CCQM-K151
Key Comparison Study on Protein Quantification
Purity-Assessed Recombinant Protein Contents in Buffer Solution using Insulin analogue

Report

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CONTENTS

INTRODUCTION

Protein-related business and technology have developed significantly in various areas including the clinical and pharmaceutical industries. This development has increased the demand for suitable protein analytical methods since determining the content of a reagent can serve as a quality control standard and may help establish safety guidelines for human use. For this reason, national metrology institutes (NMIs) are actively working on the establishment of measurement standards for protein quantification.

Following the establishment of a higher order analytical method for protein quantification, multiple stages of reduced protein such as peptides, amino acids (AAs), and elements can be analyzed and used to deduce the quantity of the original protein [1-4]. For the absolute quantification of pure-protein, isotope dilution–mass spectrometry (ID-MS) for AA analysis has been primarily adopted due to not only its straightforwardness in sample preparation but also the availability of primary AA standards and isotopic analogues required for SI-traceable calibration. For over last ten years, the Bio Analysis Working Group (BAWG) followed by the Protein Analysis Working Group (PAWG) of the Consultative Committee for Amount of Substance: Metrology in Chemistry and Biology (CCQM) have sponsored a set of three related pilot studies for the quantification of peptides in buffer solutions: CCQM-P55 angiotensin, CCQM-P55.1 four peptides, and CCQM-K115 and P55.2 c-peptide [5-6]. Based upon the results from these studies, PAWG concluded that demonstrating competency with several peptides was sufficient to document capability claims for purity-assessed peptide solutions.

Although AA-based quantification can be carried out with high accuracy and precision for a peptide/protein completely hydrolyzed into free AAs, optimization of the hydrolysis conditions is a non-trivial task and is even target-dependent. Mild hydrolysis conditions result in incomplete digestion, while strong conditions may cause a loss of AAs by unwanted side reactions [7-8]. Metrological traceability is required to produce equivalent test results across method, space, and time; this can be achieved through comparison with reference materials and reference measurement procedures [9-11]. An alternative protein quantification approach based on sulfur (S) measurements is conceivable by taking advantage of the stoichiometric presence of sulfur in the majority of proteins containing methionine (Met) and cysteine (Cys) [4, 12]. Similar to the AA-based method, the S-based method also has advantages of simple preparation and availability of primary S standard and isotope. Although the target protein will have S-containing residues, which requires sulfur impurity assessment and optimization of digestion conditions, a well-established S-based method can play the role of an orthogonal reference method along with the AA-based method for the absolute protein quantification of purity-assessed proteins.

In April 2017, CCQM approved the Key Comparison (KC) study CCQM-K151 and the parallel pilot study (P) CCQM-P191, with KRISS as the coordinating laboratory. CCQM-K151 was designed to underpin and allow NMIs and designated institutes (DIs) to assess participants' capabilities in assigning the mass fraction of a pure recombinant protein of size under 10 kDa with several disulfide bonds in a buffer solution. PAWG sponsored CCQM-K151 as well as CCQM-

P191 in collaboration with the Inorganic Analysis Working Group (IAWG). Participation in CCQM-K151 allowed NMIs and DIs to provide objective evidence that the procedures they use in finding the mass fraction value and its associated measurement uncertainty assignment of aqueous standard solutions are suitable for their intended purpose. The purpose of a standard solution produced by an NMI could be either for provision to external users as a certified reference material (CRM) or for internal use to establish the calibration hierarchy of a reference measurement procedure [8, 11, 13].

The focus of this comparison is to demonstrate the capabilities of the participants to assign the mass fraction of a purified recombinant protein in an aqueous solution. This study was conducted as a special challenge not only to demonstrate the above capability but also to underpin the established primary measurement procedure based on AA analysis with another orthogonal method based on S analysis by collaboration with IAWG. It does not demonstrate capabilities for the purity assessment of peptides, which are covered in the CCQM-K115 series. The participants were informed of the free-AA, peptide, and S interferences in the solution and were advised that the mass fraction of the target protein was approximately 3 g/kg. All of the participants in CCQM-K151 were from PAWG, and all of the participants in CCQM-P191 were from IAWG.

The sections of this report document the timeline of CCQM-K151, target measurand, characterization of the study material, participants, results, and the measurement capability claims that the results of participation in CCQM-K151 are intended to support. The Appendices reproduce the official communication materials and summarize the results provided by the participants.

The timeline for the CCQM-K151 study 'Key Comparison Study on Protein Quantification' is summarized in Table 1.

Table 1: Timeline for CCQM-K151

Date	Action
Apr 2016	Proposed to CCQM as a joint comparison between PAWG–IAWG
Apr 2017	Study proposal approved
Sep 2017	Sample characterization completed
Oct 2017	Call for participation in CCQM-K151
Jan 2018	Study samples shipped to participants from Nov 2017
Apr 2018	Results gathered and undisclosed until Oct 2018 Additional sample shipped to an omitted participant in CCQM-K151
Oct 2018	Initial report and discussion of results at PAWG/IAWG meeting
Mar 2019	Draft A report and discussion
Feb 2020	Draft B report

TBD	Final report to PAWG Chair

MEASURANDS

The test material is a kind of recombinant protein under 10 kDa that includes a few sulfide bonds in highly pure solution. The measurand is "a mass fraction of insulin, namely synthetic insulin analogue featuring 3 disulfide cross links in an aqueous solution".

The target protein was a human insulin analog, insulin aspart (Ins) [14], which is homologous with regular human insulin with the exception of a single substitution of the AA proline (Pro) by aspartic acid in position B28, and is produced by recombinant DNA technology utilizing *Saccharomyces cerevisiae* (baker's yeast) as the production organism under the name NovoLog®.

Ins has the empirical formula $C_{256}H_{381}N_{65}O_{79}S_6$ and a molecular weight of 5825.54 g/mol.

The CAS number of the target protein is 116094-23-6, and the AA sequence information in accordance with "IUPAC Condensed" is:

H-Phe-Val-Asn-Gln-His-Leu-Cys(1)-Gly-Ser-His-Leu-Val-Glu-Ala-Leu-Tyr-Leu-Val-Cys(2)-Gly-Glu-Arg-Gly-Phe-Phe-Tyr-Thr-Asp-Lys-Thr-OH.H-Gly-Ile-Val-Glu-Gln-Cys(3)-Cys(1)-Thr-Ser-Ile-Cys(3)-Ser-Leu-Tyr-Gln-Leu-Glu-Asn-Tyr-Cys(2)-Asn-OH

Participants could choose any residues as analytes, but four AA residues (Val, Leu, Ile, and Phe) were recommended. Finally, five residues including Gly were used as analytes in this study. The stoichiometric values of the five AA residues and S are as follows: 4 for Gly, 4 for Val, 6 for Leu, 2 for Ile, 3 for Phe, and 6 for S.

The primary structure of Ins is shown in Fig. 1.

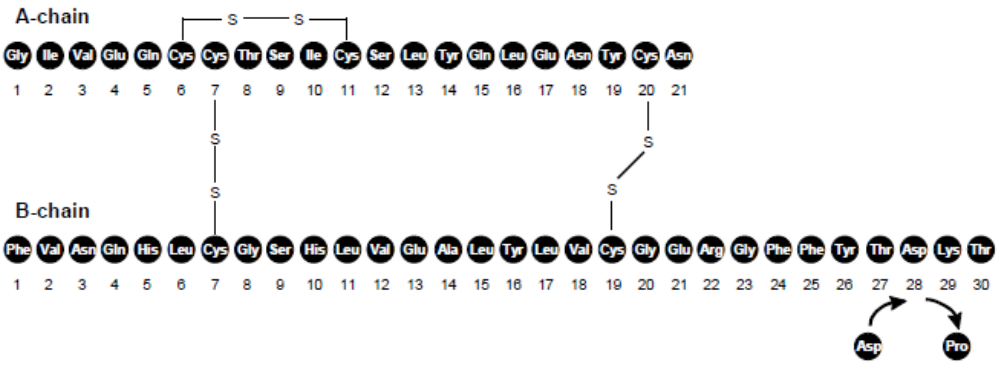

Figure 1. Primary structure of insulin aspart (Ins)

STUDY MATERIALS

Preparation of Candidate Material

A batch of commercially available therapeutic injection for diabetes mellitus containing Ins as a major constituent was used for this comparison. It is a sterile, aqueous, clear, and colorless solution that, according to the manufacturer, contains the target protein (Ins) at a concentration of about 3 g/kg and the following other components: glycerin 16 mg/mL, phenol 1.50 mg/mL, metacresol 1.72 mg/mL, zinc 19.6 µg/mL, disodium hydrogen phosphate dihydrate 1.25 mg/mL, and sodium chloride 0.58 mg/mL, with a pH of 7.2–7.6.

The samples were pooled and gently stirred at 4 °C for several hours to obtain a homogenous solution followed by filtration with a sterilized 0.22 µm nylon filter. Aliquots with about 1.5 mL of the master solution were transferred into sterilized 2 mL polyethylene (PE) cryotubes with silicone gasket seals. The resulting batch of 600 vials was separated into small cryoboxes with 5 vials each followed by resealing in an aluminized polyethylene terephthalate bag (Mylar bag®), which were stored at 4 °C. The whole process of sample handling was carried out at a clean bench in a 4 °C cold room.

For the study, 5 vials of Ins solution in PE cryotubes with silicon gasket seals were provided to the K151 participants (AA-analysis). Each contained 1.5 mL of Ins in buffer solution. Samples were distributed using temperature-controlled transport packaging with temperature data-logging devices. At least 3 of the vials were to be used in determining the results to be reported; the additional vials were for method development.

Interfering Impurity Assessment of Study Material

The purity of the target protein in the sample was the crucial point in both proposed studies to demonstrate the capability of participants in quantification. However, this study was not intended to demonstrate capabilities for the purity assessment of peptides nor the mass fraction assignment of peptides mixture in aqueous solution in order to minimize overlap with other key comparisons, namely the CCQM-K115 series[5]. Since this study focused on the absolute quantification of purity-assessed protein solution, the following impurities were screened by the coordinating lab (KRISS): 1) free AAs in the sample soup, 2) peptide-related impurities, 3) sulfur impurities, and 4) any other interference factors.

The five AA residues (Val, Leu, Ile, Phe, and Gly) used as target analytes in AA-based quantification were found to be under the detection range limit of about 3 nmol/kg in MS and LC-MS/MS analyses. For peptide-related impurities, KRISS concluded that there was no interfering impurity that could affect the results using UPLC-ESI-qTOF, and NIM kindly provided supplementary information about the presence of a deamidated form and two glycated forms in the test material (Table 2 and Fig. 2). Following discussion, these reported trace levels of glycated and Met-oxidated forms and deamidated Ins were not considered as impurities, even if they were

detected, because they are included in the quantification of the protein by AA-based analysis under the scope of this study. It was decided to present them as supplemental information following agreement by all participants. Moreover, S-containing small molecule impurities were less than 0.4 % of total S by standard addition calibration with size exclusion chromatography-ICP-MS and RPLC-ICP-MS. Consequently, there were no impurity issues in the sample.

Table 2. Possible information of protein impurities of the test material reported by NIM using ESI Q-TOF MS.

	RT	M/Z	Z	Monoisotopic mass(Da)	△	
1	29.24	1165.20643	5	5820.99		insulin
2	27.86	1230.01264	5	6145.02	324.031	Glycation*2
3	28.59	1197.60364	5	5982.98	161.986	Glycation
4	30.28	1165.40168	5	5821.97	0.97625	Deamidation
5	31.19	1150.18268	5	5745.87	-75.1187	
6	31.56	1161.79445	5	5803.93	-17.0599	
7	32.34	1164.99385	5	5819.93	-1.0629	

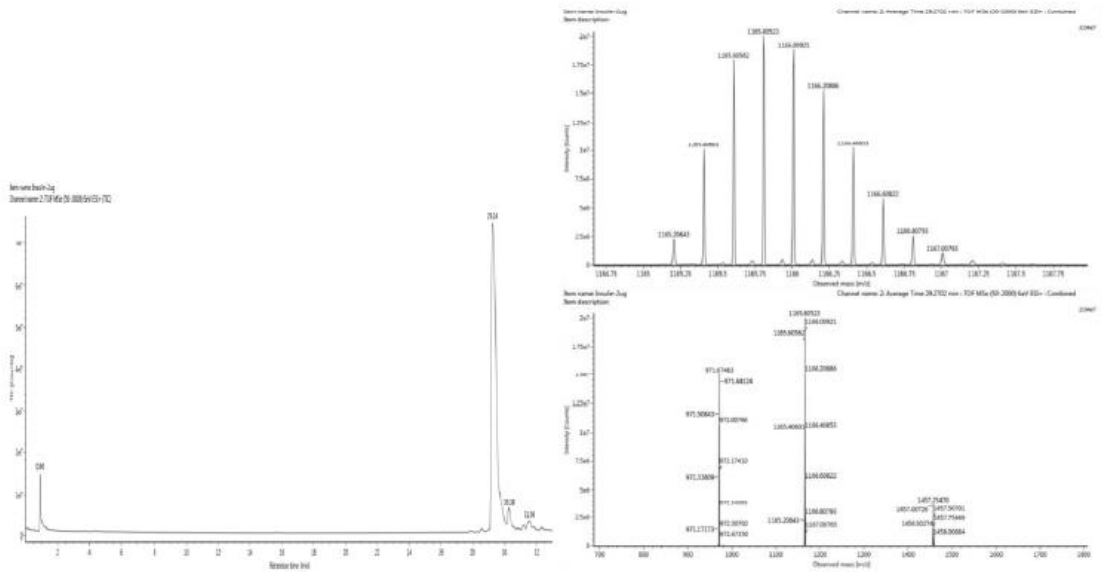

Figure 2. Total ion chromatograms and mass spectrum of protein impurities of the test material by NIM using ESI Q-TOF MS.

Homogeneity and Stability Assessment of Study Material

More than 10 vials were tested for homogeneity by both AA and S analysis. In the former, four AAs, namely Phe, Val, Leu, and Ile, were used. No statistically significant heterogeneity was found in either case; the relative standard deviations between vials were 0.22 % and 0.99 % in AA-based and S-based quantification, respectively. The sample mass used in the homogeneity tests at KRISS was about 0.1 g for AA analysis and 0.4 g for S analysis (Figure 3).

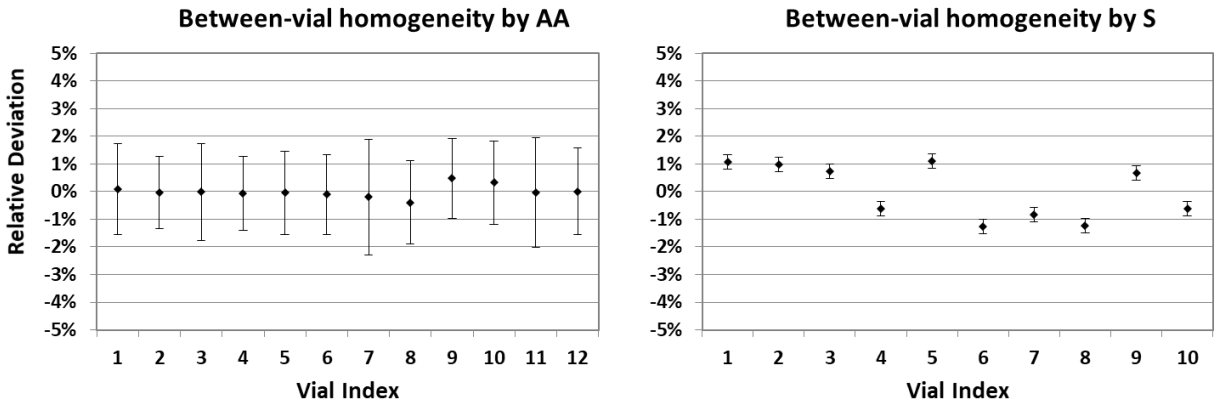

Figure 3. Homogeneity of Ins by analysis of over 10 vials via AA analysis (left) and S analysis (right).

KRISS also tested the long-term stability of the test material under the storage condition (4 °C) for 1 year at 2, 6, and 12 months after repackaging into 1.5 mL aliquots (Fig. 4). No significant trends or degradation was observed under the test periods, and thus it was concluded that no special precautions under the storage condition were necessary. While no further stability testing was conducted to simulate any unexpected issues during delivery, such as exposure to higher temperatures, the samples were distributed using temperature-controlled transport packaging with strict temperature data-logging devices.

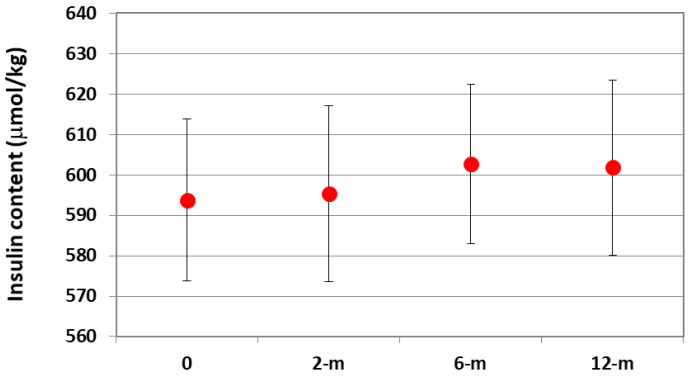

Figure 4. Long-term stability of Ins at 4 °C.

PARTICIPANTS AND INSTRUCTIONS

The call for participation in CCQM-K151 was distributed in Oct-2017 with the intent to distribute samples in Dec-2017, receive results in Mar-2018, and discuss the results at the Fall PAWG meeting in Oct-2018. The schedule of the parallel pilot study, CCQM-P191, was delayed due to further discussion about the study scope in IAWG; the call for participation in CCQM-P191 was extended to May-2018 with the intent to distribute samples in Jun-2018, and receive results in Sep-2018. Under the participants' agreement, and following the stated purpose of this study, the results of CCQM-K151 were undisclosed until Oct-2018 to first gather all results, including the extended CCQM-P191 results. Due to custom issues, the last set of test materials was delivered in Aug-2018. The initial report and discussions were conducted at the fall 2018 PAWG meeting.
Table 3 lists the institutions that participated in CCQM-K151 samples.

Table 3: List of participating NMIs/DIs in CCQM-K151

NMI or DI	Code	Contact
Health Sciences Authority, Singapore	HSA	Qinde Liu Liu_Qinde@hsa.gov.sg
Instituto Nacional de Metrologia, Qualidade e Tecnologia	INMETRO	Paulo José Miranda Beltrão pjbeltrao@inmetro.gov.br
Korea Research Institute of Standards and Science	KRISS	Ji-Seon Jeong jsjeong@kriss.re.kr
Laboratoire National de Métrologie et d'Essais	LNE	Vincent Delatour vincent.delatour@lne.fr
National Institute of Metrology, China	NIM	Wei Mi, miwei@nim.ac.cn Liqing Wu, wulq@nim.ac.cn
National Institute of Standards and Technology	NIST	Eric L Kilpatrick eric.kilpatrick@nist.gov
National Metrology Institute of Japan/National Institute of Advanced Industrial Science and Technology	NMIJ/AIST	Tomoya Kinumi t.kinumi@aist.go.jp
Physikalisch-Technische Bundesanstalt	PTB	André Henrion andre.henrion@ptb.de
National Metrology Institute of Turkey TÜBİTAK UME	UME	Merve Oztug Merve.oztug@tubitak.gov.tr

RESULTS

Participants were requested to report the mass fraction (mg/kg) of each of the test vials, including an overall combined result for three vials, as well as the mass fraction of each of the AAs. In addition to the quantitative results, participants were to describe their analytical methods, approach to uncertainty estimation, and detailed information on any isotopically labelled materials and primary reference materials used. A template for the report was shared after official registration. Descriptions of the sample preparation, analytical techniques, and calibrants used by the participants in CCQM-K151 are summarized in Tables 4–6, respectively.

Methods Used by Participants

Nine results were received from the participating institutions. All of the CCQM-K151 participants used ID-MS for AA analysis; among them, five results were hydrolyzed in the liquid-phase, and the other four results were hydrolyzed in the gas-phase. One liquid-phase result adopted microwave-assisted hydrolysis. Except UME, all participants measured four AAs, Val, Leu, Phe, and Ile, while UME measured Gly instead of Ile.

Working sample amounts varied in the range from 0.01 g to 0.7 g from the intact samples, with some participants adopting further dilution or additional preparation (e.g., addition of phenol, drying, or derivatization before/after hydrolysis). Isotopic analogues were added to the samples prior to hydrolysis by seven participants, and post-hydrolysis by two participants. All employed the isotope dilution LC-MS method with their own optimized conditions. For the AA primary reference materials, six participants (KRISS, PTB, LNE, NIST, NMIJ, and INMETRO) used pure powder CRMs produced by NMIJ. Otherwise, HSA and NIM used their own pure powder AA CRMs, and UME used a mixed-solution CRM produced by NIST.

Although the major uncertainty components varied according to each uncertainty budget, three major components were found in all results: calibration, analytical precision by repeated LC-MS analysis, and results disagreement between AA residues. Details of CCQM-K151 participants' approaches to estimating uncertainty are provided in Appendix F.

Table 4. Experimental details of the sample preparation conditions used by CCQM-K151 participants

		HSA	INMETRO	KRISS	LNE	NIM	NIST	NMIJ	PTB	UME
Working sample used (g)		0.1	0.04	0.2	0.01	0.04	0.05	0.1	0.04	0.7
Dilution		NA	NA	10	40	100	86.58	40	NA	10
Hydrolysis type		Gas-phase	Liquid-phase	Liquid-phase	Gas-phase	Liquid-phase	Gas-phase	Microwave-assisted Liquid-phase	Liquid-phase in vacuum	Gas-phase
Hydrolysis conditions	Temp (℃)	130	110	130	130	110	120	165	150	130
	Time (h)	48	120	24	40	96	21	3	65	48
	HCl conc. (M)	6	6	8	6	6	6	6	6	6
	Phenol (%)	2	0.01	no	no	no	no	0.1	0.1	0.1
Further preparations	Isotope addition before/after hydrolysis	Before	After	Before	Before	Before	After	Before	Before	Before
	Others		Sample dilution up to 50-times after hydrolysis		Dry samples before hydrolysis	Dry samples before hydrolysis, nitrogen purged	Dry samples before hydrolysis	N-Butylnicotinic acid N-hydroxysuccinimide ester derivatization after hydrolysis		Propyl cholorofor mate derivatization after hydrolysis

Table 5. Experimental details of the analytical methods used by CCQM-K151 participants

	HSA	**INMETRO**	**KRISS**	**LNE**	**NIM**	**NIST**	**NMIJ**	**PTB**	**UME**
Instrumentation	LC-MS/MS (triple quad)	LC-MS/MS (triple quad)	LC-MS/MS (triple quad)	LC-MS (quad)	LC-MS/MS (triple quad)	LC-MS/MS (triple quad)	LC-MS/MS (triple quad)	LC-MS (quad)	LC-HRMS (orbitrap)
Detection method	MRM	MRM	MRM	SIM	MRM	MRM	MRM	SIM	SIM
Target AA	VILF	VILF	VILF	VILF	VILF	VILF	VILF	VILF	GVLF
Quantification method	Double ID-MS	Double ID-MS	Exact matching double ID-MS	Double ID-MS	Double ID-MS	Double ID-MS	Double ID-MS	Double ID-MS	Double ID-MS
Calibration method	4-point linear	5-point linear	Single point	5-point linear	2-point bracketing	5-point linear	2-point bracketing	Single point	7-point linear
Separation column	Zorbax Eclipse AAA, 5 µm, 4.6 x 150 mm (Agilent)	Supelcosil C18, 5 µm, 4.6 x 250 mm (Supelco)	Acquity UPLC® BEH C18, 1.7 µm, 2.1 x 100 mm (Waters)	Acquity UPLC® BEH C18, 1.7 µm, 2.1 x 100 mm, (Waters)	KINETEX C18, 2.6 µm, 2 x 150 mm (Phenomenex)	Primesep 100, 5 µm, 2.1 x 250 mm 100A (SIELC)	Develosil C30-UG-5, 5 µm, 2 x 250 mm (Nomura Chemical)	SeQuant ZIC-HILIC 3.5 µm; 2.1 x 150 mm (Merck)	EZ:faast AAA, 4 µm, 2 x 250 mm (Phenomonex)
System name	AB SCIEX QTRAP® 5500 +Shimadzu Prominence UFLCXR	Waters Xevo TQ + Acquity UPLC I class	Waters Xevo TQ-S + Acquity UPLC	Waters Xevo TQ-MS + Acquity UPLC	AB SCIEX QTRAP® 5500 + Agilent 1200 HPLC	Agilent 6460 MS + Infinity LC	Thermo TSQ quantum MS + Shimadzu Prominence LC-20A	Agilent 1100 LC + MSD	Thermo Orbitrap-Q Exactive + Ultimate 3000

Table 6. Calibration and internal standards used by CCQM-K151 participants

		HSA	**INMETRO**	**KRISS**	**LNE**	**NIM**	**NIST**	**NMIJ**	**PTB**	**UME**
CRM	type	Pure powder	Pure powder	Pure powder	Pure powder	Pure powder	Pure powder	Pure powder	Pure powder	Mixed solution
	Manufacturer	HSA	NMIJ	NMIJ	NMIJ	NIM	NMIJ	NMIJ	NMIJ	NIST
	No.	HRM-1006A, HRM-1013A, HRM-1008A, HRM-1014A	NMIJ CRM 6012-a, 6013-a, 6014-a, 6015-a	NMIJ CRM 6012-a, 6013-a, 6014-a, 6015-a	NMIJ CRM 6012-a, 6013-a, 6014-a, 6015-a	NIM CRM for AA	NMIJ CRM 6012-a, 6013-a, 6014-a, 6015-a	NMIJ CRM 6012-a, 6013-a, 6014-a, 6015-a	NMIJ CRM 6012-a, 6013-a, 6014-a, 6015-a	SRM 2389a
ISTD	Manufacturer	Cambridge Isotope Laboratories	Cambridge Isotope Laboratories	Cambridge Isotope Laboratories	Cambridge Isotope Laboratories	Cambridge Isotope Laboratories	Cambridge Isotope Laboratories	Isotec	Cambridge Isotope Laboratories	Cambridge Isotope Laboratories
	Isotope type	$^{13}C_5$-Val $^{13}C_6$,^{15}N-Ile $^{13}C_2$-Leu Ring-$^{13}C_6$-Phe	$^{13}C_{5,}{}^{15}$N -Val $^{13}C_6$,^{15}N-Ile $^{13}C_6$,^{15}N-Leu $^{13}C_{9,}{}^{15}$N-Phe	$^{13}C_{5,}{}^{15}$N -Val $^{13}C_6$,^{15}N-Ile $^{13}C_6$,^{15}N-Leu $^{13}C_{9,}{}^{15}$N-Phe	$^{13}C_{5,}{}^{15}$N -Val $^{13}C_6$,^{15}N-Ile $^{13}C_6$,^{15}N-Leu $^{13}C_{9,}{}^{15}$N-Phe	$^{13}C_5$-Val $^{13}C_6$-Ile D_{10}-Leu $^{13}C_{9,}{}^{15}$N-Phe	$^{13}C_5$-Val $^{13}C_6$-Ile $^{13}C_6$-Leu $^{13}C_{9,}{}^{15}$N-Phe	$^{13}C_{5,}{}^{15}$N -Val $^{13}C_6$,^{15}N-Ile $^{13}C_6$,^{15}N-Leu $^{13}C_{9,}{}^{15}$N-Phe	$^{13}C_{5,}{}^{15}$N -Val $^{13}C_6$-Ile $^{13}C_6$-Leu $^{13}C_{9,}{}^{15}$N-Phe	$^{13}C_{5,}{}^{15}$N -Val $^{13}C_6$,^{15}N-Leu $^{13}C_{9,}{}^{15}$N-Phe $^{13}C_2$,^{15}N-Gly

Participant Results

CCQM-K151 results for the quantification of Ins are detailed in Table 7, with the results of the individual AAs given in Table 8 and Table 9. Figures 5 and 6 plot the Ins and AA results, respectively. Figure 7 shows the results in amount-of-substance content. The median and the standard uncertainty of the CCQM-K151 results is (3423 ± 3.3) mg/kg. The submitted results in mass fraction converted to amount-of-substance content used the following respective molecular weights of Val, Ile, Leu, Phe, and Gly: 117.15, 131.17, 131.17, 165.19, and 75.07 g/mol. The molecular weight of Ins is 5825.54 g/mol according to the atomic weights from IUPAC.

Table 7. Reported results for Ins (mg/kg)

		Insulin aspart			
	NMI	*x* (mg/kg)	*u(x)* (mg/kg)	*k*	*U(x)* (mg/kg)
K151	HSA	3390	57	2	114
	INMETRO	3449	42	2	83
	KRISS	3465	61	2.07	127
	LNE	3070	130	2	260
	NIM	3206	33	2	65
	NIST	3020	70	2	140
	NMIJ	3423	45	2	89
	PTB	3483	35	2	70
	UME	3430	138	2	277

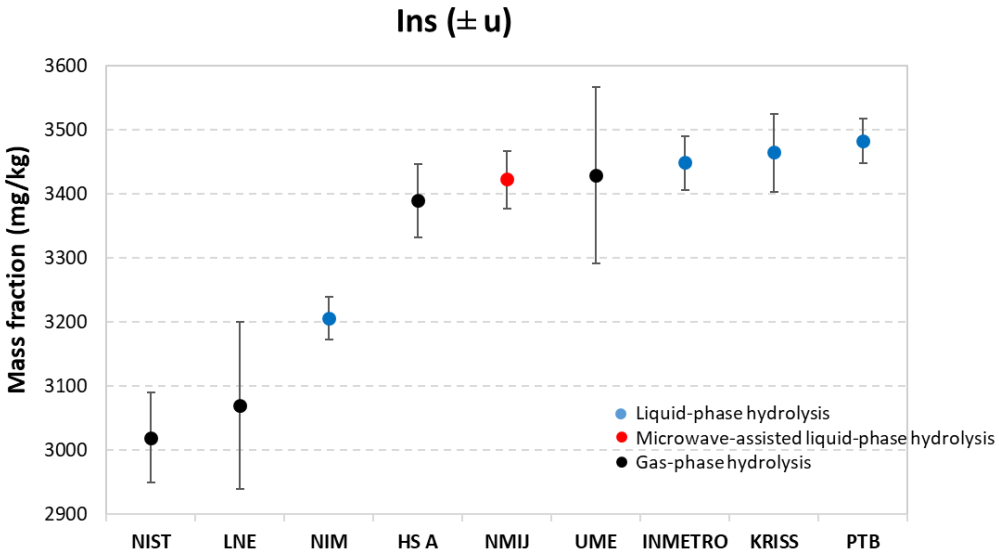

Figure 5. Reported mass fraction of Ins plotted with standard uncertainties (u) showing different hydrolysis methods.

Table 8. Summarized results of Val, Ile, and Leu AAs (in mg/kg)

	L-valine (Val)				L-isoleucine (Ile)				L-leucine (Leu)			
NMI	***x***	***u*(*x*)**	***k***	***U*(*x*)**	***x***	***u*(*x*)**	***k***	***U*(*x*)**	***x***	***u*(*x*)**	***k***	***U*(*x*)**
HSA	275.3	3.3	2	6.7	149.1	1.5	2	2.9	464.4	5.2	2	10.5
INMETRO	279.9	1.7	2	3.4	149.7	1.7	2	3.4	462.6	8.7	2	17.4
KRISS	276.1	4.4	2.0	9.0	158.8	2.2	2.1	4.5	468.6	5.9	2.2	12.8
LNE	248.3	18.8	2	37.5	137.3	15.3	2	30.5	412.0	34.0	2	67.9
NIM	258.8	4.9	2	9.8	144.1	3.1	2	6.3	433.9	10.4	2	20.9
NIST	242.4	3.1	2	6.2	125.5	3.8	2	7.6	430.4	10.1	2	20.3
NMIJ	275.3	6.9	2	13.8	153.9	4.2	2	8.5	460.7	12.6	2	25.2
PTB	279.9	3.1	2	6.3	157.4	1.6	2	3.2	468.8	5.0	2	10.2
UME	276.7	7.7	2	15.5	—	—	—	—	452.1	15.8	2	31.6
n	9				8				9			
$\bar{x}$	268.1				147.0				450.4			
s	14.4				11.2				20.2			
$\bar{u}$	7.7				6.0				14.7			
CV	5.4%				7.6%				4.5%			

n = number of results included in summary statistics; $\bar{x}$ = mean; *s* = standard deviation; CV = $100 \cdot s/\bar{x}$; $\bar{u} = \sqrt{\sum_i^n u^2(x_i)/n}$, the "average" reported uncertainty

Table 9. Summarized results of Phe and Gly AAs (in mg/kg)

	L-phenylalanine (Phe)				L-glycine (Gly)			
NMI	***x***	***u*(*x*)**	***k***	***U*(*x*)**	***x***	***u*(*x*)**	***k***	***U*(*x*)**
HSA	288.3	2.7	2	5.4				
INMETRO	295.1	2.0	2	3.9				
KRISS	293.1	4.5	2.1	9.6				
LNE	266.0	19.2	2	38.3				
NIM	272.0	4.5	2	8.9				
NIST	264.7	6.2	2	12.4				
NMIJ	293.0	9.5	2	18.9				
PTB	296.6	3.4	2	6.8				
UME	300.3	10.5	2	21.0	175.0	4.0	2	8.0
n	9				1			
$\bar{x}$	285.5				175.0			
s	13.9							
$\bar{u}$	8.6				4.0			
CV	4.9%							

n = number of results included in summary statistics; $\bar{x}$ = mean; *s* = standard deviation; CV = $100 \cdot s/\bar{x}$; $\bar{u} = \sqrt{\sum_i^n u^2(x_i)/n}$, the "average" reported uncertainty

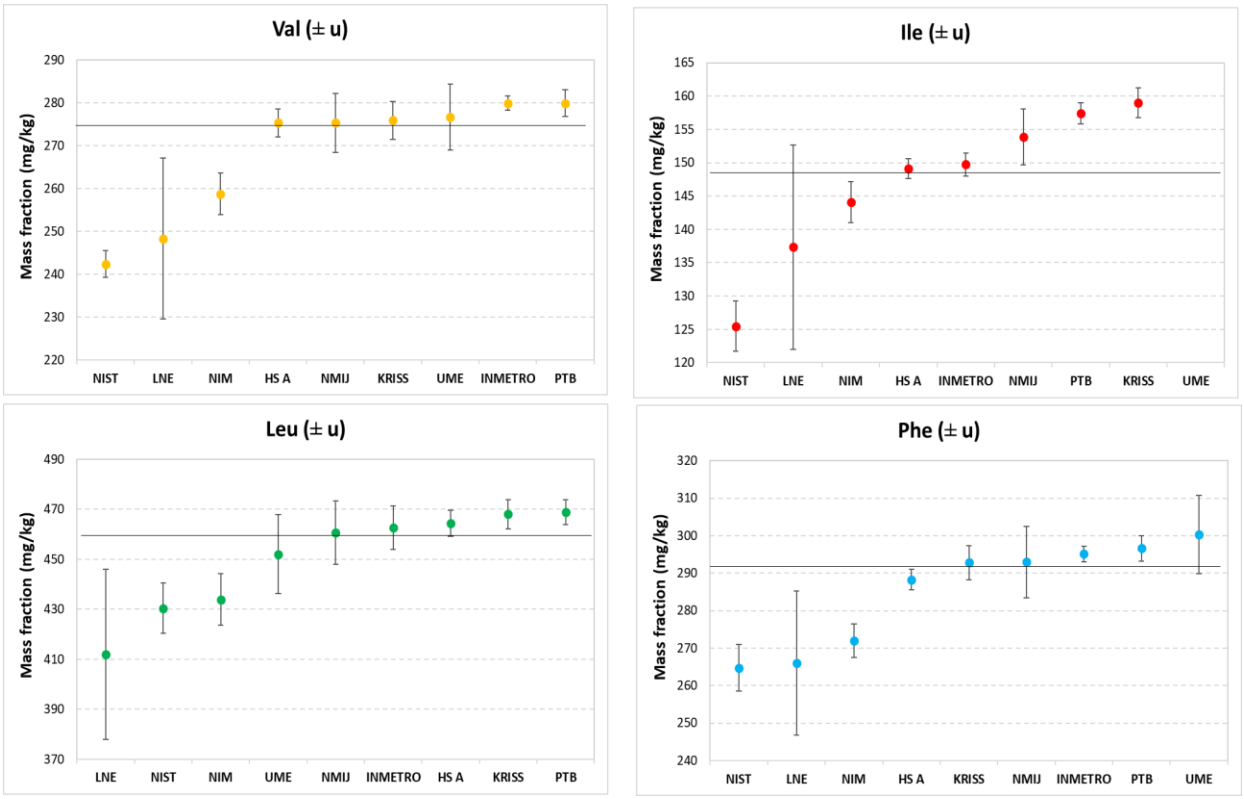

Figure 6. Reported mass fractions obtained from the analysis of the four AAs sorted by increasing value with standard uncertainties (u). The horizontal lines represent the median of each AA (UME used Gly, not Ile).

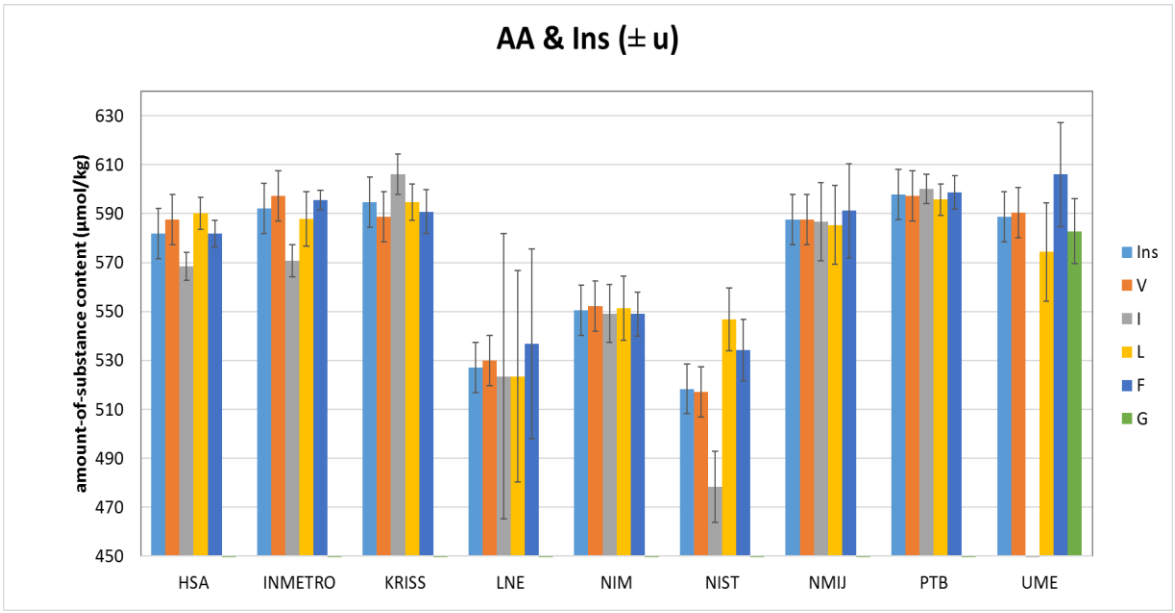

Figure 7. Reported results converted to amount-of-substance content of the AAs and Ins (in µmol/kg).

Result summary of sulfur-based quantification results

As mentioned in the Introduction, this study presented a special challenge not only in demonstrating the laboratory capability to assign the mass fraction of a target protein but also to support the established AA-based procedure with the S-based one. The S analysis proceeded as a parallel pilot study, CCQM-P191, with IAWG members. Six NMIs participated and submitted results using ID-ICP-MS, ICP-MS with external calibration, and ion chromatography. Four results measured by ID-ICP-MS were determined to be effective datasets in P191 and were subsequently processed by arithmetic mean for reference value estimation. Although CCQM-P191 report is being prepared separately (at the time of the current report's writing) with detailed data and discussion, its reference value (RV) and associated uncertainty (RU) will be summarized in this report based on the consensus of IAWG meeting in 2019 Oct. . The RV and expanded RU of the pilot study is (3489 ± 90) mg/kg with coverage factor of 3.18 at a 95 % level of confidence.

KEY COMPARISON REFERENCE VALUE (KCRV)

The documents CCQM/13-22 *Guidance note: Estimation of a consensus KCRV and associated Degrees of Equivalence*[15] describe the recommended best practices for the choice of appropriate estimators of KCRV and KCRU, depending on the range of participant results and their degree of consistency taking into account their associated measurement uncertainty.

According to the guide, results should be processed by a consistency check; here, the results of CCQM-K151 might be categorized as 'Generally consistent results with a small number of outlying values'. Indeed, there were no significant outliers in a preliminary inspection of the candidate sets using a box plot, as well as in a chi-squared test, the result of which was 15. This value indicates no clear evidence of inconsistency but does not completely rule out potential over-dispersion.

An initial discussion of the results proceeded at the PAWG meeting in Oct-2018, where it was revealed that three participants reported lower values than the other six. These three participants requested another set of test materials for additional experiments to investigate source of discrepancy in their results, and therefore were excluded from KCRV estimation in CCQM-K151. Additional sample sets were shipped to LNE, NIM, and NIST for this purpose; NIST and NIM resubmitted revised results in March and April 2019, respectively, and LNE's result was returned in July 2019. The revised results are discussed in the Additional Experiments section below.

The remaining six mutually consistent data were processed by the following estimators: 1) arithmetic mean (mean), 2) uncertainty-weighted mean (UW-mean), and 3) median. Additionally, the data were processed by 4) DerSimonian–Laird variance-weighted mean (DSL-mean), and 5)

the Bayesian approach as well. The proposed estimation results are summarized in Table 10 and Figure 8.

The reference values estimated by mean, median, and DSL-mean were in agreement with each other. According to the Guidance Note CCQM/13-22, the arithmetic mean is considered appropriate when the reported uncertainties are very similar, and further, when the arithmetic mean provides similar reference value and uncertainty estimates of the weighted mean. The dataset in this study showed mutual consistency as well as similar uncertainty, so that the estimation results were closely similar (equivalent), as shown in Table 10. It was proposed by the coordinating laboratory and agreed by the participants that the mean of the result set and its associated uncertainty was selected, respectively, as the KCRV and *u*(KCRV) for the mass fraction of Ins in CCQM-K151.

Table 10. Consensus values and their dispersion for mass fraction of Ins

Insulin aspart (Ins)

Estimator	**Consensus Value (CV),** mg/kg	**u(CV),** mg/kg	**$t_{0.05}$(n)**	**U(CV),** Mg/kg	**Relative U(CV)**	**Note**
Mean	**3440**	**13.5**	**2.57**	**35**	**1.0%**	
Median	3440	2.9	2.57	7	0.2%	u from MADe
UW-mean	3449	13.9	2.57	36	1.0%	
DSL-mean	3400	14.0	2.57	36	1.1%	
Bayesian	3400	23.0	2.57	59	1.7%	

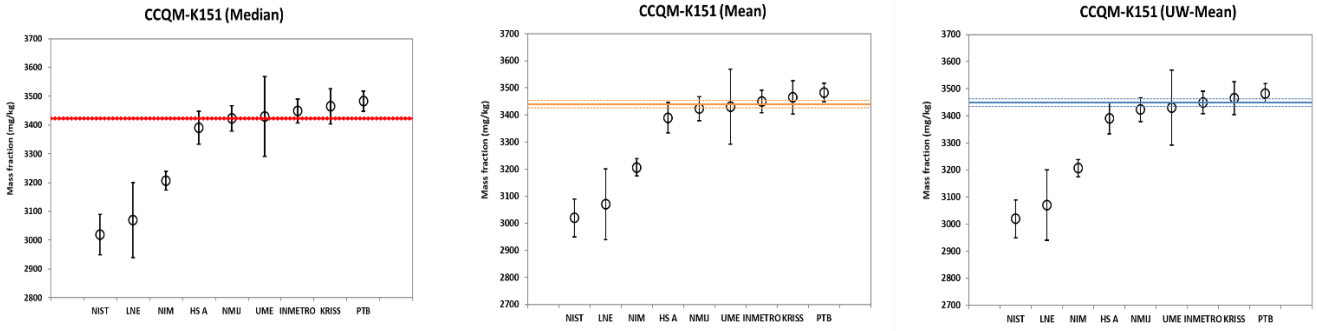

Figure 8. Results relative to proposed KCRV estimation by median (right), mean (center), and UW-mean (right). The horizontal lines represent consensus values (*CVs*) and associated standard uncertainty (*u(CV)*).

DEGREES OF EQUIVALENCE (DoE)

The absolute degrees of equivalence (DoE) of each result for Ins reported by participants in CCQM-K151 were estimated as the difference between the measured value and the KCRV: $d_i = x_i$ – KCRV with its expanded uncertainty ($U(di)$), calculated with a coverage factor (k) from the effective degree of freedom (DoF_{eff}) based on the Welch–Satterthwaite formula[16], and sum square root of the participants' uncertainty and u(KCRV):

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

The equivalence statements for CCQM-K151 based on the proposed KCRV and the associated uncertainty are given in Table 11 and graphically shown in Figure 10.

Table 11. Degrees of equivalence of participants' results for mass fraction of Ins using the mean as the KCRV estimator in CCQM-K151

NMI	d_i	$u(d_i)$	DoF_{eff}	k_i	$U(d_i)$	$\%d_i$	$\%U(d_i)$
NIST	–420.0	71.3	3927	1.96	139.8	–12.2%	4.1%
LNE	–370.0	130.7	44387	1.96	256.2	–10.8%	7.4%
NIM	–233.6	35.2	232	1.97	69.3	–6.8%	2.0%
HS A	–50.0	58.6	1790	1.96	114.9	–1.5%	3.3%
NMIJ	–17.0	46.5	710	1.96	91.3	–0.5%	2.7%
UME	–9.9	139.1	56886	1.96	272.6	–0.3%	7.9%
INMETRO	9.0	43.6	551	1.96	85.7	0.3%	2.5%
KRISS	24.9	62.8	2359	1.96	123.1	0.7%	3.6%
PTB	43.0	37.5	300	1.97	73.8	1.3%	2.1%

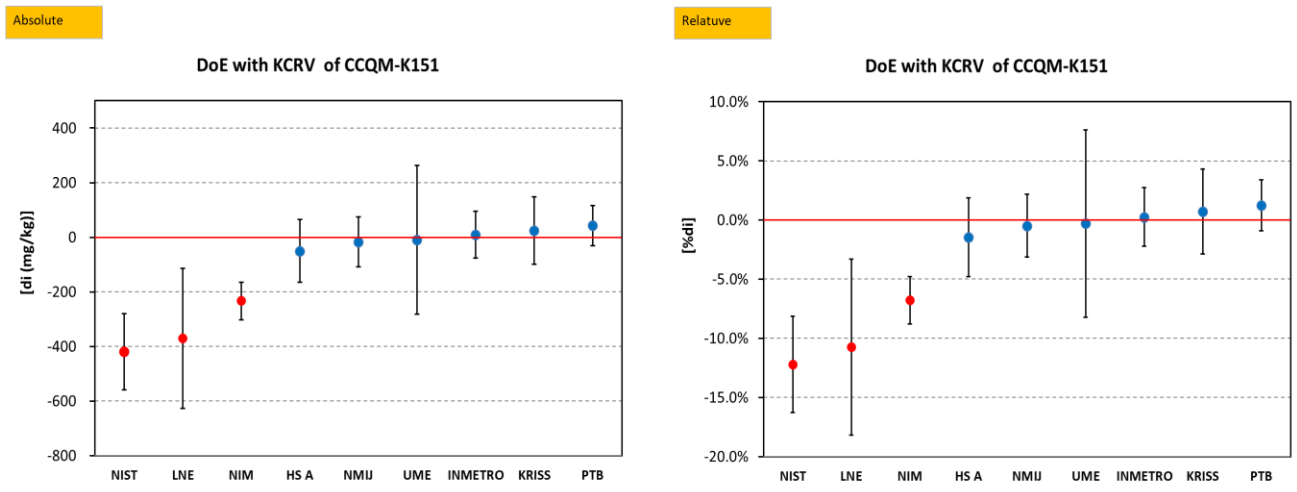

Figure 9. Degree of equivalence of all participants in absolute (left) and relative (right) scale for mass fraction of Ins using the mean as the KCRV estimator in CCQM-K151. The KCRV was estimated from the data marked with blue circles.

USE OF CCQM-K151 IN SUPPORT OF CALIBRATION AND MEASUREMENT CAPABILITY (CMC) CLAIMS

How Far the Light Shines (HFTLS)

Successful participation in this comparison demonstrates a laboratory's capabilities to assign the mass fraction of purity-evaluated recombinant proteins up to 10 kDa containing up to 3 disulfide cross-links in an aqueous calibration solution using AA-based ID-LC-MS. As previously mentioned, the comparison does not demonstrate capabilities for the purity assessment of peptides nor the mass fraction assignment of peptides mixture in aqueous solution, which requires capabilities that have been previously demonstrated in the CCQM-K115 series of comparisons.

The study protocol had a target range of mass fraction, but no exact statement about the target range in the HFTLS boundary. Each participant were to free to add any kind of sample preparation steps including dilution, derivatization, etc. All participants used different sample amounts and dilution ratios depending on their particular optimized conditions. It was noted that some of the participants demonstrated the capability for confirmatory mass fraction assignment at levels significantly lower than that nominally covered by the reported value. It is recognized that satisfactory performance in CCQM-K151 can be used by an NMI or DI to justify CMC claims for mass fraction assignments at lower levels than indicated by the KCRV.

ADDITIONAL EXPERIMENTS

The three participants that reported lower values repeated the same measurement to figure out the sources of discrepancy using another set of the same test material used in CCQM-K151. Figure 11 shows all results of CCQM-K151 including these revisions.

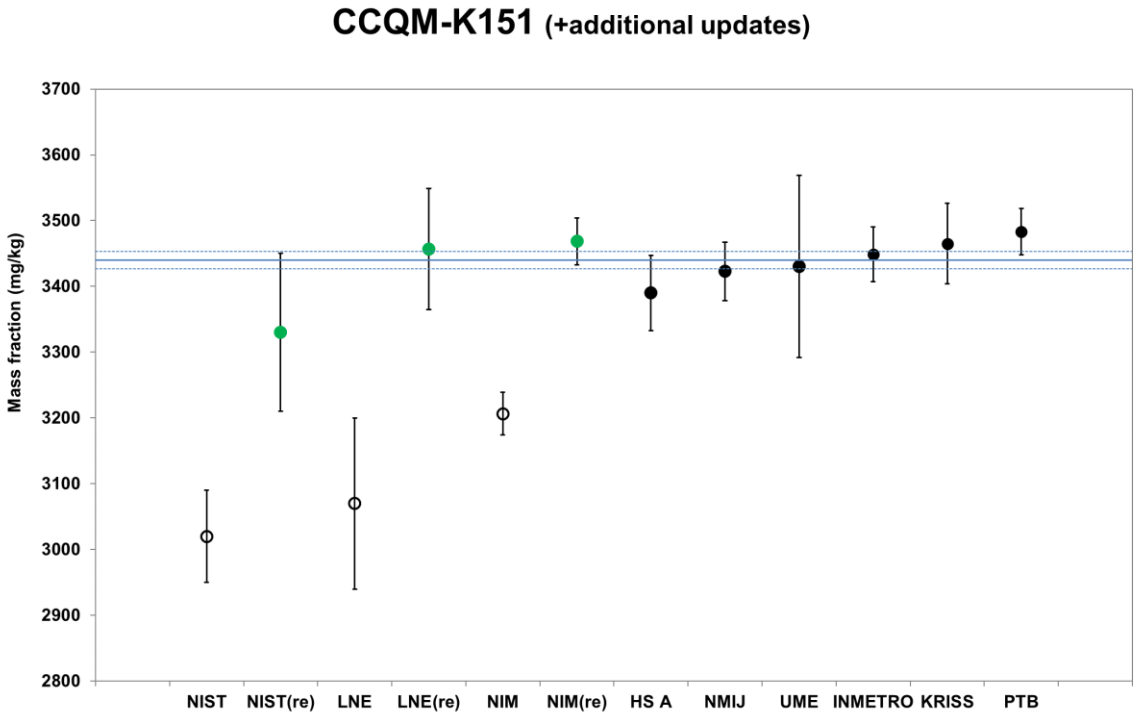

Figure 10. Plot of all results of CCQM-K151 with additional updated results by three participants. Green circles are the additional updates, and the open circles are the initially reported values. Horizontal lines are the KCRV and its standard uncertainty. The KCRV was only estimated from the data marked with black filled circles.

The revisions addressed two major issues in the original tests, namely hydrolysis condition optimization and increased sample volume preventing adsorption sampling loss. NIST revised the hydrolysis condition, and NIM and LNE revised their sampling volume.

In the initial reported result from NIST, results disagreement between the AA residues was significant; particularly the relatively low value of Ile can be regarded as a potential evidence of insufficient hydrolysis. NIST revised their hydrolysis conditions with a higher temperature and longer reaction time, as summarized in Table 11. Moreover, the isotopic analogues were added into the working sample prior to hydrolysis. Consequently, all measured values of the four AAs were increased approximately 10% from (3020 ± 140) mg/kg to (3330 ± 240) mg/kg, and

discrepancies between residues were improved to a relative standard deviation of 1.4 %, as compared to 5.7 % in the initial report. Revised data were analyzed using the Markov chain–Monte Carlo method. The overall uncertainty increased by higher repeatability variation, including between-vial variations, but remained within an acceptable range. Comparisons of NIST's two results are shown in Table 12 and Figure 12.

Table 12. Changes in NIST experimental details

		NIST (K result)	NIST (revised result)
Measured value (mg/kg)		3020	3330
Exp. U (mg/kg, *k*=2)		140	240
sample used (g)		0.05	0.06
dilution		86.6	87
hydrolysis type		Gas-phase	Gas-phase
Hydrolysis conditions	temp (℃)	**120**	**130**
	time (h)	**21**	**48**
	HCl conc. (M)	6	6
	Phenol (%)	no	no
Isotopes addition		**after-hydrolysis**	**pre-hydrolysis**

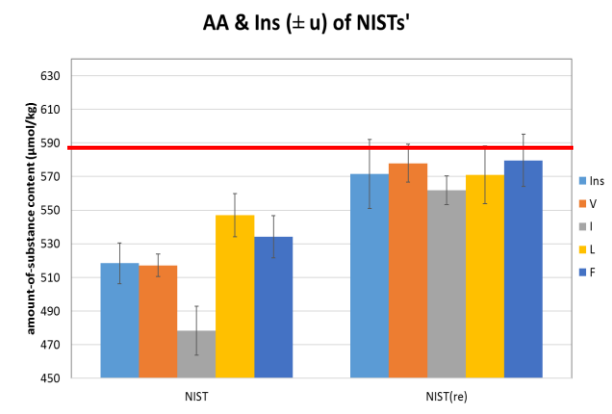

Figure 11. Original and revised results reported by NIST

LNE used the smallest amount of intact sample (0.01 g), and NIM also used quite a small amount (0.04 g) in the initial report. Both results showed similar values in all residues, but were lower than the others. This reflects potential systematic bias, rather than hydrolysis imperfection. It should be noted that even though 0.04 g was also applied by other participants (PTB and INMETRO), only NIM diluted the intact samples.

In the revision, NIM increased the sample amount used (0.2 g) as well as reduced the dilution ratio (6.8). Concerning the latter, NIM demonstrated whether the results were influenced by the dilution. Two-hundred milligram samples were diluted with 0.1 mol/L HCl into working solutions 6.8, 10, and 100 times. The results decreased with increasing dilution times. An explanation for this phenomenon might involve more protein adsorption onto the surfaces of the labware. Therefore, 6.8 was selected as the optimized sample dilution time in their revision. Results comparisons of NIM are shown in Table 13 and Figure 13.

Table 13. Changes in NIM experimental details

	NIM (K result)	NIM (revised result)
Measured value (mg/kg)	3206	3468
Exp. U (mg/kg, *k*=2)	65	71
sample used (g)	0.04	0.2
dilution	100	6.8
hydrolysis type	Liquid-phase	Liquid-phase
LC-MS syste,	AB SCIEX QTRAP ® 5500 + Agilent 1200 HPLC	**Home-made** Q-LIT V2MS system + Vanquist HPLC (Thermo)

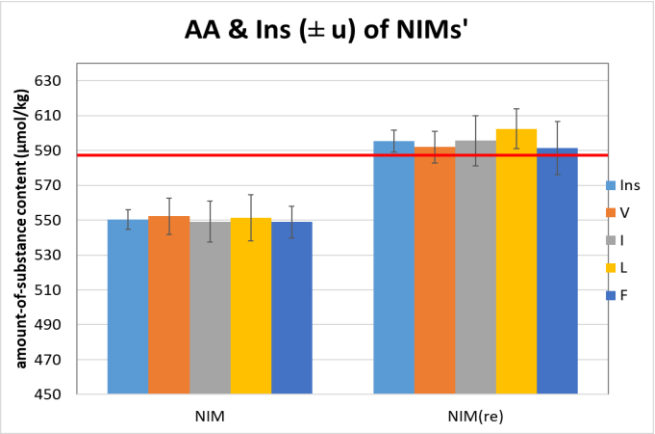

Figure 12. Original and revised results reported by NIM

Likewise, LNE increased the sample amount used up to 0.1 g, with other procedures including a 40-times dilution remaining the same as the initial report. In the revision, similar to NIM, LNE concluded that the problem issued during the first measurements was either an inhomogeneity of the sample not supported by such a low sample uptake, or adsorption of insulin on the wall of the different tubes during the sample processing, which is particularly important at such a low sample uptake, or a mix of both. Results comparison of LNE are shown in Table 14 and Figure 14.

Table 14. Changes in LNE experimental details

	LNE (K result)	LNE (revised result)
Measured value (mg/kg)	3070	3457
Exp. U (mg/kg, *k*=2)	260	184
sample used (g)	0.01	0.1
dilution	40	40
hydrolysis type	Gas-phase	Gas-phase
LC-MS conditions	Xevo TQ-MS + Acquity UPLC	Xevo TQ-MS + Acquity UPLC or Qexactive focus Thermo Fisher Scientific + U3000

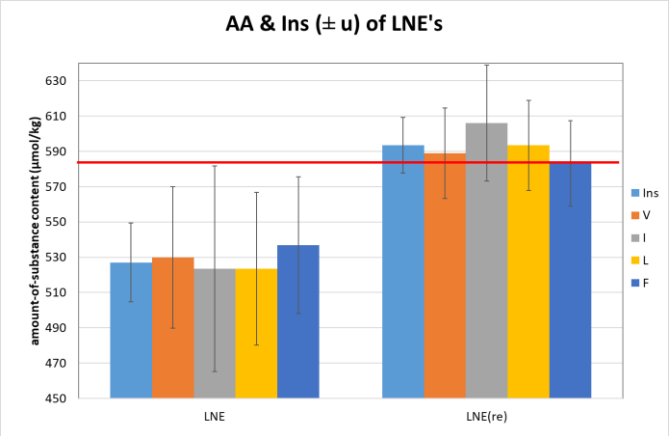

Figure 13. Original and revised results reported by LNE

CONCLUSIONS

The arithmetic mean was chosen for KCRV estimation in CCQM-K151, assessment of the mass fraction of a purified insulin analogue in aqueous solution. Six of the nine results showed mutual consistency as well as similar uncertainty, so that the estimation results by arithmetic mean, uncertainty weighted-mean, and DerSimonian–Laird were closely similar. By recommendation of a CCQM guidance note, the arithmetic mean (KCRV) proposed by the coordinating laboratory and agreed upon by the participants was 3440 mg/kg with a corresponding expanded uncertainty of ± 13.5 mg/kg. The degree of equivalence (DoE) of the dataset used in KCRV estimation showed a relative DoE under 1.5 %, showing that all results are statistically equivalent. Three participants progressed substantial experiments with further investigation into their negative-biased factor, and actively proved their measurement capability.

Human insulin analogue was selected to be representative of purity-evaluated recombinant proteins up to 10 kDa containing up to 3 disulfide cross-links using AA-based ID-LC-MS and/or sulfur-based ID-ICP/MS. The KCRV based on AA analysis will be the reference to the parallel pilot study based on S analysis, and this result underpins the complemental role both methods play in protein quantification.

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