicomponent Calibration solution		
Competency	**✓,×, or N/A**	**Specific Information**
Result for Phe content (µg/g)	$\left\|\frac{d}{U(d)}\right\|$ **= 0.02**	**489 ± 21**
Result for Leu content (µg/g)	$\left\|\frac{d}{U(d)}\right\|$ **= 0.06**	**199.5 ± 6.1**
Result for Ile content (µg/g)	$\left\|\frac{d}{U(d)}\right\|$ **= 0.07**	**215.6 ± 6.3**
Result for Pro content (µg/g)	$\left\|\frac{d}{U(d)}\right\|$ **= 0.12**	**46.6 ± 2.3**
Identification of analyte(s)	✓	*1) For LC-MSMS, a) retention time, b) molecular weight and c) mass spectrometry ion ratio* *2) For HPLC-FD, identification by a) retention time and b) excitation and fluorescence wavelengths.*
Extraction clean up of analyte(s)	N/A	
Sample concentration adjustment	N/A	
Derivatization of analyte(s) to detectable/measurable form	✓	*HPLC-FD, the amino acids derivatized using AQC (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate)*
Analytical system	✓	*1) LC-MSMS* *2) HPLC-FD*
Gravimetric procedures for preparation of calibration solutions	✓	*1) For LC-MSMS, contribution of uncertainty due to gravimetry to the combined uncertainty: Phe: 0.7%; Leu:2.0%; Ile: 2.8%; Pro: 1.7%* *2) For HPLC-FD, contribution of uncertainty due to gravimetry to the combined uncertainty: Phe: 0.8%; Leu:2.1%; Ile: 2.1%; Pro: 2.6%*
Calibration approach for value-assignment of analyte(s) in matrix	✓	*1) For LC-MSMS, quantification by IDMS and single-point calibration. Contribution of uncertainty to the combined uncertainty: Phe: 43.0%; Leu:14.3%; Ile: 15.5%; Pro: 23.6%* *2) For HPLC-FD, IS quantification and calibration. Contribution to combined uncertainty: Phe: 20.7%; Leu: 10.6%; Ile: 10 %;Pro: 13.6 %*
Verification method(s) for value-assignment of analyte(s)	✓	*Results of LC-MSMS and HPLC-FD are compared and verified with each other.*

Table 10.b : Core competency claims for CCQM-K78.a - GLHK

CCQM-K78.a	*HSA*	Polar analytes in aqueous solvent
Scope of measurement capabilities: Participation in this comparison demonstrates the laboratory's capabilities in determining the mass fraction in aqueous calibration solution of organics with molar mass of 75 g/mol to 500 g/mol having polarity *pKow* > -2 for one or more of the following: a. value assignment of a multi- component solution containing UV- and non-UV active organics at mass fractions of 50 µg/g to 500 µg/g (Phe, Leu, Ile and/or Pro reported) b. separation and quantification using chromatography (LC- or GC-systems)		
• Value assignment of Multicomponent Calibration solution		
Competency		**Specific Information**
Result for Phe content (µg/g)	$\left\|\frac{d}{U(d)}\right\|$ **= 0.42**	**483.6 ± 11.7**
Result for Leu content (µg/g)	$\left\|\frac{d}{U(d)}\right\|$ **= 0.42**	**198.1 ± 4.4**
Result for Ile content (µg/g)	$\left\|\frac{d}{U(d)}\right\|$ **= 0.10**	**214.7 ± 4.4**
Result for Pro content (µg/g)	$\left\|\frac{d}{U(d)}\right\|$ **= 0.20**	**46.7 ± 0.88**
Identification of analyte(s)	✓	LC-MS/MS method was used to identify the analytes in the sample by comparing the retention time and the m/z of the parent and daughter ions with CRMs.
Analytical system	✓	LC-MS/MS
Gravimetric procedures for preparation of calibration solutions	✓	(c) The relative uncertainties of gravimetric operations used in the preparation of calibration solutions ranged from 0.22% to 0.48%. (d) The contribution of uncertainty due to gravimetric operations to the uncertainty of the value assignment ranged from 3% to 26%.
Calibration approach for value-assignment of analyte(s) in matrix	✓	(a) IDMS method was used. (b) Four-point calibration curve was used. (c) The uncertainty due to calibration to the overall uncertainty ranged from 14% to 47%.
Verification method(s) for value-assignment of analyte(s) in sample	✓	Column 1 was an Agilent ZORBAX Eclipse AAA, 4.6 x 150 mm, 5 µm, and Column 2 was a Phenomenex Kinetex 2.6u F5 100A, 4.6 x 150 mm, 2.6 µm. Results from Column 1 were reported. Results from Column 2 were used to confirm and to estimate the MU. Pure substance CRMs of L-Phe (HRM-1014A), L-Ile (HRM-1013A), and L-Pro (HRM-1007A) from HSA, and pure substance CRM of L-Leu (CRM 6012-a) from NMIJ used as quality controls. The quality control solutions prepared by dissolving the CRMs in 0.01 mol/L HCl and measured with the comparison sample. Found to be within 1.5% of the target values.

Table 10.c : Core competency claims for CCQM-K78.a – HSA

CCQM-K78.a	*KRISS*	Polar analytes in aqueous solvent
Scope of comparison: Participation in this comparison demonstrates the laboratory's capabilities in determining the mass fraction in aqueous calibration solution of organics with molar mass of 100 g/mol to 500 g/mol having polarity *p*Kow > -2 for: **a. value assignment of a multi- component solution containing UV- and non-UV active organics at mass fractions of 50 ug/g to 500 ug/g (Phe, Leu, Ile and/or Pro reported)** **b. separation and quantification using chromatography (LC- or GC-systems)**		
• Value assignment of Multicomponent Calibration solution		
Competency		**Specific Information**
Result for Phe content (µg/g)	$\left\|\frac{d}{U(d)}\right\|$ = 0.30	**486.0 ± 9.6**
Result for Leu content (µg/g)	$\left\|\frac{d}{U(d)}\right\|$ = 0.09	**199.5 ± 4.8**
Result for Ile content (µg/g)	$\left\|\frac{d}{U(d)}\right\|$ = 0.19	**215.6 ± 3.0**
Result for Pro content (µg/g)	$\left\|\frac{d}{U(d)}\right\|$ = 0.55	**47.4 ± 1.0**
Identification of analyte(s)		*Retention time and mass spec ion ratios by ID-LC-MS/MS*
Extraction of analyte(s) from matrix	**N/A**	
Cleanup of analyte(s) from other interfering matrix components	**N/A**	
Conversion of analyte(s) of interest to detectable/measurable form	**N/A**	
Analytical system		*LC-MS/MS in MRM mode*
Calibration approach for value-assignment of analyte(s) in matrix		*ID-MS with exact matching single-point calibration*
Verification method(s) for value-assignment of analyte(s) in sample		*Additional LC-UV analysis for Phe, and the result: (487.2 ± 6.1) ug/g*

Table 10.d : Core competency claims for CCQM-K78.a – KRISS

CCQM-K78.a	*LGC*	Polar analytes in aqueous solvent
Scope of measurement capabilities: Participation in this comparison demonstrates the laboratory's capabilities in determining the mass fraction in aqueous calibration solution of organics with molar mass of 100 g/mol to 500 g/mol having polarity *pK*ow > -2 for one or more of the following: a. value assignment of a multi- component solution containing UV- and non-UV active organics at mass fractions of 50 ug/g to 500 ug/g (Phe, Leu, Ile and/or Pro reported) b. separation and quantification using chromatography (LC- or GC-systems)		
• Value assignment of Multicomponent Calibration solution		
Competency		**Specific Information**
Result for Phe content (µg/g)	$\left\|\frac{d}{U(d)}\right\|$ **= 0.07**	**488.3 ± 2.8**
Result for Leu content (µg/g)	$\left\|\frac{d}{U(d)}\right\|$ **= 0.25**	**199.2 ± 2.8**
Result for Ile content (µg/g)	$\left\|\frac{d}{U(d)}\right\|$ **= 0.13**	**214.9 ± 1.7**
Result for Pro content (µg/g)	$\left\|\frac{d}{U(d)}\right\|$ **= 0.15**	**46.8 ± 0.8**
Identification of analyte(s)	✓	*Retention time, ion ratios, NIST library match*
Extraction and clean up of analyte(s)	×	
Sample concentration adjustment	✓	*Gravimetric dilution (1+61)*
Conversion of analyte(s) of interest to detectable/measurable form	✓	*Derivatization with MTBSTFA*
Analytical system	✓	*GC-MS*
Gravimetric procedures for preparation of calibration solutions	✓	*(a) Relative uncertainty of gravimetric operations used in the preparation of calibration solutions: estimated to be less than 0.3% of the uncertainty of the standard preparation* *(b) Estimate of contribution of uncertainty due to gravimetric operations to the uncertainty of the value assignment: estimated to be less than 1% of the total uncertainty of the value assignment*
Calibration approach for value-assignment of analyte(s) in matrix	✓	*a) IDMS* *b) EM-IDMS with bracketing* *c) Estimate of contribution of uncertainty due to calibration to the uncertainty of the value assignment: estimated to be 15% of the total uncertainty of the value assignment*
Verification method(s) for value-assignment of analyte(s) in sample	✓	*Confirmation based on secondary SIM ion*

Table 10.e: Core competency claims for CCQM-K78.a – LGC

CCQM-K78.a	*LNE*	Polar analytes in aqueous solvent
Scope of comparison: Participation in this comparison demonstrates the laboratory's capabilities in determining the mass fraction in aqueous calibration solution of organics with molar mass of 100 g/mol to 500 g/mol having polarity *pK*ow > -2 for: **a. value assignment of a multi- component solution containing UV- and non-UV active organics at mass fractions of 50 ug/g to 500 ug/g (Phe, Leu, Ile and/or Pro reported)** **b. separation and quantification using chromatography (LC- or GC-systems)**		
• Value assignment of Multicomponent Calibration solution		
Competency		**Specific Information**
Result for Phe content (µg/g)	$\left\|\frac{d}{U(d)}\right\|$ **= 0.23**	**486.6 ± 8.2**
Result for Leu content (µg/g)	$\left\|\frac{d}{U(d)}\right\|$ **= 1.02**	**203.0 ± 3.1**
Result for Ile content (µg/g)	$\left\|\frac{d}{U(d)}\right\|$ **= 0.10**	**214.8 ± 3.5**
Result for Pro content (µg/g)	$\left\|\frac{d}{U(d)}\right\|$ **= 0.19**	**46.7 ± 1.0**
Identification of analyte(s)	✓	**Retention Time + Mass + Labelled IS**
Extraction of analyte(s) from matrix	N/A	
Cleanup of analyte(s) from other interfering matrix components (if used)	N/A	
Conversion of analyte(s) of interest to detectable/measurable form (if used)	N/A	
Analytical system	✓	**LC-MS**
Calibration approach for value-assignment of analyte(s) in matrix	✓	**IDMS** **5 point calibration curve**
Verification method(s) for value-assignment of analyte(s) in sample	N/A	

Table 10.f: Core competency claims for CCQM-K78.a – LNE

CCQM-K78.a	*NIM*	Polar analytes in aqueous solvent
Scope of measurement capabilities: Participation in this comparison demonstrates the laboratory's capabilities in determining the mass fraction in aqueous calibration solution of organics with molar mass of 100 g/mol to 500 g/mol having polarity *pK*ow > -2 for one or more of the following: a. value assignment of a multi- component solution containing UV- and non-UV active organics at mass fractions of 50 ug/g to 500 ug/g (Phe, Leu, Ile and/or Pro reported) b. separation and quantification using chromatography (LC- or GC-systems)		
• Value assignment of Multicomponent Calibration solution		
Competency	**✓,×, or N/A**	**Specific Information**
Result for Phe content (µg/g)	$\left\|\frac{d}{U(d)}\right\|$ **= 0.01**	**488.5 ± 4.9**
Result for Leu content (µg/g)	$\left\|\frac{d}{U(d)}\right\|$ **= 0.01**	**199.9 ± 3.1**
Result for Ile content (µg/g)	$\left\|\frac{d}{U(d)}\right\|$ **= 0.16**	**215.7 ± 3.7**
Result for Pro content (µg/g)	$\left\|\frac{d}{U(d)}\right\|$ **= 0.26**	**47.0 ± 0.5**
Identification of analyte(s) in sample	✓	LC-MS/MS was used to verify the $[M+H]^+$ ion and the corresponding daughter ions.
Extraction and clean up of analyte	N/A	
Sample concentration adjustment	✓	1:10 dilution prior to analysis.
Conversion of analyte(s) of interest	N/A	
Analytical system	✓	Shimadzu LC-20AT & waters SQ LC-MS.
Gravimetric procedures for preparation of calibration solutions	✓	(c) Pro was calibrated with BIPM pure substance Other 3 AAs all calibrated with NIMC CRMs. (d) In LC-UV for Phe: the relative uncertainty of gravimetric operations used in the preparation of calibration solutions is about 0.01%; the related linear Regression standard uncertainty is 0.22%, details please see the reporting form. (e) In LCIDMS for all the 4 AAs: relative uncertainty of gravimetric operations used in the preparation of calibration solutions is about 0.01%; the related linear regression standard uncertainty < 0.30%,
Calibration approach for value-assignment of analyte(s) in matrix	✓	Seven-point calibration used for LCUV for quantification of Phe. In LCIDMS, 7 calibration blends with isotope ratios from 0.8 to 1.15 were accurately prepared. Isotope ratio in the sample blends were controlled to be close to 1.0.
Verification method(s) for value-assignment of analyte(s) in sample	✓	A control sample of the four AAs with target concentrations was gravimetrically prepared freshly.

Table 10.g: Core competency claims for CCQM-K78.a – NIMC

CCQM-K78.a	*NIMT*	Polar analytes in aqueous solvent
Scope of measurement capabilities: Participation in this comparison demonstrates the laboratory's capabilities in determining the mass fraction in aqueous calibration solution of organics with molar mass of 100 g/mol to 500 g/mol having polarity *pK*ow > -2 for one or more of the following: a. value assignment of a multi- component solution containing UV- and non-UV active organics at mass fractions of 50 µg/g to 500 µg/g (Phe, Leu, Ile and/or Pro reported) b. separation and quantification using chromatography (LC- or GC-systems)		
• Value assignment of Multicomponent Calibration solution		
Competency	**✓,×, or N/A**	**Specific Information**
Result for Phe content (µg/g)	$\left\|\frac{d}{U(d)}\right\|$ = 0.95	**494.7 ± 6.6**
Result for Leu content (µg/g)	$\left\|\frac{d}{U(d)}\right\|$ = 1.16	**190.8 ± 7.8 ***
Result for Ile content (µg/g)	$\left\|\frac{d}{U(d)}\right\|$ = 0.35	**217.9 ± 7.8**
Result for Pro content (µg/g)	$\left\|\frac{d}{U(d)}\right\|$ = 1.90	**50.4 ± 1.8 ***
Identification of analyte(s) in sample	✓	*LC-UV for Phenylalanine* *LC-MS/MS for Phe, Leu, Ile, and Pro*
Extraction and clean up of analyte(s)	**N/A**	
Sample concentration adjustment	✓	*1. Make a dilution of 1:5 prior LC-UV detection* *2.Make a dilution of 1:500 prior LC-MS/MS detection*
Conversion of analyte(s) of interest to detectable/measurable form	**N/A**	
Analytical system	✓	*1. LC-UV for Phenylalanine* *2. LC-MS/MS for Phe, Leu, Ile, and Pro*
Gravimetric procedures for preparation of calibration solutions	✓	*LC-UV detection* *a) Rel. MU from gravimetric operation of 10% in the preparation of calibration solutions* *b) Rel. MU due to gravimetric operation on the uncertainty of the value assignment 6%* *LC-MS/MS* *a) Rel. MU from gravimetric operation of 20% in the preparation of calibration solutions* *b) Rel MU due to gravimetric operation on the uncertainty of the value assignment 1%*
Calibration approach for value-assignment of analyte(s) in matrix	✓	*1.LC-UV: 5-point external calibration curve* *2.IDMS: 5-point calibration curve*
Verification method(s) for value-assignment of analyte(s) in sample	✓	*NIST SRM 2389a as QC samples*

Table 10.h : Core competency claims for CCQM-K78.a – NIMT

* Deviation from KCRV ascribed to the non-availability of ^{13}C-labelled Leu and Pro. Only ^{13}C-labelled Phe was available for use as a surrogate in IDMS analysis.

CCQM-K78.a	*NIST*	Polar analytes in aqueous solvent
Scope of measurement capabilities: Participation in this comparison demonstrates the laboratory's capabilities in determining the mass fraction in aqueous calibration solution of organics with molar mass of 100 g/mol to 500 g/mol having polarity *pK*ow > -2 for one or more of the following: a. value assignment of a multi- component solution containing UV- and non-UV active organics at mass fractions of 50 ug/g to 500 ug/g (Phe, Leu, Ile and/or Pro reported) b. separation and quantification using chromatography (LC- or GC-systems)		
• Value assignment of Multicomponent Calibration solution		
Competency	**✓,×, or N/A**	**Specific Information**
Result for Phe content (µg/g)	$\left\|\frac{d}{U(d)}\right\|$ **= 0.34**	**492.2 ± 11.0**
Result for Leu content (µg/g)	$\left\|\frac{d}{U(d)}\right\|$ **= 0.66**	**206.0 ± 9.2**
Result for Ile content (µg/g)	$\left\|\frac{d}{U(d)}\right\|$ **= 0.56**	**220.3 ± 9.2**
Result for Pro content (µg/g)	$\left\|\frac{d}{U(d)}\right\|$ **= 1.05**	**47.8 ± 0.8**
Identification of analyte(s) in sample	✓	*Retention time matching with known internal standard and specific precursor/product ion m/z.*
Extraction and clean up of analyte(s)	**N/A**	
Sample concentration adjustment	**N/A**	
Conversion of analyte(s) of interest to detectable/measurable form	**N/A**	
Analytical system	✓	*LC-MS/MS*
Gravimetric procedures for preparation of calibration solutions	✓	*(a) Relative uncertainty of gravimetric operations used in the preparation of calibration solutions Phe 0.01 %, Leu 0.01 %, Iso 0.01 %, Pro 0.01 %* *(b) %Estimate of contribution of uncertainty due to gravimetric operations to the uncertainty of the value assignment Phe 0.42 %, Leu 0.91 %, Iso 0.92 %, Pro 0.37 % [Note: This information should NOT include a contribution from the uncertainty in the purity of the primary calibrant, information provided by the coordinating laboratory]*
Calibration approach for value-assignment of analyte(s) in matrix	✓	*a) IDMS internal standard, using external standard* *b) External 5-point calibration curve with bracketing* *c) Phe 0.60 %, Leu 1.2 %, Ile 1.3 %, Pro 0.53 %*
Verification method(s) for value-assignment of analyte(s) in sample	✓	*Use of Quality Control material - SRM 2389a Amino Acids in 0.1 mol/L Hydrochloric Acid.*

Table 10.i : Core competency claims for CCQM-K78.a – NIST

CCQM-K78.a	*NMIJ*	Polar analytes in aqueous solvent
Scope of measurement capabilities: Participation in this comparison demonstrates the laboratory's capabilities in determining the mass fraction in aqueous calibration solution of organics with molar mass of 100 g/mol to 500 g/mol having polarity *pK*ow > -2 for one or more of the following: a. value assignment of a multi- component solution containing UV- and non-UV active organics at mass fractions of 50 ug/g to 500 ug/g (Phe, Leu, Ile and Pro reported) b. separation and quantification using chromatography (LC- or GC-systems)		
• Value assignment of Multicomponent Calibration solution		
Competency	**✓,×, or N/A**	**Specific Information**
Result for Phe content (µg/g)	$\left\|\frac{d}{U(d)}\right\|$ **= 0.15**	**487.9 ± 3.8**
Result for Leu content (µg/g)	$\left\|\frac{d}{U(d)}\right\|$ **= 0.05**	**199.8 ± 1.8**
Result for Ile content (µg/g)	$\left\|\frac{d}{U(d)}\right\|$ **= 0.29**	**214.6 ± 1.8**
Result for Pro content (µg/g)	$\left\|\frac{d}{U(d)}\right\|$ **= 0.19**	**46.7 ± 1.0**
Identification of analyte(s) in sample	✓	*Retention time, mass spectrum(m/z)*
Extraction and clean up of analyte(s)	**N/A**	**N/A**
Sample concentration adjustment	**N/A**	**N/A**
Conversion of analyte(s) of interest to detectable/measurable form	✓	*Derivatization (with Ninhydrin or OPA)*
Analytical system	✓	*LC-UV, LC-FL*
Gravimetric procedures for preparation of calibration solutions	✓	*(a) Relative uncertainty of gravimetric operations in the preparation of calibration solutions 0.34 % (Phe), 0.21 % (Leu), 0.02 % (Ile) 0.18 % (Pro).* *(b) Contribution of uncertainty due to gravimetric operations to the uncertainty of the value assignment under 0.01 % in all preparations.*
Calibration approach for value-assignment of analyte(s) in matrix	✓	*a) Quantification mode: internal standard* *b) Calibration mode: single-point calibration* *c) Estimate of contribution of uncertainty due to calibration to the uncertainty of the value assignment were 20 % at Phe, 66 % at Leu, 93 % at Ile and 95 % at Pro. And the each relative uncertainties of calibration to the assignment value were 0.18 % at Phe, 0.33 % at Leu, 0.37 % at Ile and 0.85 % at Pro.*
Verification method(s) for value-assignment of analyte(s) in sample	✓	*Measurement by LC-MS and comparison of NMIJ CRMs and BIPM standards as calibrants*

Table 10.j : Core competency claims for CCQM-K78.a - NMIJ

CCQM-K78.a	*NMISA*	Polar analytes in aqueous solvent
Scope of measurement capabilities: Participation in this comparison demonstrates the laboratory's capabilities in determining the mass fraction in aqueous calibration solution of organics with molar mass of 100 g/mol to 500 g/mol having polarity *pK*ow > -2 for one or more of the following: a. value assignment of a multi- component solution containing UV- and non-UV active organics at mass fractions of 50 ug/g to 500 ug/g (Phe, Leu, Ile and/or Pro reported) b. separation and quantification using chromatography (LC- or GC-systems)		
• Value assignment of Multicomponent Calibration solution		
Competency		**Specific Information**
Result for Phe content (µg/g)	$\left\|\frac{d}{U(d)}\right\|$ **= 0.03**	**488.0 ± 14**
Result for Leu content (µg/g)	$\left\|\frac{d}{U(d)}\right\|$ **= 1.55**	**187.5 ± 8 ***
Result for Ile content (µg/g)	$\left\|\frac{d}{U(d)}\right\|$ **= 0.67**	**208.0 ± 11**
Result for Pro content (µg/g)	$\left\|\frac{d}{U(d)}\right\|$ **= 0.11**	**47.2 ± 2.9**
Identification of analyte(s) in sample	✓	Retention time compared to authentic standards, GC-TOF/MS and LC-MS/MS precursor & fragment ions
Extraction and clean up of analyte(s)	**N/A**	
Sample concentration adjustment	✓	Three different dilutions of the samples were prepared; 1:4, 1:19 and 1:49, to quantify the target analytes (at approximately 10 ug/g)
Conversion of analyte(s) of interest to detectable/measurable form	✓	Derivatisation of amino acids with MTBSTFA for GC-TOF/MS analysis
Analytical system	✓	LC-UV, LC-MS/MS and GC-TOF/MS
Gravimetric procedures for preparation of calibration solutions	✓	(a) The relative uncertainty of the calibrant, excluding the purity, between 1% and 1.3%. (b) The gravimetric operations in the preparation of the calibrant contributed 0.6% and 10% to the overall uncertainty, for external calibration and bracketing dIDMS quantification respectively.
Calibration approach for value-assignment of analyte(s) in matrix	✓	LC-UV: External 6 point calibration curve contributes an approximate 3% value assignment uncertainty. GC-TOF/MS and LC-MS/MS: IDMS bracketing (13C ILE used as internal standard for LEU). The ratio of ratios in the sample and calibration blend contributed 23% and 43% to the overall uncertainty estimate, using GC-TOF/MS and LC-MSMS analytical techniques, respectively
Verification method(s) for value-assignment of analyte(s) in sample	✓	LC-UV analysis with AQC derivatisation, results not included in the calculation of the final result or uncertainty estimation, although values agree well.

Table 10.k : Core competency claims for CCQM-K78.a – NMISA

* Deviation from the KCRV for Leu was ascribed to the non-availability of ^{13}C-labelled Leu for IDMS analysis. Good agreement with the DoE was obtained for the assignment of the other three analytes, for which labelled amino acids were obtained.

CCQM-K78.a	*NRC*	Polar analytes in aqueous solvent
Scope of measurement capabilities: Participation in this comparison demonstrates the laboratory's capabilities in determining the mass fraction in aqueous calibration solution of organics with molar mass of 100 g/mol to 500 g/mol having polarity *pK*ow > -2 for one or more of the following: a. value assignment of a multi- component solution containing UV- and non-UV active organics at mass fractions of 50 µg/g to 500 µg/g (Phe, Leu, Ile and/or Pro reported) b. separation and quantification using chromatography (LC- or GC-systems)		
• Value assignment of Multicomponent Calibration solution		
Competency		**Specific Information**
Result for Phe content (µg/g)	$\left\|\frac{d}{U(d)}\right\|$ = 0.16	**489.1 ± 3.6**
Result for Leu content (µg/g)	$\left\|\frac{d}{U(d)}\right\|$ = 0.22	**199.6 ± 1.2**
Result for Ile content (µg/g)	$\left\|\frac{d}{U(d)}\right\|$ = 0.15	**215.3 ± 1.1**
Result for Pro content (µg/g)	$\left\|\frac{d}{U(d)}\right\|$ = 0.94	**46.6 ± 0.2**
Identification of analyte(s) in sample	✓	Retention time and fragment ions
Extraction and clean up of analyte(s)	**N/A**	
Sample concentration adjustment	✓	1:20 dilution
Conversion of analyte(s) of interest to detectable/measurable form	**N/A**	
Analytical system	✓	LC-MS/MS (LC: Thermo Scientific Dionex UltiMate 3000; MS: Thermo Quantiva triple-quadrupole mass spectrometer)
Gravimetric procedures for preparation of calibration solutions	✓	(a) Relative uncertainty of gravimetric operations used in the preparation of calibration solutions: 0.04% – 0.22% RSD (b) Estimate of contribution of uncertainty due to gravimetric operations to the uncertainty of the value assignment: 3.2% – 44%
Calibration approach for value-assignment of analyte(s) in matrix	✓	a) double isotope dilution b) exact-matching single-point c) Contribution of uncertainty due to calibration to the uncertainty of the value assignment: 11% – 38%
Verification method(s) for value-assignment of analyte(s) in sample	×	

Table 10.l : Core competency claims for CCQM-K78.a – NRC

CCQM-K78.a	*PTB*	Polar analytes in aqueous solvent
Scope of measurement capabilities: Participation in this comparison demonstrates the laboratory's capabilities in determining the mass fraction in aqueous calibration solution of organics with molar mass of 100 g/mol to 500 g/mol having polarity *pK*ow > -2 for one or more of the following: c. value assignment of a multi- component solution containing UV- and non-UV active organics at mass fractions of 50 µg/g to 500 µg/g (Phe, Leu, Ile and/or Pro reported) d. separation and quantification using chromatography (LC- or GC-systems)		
• Value assignment of Multicomponent Calibration solution		
Competency		**Specific Information**
Result for Phe content (µg/g)	$\left\|\frac{d}{U(d)}\right\|$ = 0.14	**487.8 ± 5.0**
Result for Leu content (µg/g)	$\left\|\frac{d}{U(d)}\right\|$ = 0.09	**199.7 ± 2.1**
Result for Ile content (µg/g)	$\left\|\frac{d}{U(d)}\right\|$ = 0.03	**215.2 ± 2.3**
Result for Pro content (µg/g)	$\left\|\frac{d}{U(d)}\right\|$ = 0.31	**47.1 ± 0.54**
Identification of analyte(s) in sample	✓	Retention time and mass spectrum ion ratios
Extraction and clean up of analyte(s)	**N/A**	
Sample concentration adjustment	**N/A**	
Conversion of analyte(s) of interest to detectable/measurable form	**N/A**	
Analytical system	✓	LC-MS
Gravimetric procedures for preparation of calibration solutions	✓	Relative uncertainty of gravimetric procedures used in preparation of calibration solutions less than 0.1 %. Estimated contribution to the uncertainty of the final value is negligible.
Calibration approach for value-assignment of analyte(s) in matrix	✓	IDMS with single point calibration
Verification method(s) for value-assignment of analyte(s) in sample	**N/A**	

Table 10.m : Core competency claims for CCQM-K78.a - PTB

CCQM-K78.a	*UME*	Polar analytes in aqueous solvent
Scope of measurement capabilities: Participation in this comparison demonstrates the laboratory's capabilities in determining the mass fraction in aqueous calibration solution of organics with molar mass of 100 g/mol to 500 g/mol having polarity *pK*ow > -2 for one or more of the following: a. value assignment of a multi- component solution containing UV- and non-UV active organics at mass fractions of 50 ug/g to 500 ug/g (Phe, Leu, Ile and/or Pro reported) b. separation and quantification using chromatography (LC- or GC-systems)		
• Value assignment of Multicomponent Calibration solution		
Competency		**Specific Information**
Result for Phe content (µg/g)	$\left\|\frac{d}{U(d)}\right\|$ **= 0.81**	**482.6 ± 7.2**
Result for Leu content (µg/g)	$\left\|\frac{d}{U(d)}\right\|$ **= 0.23**	**200.6 ± 3.2**
Result for Ile content (µg/g)	$\left\|\frac{d}{U(d)}\right\|$ **= 0.55**	**219.0 ± 7.0**
Result for Pro content (µg/g)	$\left\|\frac{d}{U(d)}\right\|$ **= 0.21**	**47.2 ± 1.6**
Identification of analyte(s) in sample	✓	*Ion ratios, IDMS and qNMR*
Extraction and clean up of analyte(s)	**N/A**	-
Sample concentration adjustment	✓	*1:10 dilution*
Conversion of analyte(s) of interest to detectable/measurable form	✓	*Derivatization with propyl chloroformate for LC-IDMS*
Analytical system	✓	*LC-MS (HRMS)*
Gravimetric procedures for preparation of calibration solutions	✓	*(a) Relative uncertainty of gravimetric operations used in the preparation of calibrators* *Phe; 9.07x10^{-6} ; Leu: 9.07x10^{-6}* *Ile: 9.07x10^{-6} ; Pro: 9.03x10^{-6}* *(b) Estimate of contribution of uncertainty due to gravimetry to the uncertainty of the value* *Phe; 1.32x10^{-13} ; Leu: 1.32x10^{-13}* *Ile: 1.32x10^{-13} ; Pro: 8.15x10^{-13}*
Calibration approach for value-assignment of analyte(s) in matrix	✓	*a) Indicate quantification mode used)* *IDMS* *b) Indicate calibration mode used* *Calibration curve (5 point)* *c) Estimate of contribution of uncertainty due to calibration to the uncertainty of the value assignment* *Phe; 1.17x10^{-2} ; Leu: 1.2x10^{-2}* *Ile: 2.75x10^{-2} ; Pro: 2.88x10^{-2}*
Verification method(s) for value-assignment of analyte(s) in sample	✓	*qNMR (see result for CCQM-P121.a)*

Table 10.n : Core competency claims for CCQM-K78.a - UME

CCQM-K78.a	*VNIIM*	Polar analytes in aqueous solvent
Scope of measurement capabilities: Participation in this comparison demonstrates the laboratory's capabilities in determining the mass fraction in aqueous calibration solution of organics with molar mass of 100 g/mol to 500 g/mol having polarity *pK*ow> -2 for one or more of the following: a. value assignment of a multi- component solution containing UV- and non-UV active organics at mass fractions of 50 ug/g to 500 ug/g (Phe, Leu, Ile and Pro reported) b. separation and quantification using chromatography (LC- or GC-systems)		
• Value assignment of Multicomponent Calibration solution		
Competency		**Specific Information**
Result for Phe content (µg/g)	$\left\|\frac{d}{U(d)}\right\|$ **= 0.54**	**495 ± 12**
Result for Leu content (µg/g)	$\left\|\frac{d}{U(d)}\right\|$ **= 1.94**	**186.7 ± 6.7 ***
Result for Ile content (µg/g)	$\left\|\frac{d}{U(d)}\right\|$ **= 3.52**	**193.3 ± 6.2 ***
Result for Pro content (µg/g)	$\left\|\frac{d}{U(d)}\right\|$ **= 0.73**	**46.0 ± 1.1**
Identification of analyte(s)	✓	*Retention time, ions (m/z) ratio*
Extraction and clean up	**N/A**	
Sample concentration adjustment	✓	*1:50 dilution (for LC-MS)*
Conversion of analyte(s) of interest to detectable form	**N/A**	
Analytical system	✓	*LC-MS, LC-UV*
Gravimetric procedures for preparation of calibration solutions	✓	*a) Relative uncertainty of gravimetric operations used in the preparation of calibration solutions: 0.4% (for Pro, Leu, Ile) ; 0.2% (for Phe)* *b) Contribution of uncertainty due to gravimetric operations to the uncertainty of the value assignment:* *2.7% (Phe); 4.9%(Pro), 6.3 % (Leu); 11.1%(Ile)*
Calibration approach for value-assignment of analyte(s) in matrix	✓	*a) Quantification mode used:* *Internal standard (for Pro, Leu, Ile)* *External standard (for Phe))* *b) Calibration mode used: Single-point calibration* *c) Contribution of uncertainty due to calibration* *72,7% (Phe); 44,4%(Pro), 56,3% (Leu); 25,0%(Ile)*
Verification method(s) for value-assignment of analyte(s) in sample	✓	*LC-MS (for Phe)*

Table 10.o : Core competency claims for CCQM-K78.a - VNIIM

* Deviation from KCRV for Leu and Ile was ascribed to the non-availability of appropriate ^{13}C-labelled amino acids for IDMS analysis. In their absence unlabeled tryptophan was used instead as an internal standard but the method based on its use proved to be unsuitable for the analysis of Leu and Ile.

CONCLUSIONS

A very satisfactory overall level of agreement of the reported results was obtained both between participants and with the gravimetric values for each individual amino acid content in the CCQM-K78.a solution. In the few cases where agreement was not satisfactory, the participants were able to identify the cause for the inconsistency. The level of performance is consistent with that obtained in equivalent earlier comparisons, provides additional support for existing CMC claims for the assignment of organic analyte standard solutions and can be used to support future claims for standard solutions in aqueous solvent.

In particular the comparison provided additional demonstration of the trueness and precision of double IDMS-based methods for the quantification of polar analytes in aqueous solution when an isotopically labelled version of the analyte is available as the internal standard. In the few instances where another labelled material or a structurally related material was substituted as the internal standard, even if the material was a structural isomer of the analyte, a comparable level of agreement with the KCRV was not achieved. An additional observation from this specific comparison, which perhaps reflects the relative "cleanness" of the matrix and lack of interference in the ionization pathways of the analytes, was that the measurement uncertainty of results obtained by LC- or GC-IDMS methods with direct SIM quantification was generally smaller than those associated with IDMS methods using LC-MS/MS quantified against an MRM ion.

The comparison also demonstrated that established amino acid quantification techniques using pre- or post-column derivatization with UV or FLD detection can provide results with comparable levels of accuracy and precision as double IDMS-based methods.

In this case where the purity of the primary calibrators had been assigned with a relative standard uncertainty below 0.2%, results consistent with the KCRV and with relative expanded uncertainty of the assigned property in the range 1% - 2% could be realized and levels of 2%-4% could be routinely achieved. This is consistent with and supports the uncertainties claimed for existing CMCs for the assignment of the mass fraction content of standard solutions (see Figure 1).

ACKNOWLEDGEMENTS

The coordinator wishes to thank all the laboratories participating in this comparison in compliance with the study protocol and for their co-operation in providing additional information required to complete the reporting and interpretation of the comparison results.

REFERENCES

1 https://www.bipm.org/wg/CCQM/OAWG/Restricted/Finalised_Pilot_Study_Reports/CCQM-P31.doc)
2 See the result summary and link to the published Final Report for CCQM-K38 at https://kcdb.bipm.org
3 See information on the status of CCQM-K131 at https://kcdb.bipm.org
4 https://kcdb.bipm.org
5 https://www.bipm.org/cc/CCQM/Restricted/17/CCQM11-18.pdf
6 https://www.bipm.org/cc/CCQM/Restricted/19/CCQM13-22_Consensus_KCRV_v10.pdf
7 R. DerSimonian and N. Laird; *Controlled Clinical Trials*, 1986, **7**, 177–188.
8 Calculated using the "DOEmaker" Excel spreadsheet kindly provided by David Duewer (NIST). These assignments were consistent with values for the DerSimonian-Laird estimator calculated for the same dataset using the NIST Consensus Builder online app (https://consensus.nist.gov).

APPENDIX A: Homogeneity Tests on CCQM-K78.a Candidate Material

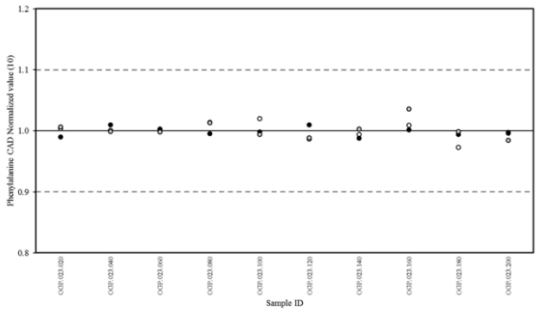


Figure 12: Phe in CCQM-K78.a by LC-CAD

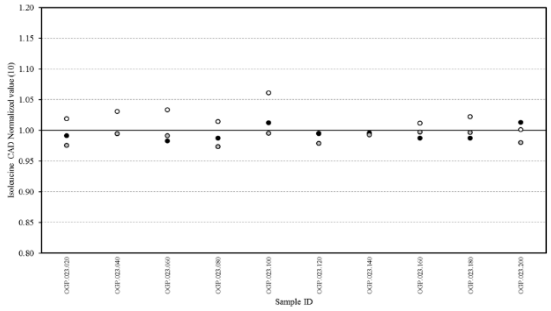


Figure 13: Ile in CCQM-K78.a by LC-CAD

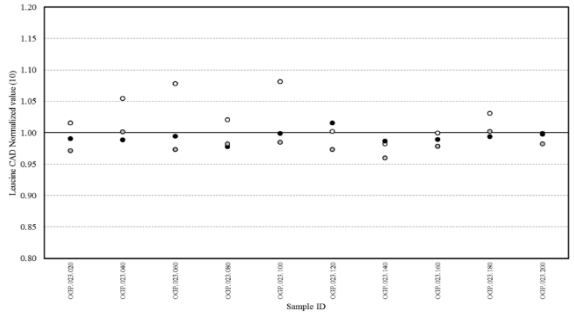


Figure 14: Leu in CCQM-K78.a by LC-CAD

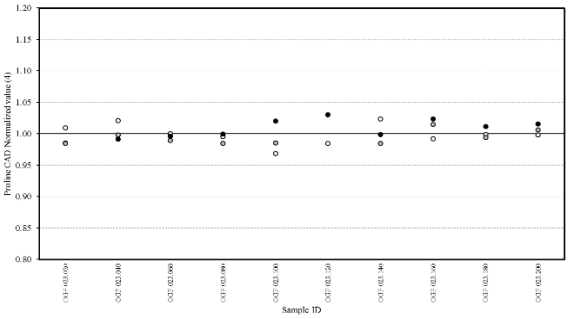


Figure 15: Pro in CCQM-K78.a by LC-CAD

Figures 12 to 15 plot the homogeneity test results by fill sequence obtained by LC-CAD after ten-fold dilution of a sample aliquot of the candidate comparison material for Phe, Ile and Leu. Figure x are the results for Pro by LC-CAD after four-fold dilution of a sample aliquot. For each vial first, second and third replicates are represented by white, black and grey circles respectively.

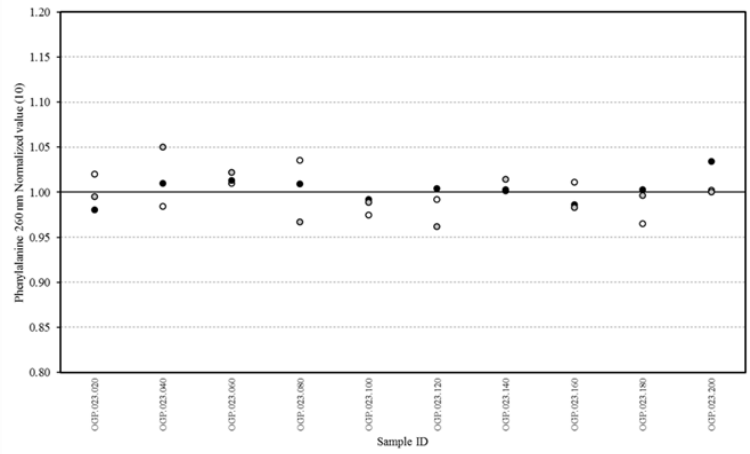


Figure 16: Homogeneity of Phe in CCQM-K78 by LC-UV

Figure 16 is a plot of the homogeneity test results for Phe obtained by LC-UV with detection at 260 nm after ten-fold dilution of a sample aliquot

APPENDIX B: Stability Tests on CCQM-K78.a Candidate Material

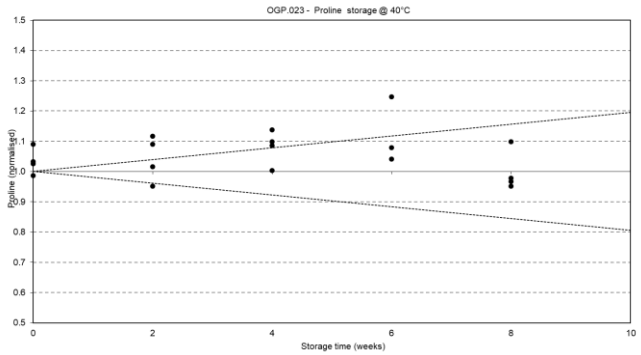


Figure 17: Pro in CCQM-K78a by LC-CAD (@ 40 °C)

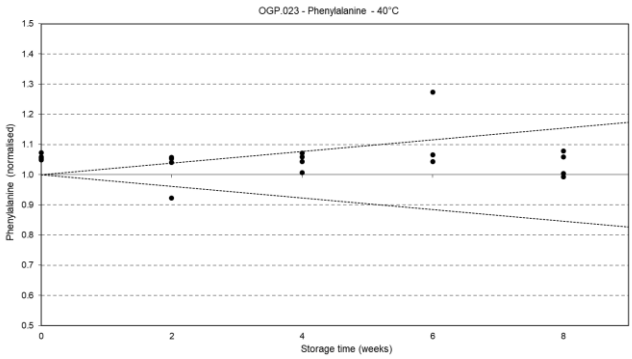


Figure 18: Phe in CCQM-K78a by LC-CAD (@ 40 °C)

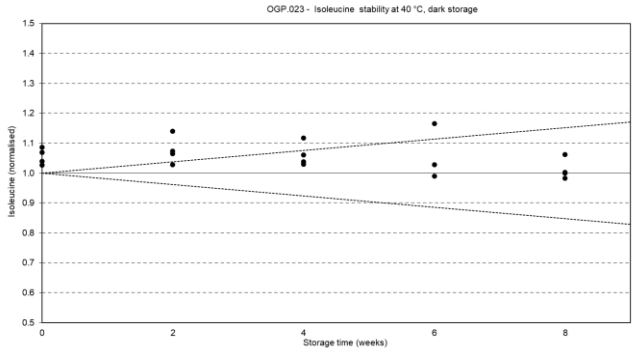


Figure 19: Ile in CCQM-K78a by LC-CAD (@ 40 °C)

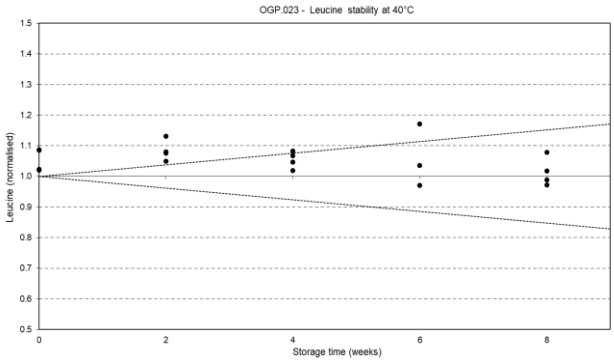


Figure 20: Leu in CCQM-K78a by LC-CAD (@ 40 °C)

Figures 17 to 20 are representative plots of the stability test results obtained by triplicate analysis by LC-CAD of aliquots taken from vials of the candidate comparison material batch subject to an isochronous stability test over eight weeks (two vials per time increment) of the effect on analyte composition of storage at 40 °C.

The results obtained for each amino acid at the other stability test conditions (22 °C, dark storage and 22 °C, ambient light) were equivalent to those shown for storage at the higher temperature.

APPENDIX C: Call for Participation and Comparison Protocol

Key Comparison CCQM-K78
High Polarity Analytes in a Multicomponent Aqueous Solution:
Mass Fraction of Amino Acids in acidic solution

Project Name: **CCQM-K78.a (Amino acids in solution)**

Comparison: **Value assignment of polar analytes in aqueous solution**

Proposed dates: 12/2016 to 3/2017

Coordination Laboratory

Bureau International des Poids et Mesures
Chemistry Department,
Pavillon de Breteuil
92312 Sèvres
France

Study Coordinator: Steven Westwood
Phone: ++33-1-45 07 70 57
Fax: ++33-1-45 34 20 21
E-mail: steven.westwood@bipm.org.

Introduction

CCQM-P31a "Polycyclic Aromatic Hydrocarbons in Solution" conducted in 2004 investigated the mass fraction assignment of the components of a standard solution in toluene containing 35 polycyclic aromatic hydrocarbons (PAHs). This was followed in 2005 with key comparison CCQM-K38 "PAHs in Solution (Toluene)", using a standard solution containing 10 PAHs. These studies, completed over ten years ago, currently underpin CMC claims for the value assignment of organic analytes in calibration solutions. CCQM-K131 "PAHs in Acetonitrile", currently in progress, is being undertaken to renew Key Comparison support for this measurement capability. The OAWG requested a complimentary comparison be conducted on the value assignment of polar organic compounds in aqueous solution. The aim of the CCQM-K78.a comparison is to permit National Measurement Institutes (NMIs) to demonstrate the validity of their procedures to assign the mass fraction content of single or multi-component polar organic analytes in aqueous calibration solutions.

Study Material

BIPM prepared a standard solution in 0.01 N HCl containing phenylalanine (Phe), leucine (Leu), isoleucine (Ile) and proline (Pro). The levels are intended to be representative of the mass fraction content of amino acids in a multicomponent standard solution provided as a reference standard for use in the calibration of amino acid analysis.

Homogeneity and Stability Assessment

BIPM has:

- demonstrated that the levels of within and between vial inhomogeneity of the mass fraction of each of the amino acid components in the solution are sufficiently small so as to not influence the validity of the comparison;
- completed an isochronous stability study to confirm that the material is sufficiently stable within the proposed time scale of the study;
- established that the amino acid content is stable in solution in the ampoule to extended exposure to light under ambient laboratory conditions;
- determined appropriate conditions for storage, transport and handling of the solution.

Reference Standards

The BIPM will provide 500 mg of a value-assigned primary calibrator for each of the amino acid analytes. These materials are to be used to establish calibration functions for the assignment of the mass fraction of each amino acid quantified in the solution.

Study Guideline

Each participant will receive four ampoules, each ampoule containing 1.2 mL of solution. Three ampoules will be required for analysis to obtain the comparison result and an additional ampoule is available for the development of measurement procedures.

The ampoules shall be stored at 4 °C prior to opening.

Gravimetric operations involving aliquots taken from the solution should be undertaken as soon as possible after opening the vial to minimize the potential for change in the analyte concentration due to evaporation of the solvent on exposure to air.

Participants are required, as a minimum, to report a single estimate of the mass fraction in the solution of the highest concentration component, phenylalanine, which is also the sole component readily detected directly by LC-UV analysis. The result should be based on combined values obtained by the measurement of at least one aliquot from each of three of the ampoules supplied (i.e. at least three independent replicates). Participants can analyze multiple aliquots per ampoule if they so choose.

Where participants have access to techniques for the quantification of amino acids that do not contain a UV-chromophore, estimates of some or all of the three additional amino acid components should also be reported. There is no restriction on the methods that may be used to assign the amino acid mass fraction content in the solution.

The "How Far The Light Shines" statement applicable to participant performance will depend on whether the participant only reports Phe content (demonstrating a capability for the quantification of polar, UV-active compounds in an aqueous calibration solution) or if the participant reports values for the non-UV active components (demonstrating a general capability for the quantification of polar compounds in an aqueous calibration solution).

Submission of Results

Each participant must provide results using the reporting sheet provided with the samples and include a completed Core Competency table. The results should be sent via email to the study coordinator (steven.westwood@bipm.org) before the submission deadline. Submitted results are considered final and no corrections or adjustments of analytical data will be accepted unless approved by the OAWG. The result must include the assigned value for the mass fraction of Phe in the solution.

Where a participant has access to appropriate measurement methodology, values should be reported for all four amino acid components in the solution. For each reported value, the standard and expanded uncertainties shall be reported with a description of the uncertainty budget. A description of the analytical procedure (GC or LC column; chromatographic conditions, quantification approach, sample chromatogram) should be provided.

Participation

All NMIs with measurement capabilities for the analysis of polar organic compounds are expected to participate in CCQM-K78. It constitutes a "Track A" Key Comparison used to demonstrate an NMI's Core Competencies for the delivery of Measurement Services to their customers and stakeholders. The ability to perform fit-for-purpose value assignments of an organic analyte mass fraction in a standard solution, either for internal use or to be made available to external users, is regarded as a core technical competency for institutes wishing to claim metrological traceability for the results of organic analysis measurement services disseminated from their institute.

Failure to participate in the comparison could result in delays in the review and approval of existing or future CMC claims by an NMI in this measurement field.

"How Far The Light Shines" Statements for CCQM-K78

i. **For participants only reporting Phe using LC-UV detection:**

Successful participation in CCQM-K78.a demonstrates the following measurement capabilities for determining the mass fraction of an organic compound containing a UV-chromophore present at a mass fraction of 500 μg/g in a multicomponent aqueous calibration solution, where the molar mass of the organic component is in the range 100 g/mol to 500 g/mol, polarity (pK_{ow}) > -2, and the value assignment of the mass fraction content of the primary calibrator for Phe is undertaken separately:

a. value assignment of a polar analyte with a UV- chromophore in aqueous solution
b. separation and quantification of a polar organic component that is readily resolved chromatographically, using an LC-UV detection method

In a case where an aliquot of the supplied comparison solution required dilution prior to analysis, the range of the mass fraction assignment capability demonstrated by the comparison participant may be revised accordingly.

ii. **For participants reporting Phe and some or all of Leu, Ile and Pro:**

Successful participation in CCQM-K78.a demonstrates the following measurement capabilities for determining the mass fraction of organic compounds present in the mass fraction range of 50 μg/μg - 500 μg/g in a multicomponent aqueous calibration solution, where the molar mass of the analytes are in the range 100 g/mol to 500 g/mol, the polarity (pK_{ow}) > -2, and the value assignment of the mass fraction content of the primary calibrators for each analyte are undertaken separately:

c. value assignment of a polar analyte in an aqueous calibration solution
d. separation and quantification of polar organic components, including those of similar chromatographic retention time (Leu/Ile)

The extent of demonstration of capabilities for mass fraction assignment, including separation and quantification, will depend on the number and nature of the reported assignments of the target analytes that are consistent with the comparison KCRVs.

In a case where an aliquot of the supplied comparison solution required dilution prior to analysis, the range of the mass fraction assignment capability demonstrated by the comparison participant may be revised accordingly.

Reporting of Results

An electronic data submission form will be supplied as an EXCEL document.

Headings include:

- Laboratory information;
- Results Table reporting the mass fraction content of each amino acid quantified in the solution (in µg/g) with the associated combined standard uncertainty of the result and the expanded uncertainty at a 95% confidence range.
- Measurement equation and uncertainty calculations for each assignment
- Short description of the procedure used for the mass fraction assignment
- Supplementary information:
    - copy of a representative chromatogram, if a tandem chromatographic method was used for the quantification

Reporting requirement:

- mass fraction of Phe in the solution in µg/g (mandatory)
- mass fraction of some or all of Leu, Ile and Pro in the solution in µg/g (encouraged)
- measurement equation for each mass fraction assignment (mandatory)
- components of the uncertainty budget for each mass fraction assignment(s) (mandatory)

Schedule

Call for participation	November 2016
Final date to register to participate	25th November 2016
Sample distribution	28th November 2016
Data due to coordinator	11th March 2017
Initial discussion of results	OAWG Meeting April 2017

Safety and Handling

The amino acid solution poses no hazards for transport or storage. There are no significant health risks involved with the handling and manipulation of the comparison material.

APPENDIX D: Summary of Participant Analytical Methods

NMI	Method 1	IS	Calibration	Method 2	IS	Calibration	Check
BIPM	LC-IDMS/MS	$^{13}C/^{15}N$-AAs	2-point				LC-CAD
EXHM	LC-IDMS/MS	$^{13}C/^{15}N$-AAs	4-point	LC-MS/MS	$^{13}C/^{15}N$-AAs	4-point	LC-UV/FLD for Phe
GLHK	LC-FLD	Norvaline	1-point	LC-IDMS/MS	$^{13}C/^{15}N$-AAs	1-point	
HSA	LC-IDMS/MS	$^{13}C/^{15}N$-AAs	4-point				
KRISS	LC-IDMS/MS	$^{13}C/^{15}N$-AAs	1-point				
LGC	GC-MS	$^{13}C/^{15}N$-AAs	Bracketed				qNMR
LNE	LC-IDMS	$^{13}C/^{15}N$-AAs	5-point				
NIMC	LC-IDMS/MS	$^{13}C/^{15}N$-AAs	7-point	LC-UV (Phe)		7-point	LC-CIDCD
NIMT	LC-IDMS/MS	$^{13}C/^{15}N$-AAs	5-point	LC-UV (Phe)		5-point	
NIST	LC-IDMS/MS	$^{13}C/^{15}N$-AAs					
NMIJ	LC-FLD	Norvaline & Sarcosine	1-point	LC-UV (All)	L-Asn	1-point	
NMISA	GC-TOFMS	$^{13}C/^{15}N$-AAs	Bracketed	LC-IDMS/MS	$^{13}C/^{15}N$-AAs	Bracketed	LC-UV (Phe)
NRC	LC-IDMS	$^{13}C/^{15}N$-AAs	1-point				
PTB	LC-IDMS	$^{13}C/^{15}N$-AAs	1-point				
UME	LC-IDMS	$^{13}C/^{15}N$-AAs	5-point				qNMR
VNIIM	LC-MS	L-Trp	1-point	LC-UV (Phe)	L-Trp	1-point	

Note: All LC-IDMS and LC-IDMS/MS methods were carried out using an exact matching approach against isotopologues of the assigned amino acid analyte except NIMT ($^{13}C_6$-Phe used for IDMS of all analytes) and NMISA ($^{13}C_n$-Leu not available for assignment of Leu content).

APPENDIX E: Summary of Participants' Analytical Information

The following Tables summarize the detailed information about the analytical procedures each participant provided in their "Analytical Information" worksheets. The presentation of the information in many entries has been consolidated and standardized.

The participant's measurement uncertainty statements are provided in Appendix F.

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 BIPM or other participants in this Key Comparison, nor does it imply that the equipment, instruments, or materials are necessarily the best available for the purpose.

Table E-1: Summary of Sample Size, Pre-treatment and Analytical System for CCQM-K78.a

Institute	Sample size (g)	Pre-treatment	Analytical System
BIPM	0.3 (Pro, Ile and Leu) 0.2 (Phe)	Pro, Ile and Leu: 1:500 dilution, labelled IS mix then water Phe: 1:500 dilution, first HCl 0,01N then IS mix then water	Sciex QTrap Triple quad MS/MS
EXHM	0.1 g	Dil. 1:10 in ACN for LC-UV/FLD Dil. 1:100 - 1:10000 in ACN/ H2O 82.5/17.5 for LC-MS/MS	Agilent 1260 (LC-UV) Thermo LC - Thermo Quantum Ultra AM MS/MS (LC-IDMS)
GLHK	0.05 (LC-MS/MS) 0.1 (LC-FLD)	None for LC-MS/MS Derivatize with AQC for LC-FLD	LC-MS/MS LC-FLD
HSA	0.1	spiked with IS standard. mixed by vortexing and diluted to 300 ng/g (Phe), 630 ng/g (Leu), 140 ng/g (Ile) 150 ng/g (Pro) before LC-MS/MS measurement	AB Sciex Qtrap 5500 MS/MS & Shimadzu Prominence UFLC XR LC system
KRISS	0.5	None	Waters Acquity UPLC Waters Xevo TQ-S ESI-QQQ MS system
LGC	0.8	62 fold dilution gravimetrically with 10 mM HCl. Blend with labelled AA internal standards. Evaporate to dryness in vacuo, derivatise residue with MTBSTFA	Agilent 7890A GC with 5975C MSD
LNE	0.05	1:14	LC-MS
NIMC	0.2	1:10	LC-IDMS
NIMT	0.4		LC-IDMS and LC-UV (Phe only)
NIST	0.14	1:1 with internal standard mixture	Agilent Infinity 1290 UPLC coupled to Agilent 6460 mass spectrometer

Institute	Sample size (g)	Pre-treatment	Analytical System
NMIJ	0.5	Asp spiked as IS before measurement. 1:1 ratio.	LC-UV after derivatization with ninhydrin LC-FLD after derivatization with OPA
NMISA	0.1	Dilution 1:5 (Pro), 1:20 (Leu and Ile), 1:50 (Phe)	GC-TOF/MS, LC-MS/MS and LC-UV (Phe)
NRC	0.05	1:20	Thermo Scientific Dionex UltiMate 3000 LC Thermo Quantiva triple-quadrupole MS
PTB	0.05	IS added gravimetrically to aliquot of sample. HCl removed at 108°C under N2. Residue diluted for LC/MS with ACN	LC-MS
UME	0.1	Diluted with 0.01 N HCl (aq) gravimetrically. Derivatized with propyl chloroformate	Thermo Q Exactive, Orbitrap LC/MS)
VNIIM	0,1 (for Phe); 0,2 (for others)	1:50 (for LC-MS method)	LC- ESI MS (for Pro, Leu, Ile) LC-UV, 257 nm (for Phe)

Table E-2: Summary of Analytical Techniques for CCQM-K78.a

Institute	Column	Detection Method and Chromatographic Conditions	SIM/MRM for MS methods
BIPM	Capcell PAK C18 MG S-5 250*4.6 mm, 5 µm (Shiseido)	**LC-IDMS** Phase: A= Water + 0.1% Formic Acid 89% Phase B= Methanol + 0.1% Formic Acid 11%, Isocratic; Flow: 1 mL/min; Col. Temp.: 40°C Inj: 10 µL	Pro 116.1>70.1; Pro* 121.1>74.1 Ile 132.2>69.1; Ile* 138.2> 74.1 Leu 132.2>86.2; Leu* 138.2>92.2 Phe 166.2>120.1; Phe* 176.6>129.2
EXHM	Sequant ZIC-HILIC 150 x 2,1 mm, 5 µm, 200 Å	**LC-IDMS/MS for assignment and LC-UV/FLD for verification** Isocratic - 82,5 % acetonitrile / 17,5 % 10 mM acetic acid 5 mM ammonium formate (UV/FLD and MS/MS analysis)	Pro 116 >70q and 68 Ile 132 >86q and 69q Leu 132 >86q and 43q Phe 166 >120q and 103
GLHK	LC-MS/MS: Grace Alltima, C18, 250 x 2.1 mm, 5 µm LC-FLD: Waters Nova-Pak, C18, 150 x 3.9 mm, 4 µm	**LC-MS/MS:** Phase A= 0.05% TFA in 30% ACN/H_2O ; Phase B= 0.05% TFA in 60% ACN/H2O 100% B to 100% A (15 min), hold, return to 100% B **LC-FLD**: Phase: A= 60% ACN/H_2O ; Phase B= pH 5.05 buffer using NaOAc/Et_3N Gradient: 100% B to 68% A (46 min), to 100% A to 100% B Col. Temp. 37 °C, Excitation 250 nm, Emission 395 nm	Pro 116>70 ; Pro* 122>75 Ile 132>83 ; Ile* 139>92 Leu 132>86 ; Leu* 138>91 Phe 166>120 ; Phe* 176>129
HSA	Col. 1: Agilent ZORBAX Eclipse, 4.6 x 150 mm, 5 µm Col. 2: Phenomenex Kinetex 4.6 x 150 mm, 2.6 µm	**LC-IDMS** Mobile Phase A: 0.1% TFA in water Mobile Phase B: 0.1% TFA in acetonitrile Gradient: 10% to 30% mobile phase B (Column 1) 8% to 30% mobile phase B (Column 2).	Pro 116.0/70.0; Pro* 122.1/75.0 Ile 132.1/86.0 (q), 69.1; Ile* 139.2/92.1 (q), 73.9 Leu 132.1/86.0 (q), 44.0 Leu* 134.3/87.1 (q), 44.9 Phe 166.1>120.2 (q), 103.0 Phe* 171.0>125.0 (q), 106.0
KRISS	Capcellcore ADME, 2.1 x 150 mm, 2.7 µm (Shiseido)	**LC-IDMS** Mobile phases: A; 0.1% TFA in H_2O, B; 0.1% TFA in ACN Flow rate: 0.5 mL/min, column temp.: 40 ℃, inj. vol.: 1 µL Gradient: A 98.0 %, B 2.0 %, to A 30.0 %, B 70.0 %	Pro 117.9>71.9; Pro* 123.9>76.9, Ile & Leu 131.9>86.0; Ile* & Leu* 138.9>92.0 Phe 165.9>120.0, ; Phe* 171.9>126.0
LGC	Restek Rxi-5HT, 30 m, 0.25 mm ID, 0.25 um	**GC-IDMS** Helium carrier gas at 1mL/min; constant flow mode Oven: 130°C for 3min, 25°C/min to 200°C, 120°C/min to 340°C, hold for 5 min Injector 350°C; Inj Col 1uL (split) Transfer line 300 °C	SIM ions (quantification/ qualifier) Pro 258 / 286 ; Pro* 263 / 292 Ile 302 / 274 ; Ile* 309 /280 Leu 302 / 274 ; Leu* 309 / 280 Phe 308 / 234; Phe* 317/ 243

Institute	Column	Detection Method and Chromatographic Conditions	SIM/MRM for MS methods
LNE	Waters Acquity BEH reverse phase C18	**LC-IDMS** Isocratic: H2O / ACN / TFA (99 / 1 / 0.1)	SIM for quantification Pro 116.1 ; Pro* 121.1 Leu 132.1 ; Leu* 138.1 Ile 132.1 ; Ile* 138.1 Phe 166.1 ; Phe* 176.1
NIMC	AcclaimTMRSLC PA2 Polar Advantage 100, 2.1*250mm, 2.2 µm (Thermo Fisher)	**LC-IDMS** Isocratic gradient: Mobile phase: 95:5(v:v) H_2O + 0.3% TFA : ACN Injection volume 5µL; Flow rate:0.2mL/min	SIM for quantification Pro 116.1; Pro* 121.2 Leu 132.2; Leu* 138.2 Ile 132.1; Ile* 139.2 Phe 166.2; Phe* 174.2
NIMT	Intrada Amino Acid column, 3µm, 150 x 3.0 mm, Imtakt (LC-IDMS) Clipeus C8 5 µm, 150 x 3.0 mm (LC-UV)	**LC-IDMS** Gradient: 0 % B -17 %B - 100 %B Phase A: ACN/THF/ 25 mM NH_4CHO_2/CHO_2H: 9/75/16/0.3 (v/v/v/v) ; Phase B: ACN/ 100 mM NH_4 CHO_2: 20/80 (v/v) Column oven: 40°C; flow rate: 0.4 mL/min; Inj: 5 µL **LC-UV** Mobile phase: 10% methanol in water flow rate: 01 mL/min; Inj: 5 µL ; Detection @ 210 nm	Pro 116.05 >70.00 Ile 132.10 >86.05 Leu 132.10 >86.05 Phe 166.11 >120.07 Phe* 172.16 >126.10
NIST	SIELC Primesep 100 mixed-mode (ion-exclusion and reverse phase) analytical column (2.1 x 250 mm, 5 µm particles, 100 Å pores)	**LC-IDMS** Phase A: H_2O, ACN,TFA - 79.95%, 20% and 0.05 %, Phase B: H_2O, ACN,TFA - 79.55%, 20% and 0.45 %, Flow rate of 200 µL/min. Gradient: 0 % to 50% B over 20 minutes. Column Temp: 15 °C. Injection volume 5 µL.	Pro: A 116.1>70.1; B 116.1>43.1 Pro*: A 122.0>75.1; B 122.0>46.1 Leu/Ile: A 132.1>86.1; B 132.1>44.1, Leu*/Ile*A 138.1>91.1; B 138.1>46.1 Phe 166.2>103.1, Phe* 176.1>111.1
NMIJ	LC-UV: Packed twin column for high res biological fluid analysis-Li, 5 µm × 4.6 mm I.D. × 120 mm (Hitachi) LC-FL:Shim-pack Amino-Li, 5 µm × 6.0 mm I.D.× 100 mm (Shimadzu)	**LC-UV**: Gradient of 4 commercial buffer solns (L-8500-PF1/2/3/4) from Mitsubishi Chem Reaction solution Ninhydrin/LiOAC buffer (1 :1) Reaction 135 °C; Detn.: 570 nm (Phe,Ile,Leu) ; 440 nm Pro **LC-FLD**: Mobile phases A: 0.15 M Li citrate; B: 0.3 M Li citrate/ 0.2 M Boric acid ; C : 0.2 M LiOH ; D : H_2O Gradient : 100% A to 100% B to 100% C. Rinse with 100% D then return to 100 % A; Post column derivatization with OPA; Excitation @ 350 nm, Emission @ 450 nm	

Institute	Column	Detection Method and Chromatographic Conditions	SIM/MRM for MS Methods		
NMISA	GC column - Restek Rxi-5SilMS 20m, 0.18 mm ID, 0.18 µm df LC Column - Acquity UPLC BEH HILIC 1.7 µm, 2.1 x 150 mm	**GC-TOFMS** Initial oven temperture held at 100 °C for 1 min and ramped at 20 °C/min to 320°C, 1 min hold **LC-MS/MS** Organic solvent (95%) 0.1 % formic acid in acetonitrile pumped at 0.35 mL/min for 5 min, followed by ramp to 5 mM ammonium acetate (90%) and subsequently re-equlibrated to starting conditions		GC-TOFMS	LC-MS/MS
			Pro	184 & 258	116 > 70.1
			Pro*	188 & 262	120.7 >73.9
			Ile	200	132.1 > 69.2
			Ile*	205	137.7 > 90.9
			Leu	200	132.1 > 86.1
			Phe	234 & 336	161.1 > 120.1
			Phe*	235 & 337	167 > 121
NRC	Sequant ZIC-HILIC, 100 x 2.1mm, 3.5 µm, PEEK, 100A	**LC-IDMS** Phase A: 10mM NH_4OAc adjusted to pH 3.5 in H_2O Phase B: ACN ; Isocratic elution with 90% B Flow rate 0.25mL/min ; Col. Temp. 35 °C; Inj. Vol 1 µL	Pro	116.1>70.1; Pro* 121.1>74.2	
			Ile	132.1>69.2; Ile* 138.1> 74.2	
			Leu	132.2>86.2; Leu* 138.2>92.2	
			Phe	166.1>120.2; Phe* 172.1>126.2	
PTB	SeQuant ZIC-HILIC 3,5µm; 150 x 2,1 mm	**LC-IDMS** Isocratic elution: 80:20 (v/v) acetonitrile / 5 mM ammonium acetate ; flow rate: 0,1 ml / min	SIM Mode for quantification		
			Pro	116.1 ; Pro*	122.1
			Ile	132.1 ; Ile*	138.1
			Leu	132.1 ; Leu*	138.1
			Phe	166.1 ; Phe*	176.1
UME	Phenomenex EZ:Fast 4 µm AAA-MS (250 x 2.0 mm i.d.).	**LC-IDMS** (as propyl chloroformate derivative) Inj. 2 µL. Run time: 22.0 min. Phase A: MeOH: H_2O (0,01 M NH_4HCO_2) (1:1), B: MeOH (0,01 M NH4 HCO_2) Flow rate 0.25 mL/min, Column temp. 40 °C.	SIM Mode for quantification		
			Ile-PC	260.186 ; Ile-PC*	261.183
			Leu-PC	260.184; Leu-PC*	261.184
			Phe-PC	294.168 ; Phe-PC*	299.198
VNIIM	LC-MS: YMC Hydrosphere C18, 100x4,6 mm ID, 3µm LC-UV: Eclipse XDB- C18, 150x4,6 mm ID, 5µm	**LC-MS:** Phase: A - 0,1% aq HFBA; B - 0,1% HFBA in ACN Gradient: 0 min 0% B; 15 min 15% B; 17 min 16% B; 18 min 0% B; 25 min 0% B Inj. vol. 1 µl; Flow-rate 0.8 ml/min **LC-UV:** Phase: A: 0,1% aq HFBA; B: ACN Isocratic; 86%A 14%B Inj. vol. 3 µl; Flow-rate 1,0 ml/min	SIM Mode for quantification		
			Pro	116;	
			Ile	132	
			Leu	132	
			Phe	166	

Table E-3: Summary of Calibrants and Standards for CCQM-K78.a

Institute	Calibration	Calibrants	Internal Standards
BIPM	Bracketed double IDMS/MS	As supplied by BIPM.	^{13}C, ^{15}N-labelled Phe, Leu, Ile and Pro purchased from CIL
EXHM	Single point exact matching external IDMS/MS	As supplied by BIPM.	^{15}N-Ile, ^{15}N-Leu - $^{15}N^{13}C$-Phe (supplied by University of Patras) added during calibrant and sample dilution
GLHK	Single point double IDMS/MS	As supplied by BIPM.	^{13}C-labelled Phe, Leu, Ile and Pro (CIL) for LC-MS/MS Norvaline (Agilent) for Phe/Leu/Ile by LC-FLD Sarcosine (Agilent) for Pro by LC-FLD
HSA	Four-point	As supplied by BIPM.	$^{2}H_5$- Phe, $^{13}C_2$- Leu, $^{13}C_n$,^{15}N-Ile and Pro (purchased from CIL)
KRISS	Single-point exact matching double IDMS/MS	As supplied by BIPM.	Pro*; $^{13}C_5$; ^{15}N, Ile*; $^{13}C_6$; ^{15}N, Leu*;$^{13}C_6$, 98%; ^{15}N, 100%, Phe*; $^{13}C_9$, 98%; ^{15}N, 98% (purchased from CIL)
LGC	Bracketed exact matched double IDMS	As supplied by BIPM.	Leu-$^{13}C_6$,^{15}N; Ile-$^{13}C_6$; Pro-$^{13}C_5$,^{15}N; Phe-$^{13}C_9$,^{15}N; (purchased from CIL)
LNE	5 point linear regression / matching IDMS	As supplied by BIPM.	Ile-$^{13}C_6$ (CLM-2248); Leu-$^{13}C_6$ (CLM-2262); Phe-$^{13}C_9$ ^{15}N (CNLM-575); Pro-$^{13}C_5$ (Ref CLM-2260). (purchased from CIL)
NIMC	Linear Regression (LC) 7-point bracketing IDMS	As supplied by BIPM.	Phe-D_8, Leu- $^{13}C_6$, Ile-$^{13}C_6$, $^{15}N_1$,Pro-$^{13}C_5$ (purchased from CIL)
NIMT	5-point (IDMS/MS & UV)	As supplied by BIPM.	Internal standard was L-Phe, ($^{13}C_6$, 99%) (purchased from CIL)
NIST	5-point bracketing IDMS/MS. Two calibration sets for each amino acid from separate stock solutions.	As supplied by BIPM.	$^{1}5N$ and ^{13}C -stable isotope labeled amino acids purchased from CIL. Mixed directly with sample gravimetrically prior to analysis.
NMIJ	Internal standard calibration	As supplied by BIPM.	Asp solution prepared from NMIJ CRM 6027-a

Institute	Calibration	Calibrants	Internal Standards
NMISA	External calibration (LC-UV) and bracketing IDMS (GC-TOFMS) and IDMS/MS (LC-MS/MS).	As supplied by BIPM.	$^{13}C_3$ Phe (Lot-PR18416), $^{13}C_6$ Ile (Lot-PR1600513), $^{13}C_5$ Pro (Lot-PR18738) (purchased from CIL)
NRC	Exact matching double isotope dilution (IDMS) 3 aliquots per ampoule. 2 primary standard gravimetric preps per AA.	As supplied by BIPM.	$^{13}C_6$-Phe,$^{13}C_6$-Leu,$^{13}C_6$-Ile,$^{13}C_5$-Pro (purchased from CIL) Added at last step
PTB	IDMS with single-point calibration	As supplied by BIPM.	L-Phe-$^{13}C_9$,^{15}N; L-Leu-$^{13}C_6$; L-Ile-$^{13}C_6$; L-Pro-$^{13}C_5$,^{15}N (purchased from CIL)
UME	IDMS with 5 point calibration	As supplied by BIPM.	L-Phe (Ring-D5, 98%); L-Leu (1-^{13}C, 99%); L-Ile (^{15}N, 98%); L-Pro (^{15}N, 98%) [purchased from CIL]
VNIIM	Pro, Leu, Ile: IS calibration (IS = L-Trp); single-point; Phe: External calibration, single-point	As supplied by BIPM.	L-Trp from Sigma-Aldrich cat.# T0254-1g (>98 %

Table E-4 Verification Methods Reported for CCQM-K78.a Value Assignments

Institute	Result Verification
BIPM	Independent check of the assigned value using as calibrators an alternative set of amino acid pure materials value-assigned in house by BIPM
EXHM	Methods used gave results consistent with the certified values when applied to the NIST SRM 2389a Amino Acids in 0.1 mol/L Hydrochloric Acid
HSA	Pure substance CRMs of L-Phe (HRM-1014A), L-Ile (HRM-1013A), and L-Pro (HRM-1007A) from HSA, and pure substance CRM of L-Leu (CRM 6012-a) from NMIJ, were used to prepare a standard solution for uses as quality a control/ verification material.
NIMC	Checked using a control sample prepared from CRM pure materials assigned by NIMC for Phe (GBW09235), Leu (GBW09237), Ile (GBW092378) and Pro (GBW(E)100084)
NIMT	Methods used gave results consistent with the certified values when applied to the NIST SRM 2389a Amino Acids in 0.1 mol/L Hydrochloric Acid
NIST	Methods used gave results consistent with the certified values when applied to the NIST SRM 2389a Amino Acids in 0.1 mol/L Hydrochloric Acid
NMIJ	NMIJ compared the purity of the supplied standard materials with those of NMIJ's amino acid CRMs. No significant difference was observed between concentrations of single component solutions prepared from respective standards.
NMISA	LC-UV analysis for each component after prederivatisation with AQC gave results consistent with the values obtained by LC-MS/MS and GC-TOFMS
UME	Values obtained were consistent with independent assignments by qNMR

APPENDIX F: Summary of Participant Uncertainty Estimation Approaches

The following are text excerpts and/or pictures of the uncertainty-related information provided by the participants in the reporting form.

BIPM

For replicate analysis of an individual double IDMS mixture, where the measurement equation is

$$W_x = \left(\frac{m_{solute}}{m_{final}}\right)_{stock} * \left(\frac{m_{stock}}{m_{final}}\right)_{working} * \frac{m_z}{m_{yc}} * \frac{m_y}{m_x} * \frac{R'_B}{R'_{Bc}} * P$$

and:

W_x	Sample Mass Fraction
m_{solute}	Mass of the analyte weighed in the preparation of the stock solution
$m_{final(stock)}$	Final mass of the stock solution
m_{stock}	Mass of the stock solution weighed in the preparation of the working solution
$m_{final(working)}$	Final mass of the working solution
m_z	Mass of the analyte solution added to the standards
m_{yc}	Mass of the IS solution added to the standards
m_y	Mass of IS solution added to the samples
m_x	Mass of sample used
R'_B	Analyte/IS ratio measured in the sample
R'_{Bc}	Analyte/IS ratio measured in the standard
P	Mass fraction purity of calibration standard

MU budget for an individual result (for duplicate analysis for Leu of two samples prepared from aliquots from one ampoule) are shown. The individual masses were similar but non-identical for the two sample preparations and the four results obtained were normalized for combination into the MU budget.

Uncertainty sources	Value	Standard Uncertainty	Sensitivity Coefficient	Uncertainty Component
m solute (stock) [mg]	25.322	3.46E-03	7.519	0.026
m final (stock) [g]	25.112	6.36E-04	-7.582	0.005
m stock (working) [mg]	199.418	5.46E-03	0.955	0.005
m final (working) [g]	0.996	2.17E-05	-191.103	0.004
Purity of calibrator [kg/kg]	0.998	0.002	190.728	0.381
Analyte soln. mass - mz [mg]	308.54	3.42E-03	0.617	0.002
IS mass in the standards - myc [mg]	324.26	3.42E-03	-0.587	0.002
IS mass in the samples - my [mg]	312.20	3.42E-03	0.610	0.002
Sample mass - mx [mg]	309.25	3.42E-03	-0.616	0.002
R'B/R'Bc (repeatability)	0.98	0.0244	193.545	4.728
w_x (µg/g)	**198.3 ± 13.2**		**u**	**4.743**
			k	**2.78**
			$U_{95\%}$	**13.2**

However the values derived from analysis of each of the three individual ampoules were not consistent within their assigned uncertainties. For value assignment for the comparison, the assignments for each of the three ampoules were used and the mid-range of these values, taking into account their expanded uncertainties, was the assigned value. The associated standard uncertainty of the reported result assumed a rectangular distribution over this range.

EXHM:

For the reverse IDMS experiments, the measurement equation is:

$$w_{A,S} = w_{A,C} \frac{m_{S,dil}}{m_{S,in}} \times \frac{m_{C,in}}{m_{C,dil}} \times \frac{m_{is,S}}{m_{D,S}} \times \frac{m_{A,C}}{m_{is,C}} \times \frac{R_S}{R_C}$$

where
- $w_{A,S}$ = dry mass fraction of the analyte (LEU, ILE, and PHE) in the sample, ($\mu g/kg$)
- $w_{A,C}$ = mass fraction of the amino acid in the calibrant solution, ($\mu g/kg$)
- $m_{S,in}$ = the mass of sample in the diluted sample (g)
- $m_{S,dil}$ = the total mass of the diluted sample (g)
- $m_{C,in}$ = the mass of sample in the diluted calibrant (g)
- $m_{C,dil}$ = the total mass of the diluted calibrant (g)
- $m_{is,S}$ = mass of internal standard solution added to sample blend, (g)
- $m_{D,S}$ = mass of diluted test material in sample blend, (g)
- $m_{A,C}$ = mass of the calibrant solution added to calibration blend, (g)
- $m_{is,C}$ = mass of internal standard solution added to calibration blend, (g)
- R_S = measured peak area ratio of the selected ions in the sample blend
- R_C = measured peak area ratio of the selected ions in the calibration blend

The equation used to estimate standard uncertainty is:

$$u(w_{BS}) = \sqrt{(s_R)^2 + \sum \left(C_j u(m_i)\right)^2 + \sum \left(C_j u(R_i)\right)^2 + \left(C_j u(w_{MC})\right)^2}$$

where s_R is the standard deviation under reproducibility conditions, n the number of determinations and C_j the sensitivity coefficients associated with each uncertainty component (masses, ion ratios and calibrant concentration). The uncertainty of the peak area ratios was considered to have been included in the estimation of method precision.

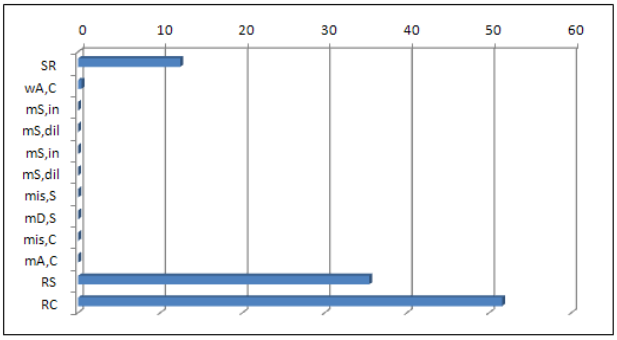
Uncertainty estimation was carried out according to JCGM 100: 2008. The standard uncertainties were combined as the sum of the squares of the product of the sensitivity coefficient (obtained by partial differentiation of the measurement equation) and standard uncertainty to give the square of the combined uncertainty. The square root of this value was multiplied by a coverage factor (95% confidence interval) from the t-distribution at the total effective degrees of freedom obtained from the Welch-Satterthwaite equation to give the expanded uncertainty.

The uncertainty budgets for LEU, ILE, and PHE are shown in the following page

EXHM (ctd) : Leucine

uncertainty component (typical values)	symbol	value	sensitivity coefficient	standard uncertainty	relative uncertainty	$C_i \times u_i$	$(C_i \times u_i)^2$
method precision	s_R	200,634	1,000	1,12369	0,0056	1,1237	1,2627
mass fraction of LEU in the calibration solution, (µg/g)	$w_{A,C}$	200,699	1,000	0,21200	0,0011	0,2119	0,0449
the mass of calibrant in the dilute calibration solution (g)	$m_{S,in}$	0,09944	2017,6	0,00002	0,0002	0,0392	0,0015
the total mass of the diluted calibration solution (g)	$m_{S,dil}$	0,79522	-252,3	0,00002	0,0000	-0,0057	0,0000
the mass of sample in the diluted sample (g)	$m_{S,in}$	0,10098	-1986,9	0,00002	0,0002	-0,0386	0,0015
the total mass of the diluted sample (g)	$m_{S,dil}$	0,80924	247,9	0,00002	0,0000	0,0056	0,0000
mass of LEU-^{15}N solution added to sample blend, (g)	$m_{is,S}$	0,07852	2555,2	0,00002	0,0002	0,0494	0,0024
mass of diluted test material in sample blend, (g)	$m_{D,S}$	0,07860	-2552,6	0,00002	0,0002	-0,0494	0,0024
mass of LEU solution added to calibration blend, (g)	$m_{is,C}$	0,07893	2541,9	0,00002	0,0002	0,0492	0,0024
mass of LEU-^{15}N solution added to calibration blend, (g)	$m_{A,C}$	0,07872	-2548,7	0,00002	0,0002	-0,0493	0,0024
measured peak area ratio of the selected ions in the sample blend	R_S	0,905	221,7	0,00856	0,0095	1,8980	3,6023
measured peak area ratio of the selected ions in the calibration blend	R_C	0,909	-220,8	0,01037	0,0114	-2,2896	5,2424

result (µg/g)	200,63
combined standard uncertainty (µg/g)	3,19
relative uncertainty (%)	1,59
effective degrees of freedom	
coverage factor	2,0
expanded uncertainty (µg/kg)	6,38



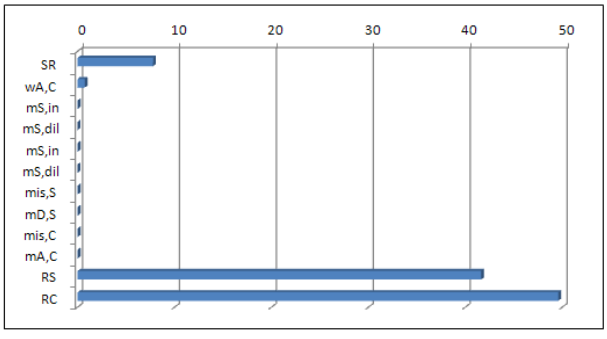
MEASUREMENT EQUATION

$$w_{A,S} = w_{A,C} \frac{m_{S,dil}}{m_{S,in}} \times \frac{m_{C,in}}{m_{C,dil}} \times \frac{m_{is,S}}{m_{D,S}} \times \frac{m_{A,C}}{m_{is,C}} \times \frac{R_S}{R_C}$$

Isoleucine

uncertainty component (typical values)	symbol	value	sensitivity coefficient	standard uncertainty	relative uncertainty	$C_i \times u_i$	$(C_i \times u_i)^2$
method precision	s_R	213,578	1,000	0,72713	0,0034	0,7271	0,5287
mass fraction of ILE in the calibration solution, (µg/g)	$w_{A,C}$	211,008	1,012	0,22173	0,0011	0,2244	0,0504
the mass of calibrant in the dilute calibration solution (g)	$m_{S,in}$	0,09944	2147,8	0,00002	0,0002	0,0417	0,0017
the total mass of the diluted calibration solution (g)	$m_{S,dil}$	0,79522	-268,6	0,00002	0,0000	-0,0060	0,0000
the mass of sample in the diluted sample (g)	$m_{S,in}$	0,10098	-2115,1	0,00002	0,0002	-0,0411	0,0017
the total mass of the diluted sample (g)	$m_{S,dil}$	0,80924	263,9	0,00002	0,0000	0,0059	0,0000
mass of ILE-^{15}N solution added to sample blend, (g)	$m_{is,S}$	0,07852	2720,0	0,00002	0,0002	0,0526	0,0028
mass of diluted test material in sample blend, (g)	$m_{D,S}$	0,07860	-2717,3	0,00002	0,0002	-0,0525	0,0028
mass of ILE solution added to calibration blend, (g)	$m_{is,C}$	0,07893	2705,9	0,00002	0,0002	0,0523	0,0027
mass of ILE-^{15}N solution added to calibration blend, (g)	$m_{A,C}$	0,07872	-2713,1	0,00002	0,0002	-0,0525	0,0028
measured peak area ratio of the selected ions in the sample blend	R_S	0,962	222,0	0,00757	0,0079	1,6801	2,8227
measured peak area ratio of the selected ions in the calibration blend	R_C	0,954	-223,9	0,00819	0,0086	-1,8335	3,3619

result (µg/g)	213,58
combined standard uncertainty (µg/g)	2,60
relative uncertainty (%)	1,22
effective degrees of freedom	
coverage factor	2
expanded uncertainty (µg/kg)	5,21



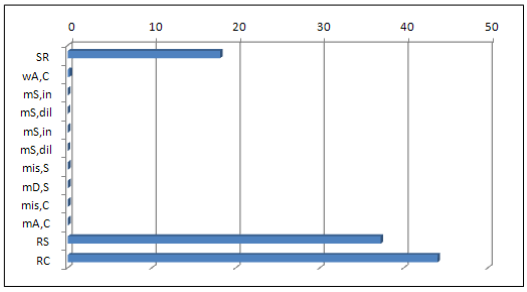
MEASUREMENT EQUATION

$$w_{A,S} = w_{A,C} \frac{m_{S,dil}}{m_{S,in}} \times \frac{m_{C,in}}{m_{C,dil}} \times \frac{m_{is,S}}{m_{D,S}} \times \frac{m_{A,C}}{m_{is,C}} \times \frac{R_S}{R_C}$$

EXHM (ctd): Phenylalanine

uncertainty component (typical values)	symbol	value	sensitivity coefficient	standrard uncertainty	relative uncertainty	$C_i \times u_i$	$(C_i \times u_i)^2$
method precision	s_R	492,210	1,000	1,96421	0,0040	1,9642	3,8581
mass fraction of PHE in the calibration solution, ($\mu g/g$)	$w_{A,C}$	487,014	1,011	0,22173	0,0005	0,2241	0,0502
the mass of calibrant in the dilute calibration solution (g)	$m_{S,in}$	0,09944	4949,8	0,00002	0,0002	0,0962	0,0092
the total mass of the diluted calibration solution (g)	$m_{S,dil}$	0,79522	-619,0	0,00002	0,0000	-0,0139	0,0002
the mass of sample in the diluted sample (g)	$m_{S,in}$	0,10098	-4874,3	0,00002	0,0002	-0,0947	0,0090
the total mass of the diluted sample (g)	$m_{S,dil}$	0,80924	608,2	0,00002	0,0000	0,0137	0,0002
mass of PHE-$^{13}C_9{}^{15}N$ solution added to sample blend, (g)	$m_{is,S}$	0,07852	6268,6	0,00002	0,0002	0,1212	0,0147
mass of diluted test material in sample blend, (g)	$m_{D,S}$	0,07860	-6262,2	0,00002	0,0002	-0,1211	0,0147
mass of PHE solution added to calibration blend, (g)	$m_{is,C}$	0,07893	6236,0	0,00002	0,0002	0,1206	0,0145
mass of PHE-$^{13}C_9{}^{15}N$ solution added to calibration blend, (g)	$m_{A,C}$	0,07872	-6252,7	0,00002	0,0002	-0,1209	0,0146
measured peak area ratio of the selected ions in the sample blend	R_S	0,439	1121,6	0,00251	0,0057	2,8153	7,9259
measured peak area ratio of the selected ions in the calibration blend	R_C	0,436	-1129,4	0,00271	0,0062	-3,0606	9,3670

result (µg/g)	492,21
combined standard uncertainty (µg/g)	4,61
relative uncertainty (%)	0,94
effective degrees of freedom	
coverage factor	2
expanded uncertainty (µg/kg)	9,23



MEASUREMENT EQUATION

$$w_{A,S} = w_{A,C} \frac{m_{S,dil}}{m_{S,in}} \times \frac{m_{C,in}}{m_{C,dil}} \times \frac{m_{is,S}}{m_{D,S}} \times \frac{m_{A,C}}{m_{is,C}} \times \frac{R_S}{R_C}$$

GLHK:

Leucine	Value x	Standard uncertainty $u(x_i)$	Relative standard uncertainty $u(x_i)/x_i$
Mass fraction of spike solution	200.67	3.09E-01	1.54E-03
Weight of internal standard in sample blend	0.79486	5.30E-05	6.67E-05
Weight of sample	0.05046	5.30E-05	1.05E-03
Weight of internal standard in calibration blend	0.80102	5.30E-05	6.62E-05
Weight of standard added to calibration blend	0.05039	5.30E-05	1.05E-03
Isotope ratio of sample	1.031	2.62E-03	2.54E-03
Isotope ratio ofcalibration blend	1.027	2.51E-03	2.45E-03
Run to run variability	1.000	1.01E-02	1.01E-02
Combined uncertainty (ug/g)			2.17
Expanded uncertainty (ug/g)			4.34
Relative Expanded Uncertainty (%)			2.18

Isoleucine	Value x	Standard uncertainty $u(x_i)$	Relative standard uncertainty $u(x_i)/x_i$
Mass fraction of spike solution	212.66	3.43E-01	1.61E-03
Weight of internal standard in sample blend	0.79486	5.30E-05	6.67E-05
Weight of sample	0.05046	5.30E-05	1.05E-03
Weight of internal standard in calibration blend	0.80102	5.30E-05	6.62E-05
Weight of standard added to calibration blend	0.05039	5.30E-05	1.05E-03
Isotope ratio of sample	0.996	1.98E-03	1.99E-03
Isotope ratio ofcalibration blend	0.977	2.28E-03	2.33E-03
Run to run variability	1.000	8.81E-03	8.81E-03
Combined uncertainty (ug/g)			2.06
Expanded uncertainty (ug/g)			4.12
Relative Expanded Uncertainty (%)			1.92

Proline	Value x	Standard uncertainty $u(x_i)$	Relative standard uncertainty $u(x_i)/x_i$
Mass fraction of spike solution	47.49	1.19E-01	2.50E-03
Weight of internal standard in sample blend	0.79486	5.30E-05	6.67E-05
Weight of sample	0.05046	5.30E-05	1.05E-03
Weight of internal standard in calibration blend	0.80102	5.30E-05	6.62E-05
Weight of standard added to calibration blend	0.05039	5.30E-05	1.05E-03
Isotope ratio of sample	0.989	5.72E-03	5.78E-03
Isotope ratio ofcalibration blend	0.988	6.73E-03	6.82E-03
Run to run variability	1.000	1.68E-02	1.68E-02
Combined uncertainty (ug/g)			0.90
Expanded uncertainty (ug/g)			1.80
Relative Expanded Uncertainty (%)			3.86

Phenylalanine	Value x	Standard uncertainty $u(x_i)$	Relative standard uncertainty $u(x_i)/x_i$
Mass fraction of spike solution	487.80	6.09E-01	1.25E-03
Weight of internal standard in sample blend	0.77478	5.30E-05	6.85E-05
Weight of sample	0.04953	5.30E-05	1.07E-03
Weight of internal standard in calibration blend	0.77639	5.30E-05	6.83E-05
Weight of standard added to calibration blend	0.04964	5.30E-05	1.07E-03
Isotope ratio of sample	1.101	8.08E-03	7.34E-03
Isotope ratio ofcalibration blend	1.102	7.22E-03	6.55E-03
Run to run variability	1.000	1.15E-02	1.15E-02
Combined uncertainty (ug/g)			7.45
Expanded uncertainty (ug/g)			14.89
Relative Expanded Uncertainty (%)			3.05

Leucine	Value x	Standard uncertainty $u(x_i)$	Relative standard uncertainty $u(x_i)/x_i$
Mass fraction of standard solution	199.70	3.09E-01	1.55E-03
Weight of internal standard in sample blend	0.10057	5.30E-05	5.27E-04
Weight of sample	0.09817	5.30E-05	5.40E-04
Weight of internal standard in calibration blend	0.10048	5.30E-05	5.28E-04
Weight of standard added to calibration blend	0.09776	5.30E-05	5.43E-04
Relative response factor of measurand in sample blend	0.395	3.93E-04	9.94E-04
Relative response factor of measurand in calibration blend	0.392	3.09E-04	7.87E-04
Factor of derivatization	0.997	2.58E-03	2.59E-03
Run to run variability	1.000	9.98E-03	9.98E-03
Combined uncertainty (ug/g)			2.11
Expanded uncertainty (ug/g)			4.22
Relative Expanded Uncertainty (%)			2.11

Isoleucine	Value x	Standard uncertainty $u(x_i)$	Relative standard uncertainty $u(x_i)/x_i$
Mass fraction of standard solution	214.96	3.43E-01	1.60E-03
Weight of internal standard in sample blend	0.10057	5.30E-05	5.27E-04
Weight of sample	0.09817	5.30E-05	5.40E-04
Weight of internal standard in calibration blend	0.10048	5.30E-05	5.28E-04
Weight of standard added to calibration blend	0.09776	5.30E-05	5.43E-04
Relative response factor of measurand in sample blend	0.416	6.04E-04	1.45E-03
Relative response factor of measurand in calibration blend	0.412	4.13E-04	1.00E-03
Factor of derivatization	0.998	2.32E-03	2.33E-03
Run to run variability	1.000	1.04E-02	1.04E-02
Combined uncertainty (ug/g)			2.38
Expanded uncertainty (ug/g)			4.77
Relative Expanded Uncertainty (%)			2.20

Proline	Value x	Standard uncertainty $u(x_i)$	Relative standard uncertainty $u(x_i)/x_i$
Mass fraction of standard solution	48.51	1.19E-01	2.44E-03
Weight of internal standard in sample blend	0.10057	5.30E-05	5.27E-04
Weight of sample	0.09817	5.30E-05	5.40E-04
Weight of internal standard in calibration blend	0.10048	5.30E-05	5.28E-04
Weight of standard added to calibration blend	0.09776	5.30E-05	5.43E-04
Relative response factor of measurand in sample blend	1.002	1.45E-03	1.45E-03
Relative response factor of measurand in calibration blend	1.018	9.68E-04	9.51E-04
Factor of derivatization	0.999	4.62E-03	4.62E-03
Run to run variability	1.000	1.41E-02	1.41E-02
Combined uncertainty (ug/g)			0.71
Expanded uncertainty (ug/g)			1.41
Relative Expanded Uncertainty (%)			3.04

Phenylalanine	Value x	Standard uncertainty $u(x_i)$	Relative standard uncertainty $u(x_i)/x_i$
Mass fraction of standard solution	494.35	6.09E-01	1.23E-03
Weight of internal standard in sample blend	0.10057	5.30E-05	5.27E-04
Weight of sample	0.09817	5.30E-05	5.40E-04
Weight of internal standard in calibration blend	0.10048	5.30E-05	5.28E-04
Weight of standard added to calibration blend	0.09776	5.30E-05	5.43E-04
Relative response factor of measurand in sample blend	0.941	1.04E-03	1.11E-03
Relative response factor of measurand in calibration blend	0.942	1.32E-03	1.40E-03
Factor of derivatization	0.995	5.86E-03	5.89E-03
Run to run variability	1.000	1.25E-02	1.25E-02
Combined uncertainty (ug/g)			6.86
Expanded uncertainty (ug/g)			13.72
Relative Expanded Uncertainty (%)			2.80

HSA

The mass fraction of each measurand calculated based on equation (1):

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

where

C_X = mass fraction of measurand in the sample solution
M_X = mass of sample solution (determined by weighing)
M_Y = mass of isotope standard solution (determined by weighing)
W_Y = mass of the isotope labeled standard spiked into the sample solution (equals to $M_Y \times C_Y$)
R_B = peak area ratio of sample blend (determined by LC-MS/MS measurements)
C_Y = concentration of isotope labeled standard solution (determined by weighing and from purity of the isotope labeled standard)
m = gradient of the slope of linear regression plot (determined by the linear fit of the isotope mass ratio and the peak area ratio of the calibration blends)
b = intercept on y axis of the linear regression plot (determined by the linear fit of the isotope mass ratio and the peak area ratio of the calibration blends)

Considering $R_M = mR_B + b$, and let $R_M = R_M' C_Y / C_Z$, Equation (1) becomes:

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

where

R_M = isotope mass ratio in sample blend
C_Z = concentration of measurant in the calibration standard solution

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

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

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

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

$$\frac{\partial C_X}{\partial F_C} = \frac{C_X}{F_C} \quad \frac{\partial C_X}{\partial F_I} = \frac{C_X}{F_I}$$

The standard uncertainty of each component was calculated as follows:

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

(2) F_P: The standard deviation of the results 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).

HSA (ctd)

(4) F_C: The standard deviation of the difference of the results using two columns 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 columns divided by 2 (for significant difference using t-test).
(5) C_Z: The purity and uncertainty of the calibration standards from BIPM with the uncertainty of weighing for 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 RM' was calculated using equation (4):

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

where

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

R_B = peak area ratio of sample blend

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

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

N = injection time for each sample

R_{Mc} = isotope mass ratio in calibration blends

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

The combined standard uncertainty was calculated using equation (5):

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

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 coverage factor (k = 2) for 95% confidence level.

HSA (ctd)

Table 1 Uncertainty budget of phenylalanine						
	x	**u(x)**	**u(x)/(x) (%)**	**dCx/dx**	**c^2 . $u(x)^2$**	**% contribution**
M_X (g)	0.0994	0.000127	0.128	4863.3	0.3832	1.12
M_Y(g)	0.0978	0.000127	0.130	4944.1	0.3960	1.16
C_Z(mg/g)	2029.53	4.49367	0.221	0.2383	1.1467	3.36
R_M'	0.95884	0.00793	0.827	504.39	16.0017	46.87
F_P(mg/g)	483.6	3.38689	0.700	1.0	11.4710	33.60
F_C(mg/g)	483.6	1.82394	0.377	1.0	3.3267	9.74
F_I(mg/g)	483.6	1.18915	0.246	1.0	1.4141	4.14
Table 2 Uncertainty budget of leucine.						
	x	**u(x)**	**u(x)/(x)**	**dCx/dx**	**c2 . u(x)2**	
M_X (g)	0.0992	0.000127	0.128	1996.0	0.0645	1.44
M_Y(g)	0.0980	0.000092	0.094	2020.7	0.0345	0.77
C_Z(mg/g)	1757.2	5.68187	0.323	0.1127	0.4103	9.16
R_M'	0.97937	0.00486	0.497	202.26	0.9682	21.62
F_P(mg/g)	198.1	1.58388	0.800	1.0	2.5087	56.01
F_C(mg/g)	198.1	0.59119	0.298	1.0	0.3495	7.80
F_I(mg/g)	198.1	0.37885	0.191	1.0	0.1435	3.20
Table 3 Uncertainty budget of isoleucine.						
	x	**u(x)**	**u(x)/(x)**	**dCx/dx**	**c2 . u(x)2**	
M_X (g)	0.0992	0.000127	0.128	2165.0	0.0759	1.57
M_Y(g)	0.0988	0.000092	0.093	2172.1	0.0399	0.82
C_Z(mg/g)	1511.7	4.83190	0.320	0.1420	0.4710	9.72
R_M'	0.97240	0.00464	0.477	220.81	1.0486	21.64
F_P(mg/g)	214.7	1.49605	0.697	1.0	2.2382	46.19
F_C(mg/g)	214.7	0.73320	0.341	1.0	0.5376	11.09
F_I(mg/g)	214.7	0.65926	0.307	1.0	0.4346	8.97
Table 4 Uncertainty budget of proline						
	x	**u(x)**	**u(x)/(x)**	**dCx/dx**	**c2 . u(x)2**	
M_X (g)	0.0992	0.000127	0.128%	470.76	0.00359	1.84%
M_Y(g)	0.0980	0.000092	0.094%	476.57	0.00192	0.98%
C_Z(mg/g)	1025.3	4.89760	0.478%	0.0456	0.04980	25.53%
R_M'	0.98342	0.00351	0.356%	47.51	0.02773	14.21%
F_P(mg/g)	46.72	0.29811	0.638%	1.0	0.08887	45.55%
F_C(mg/g)	46.72	0.15228	0.326%	1.0	0.02319	11.89%

KRISS

Measurement equation:

$$C_x = \frac{R_{a2}}{R_{a1}} \times \frac{R_{m1}}{R_{m2}} \times \frac{C_s}{f_d}$$

C_x: mass fraction of analyte (mg/kg)
R_{a2}: area ratio of sample (analyte/internal standard form)
R_{a1}: area ratio of standard (analyte/internal standard form)
R_{m1}: mass ratio of isotope ratio standard (standard solution/ IS solution)
R_{m2}: mass ratio of sample (sample/ IS solution)
C_s: mass fraction of analyte in standard (mg/kg)
f_d: dilution factor (dilution ratio) of sample

Uncertainty breakdown

Category	Factor	Val	Ile	Leu	Phe	Phe*
Systematic u (u_{std})	Uncertainty of purity of primary reference material	0.05%	0.10%	0.10%	0.05%	0.05%
	Uncertainty of gravimetric preparation for standard solutions	0.40%	0.34%	0.52%	0.44%	0.31%
	Uncertainty of gravimetric mixing for calibration isotope standard mixtures	0.42%	0.42%	0.42%	0.42%	-
	Area ratio of native/isotope for the calibration standard mixture, observed by LC-MS	0.71%	0.10%	0.79%	0.61%	0.42%
Random u (u_{sam})	Measurement of sample soluteons including homogeneity (s^2/n)	0.02%	0.06%	0.03%	0.03%	0.14%
u_{com}	$\sqrt{(u_{std}^2 + u_{sam}^2)}$	0.92%	0.56%	1.04%	0.86%	0.54%
v_{eff}	Welch-Satterthwaite formula	8	6	8	8	24
$k(>95\%)$	t-table	2.31	2.45	2.31	2.31	2.06
U_{exp}	$k \times u_{com}$	2.12%	1.37%	2.39%	1.98%	1.12%

* Measured result of Phe by LC-UV

Systematic uncertainty: Uncertainties of weighing and mixtures with isotopes calculated from the standard deviation of the response factor (RF) of repetitively prepared standard solutions and the standard uncertainty of RF of sub-samples from the same standard solutions, respectively.
Random uncertainty: Square and divided into sample numbers of standard deviations of multiple measurement results from three individual samples (s^2/n)
Combined standard uncertainties obtained by combining systematic and random uncertainty
Expanded uncertainty calculated using a coverage factor (k) of 95% confidence level of the t-distribution with effective degrees of freedom (v_{eff}) obtained from Welch-Satterthwaite formula.

LGC

Measurement equation for amino acid (AA) mass fraction in each replicate blend from each sample:

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

where:

w_z	=	mass fraction of the calibration blend.
u_{wz}	=	standard uncertainty associated with preparation of the calibration blend.
m_x	=	mass of sample used.
u_{mx}	=	uncertainty associated with the mass of sample used.
m_y	=	mass of labelled AA added to the sample.
u_{my}	=	uncertainty associated with the mass of labelled AA added to the sample.
m_z	=	mass of AA added to the calibration blend.
u_{mz}	=	uncertainty associated with the mass of AA added to the calibration blend.
m_{yc}	=	mass of labelled AA added to the calibration blend
u_{myc}	=	uncertainty associated with the mass of labelled AA added to the calibration blend.
R'_B/R'_{BC}	=	average ratio of the measured ratio of natural to labelled AA in the sample blend R'_B and in the calibration blend R'_{Bc} (n=5)
$u_{R'B/R'BC}$	=	standard deviation of the R'_B/R'_{Bc}
df	=	dilution factor for the gravimetric dilution of the sample
u_{df}	=	uncertainty associated with the dilution factor

uncertainty budget:

$$u_{w_x} = W_x \sqrt{\left(\frac{u_{wz}}{w_z}\right)^2 + \left(\frac{u_{m_x}}{m_x}\right)^2 + \left(\frac{u_{m_y}}{m_y}\right)^2 + \left(\frac{u_{m_z}}{m_z}\right)^2 + \left(\frac{u_{m_{yc}}}{m_{yc}}\right)^2 + \left(\frac{u_{R'_B/R'_{Bc}}}{R'_B/R'_{Bc}}\right)^2 + \left(\frac{u_{df}}{df}\right)^2}$$

Reported mass fraction calculated as the average of 3 replicate blends each from the 3 samples (n=9).

Combined uncertainty was calculated as:

Square root(sum of squares) of average uncertainty per replicate result u_w and b_{var}, where

b_{var} = standard deviation of the mass fractions w_x per replicate result.

LNE

Measurement equation:

$$C_s = \frac{Q \cdot m_{spike} \cdot C_{spike}}{m_s} \cdot F_D$$

C_s: amino acid mass fraction in the sample.
m_s, m_{spike}: mass of diluted sample and spike, respectively.
F_D: sample dilution factor.
Q: mass ratio of natural to labelled aminoacid calculated from the calibration function
Cspike: amino acid mass fraction of the spike

Uncertainty budget:

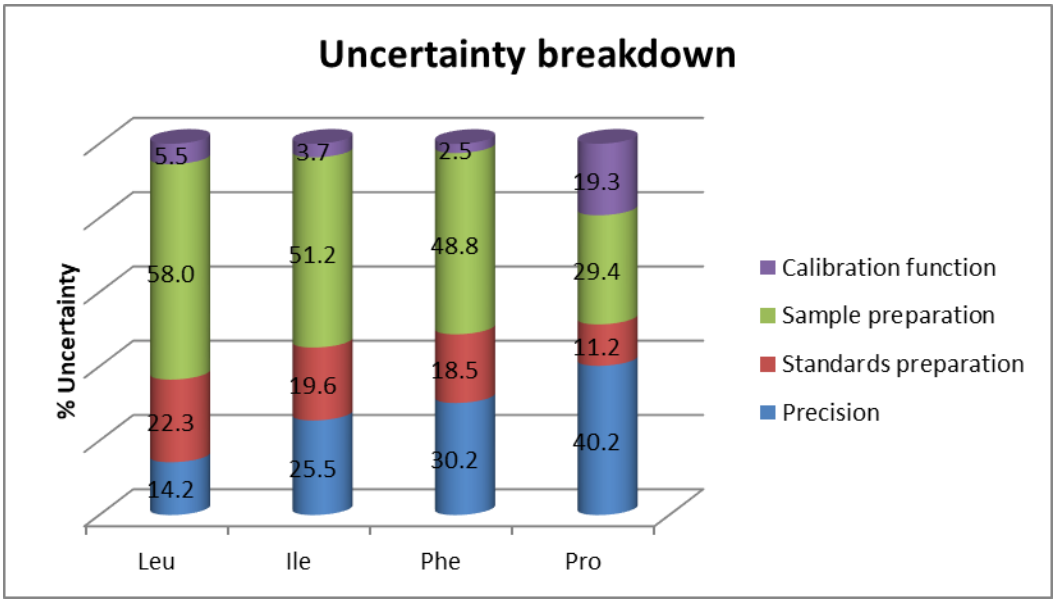
The following uncertainty components were combined following the rules for uncertainty propagation applied to the measurement equation above.

$$u(Q) = Q \cdot \sqrt{\left(\frac{u(Q_{lin})}{Q_{lin}}\right)^2 + u(Q_{cal})^2}$$

$u(Q_{lin})$: uncertainty of the mass ratio calculated through the calibration regression model.
$u(Q_{cal})$: uncertainty associated to the weighing in the preparation of the standards and their purity.
$u(m_s)$, $u(m_{spike})$: uncertainties associated to weighing the sample and spike.
$u(F_D)$: uncertainty associated to weighing in the sample predilution.
$u(C_{spike})$: negligible in matching IDMS.

Standard uncertainty (u) of the reported amino acid mass fraction includes a precision component (u_{rep}), which is the standard deviation of the mass fraction values of the replicates measured.

$$u = \sqrt{u(C_s)^2 + {u_{rep}}^2}$$



NIMC

Uncertainty Budget ofwith LCIDMS																			
Steps	Uncertainty Components	Phenylalanine				Isoleucine				Leucine				Proline				Type of uncertainty
		Value *X*	Unit	Standard uncertainty *u(x)*	Relative uncertainty *u(x)/x*	Value *X*	Unit	Standard uncertainty *u(x)*	Relative uncertainty *u(x)/x*	Value *X*	Unit	Standard uncertainty *u(x)*	Relative uncertainty *u(x)/x*	Value *X*	Unit	Standard uncertainty *u(x)*	Relative uncertainty	
Step1 Sample Diluted	balance 1 (readability 0.01 mg)	0.19537	g	1.1547E-05	5.91033E-05	0.19537	g	1.1547E-05	5.91033E-05	0.19537	g	1.1547E-05	5.9103E-05	0.19537	g	1.1547E-05	5.9103E-05	Type B
	balance 1 (readability 0.01 mg)	1.93438	g	1.1547E-05	5.96936E-06	1.93438	g	1.1547E-05	5.96936E-06	1.93438	g	1.1547E-05	5.9694E-06	1.93438	g	1.1547E-05	5.9694E-06	Type B
Step 2 Preparare Stock solution	balance 2(readability 0.001 mg)	9.717	mg	1.1547E-06	1.18833E-07	7.802	mg	1.1547E-06	1.48001E-07	9.792	mg	1.1547E-06	1.1792E-07	10.576	mg	1.1547E-06	1.0918E-07	Type B
	balance 1 (readability 0.01 mg)	47.24608	g	1.1547E-05	2.44401E-07	34.19494	g	1.1547E-05	3.37682E-07	51.75783	g	1.1547E-05	2.231E-07	60.30682	g	1.1547E-05	1.9147E-07	Type B
	Purity of Phenylalanine	99.8±0.2	%		0.20%	99.7±0.15	%		0.15%	99.7±0.2	%		0.20%	999±1	mg/kg		0.05%	Type B
Step 3 preparation of Mix AAs	balance 1 (readability 0.01 mg)	2.19871	g	1.1547E-05	5.25E-06	0.86478	g	1.1547E-05	1.33525E-05	0.97336	g	1.1547E-05	1.1863E-05	0.24059	g	1.1547E-05	4.7995E-05	Type B
	balance 1 (readability 0.01 mg)	5.71337	g	1.1547E-05	2.02E-06	5.71337	g	1.1547E-05	2.02105E-06	5.71337	g	1.1547E-05	2.021E-06	5.71337	g	1.1547E-05	2.021E-06	Type B
Step 4 preparation of Calibration solution	balance 1 (readability 0.01 mg)	0.33834	g	1.1547E-05	3.41E-05	0.33834	g	1.1547E-05	3.41284E-05	0.33834	g	1.1547E-05	3.4128E-05	0.33834	g	1.1547E-05	3.4128E-05	Type B
	balance 1 (readability 0.01 mg)	0.36148	g	1.1547E-05	3.19E-05	0.36148	g	1.1547E-05	3.19437E-05	0.36148	g	1.1547E-05	3.1944E-05	0.36148	g	0	3.1944E-05	Type B
Step 5 LCIDMS Measurement	Linear Regression				0.20%				0.27%				0.30%				0.10%	Type B
	RSD1				0.42%				0.50%				0.56%				0.33%	Type A
		Average(mg/kg)			488.69	Average(mg/kg)			215.19	Average(mg/kg)			199.91	Average(mg/kg)			46.97	
		Combined Uncertainty(%)			2.48	Combined Uncertainty(%)			1.26	Combined Uncertainty(%)			1.33	Combined Uncertainty(%)			0.16	
		k			2	*k*			2	*k*			2	*k*			2	
		Expand Uncertainty(%)			4.96	Expand Uncertainty(%)			2.53	Expand Uncertainty(%)			2.65	Expand Uncertainty(%)			0.32	

Uncertainty Budget of Phenylalanine with LCUV						
	Uncertainty Components	Value *X*	Unit	Standard uncertainty *u(x)*	Relative uncertainty *u(x)/x*	Type of uncertainty
Step1 Sample Diluted	balance 1 (readability 0.01 mg)	0.19537	g	1.1547E-05	5.91E-05	Type B
	balance 1 (readability 0.01 mg)	1.93438	g	1.1547E-05	5.97E-06	Type B
Step 2 Preparare Stock solution	balance 2(readability 0.001 mg)	10.283	mg	1.1547E-06	1.12E-07	Type B
	balance 1 (readability 0.01 mg)	50.06372	g	1.1547E-05	2.31E-07	Type B
	Purity of Phenylalanine	99.8±0.4			0.20%	
Step 3 preparation of working solution	balance 1 (readability 0.01 mg)	0.88473	g	1.1547E-05	1.31E-05	Type B
	balance 1 (readability 0.01 mg)	0.19244	g	1.1547E-05	6.00E-05	Type B
Step 4 LCUV Measurement	Linear Regression				0.22%	Type B
	RSD1				0.42%	Type A
		Average(mg/kg)			488.60	
		Combined Uncertainty(mg/kg)			2.52	
		k			2	
		Expand Uncertainty(mg/kg)			5.04	

NIMT

Measurement equation:

$$w_x = F_{std} \cdot w_0 \cdot w_{y(x)} \cdot \frac{m_{y(x)}}{m_x}$$

w_x = Mass fraction of measurand in sample, ug/g

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

w y(x) = Mass fraction of internal standard, ug/g

m y(x) = Mass of internal standard spiked into sample, g

mx = Mass of sample, g

Proline using LC-MS/MS			Combination of Uncertainties			
Cx =	50.376	ug/g	Factor	Values	Uncertainties	
u(x) =	0.907	ug/g		x	u(x)	u(x)/(x)
u(x)/x =	1.80%		Measurement equation factors			
Veff(total) =	10.618		Method Precisior	1	0.01310153	0.01310153
k=	2.23	(@ 95% level)	w0	0.504796265	0.000581148	0.001151252
U(x) =	2.021		wy(x)	39.7739379	0.417295775	0.010491689
%U(x) =	4.01%		my(x)	0.01982	7.39047E-05	0.003728795
			mx	0.04009	6.01041E-05	0.001499229
			Calibrant type B	502.9921706	2.501400255	0.00497304
			Calibrant type A	14.55465461	0.007481546	0.000514031

Phenylalanine using LC-MS/MS			Combination of Uncertainties			
Cx =	494.399	ug/g	Factor	Values	Uncertainties	
u(x) =	9.695	ug/g		x	u(x)	u(x)/(x)
u(x)/x =	1.96%		Measurement equation factors			
Veff(total) =	51.872		Method Precisior	1	0.010773703	0.010773703
k=	2.01	(@ 95% level)	w0	4.944157758	0.057936279	0.011718129
U(x) =	19.463		wy(x)	40.06704798	0.420175608	0.010486812
%U(x) =	3.94%		my(x)	0.019935	7.39047E-05	0.003707285
			mx	0.03962	6.01041E-05	0.001517014
			Calibrant type B	1005.818243	2.215269342	0.002202455
			Calibrant type A	144.6070436	0.074874799	0.000517781

Combination of Uncertainties of Phe using LC-UV detection				Uncertainty Analysis Results	
Factor	Values	Uncertainties		Cx = 494.7	ug/g
	x	u(x)	u(x)/(x)	u(x) = 3.227	ug/g
Method Precisior	1	0.003	0.003	u(x)/x = 0.652%	
w0	99.283	0.432	0.004	Veff(total) = 287.4	
mx	0.040	0.000	0.002	k= 1.97	(@ 95% level)
Calibrant type B	999.134	3.320	0.003	U(x) = 6.353	
Calibrant type A	122.100	0.063	0.001	%U(x) = 1.28%	

$$w_x = w_0 \times \frac{d}{m_x}$$

wx = Mass fraction of measurand in sample, ug/g

w0 = Result from linear calibration curve, ug/g

mx = Mass of sample, g

d = Mass of testing solution, g

NIST

A hierarchical Bayesian analysis was performed for statistical assessment of uncertainties. For each of the four measurands, there were sets of 12 measurements (3 samples, 4 replicates) per analyst, 2 calibration sets per amino acid, 2 transitions per amino acid, and duplicate injections, with their standard uncertainties. These were combined using a random effects meta-analysis model which accounted for the uncertainty in each measurement as well as for the between sample uncertainty. The 12 measurements in each set were highly correlated (around 0.98) and so the standard uncertainties of the combined estimates did not fully benefit from the replication, in other words, the replication did not have the usual effect of reducing the uncertainty by division by the square root of the number of replicates.

	Individual components of uncertainty, %			
	reproducibility	calibration	gravimetric procedures	calibrant and injection effect
Phe	0.27	0.60	0.42	0.78
Leu	0.3	1.2	0.91	1.6
Ile	0.3	1.3	0.92	1.3
Pro	0.25	0.53	0.37	0.50

NMIJ

Concentration of analyte in the sample solution was calculated by the following equation.

$$C_{sample} = \frac{A_{sample}}{A_{std}} \times C_{std}$$

C; Concentration (mg/kg), A; Area of HPLC sample; CCQM sample, std; standard solution

The reported value was calculated as arithmetic mean of Ninhydrin method and OPA method. The uncertainty due to difference of methods was considered.

$$C'_{sample} = \frac{C_{sample,\ Ninhydrin} + Csampl_{e,\ OPA}}{2}$$

The budget table of Ninhydrin method except for common uncertainty sources in OPA method

Ninhydrin method		Phe	Leu	Ile	Pro
Source of uncertainty		Relative uncertainty (%)			
Precision of sample measurement	Precision	0.11	0.13	0.11	0.29
	Ampoule	0.08	0.10	0.08	0.23
	Sample preparation	0.01	0.00	0.03	0.00
	Measurement	0.08	0.08	0.06	0.17
Standard solution	Measurement	0.09	0.09	0.01	0.14
Combined uncertainty		0.14	0.16	0.11	0.32

NMIJ (ctd)

The budget table of OPA method except for common uncertainty sources in Ninhydrin method

OPA method		Phe	Leu	Ile	Pro
Source of uncertainty		Relative uncertainty (%)			
Precision of sample measurement	precision	0.07	0.07	0.13	0.26
	Ampoule	0.03	0.04	0.11	0.00
	Sample preparation	0.00	0.00	0.00	0.00
	Measurement	0.07	0.06	0.07	0.26
Standard solution	Measurement	0.20	0.27	0.19	1.04
Combined uncertainty		0.21	0.28	0.23	1.07

Calculation of arithmetic means

	Phe		Leu		Ile		Pro	
	Value	Standard uncertainty	Value	Standard uncertainty	Value	Standard uncertainty	Value	Standard uncertainty
	mg/kg		mg/kg		mg/kg		mg/kg	
The results of Ninhydrin method	488.7	0.7	200.5	0.3	215.4	0.2	47.1	0.2
The results of OPA method	487.1	1.0	199.2	0.6	213.8	0.5	46.3	0.5
The difference between methods		0.80		0.63		0.79		0.37
Arithmetic mean*	487.9	0.9	199.8	0.7	214.6	0.8	46.7	0.4

*Included the uncertainty of between methods

The budget table for reported values

		Phe	Leu	Ile	Pro
Source of uncertainty			Relative uncertainty (%)		
Precision of sample measurement	Combined uncertainty(Weighted mean)	0.176	0.327	0.374	0.846
Standard solution	Precision of preparation	0.340	0.210	0.020	0.180
	Balance	0.005	0.006	0.006	0.005
	Weigh of standard	0.005	0.005	0.005	0.005
	Weigh of solvent	0.0001	0.0001	0.0001	0.0001
	Dilution	0.0015	0.0027	0.0025	0.0022
	Buoyancy correction	Negligible	Negligible	Negligible	Negligible
	Purity of standard	0.050	0.101	0.101	0.050
Sample preparation	Balance	0.007	0.007	0.007	0.007
	Buoyancy correction	Negligible	Negligible	Negligible	Negligible
	Dilution	0.007	0.007	0.007	0.007
Combined standard uncertainty		0.39	0.40	0.39	0.87

Reported values from NMIJ

Phe		Leu		Ile		Pro	
Value	Expanded uncertainty	Value	Expanded uncertainty	Value	Expanded uncertainty	Value	Expanded uncertainty
mg/kg	(k = 2), mg/kg	mg/kg	(k = 2), mg/kg	mg/kg	(k = 2), mg/kg	mg/kg	(k = 2), mg/kg
487.9	3.8	199.8	1.8	214.6	1.8	46.7	1.0

NMIJ also used LC/MS for the determination, but the repeatability of LC/MS were worse than those of above reported methods. Thus, we did not combine the results obtained by LC/MS, although their results corresponded to each other in the range of uncertainty.

1 Uncertainty estimation of amino acids using LC-MS/MS detection and bracketing quantification

LC-MS/MS dIDMS bracketing PHE	PHENYLALANINE	(LC-MS/MS)	490.28	ug/g			
			x	u	u/x	u/x2	% contribution to the overall uncertainty
	Wz -purity	BIPM CRM certificate	1.00	0.00100	0.001001	1E-06	0.45%
	Wz -grav	gravimetric operations in preparation of the calibrant	100.46	0.48695	0.004847	2.35E-05	10.61%
	mz	CRM added to cal blend. Mass balance certificate, weighing ESDM	0.05	0.00000	1.1E-06	1.21E-12	0.00%
	my	Isotope in sample, ESDM	0.05	0.00009	0.001772	3.14E-06	1.42%
	myc	Isotope in Cal, ESDM	0.10	0.00020	0.002013	4.05E-06	1.83%
	mx	ESDM across 100mg aliquots	0.10	0.00030	0.002983	8.9E-06	4.02%
	Df	gravimetric dilution operations	0.02	0.00007	0.003723	1.39E-05	6.26%
	R_B/R_{BC}	ESDM	1.02	0.00840	0.008248	6.8E-05	30.71%
	Precision	ESDM of repeat measurements	490.28	4.87914	0.009952	9.9E-05	44.71%
						0.00022	
						7.30	u
						15.47	U (k= 2.12)
						3.16	Rel U
LC-MS/MS dIDMS bracketing LEU	LEUCINE	(LC-MS/MS)	184.75	ug/g			
			x	u	u/x	u/x2	% contribution to the overall uncertainty
	Wz -purity	BIPM CRM certificate	1.00	0.00100	0.001005	1.01E-06	0.49%
	Wz -grav	gravimetric operations in preparation of the calibrant	100.93	0.63835	0.006325	4E-05	19.49%
	mz	CRM added to cal blend. Mass balance certificate, weighing ESDM	0.05	0.00000	1.1E-06	1.21E-12	0.00%
	my	Isotope in sample, ESDM	0.05	0.00009	0.001772	3.14E-06	1.53%
	myc	Isotope in Cal, ESDM	0.10	0.00020	0.002013	4.05E-06	1.97%
	mx	ESDM across 100mg aliquots	0.10	0.00030	0.002983	8.9E-06	4.34%
	Df	gravimetric dilution operations	0.05	0.00014	0.002896	8.39E-06	4.09%
	R_B/R_{BC}	ESDM	1.00	0.00968	0.009697	9.4E-05	45.82%
	Precision	ESDM of repeat measurements	184.75	1.24916	0.006761	4.57E-05	22.27%
						0.00021	
						2.65	u
						5.74	U (k= 2.17)
						3.11	%Rel U
LC-MS/MS dIDMS bracketing ILE	ISOLEUCINE	(LC-MS/MS)	204.33	ug/g			
			x	u	u/x	u/x2	% contribution to the overall uncertainty
	Wz -purity	BIPM CRM certificate	0.99	0.00100	0.001006	1.01E-06	0.19%
	Wz -grav	gravimetric operations in preparation of the calibrant	100.46	0.67369	0.006706	4.5E-05	8.38%
	mz	CRM added to cal blend. Mass balance certificate, weighing ESDM	0.05	0.00000	1.1E-06	1.21E-12	0.00%
	my	Isotope in sample, ESDM	0.05	0.00009	0.001772	3.14E-06	0.59%
	myc	Isotope in Cal, ESDM	0.10	0.00020	0.002013	4.05E-06	0.76%
	mx	ESDM across 100mg aliquots	0.10	0.00030	0.002983	8.9E-06	1.66%
	Df	gravimetric dilution operations	0.05	0.00014	0.002896	8.39E-06	1.56%
	R_B/R_{BC}	ESDM	1.06	0.01727	0.016338	0.000267	49.75%
	Precision	ESDM of repeat measurements	204.33	2.88342	0.014112	0.000199	37.12%
						0.00054	
						4.73	u
						10.56	U (k= 2.23)
						5.17	%Rel U
LC-MS/MS dIDMS bracketing PRO	PROLINE	(LC-MS/MS)	48.32	ug/g			
			x	u	u/x	u/x2	% contribution to the overall uncertainty
	Wz -purity	BIPM CRM certificate	1.00	0.00100	0.001001	1E-06	0.13%
	Wz -grav	gravimetric operations in preparation of the calibrant	103.35	0.56580	0.005475	3E-05	3.84%
	mz	CRM added to cal blend. Mass balance certificate, weighing ESDM	0.05	0.00000	1.1E-06	1.21E-12	0.00%
	my	Isotope in sample, ESDM	0.05	0.00009	0.001772	3.14E-06	0.40%
	myc	Isotope in Cal, ESDM	0.10	0.00020	0.002013	4.05E-06	0.52%
	mx	ESDM across 100mg aliquots	0.10	0.00030	0.002983	8.9E-06	1.14%
	Df	gravimetric dilution operations	0.20	0.00118	0.005851	3.42E-05	4.39%
	R_B/R_{BC}	ESDM	1.02	0.01883	0.018495	0.000342	43.87%
	Precision	ESDM of repeat measurements	48.32	0.91218	0.018879	0.000356	45.71%
						0.00078	
			min	max		1.35	u
			45.30721	51.32845		3.01	U (k= 2.23)
						6.23	%Rel U

2 Uncertainty estimation of amino acids using GC-TOF/MS detection (after MTBSTFA derivatisation) and bracketing

GC-TOF/MS dIDMS bracketing PHE	PHENYLALANINE	(GC-TOF/MS)	488.88	ug/g			
			x	u	u/x	u/x2	% contribution to the overall uncertainty
	Wz -purity	BIPM CRM certificate	1.00	0.00100	0.001001	1E-06	0.48%
	Wz -grav	gravimetric operations in preparation of the calibrant	100.46	0.48695	0.004847	2.35E-05	11.37%
	mz	CRM added to cal blend. Mass balance certificate, weighing ESDM	0.05	0.00000	1.1E-06	1.21E-12	0.00%
	my	Isotope in sample, ESDM	0.05	0.00009	0.001772	3.14E-06	1.52%
	myc	Isotope in Cal, ESDM	0.10	0.00020	0.002013	4.05E-06	1.96%
	mx	ESDM across 100mg aliquots	0.10	0.00030	0.002983	8.9E-06	4.31%
	Df	gravimetric dilution operations	0.02	0.00007	0.003723	1.39E-05	6.71%
	R_B/R_{BC}	ESDM	1.00	0.00707	0.007055	4.98E-05	24.09%
	Precision	ESDM of repeat measurements	488.88	4.94719	0.01012	0.000102	49.56%
						0.00021	
						7.03	u
						14.86	U (k= 2.12)
						3.04	%Rel U
GC-TOF/MS dIDMS bracketing LEU	LEUCINE	(GC-TOF/MS)	190.27	ug/g			
			x	u	u/x	u/x2	% contribution to the overall uncertainty
	Wz -purity	BIPM CRM certificate	1.00	0.00100	0.001005	1.01E-06	0.19%
	Wz -grav	gravimetric operations in preparation of the calibrant	100.93	0.63835	0.006325	4E-05	7.48%
	mz	CRM added to cal blend. Mass balance certificate, weighing ESDM	0.05	0.00000	1.1E-06	1.21E-12	0.00%
	my	Isotope in sample, ESDM	0.05	0.00009	0.001772	3.14E-06	0.59%
	myc	Isotope in Cal, ESDM	0.10	0.00020	0.002013	4.05E-06	0.76%
	mx	ESDM across 100mg aliquots	0.10	0.00030	0.002983	8.9E-06	1.66%
	Df	gravimetric dilution operations	0.05	0.00014	0.002896	8.39E-06	1.57%
	R_B/R_{BC}	ESDM	0.98	0.00845	0.008614	7.42E-05	13.88%
	Precision	ESDM of repeat measurements	190.27	3.78089	0.019871	0.000395	73.87%
						0.00053	
						4.40	u
						10.05	U (k= 2.28)
						5.28	%Rel U
GC-TOF/MS dIDMS bracketing ILE	ISOLEUCINE	(GC-TOF/MS)	211.58	ug/g			
			x	u	u/x	u/x2	% contribution to the overall uncertainty
	Wz -purity	BIPM CRM certificate	0.99	0.00100	0.001006	1.01E-06	0.20%
	Wz -grav	gravimetric operations in preparation of the calibrant	100.46	0.67369	0.006706	4.5E-05	8.95%
	mz	CRM added to cal blend. Mass balance certificate, weighing ESDM	0.05	0.00000	1.1E-06	1.21E-12	0.00%
	my	Isotope in sample, ESDM	0.05	0.00009	0.001772	3.14E-06	0.63%
	myc	Isotope in Cal, ESDM	0.10	0.00020	0.002013	4.05E-06	0.81%
	mx	ESDM across 100mg aliquots	0.10	0.00030	0.002983	8.9E-06	1.77%
	Df	gravimetric dilution operations	0.05	0.00014	0.002896	8.39E-06	1.67%
	R_B/R_{BC}	ESDM	1.01	0.00584	0.005782	3.34E-05	6.65%
	Precision	ESDM of repeat measurements	211.58	4.22317	0.01996	0.000398	79.32%
						0.00050	
						4.74	u
						10.83	U (k= 2.28)
						5.12	%Rel U
GC-TOF/MS dIDMS bracketing PRO	PROLINE	(GC-TOF/MS)	46.05	ug/g			
			x	u	u/x	u/x2	% contribution to the overall uncertainty
	Wz -purity	BIPM CRM certificate	1.00	0.00100	0.001001	1E-06	0.12%
	Wz -grav	gravimetric operations in preparation of the calibrant	103.35	0.56580	0.005475	3E-05	3.64%
	mz	CRM added to cal blend. Mass balance certificate, weighing ESDM	0.05	0.00000	1.1E-06	1.21E-12	0.00%
	my	Isotope in sample, ESDM	0.05	0.00009	0.001772	3.14E-06	0.91%
	myc	Isotope in Cal, ESDM	0.10	0.00020	0.002013	4.05E-06	0.49%
	mx	ESDM across 100mg aliquots	0.10	0.00030	0.002983	8.9E-06	1.08%
	Df	gravimetric dilution operations	0.20	0.00118	0.005851	3.42E-05	4.16%
	R_B/R_{BC}	ESDM	0.99	0.01244	0.012547	0.000157	41.59%
	Precision	ESDM of repeat measurements	46.05	0.44351	0.009631	9.28E-05	48.00%
						0.00033	
						0.84	u
						1.89	U (k= 2.25)
						4.11	%Rel U

NMISA (ctd)

3. Uncertainty of phenylalanine determination by UV detection and external calibration

external calibration UV detection (PHE)

		484.88	ug/g UV PHE			
		x	u	u/x	u/x2	% contribution to the overall uncertainty
CRM-purity	CRM	1.00	0.001	0.001001	1E-06	0.22%
CRM-gravimetri	mass balance certificate, repeat weighing ESDM	210.83	0.341483	0.00162	2.62E-06	0.59%
Df	gravimetric operations in sample dilution	0.20	0.000205	0.001024	1.05E-06	0.23%
precision	ESDM from repeat measurement	484.88	10.04753	0.020722	0.000429	96.10%
Sy/x	error of the calibration curve	484.88	1.732467	0.003573	1.28E-05	2.86%
				sum	0.000447	ug/g
					10.25	u
					33.90	U (k=3.307)
					6.99	% Rel U

4 The final combined expanded uncertainty reported was determined using the following equation

$$U_{95}(\bar{Y}) = 2 \times \sqrt{\frac{\left(\sum_{j=1}^{N}(Y_j - \bar{Y})^2/N - 1\right) + \left(\sum_{j=1}^{N}\left[\frac{U_{95}(Y_j)}{2}\right]^2/N\right)}{N}}$$

Where j denotes the various methods used in determining the mean;
Y the concentration calculated (ug/g) and
N the number of estimates from different analytical measurements

NRC

Measurement equation:

$$w_A = w_{A*} \cdot \frac{r_{A*} - r_{A^*B}}{r_{A^*B} - r_B} \cdot \frac{r_B - r_{AB}}{r_{AB} - r_{A*}} \cdot \frac{m_{A^*(A^*B),aq}}{m_{B(A^*B),aq}} \cdot \frac{m_{B(AB),aq}}{m_{A(AB),aq}}$$

Symbol	Name
A	Analyte in the sample (natural isotopic composition)
A*	Primary reference standard (natural isotopic composition)
B	Analyte in the isotopic standard (isotopically enriched composition)
AB	Blend of sample A and isotopic standard B
A*B	Blend of primary standard A* and isotopic standard B
$m_{A(AB)}$	Mass of sample A in the blend AB
$m_{B(AB)}$	Mass of isotopic standard B in the blend AB
$m_{A*(A*B)}$	Mass of primary standard A* in the blend A*B
$m_{B(A*B)}$	Mass of isotopic standard B in the blend A*B
r_{AB}	Isotope ratio in the blend AB
r_{A*B}	Isotope ratio in the blend A*B
r_{A*}	Isotope ratio in the standard A*
r_B	Isotope ratio in the spike B
w_A	Mass fraction of A in the sample
w_{A*}	Mass fraction of A in the primary standard

Uncertainty budget

			Contribution (%)			
Component	**Description**	**Type**	**Pro**	**Leu**	**Ile**	**Phe**
$u(m_{A(AB)})$	- Weighing of sample A in the blend AB	A	0.3	0.5	2	0.7
$u(m_{B(AB)})$	- Weighing of sample B in the blend AB	A	0.3	0.5	2	0.7
$u(m_{A*(A*B)})$	- Weighing of sample A* in te blend A*B	A	0.3	0.5	2	0.6
$u(m_{B(A*B)})$	- Weighing of sample B in the blend A*B	A	0.3	0.5	2	0.6
$u(r_{AB})$	- Measuring isotope ratio in the blend AB	A	39	48	40.5	69
$u(r_{A*B})$	- Measuring isotope ratio in the blend A*B	A	11	38	35	26
$u(r_{A*})$	- Isotope ratio in A*	B	ins.	ins.	ins.	ins.
$u(r_B)$	- Isotope ratio in B	B	ins.	ins.	ins.	ins.
$u(w_{A*})$	- Weighing of A*	A	43	1	0.5	0.5
$u(A^*_{purity})$	- Purity of primary standard A*	A	6	11	16	1.9

Individual uncertainties were combined analytically according to the JCGM Guide 100.

PTB

Measurement equation:

Results were calculated using the double IDMS equation:

$$w_{sample} = w_{calib} \cdot \frac{m_{calib} \cdot m_{spike(sample)} \cdot R_{M(calib)}}{m_{sample} \cdot m_{spike(calib)} \cdot R_{M(sample)}}$$

where:

w_{sample} = mass fraction of analyte in sample

w_{calib} = mass fraction of analyte in the calibration standard solution used to prepare calibration blend

m_{calib} = mass of the calibration standard solution added to calibration blend

m_{sample} = mass of sample added to sample blend

$m_{spike(calib)}$ = mass of internal standard solution added to calibration blend

$m_{spike(sample)}$ = mass of internal standard solution added to sample blend

$R_{M(calib)}$ = measured isotope ratio in internal standard/standard calibration blend

$R_{M(sample)}$ = measured isotope ratio in internal standard/sample blend

UME

Measurement equation:

$$C_{sample} = \frac{Area_{sample}}{Area_{IS}} \frac{C_{Std}}{C_{IS}} \frac{Area_{STD-IS}}{Area_{Sample}} C_{Sample-IS}$$

The uncertainty budgets for the LC-MS methods are given below.

Uncertainty budget of Phenylalanine (LC-MS)				
		Value	u(x)	u(x)/x
Weighing of sample (mg)		25	1,47E-04	5,88E-06
Weighing of IS (mg)		25	1,57E-05	6,28E-07
Standard stock solution (mg/kg)		5000	5,01E+00	1,00E-03
Internal stock solution (mg/kg)		5000	1,53E+01	3,06E-03
Intermediate precision		100	1,31E-01	1,31E-03
Recovery		100	2,33E-03	2,33E-05
Repeatability		100	1,23E-01	1,23E-03
Calibration graph		1,8	1,17E-02	6,48E-03
				7,46E-03
Result (mg/g)	482,94			
Combined uncertainty		3,60		
Expanded uncertainty		7,20		
% Relative uncertainty		1,49		
% Relative standard uncertainty		0,75		

Uncertainty budget of Leu (LC-MS)				
		Value	u(x)	u(x)/x
Weighing of sample (mg)		25	1,47E-04	5,88E-06
Weighing of IS (mg)		25	1,57E-05	6,28E-07
Standard stock solution (mg/g)		2500	5,02E+00	2,01E-03
Internal stock solution (mg/g)		2500	7,65E+00	3,06E-03
Intermediate precision		100	1,58E-01	1,58E-03
Recovery		100	5,44E-03	5,44E-05
Repeatability		100	1,95E-01	1,95E-03
Calibration graph		1,8	1,22E-02	6,75E-03
				8,08E-03
Result (mg/g)	197,49			
Combined uncertainty		1,60		
Expanded uncertainty		3,19		
% Relative uncertainty		1,62		
% Relative standard uncertainty		0,81		

Uncertainty budget of ILE (LC-MS)				
		Value	u(x)	u(x)/x
Weighing of sample (mg)		25	1,47E-04	5,88E-06
Weighing of IS (mg)		25	1,57E-05	6,28E-07
Standard stock solution (mg/g)		2500	5,03E+00	2,01E-03
Internal stock solution (mg/g)		2500	7,65E+00	3,06E-03
Intermediate precision		100	1,87E-01	1,87E-03
Recovery		100	4,75E-03	4,75E-05
Repeatability		100	2,17E-01	2,17E-03
Calibration graph		1,8	2,75E-02	1,53E-02
				1,60E-02
Result (mg/g)	219,98			
Combined uncertainty		3,51		
Expanded uncertainty		7,03		
% Relative uncertainty		3,20		
% Relative standard uncertainty		1,60		

Uncertainty budget of Pro (LC-MS)				
		Value	u(x)	u(x)/x
Weighing of sample (mg)		10	1,47E-04	1,47E-05
Weighing of IS (mg)		10	1,57E-05	1,57E-06
Standard stock solution (mg/g)		1000	1,00E+00	1,00E-03
Internal stock solution (mg/g)		1000	3,06E+00	3,06E-03
Intermediate precision		100	1,10E-01	1,10E-03
Recovery		100	1,74E-02	1,74E-04
Repeatability		100	5,25E-01	5,25E-03
Calibration graph		1,8	2,88E-02	1,60E-02
				1,72E-02
Result (mg/g)	47,39			
Combined uncertainty		0,81		
Expanded uncertainty		1,63		
% Relative uncertainty		3,44		
% Relative standard uncertainty		1,72		

VNIIM

Combined standard uncertainty of mass fraction of **Phe (by LC-UV)**:

Standard uncertainty of preparation of calibration solutions

Standard uncertainty of calibration

Standard deviation of measurement results (n=3)

Source of uncertainty	Type of evaluation	$u(x_i)$, % (relative)
Standard uncertainty of pure materials	B	0.1
Preparation of calibration solutions	B	0.2
Calibration	B	1.04
Standard deviation of measurement results	A	0.61
Combined Standard Uncertainty		**1.23**
Expanded Uncertainty (*k***= 2)**		**2.5**

Combined standard uncertainty of mass fraction of **Pro, Leu, Ile (by LC-MS)**:

Standard uncertainty of preparation of calibration solutions

Standard uncertainly of calibration

Standard uncertainly of IS addition (by weight)

Standard uncertainly of sample weighing

Standard deviation of measurement results (n=8)

Source of uncertainty	Type of evaluation	**Pro** $u(x_i)$, % (relative)	**Leu** $u(x_i)$, % (relative)	**Ile** $u(x_i)$, % (relative)
Standard uncertainty of pure materials	B	0.1	0.1	0.1
Preparation of calibration solutions	B	0.4	0.4	0.4
Calibration	B	1.2	1.2	0.6
Sample weighting	B	0.02	0.02	0.02
Internal standard addition	B	0.4	0.3	0.3
Standard deviation of measurement results	A	1.2	0.9	0.9
Combined Standard Uncertainty		**1.8**	**1.6**	**1.2**
Expanded Uncertainty (*k***= 2)**		**3.6**	**3.2**	**2.4**

APPENDIX G: Core Competency Table Template

CCQM-K78.a	*NMI Acronym*	Polar analytes in aqueous solvent
Scope of comparison: Participation in this comparison demonstrates the laboratory's capabilities in determining the mass fraction in aqueous calibration solution of organics with molar mass of 100 g/mol to 500 g/mol having polarity *pKow* > -2 for one or more of the following: **a. value assignment of a single component solution containing a UV-active organic present at a mass fraction above 500 µg/g (Phe only reported)** **b. value assignment of a multi- component solution containing UV- and non-UV active organics at mass fractions of 50 µg/g to 500 µg/g (Phe, Leu, Ile and/or Pro reported)** **c. separation and quantification using chromatography (LC- or GC-systems)**		
• Value assignment of Multicomponent Calibration solution		
Competency	**✓,×, or N/A**	**Specific Information**
Result for Phe content (µg/g)		
Result for Leu content (µg/g)		
Result for Ile content (µg/g)		
Result for Pro content (µg/g)		
Identification of analyte(s) in sample		*Indicate method(s) used to identify analyte(s) in the sample (e.g., Retention time, ion ratios, other)*
Extraction and clean up of analyte(s) from matrix (if used)		*Indicate extraction and/or cleanup technique(s) used, if any, (e.g. SPE, LC fractionation, other)*
Sample concentration adjustment (if used)		*Indicate extent of dilution or concentration of the sample prior to analysis (e.g.1:10 dilution).*
Conversion of analyte(s) of interest to detectable/measurable form (if used)		*Indicate chemical transformation method(s), if any, (i.e., derivatization, other)*
Analytical system		*Indicate analytical system (i.e., LC-MS/MS, LC-UV, GC-MS, LC-FD, other)*
Calibration approach for value-assignment of analyte(s) in matrix		*a) Indicate quantification mode used* *b) Indicate calibration mode used*
Verification method(s) for value-assignment of analyte(s) in sample		*Indicate any confirmative method(s) used, if any.*

Instructions:

- Insert the acronym for your NMI in the top cell of the middle column
- The left hand column does not require input
- The middle column requires placing a tick or cross or to say the entry is not applicable (N/A) for each competency listed
- For each entry of a tick in a cell of the middle column, enter the information requested in blue in each corresponding cell of the right hand column