CCQM-P216

PAWG Pilot Study on Quantification of SARS-CoV-2 Monoclonal Antibody

Part 1

Final Report

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ACRONYMS

AA	Amino acid
AAA	Amino acid analysis
Anti-S IgG mAb	Antibody against spike glycoprotein of SARS-CoV-2
ANOVA	One-way analysis of variance
CCQM	Consultative Committee for Amount of Substance: Metrology in Chemistry and Biology
COVID-19	Coronavirus Disease 2019
D	Difference from the reference value
DI	Designated Institute
F/T	Freeze/thaw cycle
F, Phe	Phenylalanine
ID-MS	Isotope dilution mass spectrometry
IgG	Immunoglobulin G
I, Ile	Isoleucine
RV	Reference Value
L, Leu	Leucine
LC	Liquid chromatography
mAb	Monoclonal antibody
MALS	Multiangle light scattering
MS	Mass spectrometry
NMI	National Metrology Institute
PAWG	Protein Analysis Working Group
PEP	Peptide
P, Pro	Proline
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SDS-PAGE	Sodium dodecyl sulphate–polyacrylamide gel electrophoresis
SEC	Size exclusion chromatography
UV	Ultraviolet (detection)
V, Val	Valine

SYMBOLS

D_i	Difference from the reference value
k	Coverage factor
n	Number of quantity values in a series of quantity values
RV	Reference value
$u(x_i)$	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	i^{th} member of a series of quantity values
w_i	Mass fraction of analyte in kg/kg or subunits thereof in a given matrix

INTRODUCTION

Monoclonal antibodies (mAbs) are immune system proteins that are created in the laboratory. The mAbs have tremendous applications in the field of diagnostics, therapeutics and targeted drug delivery systems. Comprehensive analysis of monoclonal antibody therapeutics is no easy task. These molecules embody various complex attributes, the characterization of which is a long and arduous process [1-2].

The potential applications and benefits of these complex molecules have increased the demand for harmonization and standardization of suitable analytical methods for mAb proteins. For this reason, it is important to build capacity and demonstrate equivalence across NMIs/DIs in methods that can be used for antibody characterization and quantitation [3-6].

The global COVID-19 pandemic, which as of 1 October 2021 had infected over 233 million people, as reported by WHO [7], has also led to increased focus on antibody quantitation methods. IgG are among the immunoglobulins produced by the immune system to provide protection against SARS-CoV-2. Anti-SARS-CoV-2 IgG can therefore be detected in samples from affected patients. Antibody tests can show whether a person has been exposed to the SARS-CoV-2, and whether or not they potentially show lasting immunity to the disease. With the constant spread of the virus and the high pressure of re-opening economies, antibody testing plays a critical role in the fight against COVID-19 by helping healthcare professionals to identify individuals who have developed an immune response, either via vaccination or exposure to the virus. Many countries have launched large-scale antibody testing for COVID-19 [8-9]. The development of measurement standards for the antibody detection of SARS-CoV-2 is critically important to deal with the challenges of the COVID-19 pandemic. In this study, the SARS-CoV-2 monoclonal antibody is being used as a model system to build capacity in methods that can be used in antibody quantification.

The following sections of this report document the timeline of CCQM-P216, the measurands, study material, participants, and results. The Appendices reproduce the official communication materials and summaries of information about their results provided by the participants.

TIMELINE

Table 1: lists the timeline for CCQM-P216.

Date	Action
March 2020	Initial discussion of the proposal
Apr-July 2020	Draft protocol discussion
May 2020	Coordination laboratory confirmation (NIM, NRC, BIPM)
July 2020	PAWG authorized CCQM- P216
Aug 2020	Protocol approved
Sep 2020	Call for participation 30th September 2020, final date to register to participate
Oct 2020	Samples distribution
Nov 2020	Study samples shipped to the participants
Apr 2021	The first round results submitted by 9th April 2021 Initial discussion of the first round results at PAWG meeting
May 2021	Discussion of the P216 SARS-CoV-2 antibody study
Sep 2021	Reporting of the optional measurands
Oct 2021	Discussion of the second round results at PAWG Meeting

MEASURANDS

The study material is a recombinant humanized IgG monoclonal antibody against Spike glycoprotein of SARS-CoV-2 (Anti−S IgG mAb) in solution. The molecular weight of Anti−S IgG mAb is 149 kDa, with 16 disulfide cross links. The number of amino acids in one heavy chain is 449, including 12 phenylalanines (Phe), 42 valines (Val), 30 leucines (Leu), 14 isoleucines (Ile), and 36 prolines (Pro); the number of amino acids in one light chain is 220, including 7 phenylalanines (Phe), 16 valines (Val), 17 leucines (Leu), 7 isoleucines (Ile), and 11 prolines (Pro). The Sequence information of Anti−S IgG mAb protein is given below.

The sequence of constant region of the light chain is as follows:

RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQE
SVTEQDSKDSTYSLSSTLT LSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC*

The sequence of constant region of the heavy chain is:

ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPA
VLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCP
PCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGV
EVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTI
SKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENN
YKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLS
PGK*

(*for termination)

The proposed measurands are: a) Mass fraction of different AAs in the materials; b) Mass fraction of proteotypic peptides belonging to the constant region of the mAb.

Participants should consider using the methods named below, but NMIs are welcome to perform additional methods of their choice. A description of the measurement methods used by the participating laboratory should be submitted as part of their results.

The measurement methods to be considered for use include:
1) Isotope dilution mass spectrometry (ID-MS) for amino acid analysis;
2) Peptide-based MS quantification in the constant region of the mAb.
The following peptides are candidate suggestions from the constant region of Anti-S IgG mAb:

A) ALPAPIEK
B) GPSVFPLAPSSK
C) STSGGTAALGCLVK
D) DSTYSLSSTLTLSK

STUDY MATERIALS

Preparation of Candidate Material

A recombinant humanized IgG monoclonal antibody against Spike glycoprotein of SARS-CoV-2 (Anti−S IgG mAb) in solution was used for this comparison. The buffer solution is 77 mM citrate acid (0.1 M citrate acid was adjusted to pH 6.7 with 1 M Tris) with 0.5 % Glycine, pH 6.7. The nominal mass fraction of the target Anti−S IgG mAb

protein is estimated at 0.5 mg/g. The antibody was screened against the S1 fragment of SARS-CoV-2 Spike glycoprotein, then humanized and expressed by 293T cell lines.

Five vials of Anti−S IgG mAb solution in PE cryotube with silicon gasket seal were provided to CCQM-P216 participants (AA/PEP-analysis). Each contains 0.2 mL of a solution of Anti−S IgG mAb in buffer solution. Samples were distributed using temperature controlling transport packaging with temperature data logging devices. At least three of these vials were to be used in determining the results to be reported; the additional samples were for method development.

Impurity Assessment of the Study Material

The purity of Anti−S IgG mAb was determined by SDS-PAGE analysis, SEC-HPLC and LC-MS/MS analysis. Impurity protein bands were not detected in the SDS-PAGE analysis. And no peak of dimer impurity or other impurities was observed with UV detection by SEC-HPLC analysis. The peak representing the potential dimer impurity was very small by MALS detection which is lower than 1 %. The free amino acids in the sample solution were checked by LC-MS/MS. No free amino acids of Phe, Val, Leu or Ile were detected. Also, no free peptides of ALPAPIEK or GPSVFPLAPSSK were detected in the solution. Consequently, there are no significant impurities of concern in the sample.

Homogeneity and Stability Assessment of the Study Material

Homogeneity

The homogeneity of the study material was assessed by performing three replicate measurements (sub-samplings) of 15 units. The homogeneity was evaluated by amino acid ID-MS. Data was analyzed using ANOVA. No differences in the within- and between-sample variances could be detected by the F-tests at the 95 % confidence level (Figure 1). Therefore, the material was deemed to be homogeneous. The u_{bb} of 0.19 % was adopted as an estimate for the uncertainty contribution for homogeneity. The sample size of the material used for homogeneity test was approximately 20 mg of sample solution per analysis.

The homogeneity was also evaluated by SEC-UV. No differences in the within- and between-sample variances could be detected by the F-tests at the 95 % confidence level

(Figure2). The u_{bb} for the uncertainty contribution from homogeneity analyzed by SEC-UV was 0.2 %.

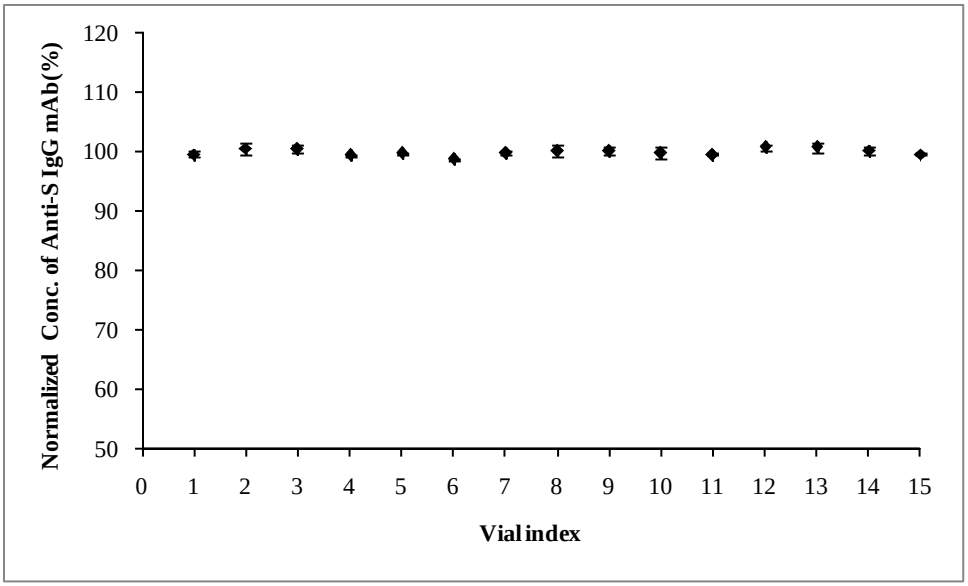

Figure 1: Homogeneity of Anti-S IgG mAb (by amino acid ID-MS) – Filling sequence

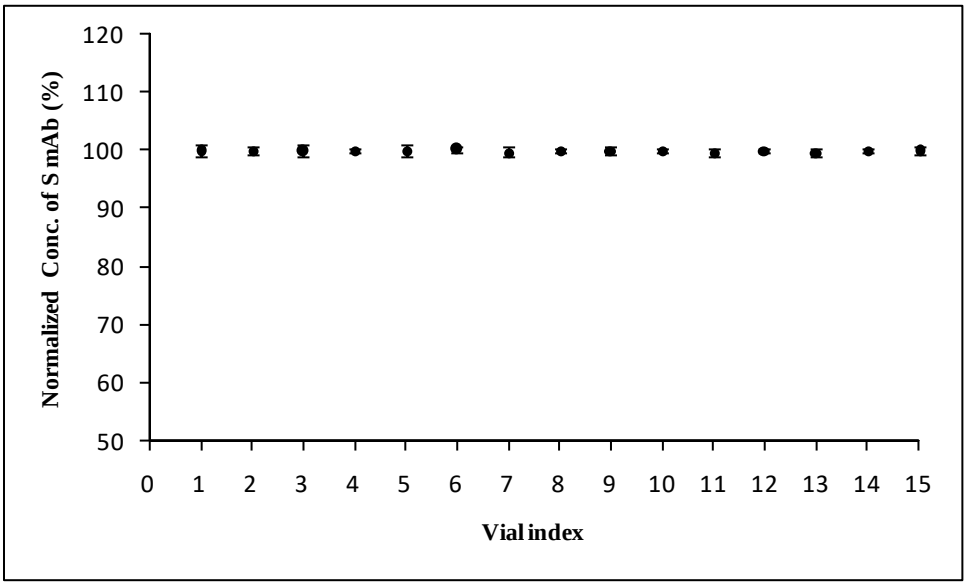

Figure 2: Homogeneity of Anti-S IgG mAb (by SEC-UV) – Filling sequence

Stability

A short-term stability study was performed by incubation of study materials at4 °C and 25 °C for 1 and 7 days. Stability was assessed by amino acid ID-MS and SEC-UV analysis. The effect of storage temperatures on the mass fractions of Anti-S IgG mAb by amino acid ID-MS and peak area of Anti-S IgG mAb by SEC-UV analysis is shown in Figures 3-6. No obvious trend was observed by statistical analysis (linear regressions).

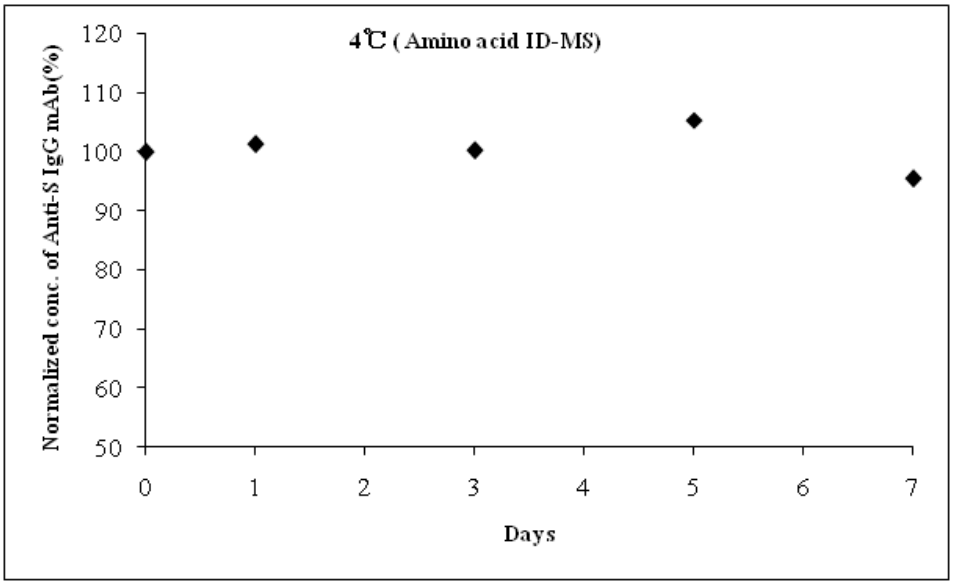

Figure 3: Short-term stability study of Anti-S IgG mAb at 4 °C (by amino acid ID-MS)

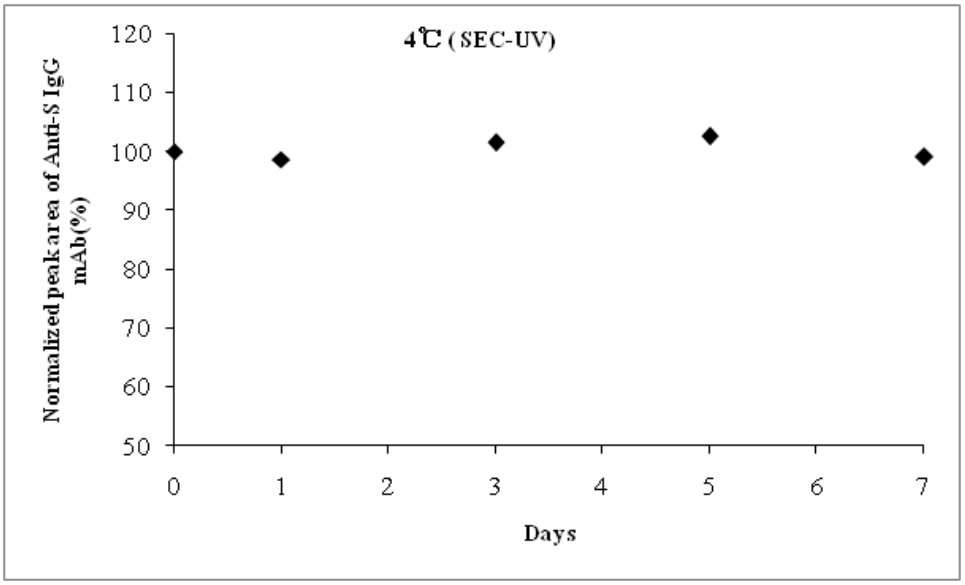

Figure 4: Short-term stability study of Anti-S IgG mAb at 4 °C (by SEC-UV)

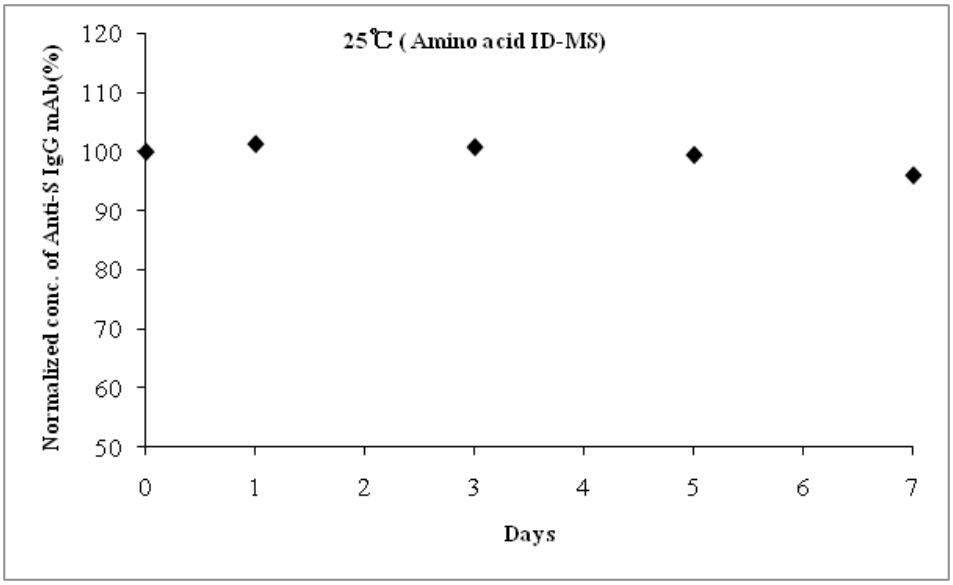

Figure 5: Short-term stability study of Anti-S IgG mAb at 25 °C (by amino acid ID-MS)

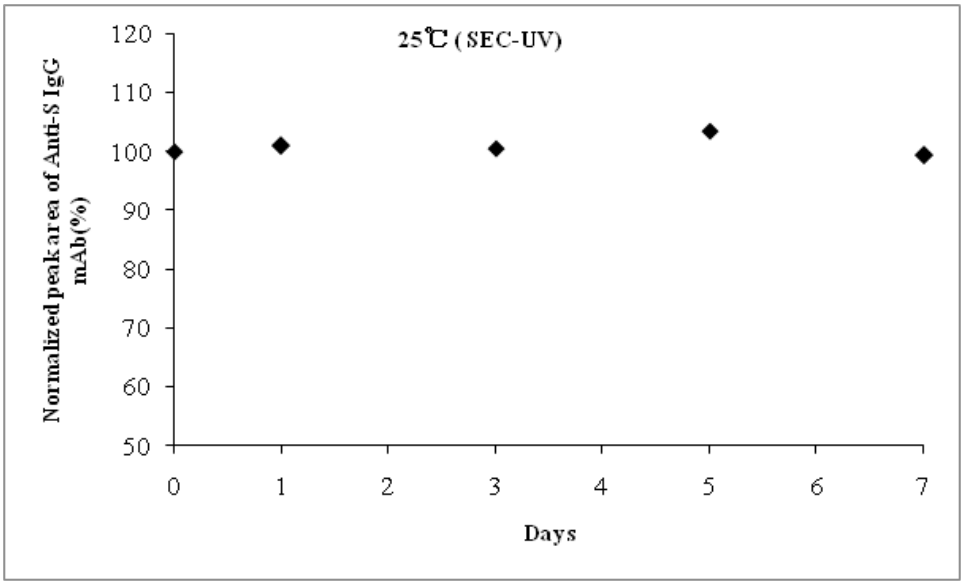

Figure 6: Short-term stability study of Anti-S IgG mAb at 25 °C (by SEC-UV)

Freeze/thaw (F/T) cycle stability was also determined. F/T samples were tested after undergoing an additional one to five F/T cycles with freeze temperature at -70 °C. The stability was assessed by SEC-UV analysis and amino acid ID-MS. The effect of F/T cycle on the mass fractions of Anti-S IgG mAb by amino acid ID-MS is shown in Figure 7. No obvious trend was observed by statistical analysis (linear regressions). The uncertainty component from five F/T cycles by amino acid ID-MS analysis was 0.65 %.

The effect of F/T cycle on peak area of Anti-S IgG mAb by SEC-UV analysis is shown in Figure 8. No obvious trend was observed by statistical analysis. The uncertainty component from five F/T cycles by SEC-UV analysis was 2.36 %.

Freeze/thaw cycle stability study of Anti-S IgG mAb from one to five were shown in Figure 7 and Figure 8.

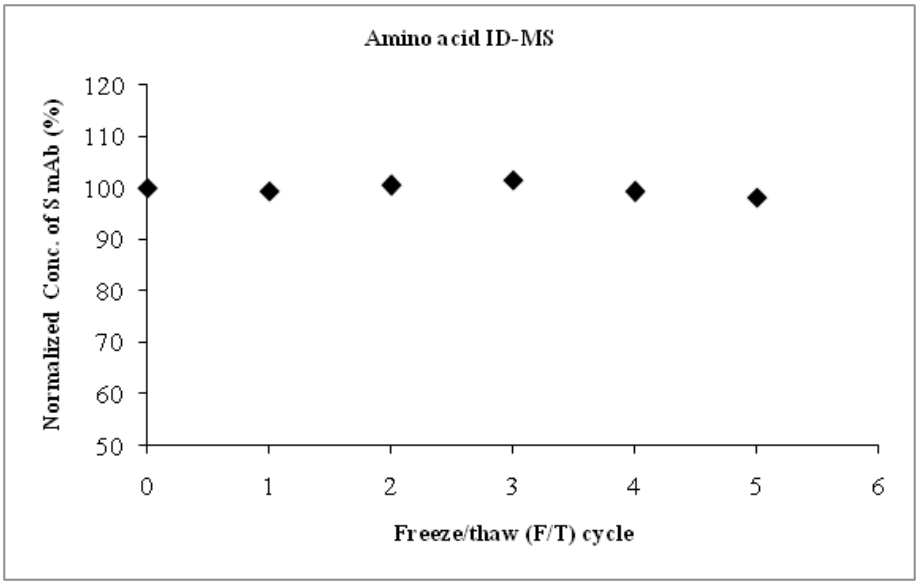

Figure 7: Freeze/thaw cycle stability study of Anti-S IgG mAb (by amino acid ID-MS)

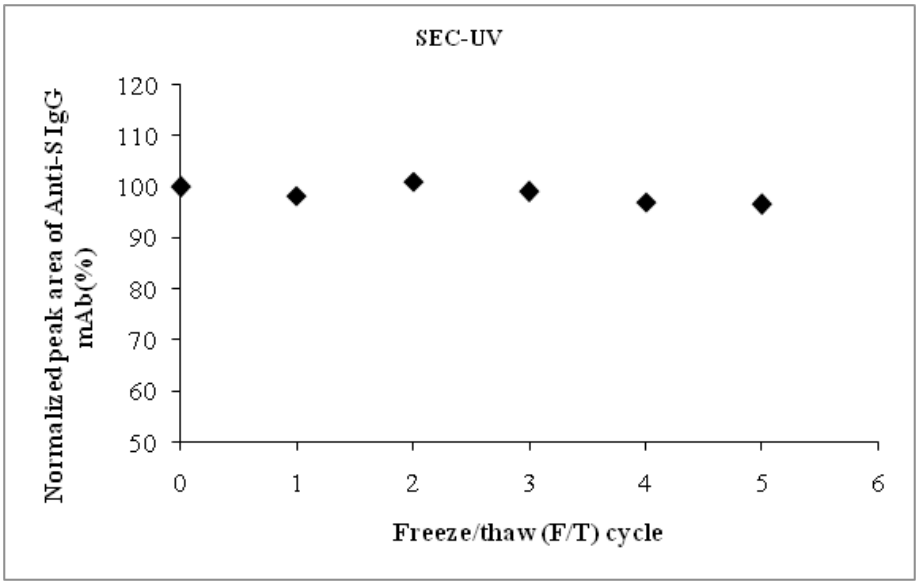

Figure8: Freeze/thaw cycle stability study of Anti-S IgG mAb (by SEC-UV)

Along-term stability study was performed at NIM prior to shipment of the study materials, with analysis of two units performed every two months. The study material was stored at the reference temperature of -70 °C.

The long-term stability was analyzed by amino acid ID-MS (see Figure 9-1) and SEC-UV (see Figure 9-2). No obvious trend was observed by statistical analysis (linear regressions). The u_s of 0.73 % was adopted as an estimate for the uncertainty component from 7 months long term stability. The coordinating laboratory will continue monitoring stability every two months until completion of the study.

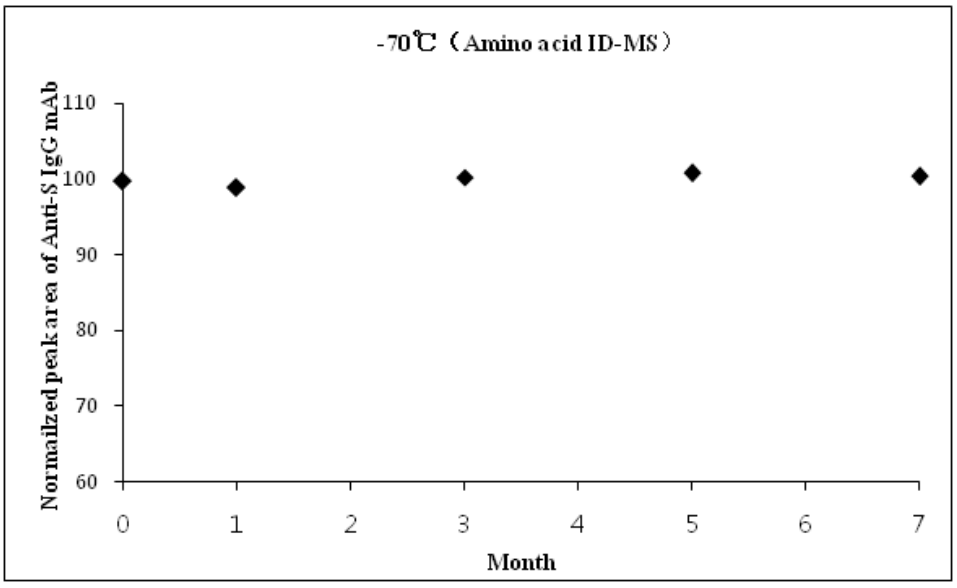

Figure 9-1: Long term stability (Amino acid ID-MS)

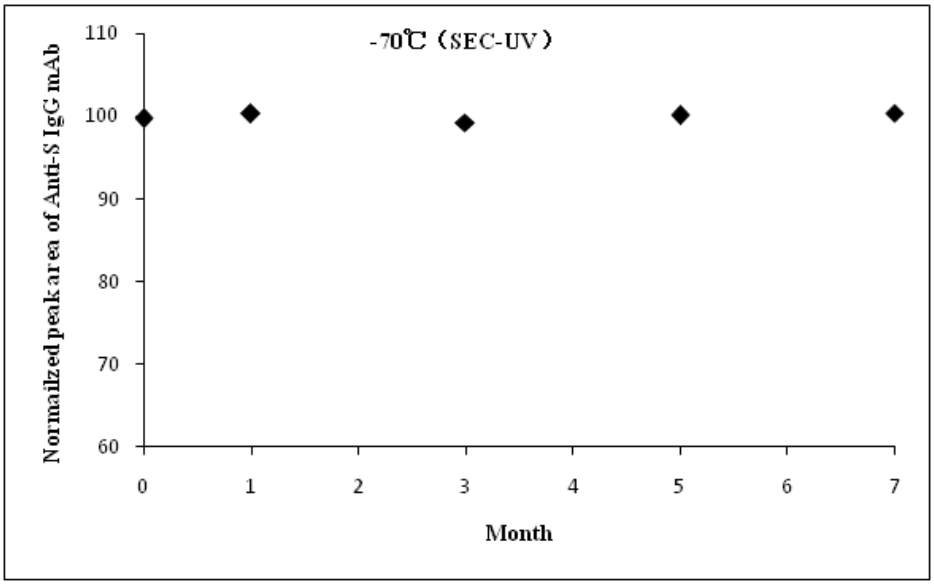

Figure 9-2: Long term stability (SEC-UV)

SAMPLE DISTRIBUTION

Five vials of Anti-S IgG mAb solution were provided to each participant for the first round of measurements. Samples were distributed using boxes equipped with temperature data-logging devices. Participants were asked to return the temperature indicator form acknowledging receipt of the samples and to advise the coordinator if any obvious damage had occurred to the vials during shipping. The sample shipped to INMETRO on 21 February 2021vwas only delivered on 22 March 2021 due to customs issues. The coordinator has verified that all the temperature indicators inside the shipping container had not registered a temperature in excess of -70 °C during the transport process. Table 2 provides an overview of the NMIs/DIs that received CCQM-P216 samples and corresponding shipping conditions.

Table 2 lists the institutions that received CCQM-P216 samples.

NMI or DI	Code	Contact	Shipped	Delivered	Temperature data
Health Sciences Authority, Singapore	HSA	Qinde Liu, Tang Lin Teo	14-Nov-20	16-Nov-20	YES
TÜBİTAK/UME National Metrology Institute, Turkey	UME	MerveÖztuğ	14-Nov-20	17-Nov-20	YES
National Research Council Canada, Canada	NRC	Jeremy Melanson	14-Nov-20	18-Nov-20	YES
National Metrology Institute of Japan/National Institute of Advanced Industrial Science and Technology, Japan	NMIJ/AIST	TomoyaKinumi	14-Nov-20	18-Nov-20	YES
BundesanstaltfürMaterialforschung und -Prüfung, Germany	BAM	Carsten Jaeger	14-Nov-20	25-Nov-20	YES
Bureau International des Poids et Mesures, in France	BIPM	Ralf D. Josephs	14-Nov-20	16-Nov-20	YES
Laboratory of the Government Chemist, United Kingdom	LGC	Chris Mussell	14-Nov-20	17-Nov-20	YES
Laboratoire National de Métrologie et d'Essais, France	LNE	Vincent Delatour,	14-Nov-20	16-Nov-20	YES
Korea Research Institute of Standards and Science, Korea	KRISS	J. Eugene Lee	14-Nov-20	23-Nov-20	YES
Physikalisch-TechnischeBundesanstalt, Germany	PTB	Gavin O'Connor	12-Dec-20	14-Dec-20	YES
Instituto Nacional de Metrologia, Qualidade e Tecnologia, Brazil	INMETRO	Paulo José Miranda da Silva IwakamiBeltrão	26-Feb-21	22-Mar-21	YES
National institute of metrology, China	NIM	Xinhua Dai, Wei Mi	/	/	/

RESULTS

In the first round of measurements participants were requested to report the mass fraction (mg/kg) of each target amino acid and peptide in the constant region in vials. In addition to the quantitative results, participants were to describe their analytical methods, approach to uncertainty estimation, and detailed information of any isotopically-labelled materials, and primary reference materials used. A template for the report was shared as "the CCQM-P216: Quantification of SARS-CoV-2 monoclonal antibody in solution, Data Submission Form".

Methods Used by Participants

The well-established ID-MS for amino acid analysis and peptide analysis was encouraged, but participants were free to use any suitable method for quantification of target amino acids and peptides. Participants have been were asked to include a detailed description of the measurement procedure used.

Twelve CCQM-P216 result reports were received from 12 NMIs/DIs that received samples. NIM China used a commercial mass spectrometer and a home-built Q-LIT V5 mass spectrometer to perform MS/MS acquisition. Consequently, two sets of data reported in the report: one was marked as NIM, and the other was marked as NIM_Q-LIT V5.

Eleven participants of CCQM-P216 used ID-MS for AA analysis. Among them, 9 participants measured 5 AAs Leu, Val, Phe, Ile, and Pro. NIM and INMETRO measured 4 AAs Leu, Val, Phe, and Ile. BAM measured 2 AAs Leu and Phe.

Nine institutions used ID-MS analysis for proteotypic peptides in the constant region of the mAb. Eight participants measured the peptide ALPAPIEK, while seven participants measured the peptide GPSVFPLAPSSK. LNE also measured the peptide STSGGTAALGCLVK, and UME measured the peptide DSTYSLSSTLTLSK.

Brief descriptions of the analytical methods used by the CCQM-P216 participants, including sample preparation, analytical technique, calibrants, and quantification approach are summarized in Table 3-8.

Table 3: The summary of hydrolysis method

Participant		HSA	UME	NIM	NIM Q-LIT V5	BAM	BIPM	LNE	INMETRO	NMIJ	NRC	LGC	PTB
Sample amount used		21 mg	100 µL	20 mg	20 mg	10 µL	10 mg	50 mg	40 mg	60 mg	50 mg		
Hydrolysis method	**Type**	Gas-phase	Gas-phase	Liquid-phase	Liquid-phase	Acidic hydrolysis	Microwave-assisted vapor phase	Gas-phase	Liquid-phase	Microwave-assisted Liquid phase	Liquid-phase	Microwave assisted	Liquid-phase
	Temp (℃)	130	130	110	110	150	150 (150 W)	130		160	110	160	150
	Time (h)	24	48	48	48	1	5/6	72	72	3	72	3	65
	HCl conc. (M)	6	6	6	6	6	6	6	6	6	6	6	6
	Phenol (%)	0.1	Yes	No	No	No	No	No	0.1	0.1	No	No	0.1
Further preparation steps	**Isotopes addition before/after hydrolysis**	before	before	before	before	before	before	before	after	before	before	before	before
	Other	/	/	/	/	/	/	/	/	/	/	/	/

Table 4: The summary of used instruments and conditions for amino acid analysis

Participant	HSA	UME	NIM	NIM_ Q-LIT V5	BAM	BIPM	LNE	INMETRO	NMIJ	NRC	LGC	PTB
Analytical instrumentation used	LC-MS-MS	HR-LC-MS	LC–MS/MS	LC-MS/MS	LC-MS	LC-MS/MS	LC-MS	LC-MS/MS	LC-MS/MS	LC-MS/MS	GC-MS/MS	LC-MS/MS
Analytical instrumentation name	SCIEX Triple Quad 6500+ -Agilent 1290 HPLC	OrbiTrap-Q Exactive HR-MS -3000 UHPLC system	AB Sciex QTRAP 5500 -Agilent 1200 HPLC	Q-LIT V5 MS -system Shimadzu HPLC system	SciexTriple TOF 6600 -Agilent 1290 UHPLC	AB Sciex QTrap 6500+ -Exion LC	Qexactive focus -U3000 HPLC	Xevo TQS Waters UPLC	TSQ -LC-20A HPLC system	Quantiva triple-quadrupole –Dionex UltiMate 3000	Agilent 7010 triple quadrupole-Agilent 7890B GC	Agilent 1100 LC-MSD
Detection method used	MRM	Full MS	MRM	MRM	Full scan	SRM	SIM	MRM	SRM	MRM	dMRM	MRM
Chromatographic Column	Agilent ZORBAX Eclipse AAA, 4.6 × 75mm, 3.5 µm	PhenomonexEZ :faast 4u AAA, 2.0 mm × 250 mm	KINETEX C18 column ,150 × 2.1 mm, 2.6 µm	Waters ACQUITY UPLC HSS T3 column ,100 mm × 2.1 mm, 1.8µm	BEH Amide, Waters, 2.1 × 75 mm, 1.7 µm	Primesep 100 (Mixed-mode column by SIELC), 250 × 4.6 mm	Acquity UPLC® BEH C18, 1.7 µm, 2.1 × 100 mm	Waters Acquity UPLC HSS T3 1.8 µm, 2.1 × 100 mm	Develosil C30-UG-5, 5 µm, 2.0 mm × 250 mm	Nucleodur HILIC, 100 × 2.0 mm, 3 µm, 100 Å	Zebron ZB-5HT Inferno GC column, (30 m × 0.25 mm, 0.25 mm film thickness) with a Z-guard retention gap (2 m × 0.53 mm ID)	SeQuant ZIC-HILIC 3.5 µm, 150 × 2.1 mm (Merck)

Table 5: The summary of used Calibration standards and Internal standards for amino acid analysis

Participant	HSA	UME	NIM	NIM_ Q-LIT V5	BAM	BIPM	LNE	INMETRO	NMIJ	NRC	LGC	PTB
Target AA	V, P, L, I, F	V, P, L, I, F	V, I, L, F	V, L, I, F	L, F	V, P, L, I, F	V, P, L, I, F	V, L, I, F	V, P, L, I, F	V, P, L, I, F	V, P, L, I, F	V, P, L, I, F
Calibration standards	HRM -1006A -1007A -1008A -1013A -1014A	NMIJ 6015-a 6016-a 6012-a 6013-a 6014-a	GBW 09236 0928 09237 09235	GBW 09236 0928 09237 09235	NIST SRM 84l NIST SRM 350b	OGP.015 OGP.019 OGP.018 OGP.017 OGP.016	NMIJ 6015-a 6016-a 6012-a 6013-a 6014-a	GBW 09236 09237 09238 09235	NMIJ 6015-a 6016-a 6012-a 6013-a 6014-a	NRC APHE-1: ALEU-1 APRO-1	NMIJ 6015-a 6016-a 6012-a 6013-a 6014-a	NMIJ 6015-a 6012-a 6013-a NRC APRO-1 APHE-1
Internal standards used	$^{13}C_5$-Val $^{13}C_5{}^{15}N$-Pro $^{13}C_6$-Leu $^{13}C_6{}^{15}N$-Ile Ring-$^{13}C_6$Phe	$^{13}C_5{}^{15}N$-Val $^{13}C_5{}^{15}N$-Pro $^{13}C_6{}^{15}N$-Leu ^{15}N-Ile $^{13}C_9{}^{15}N$-Phe	$^{13}C_5$-Val $^{13}C_6$-Leu $^{13}C_6$-Ile $^{13}C_9$-Phe	$^{13}C_5$-Val $^{13}C_6$-Leu $^{13}C_6$-Ile $^{13}C_9$-Phe	$^{13}C_6{}^{15}N$-Leu $^{13}C_9{}^{15}N$-PHE	$^{13}C_5{}^{15}N$-Val $^{13}C_5$-Pro $^{13}C_6{}^{15}N$-Leu $^{13}C_6$-Ile $^{13}C_9$-Phe	$^{13}C_9{}^{15}N$-Val $^{13}C_5$-Pro $^{13}C_6$-Leu $^{13}C_6$-Ile $^{13}C_5{}^{15}N$-Phe	$^{13}C_5$-Val $^{13}C_6$-Leu $^{13}C_6$-Ile $^{13}C_9$-Phe	$^{13}C_5{}^{15}N$-Val $^{13}C_5{}^{15}N$-Pro $^{13}C_6{}^{15}N$-Leu $^{13}C_6{}^{15}N$-Ile $^{13}C_9{}^{15}N$-Phe	$^{13}C_5$-Val$^{13}C_5$ -Pro $^{13}C_6$-Leu, $^{13}C_6$-Ile Ring-$^{13}C_6$-Phe	$^{13}C_5{}^{15}N$-Val^{13} $C_5{}^{15}N$-Pro $^{13}C_6{}^{15}N$-Leu $^{13}C_6$-Ile $^{13}C_9{}^{15}N$-Phe $^{13}C_3{}^{15}N$-Ala	$^{13}C_5{}^{15}N$-Val $^{13}C_5$-Pro $^{13}C_6$-Leu $^{13}C_6$-Ile $^{13}C_9{}^{15}N$-Phe

Table 6: The summary of peptides analysis method

Participant		**HSA**	**UME**	**NIM**	**NIM Q-LIT V5**	**BAM**	**BIPM**	**LNE**	**INMETRO**	**KRISS**
Sample amount used for analysis		26 mg	30 µL	25 mg	25 mg	25 µL	40-50 mg	6 mg	25 mg	
Further preparation steps	**Isotopes addition before/after denaturing**	before	/	before	before	/	before	after	after	before
	Others	After enzymatic digestion, acetonitrile was added to the mixture to obtain about 10 % acetonitrile solution for LC-MS/MS analysis.	/	The enzymatic digested samples were dried in vacuum and the residue was dissolved in 0.1 % (v/v) FA for HPLC-IDMS analysis.	The enzymatic digested samples were dried in vacuum and the residue was dissolved in 0.1 % (v/v) FA for HPLC-IDMS analysis.	NISTmAb diluted in same buffer as study material (77 mM citrate pH 6.7, 0.5 % glycine)	Reduction with 0.4 mM TCEP. Alkylation with 1 mM iodoacetamide. Rapid digestion Trypsin / Lys C kit (Promega VA1061) containing buffer and Trypsin / Lys C enzyme mixture. Enzyme / substrate ratio used: 1/5. Digestion at 37 °C for 0.5 h followed by 3 h at 70 °C.	Trypsin digestion was performed in a final concentration of 1M urea. After 24h of digestion samples were subjected to solid phase extraction (SPE), then evaporated by Speed-Vac and resuspended in 20 µL of 0.1 % formic acid in water.	/	/

Table 7: The summary of used instruments and conditions for peptide analysis

Participant	HSA	UME	NIM	NIM_Q-LIT V5	BAM	BIPM	LNE	INMETRO	KRISS
Analytical instrumentation used	LC-MS/MS	LC-MS	LC-MS/MS	LC-MS/MS	LC-MS	LC-MS/MS	LC-MS	LC-MS/MS	LC-MS/MS
Analytical instrumentation name	SCIEX Triple Quad 6500+ Mass Spectrometer coupled with Agilent 1290 Infinity II HPLC	OrbiTrap-Q Exactive HR-MS-3000 UHPLC system.	AB Sciex QTRAP 5500 MS system -Agilent 1200 series HPLC system	Home-made Q-LIT V5 MS system (NIM, P.R. China) -Shimadzu LC-20A HPLC system	SciexTripleTOF 6600 -Agilent 1290 UHPLC	AB Sciex Qtrap 6500+ -Exion LC	Qexactive focus -U3000 HPLC	XevoTQSWaters UPLC	Orbitrap Fusion Eclipse -EASY-nLC 1000
Detection method used	MRM	Full MS	MRM	MRM	Full scan	MRM	PRM	MRM	PRM
Chromatographic Column	Agilent ZORBAX 300SB–C18, 2.1 × 50 mm, 3.5 µm	Aeris Peptide C18 column ,250 × 2 mm, 3,6 µm	AerisXB-C18 column ,150 × 2.1 mm, 3.6 µm(Phenomenex)	Waters ACQUITY UPLC HSS T3 column, 100 mm × 2.1 mm, 1.8 µm	Pinnacle DB Aqueous C18, Restek, 100 × 2.1 mm, 1.9 µm	Phenomenexbio Zen 2.6 µm Peptide XB-C18, 150 × 4.6 mm	Acclaim Pepmap 100, C18, 1 × 250 mm (Thermo Fisher Scientific)	Oasis HLB Direct Connect HP (20 µm, 2.1 × 30 mm) directly connected to a Waters Acquity UPLC BEH C18 (1.7 µm 2.1 × 50 mm) Column	In-house made Laser pulled fused-silica capillary, (15cm, 75µm ID) packed with C18 beads (10 nm, Bonna-Agela)

Table 8: Summary Calibration standards and Internal standards used for peptide analysis

Participant	HSA	UME	NIM	NIM_Q-LIT V5	BAM	BIPM	LNE	INMETRO	KRISS
Target PEP	APAPIEK	ALPAPIEK GPSVFPLAPSSK DSTYSLSSTLTLSK	ALPAPIEK GPSVFPLAPSSK	ALPAPIEK GPSVFPLAPSSK	ALPAPIEK	ALPAPIEK GPSVFPLAPSSK	ALPAPIEK GPSVFPLAPSSK STSGGTAALGCLVK	ALPAPIEK GPSVFPLAPSSK	GPSVFPLAPSSK
Calibration standards	NIST RM 8671	ALPAPIEK/ GPSVFPLAPSSK/ DSTYSLSSTLTLSK	ALPAPIEK/ GPSVFPLAPSSK	ALPAPIEK/ GPSVFPLAPSSK	NIST Monoclonal Antibody Reference Material 8671	ALPAPIEK/ GPSVFPLAPSSK	ALPAPIEK/ GPSVFPLAPSSK/ STSGGTAALGCLVK	ALPAPIEK/ GPSVFPLAPSSK	GPSVFPLAPSSK
Internal standards used	A(L-$^{13}C_6$,^{15}N)PAPIEK	ALPAPIE(K-$^{13}C_6$,$^{15}N_2$); GPSVFPLAPSS(K-$^{13}C_6$,$^{15}N_2$); DSTYSLSSTLTLS(K-$^{13}C_6$,$^{15}N_2$)	A(L-$^{13}C_6$,^{15}N)PAPIEK; GPSVFP(L-$^{13}C_6$,^{15}N)APSSK	A(L-$^{13}C_6$,^{15}N)PAPIEK; GPSVFP(L-$^{13}C_6$,^{15}N)APSSK	ALPAPIE(K-$^{13}C_6$,$^{15}N_2$)	ALPAPIEK(^{13}C,^{15}N) ;GPSVFPLAPSSK(^{13}C,^{15}N)	ALPAPIE(K-$^{13}C_6$,$^{15}N_2$); GPSVFPLAPSS(K-$^{13}C_6$,$^{15}N_2$); STSGGTAALGCLV(K-$^{13}C_6$,$^{15}N_2$)	A(L-$^{13}C_6$$^{15}N$)PAPIEK; GPSVFP(L-$^{13}C_6$$^{15}N$) APSSK	GPSVFPLAPSS(K-$^{13}C_6$,$^{15}N_2$)

Participant Results

The CCQM-P216 results for mass fraction of target amino acids are detailed in Table 9-13 and presented graphically in Figure 10-14.

The CCQM-P216 results for mass fraction of peptides are detailed in Table 14-16 and Figure 15-16.

The submitted results in mass fraction of different AAs converted to mass fraction of anti-S IgG mAb and presented in Figure 17.

The submitted results in mass fraction of different PEPs were converted to mass fraction of anti-S IgG mAb and presented in Figure 18.

Table 9: Results for CCQM-P216: mass fraction of **Leucine** in the anti-S IgG mAb material and uncertainties as received

Participant	**Leucine**			
	Mass fraction (mg/kg)	**Standard uncertainty (*u*) (mg/kg)**	**Coverage Factor (*k*)**	**Exp. *Unc*. (*U*) for approximately 95 % confidence interval (mg/kg)**
HSA	44.80	0.78	2.31	1.80
UME	32.77	0.89	2	1.77
NIM	36.09	0.91	2	1.82
NIM_Q-LIT V5	37.46	0.5	2	1.0
BAM	42.267	1.993	1.98	3.955
BIPM	32.8	2.6	2	5.2
LNE	31.2	1.2	2	2.3
INMETRO	39.08	0.65	2	1.31
NMIJ/AIST	35.49	0.31	2	0.62
LGC	37.0	1.7	2	3.3
PTB	34.18	0.52	2	1.03
NRC	39.1	0.8	2	1.6
HSA _corrected	35.40	0.62	2.31	1.42

Table 10: Results for CCQM-P216: mass fraction of **Valine** in the anti-S IgG mAb material and uncertainties as received

Participant	**Valine**			
	Mass fraction (mg/kg)	**Standard uncertainty (*u*) (mg/kg)**	**Coverage Factor (*k*)**	**Exp. *Unc*. (*U*) for approximately 95 % confidence interval (mg/kg)**
HSA	49.78	0.87	2.31	2.02
UME	36.15	0.69	2	1.38
NIM	39.78	0.88	2	1.76
NIM_Q-LIT V5	39.37	0.8	2	1.6
BAM	/	/	/	/
BIPM	35.8	3.0	2	6.0
LNE	38.1	1.3	2	2.6
INMETRO	41.40	1.12	2	2.24
NMIJ/AIST	39.02	0.47	2	0.94
LGC	40.6	1.0	2	2.0
PTB	36.28	0.43	2	0.86
NRC	42.1	0.9	2	1.8
HSA _corrected	39.26	0.69	2.31	1.59

Table 11: Results for CCQM-P216: mass fraction of **Phenylalanine** in the anti-S IgG mAb material and uncertainties as received

Participant	**Phenylalanine**			
	Mass fraction (mg/kg)	**Standard uncertainty (*u*) (mg/kg)**	**Coverage Factor (*k*)**	**Exp. *Unc*. (*U*) for approximately95 % confidence interval (mg/kg)**
HSA	22.85	0.40	2.26	0.91
UME	16.59	0.43	2	0.86
NIM	18.66	0.47	2	0.94
NIM_Q-LIT V5	18.90	0.33	2	0.66
BAM	21.612	0.848	1.98	1.683
BIPM	16.5	1.3	2	2.6
LNE	15.9	0.66	2	1.3
INMETRO	19.35	0.37	2	0.73
NMIJ/AIST	18.69	0.50	2	1.01
LGC	18.7	1.0	2	1.9
PTB	17.28	0.36	2	0.72
NRC	19.7	0.4	2	0.8
HSA _corrected	18.06	0.32	2.26	0.72

Table 12: Results for CCQM-P216: mass fraction of **Isoleucine** in the anti-S IgG mAb material and uncertainties as received

Participant	**Isoleucine**			
	Mass fraction (mg/kg)	**Standard uncertainty (*u*) (mg/kg)**	**Coverage Factor (*k*)**	**Exp. *Unc*. (*U*) for approximately 95 % confidence interval (mg/kg)**
HSA	19.53	0.37	2	0.74
UME	15.14	0.32	2	0.65
NIM	16.02	0.56	2	1.12
NIM_Q-LIT V5	16.56	0.31	2	0.62
BAM	/	/	/	/
BIPM	13.2	2.0	2	4.0
LNE	15.3	0.61	2	1.2
INMETRO	17.64	0.25	2	0.49
NMIJ/AIST	16.07	0.21	2	0.43
LGC	15.7	1.3	2	2.6
PTB	15.38	0.31	2	0.62
NRC	17.9	0.4	2	0.8
HSA _corrected	15.05	0.29	2.0	0.57

Table 13: Results for CCQM-P216: mass fraction of **Proline** in the anti-S IgG mAb material and uncertainties as received

Participant	**Proline**			
	Mass fraction (mg/kg)	**Standard uncertainty (*u*) (mg/kg)**	**Coverage Factor (*k*)**	**Exp. *Unc*. (*U*) for approximately 95 % confidence interval (mg/kg)**
HSA	41.79	0.74	2.26	1.67
UME	30.07	0.57	2	1.14
NIM	/	/	/	/
NIM_Q-LIT V5	/	/	/	/
BAM	/	/	/	/
BIPM	29.1	1.2	2	2.4
LNE	26.5	1.2	2	2.3
INMETRO	/	/	/	/
NMIJ/AIST	31.23	0.41	2	0.82
LGC	32.7	1.1	2	2.1
PTB	29.90	0.44	2	0.89
NRC	34.2	0.8	2	1.6
HSA _corrected	30.93	0.54	2.26	1.23

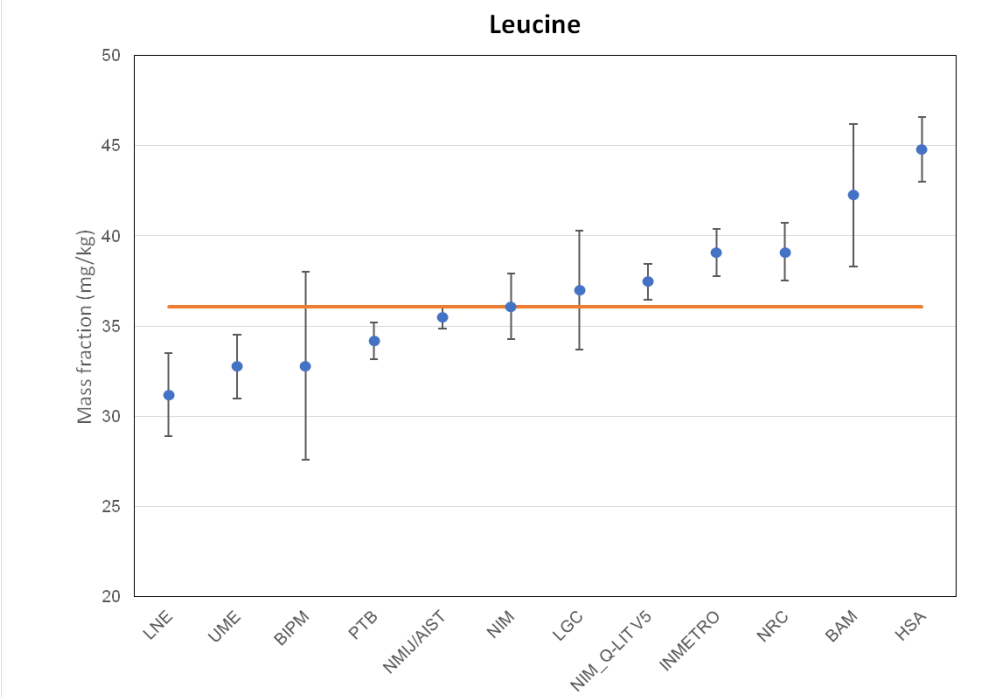

Figure 10: Mass fraction of Leucine in the anti-S IgG mAb material reported by participants in CCQM-P216 - sorted by increasing value plotted with expanded uncertainties (*U*) at a confidence level of approximately 95 %. The horizontal lines represent the median of Leucine.

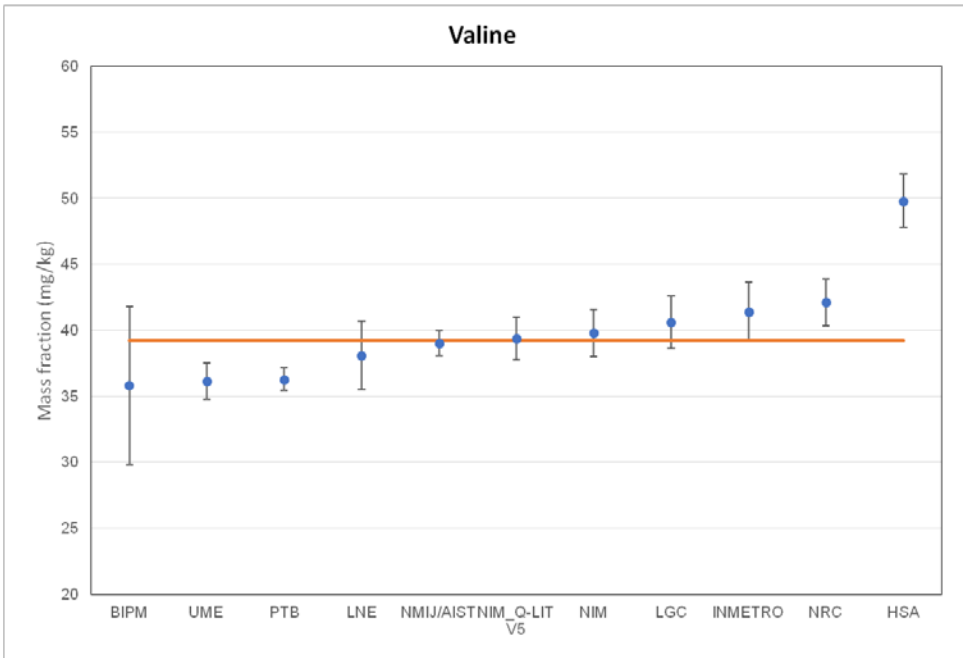

Figure 11: Mass fraction of Valine in the anti-S IgG mAb material reported by participants in CCQM-P216 - sorted by increasing value plotted with expanded uncertainties (*U*) at a confidence level of approximately 95 %. The horizontal lines represent the median of Valine.

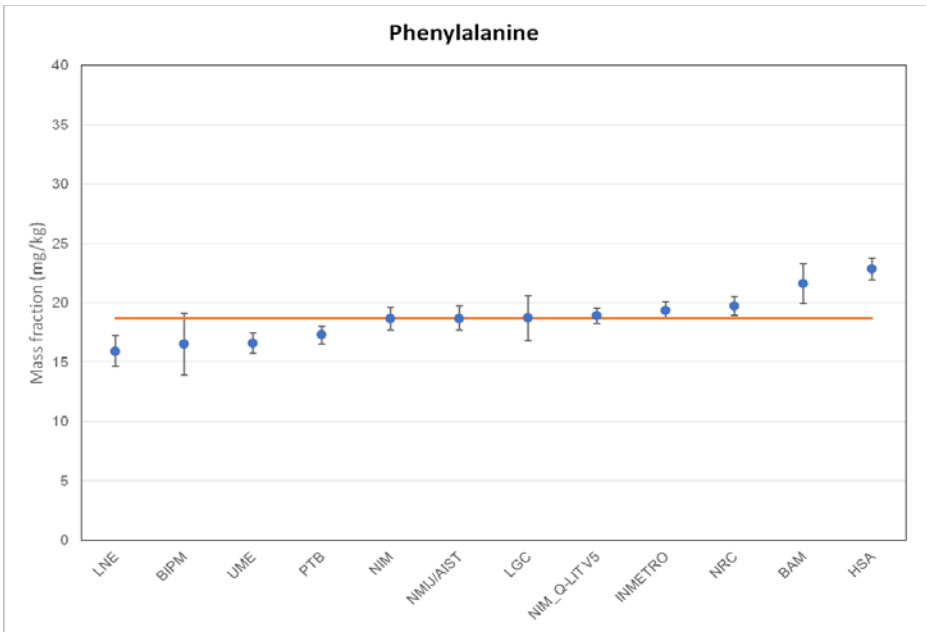

Figure 12: Mass fraction of Phenylalanine in the anti-S IgG mAb material reported by participants in CCQM-P216 - sorted by increasing value plotted with expanded uncertainties (*U*) at a confidence level of approximately 95 %. The horizontal lines represent the median of Phenylalanine.

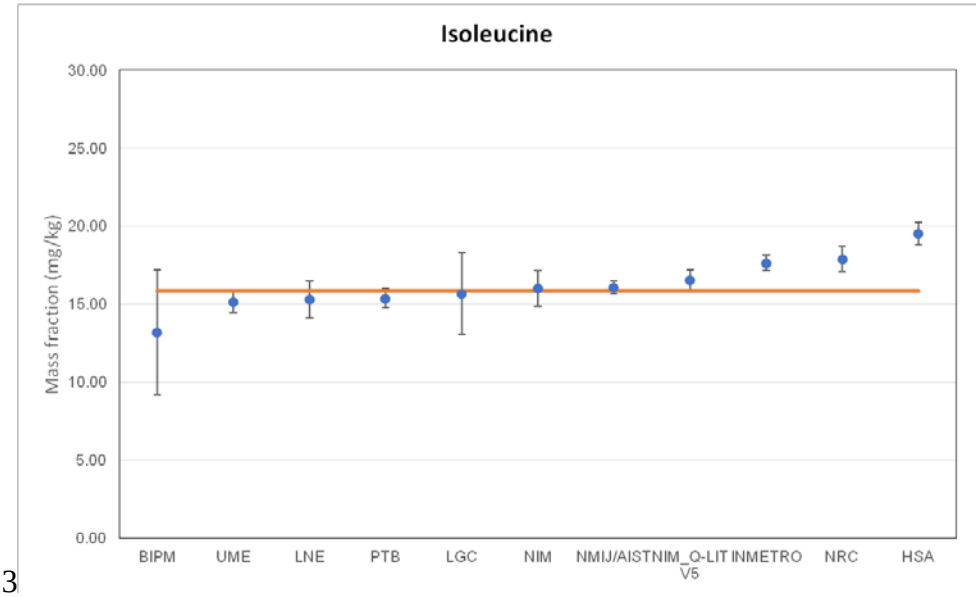

Figure 13: Mass fraction of Isoleucine in the anti-S IgG mAb material reported by participants in CCQM-P216 - sorted by increasing value plotted with expanded uncertainties (*U*) at a confidence level of approximately 95 %. The horizontal lines represent the median of Isoleucine.

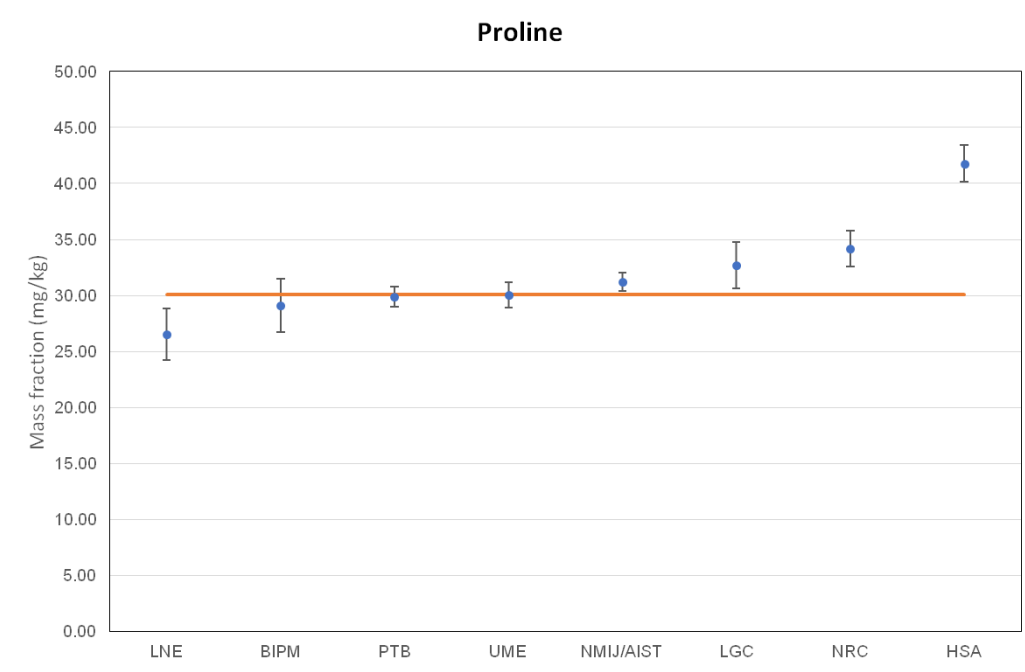

Figure 14: Mass fraction of Proline in the anti-S IgG mAb material reported by participants in CCQM-P216 - sorted by increasing value plotted with expanded uncertainties (*U*) at a confidence level of approximately 95 %. The horizontal lines represent the median of Proline.

Table 14: Results for CCQM-P216: mass fraction of peptide **ALPAPIEK** in the anti-S IgG mAb material and uncertainties as received

Participant	**ALPAPIEK**			
	Mass fraction (mg/kg)	**Standard uncertainty (*u*) (mg/kg)**	**Coverage Factor (*k*)**	**Exp. *Unc*. (*U*) for approximately 95 % confidence interval (mg/kg)**
HSA	5.54	0.29	2	0.58
UME	5.14	0.15	2	0.3
NIM	4.85	0.17	2	0.34
NIM_Q-LIT V5	4.92	0.14	2	0.28
BAM	5.743	0.332	1.99	0.659
BIPM	4.39	0.23	2	0.46
LNE	4.59	0.26	2	0.53
INMETRO	5.12	0.13	2	0.30
KRISS	/	/	/	/

Table 15: Results for CCQM-P216: mass fraction of peptide **GPSVFPLAPSSK** in the anti-S IgG mAb material and uncertainties as received

Participant	**GPSVFPLAPSSK**			
	Mass fraction (mg/kg)	**Standard uncertainty (*u*) (mg/kg)**	**Coverage Factor (*k*)**	**Exp. *Unc*. (*U*) for approximately 95 % confidence interval (mg/kg)**
HSA	/	/	/	/
UME	7.87	0.28	2	0.57
NIM	6.90	0.35	2	0.70
NIM_Q-LIT V5	7.02	0.21	2	0.42
BAM	/	/	/	/
BIPM	6.22	0.20	2	0.40
LNE	5.35	0.32	2	0.64
INMETRO	7.75	0.52	2	1.0
KRISS	3.19	0.0893	2.78	0.24

Table 16: Results for CCQM-P216: mass fraction of peptide **DSTYSLSSTLTLSK or STSGGTAALGCLVK** in the anti-S IgG mAb material and uncertainties as received

Participant	**DSTYSLSSTLTLSK**			
	Mass fraction (mg/kg)	**Standard uncertainty (*u*) (mg/kg)**	**Coverage Factor (*k*)**	**Exp. *Unc*. (*U*) for approximately 95 % confidence interval (mg/kg)**
UME	8.91	0.39	2	0.77
Participant	**STSGGTAALGCLVK**			
	Mass fraction (mg/kg)	**Standard uncertainty (*u*) (mg/kg)**	**Coverage Factor (*k*)**	**Exp. *Unc*. (*U*) for approximately 95 % confidence interval (mg/kg)**
LNE	4.26	0.22	2	0.44

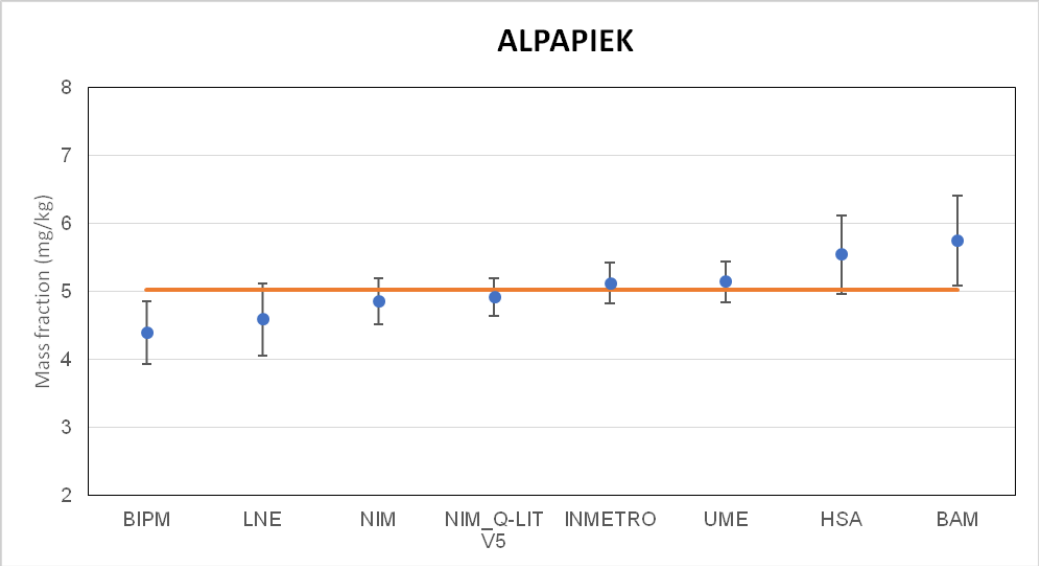

Figure 15: Mass fraction of proteotypic peptide **ALPAPIEK** in the anti-S IgG mAb material reported by participants in CCQM-P216 - sorted by increasing value plotted with expanded uncertainties (*U*) at a confidence level of approximately95 %. The horizontal lines represent the median of peptide **ALPAPIEK**.

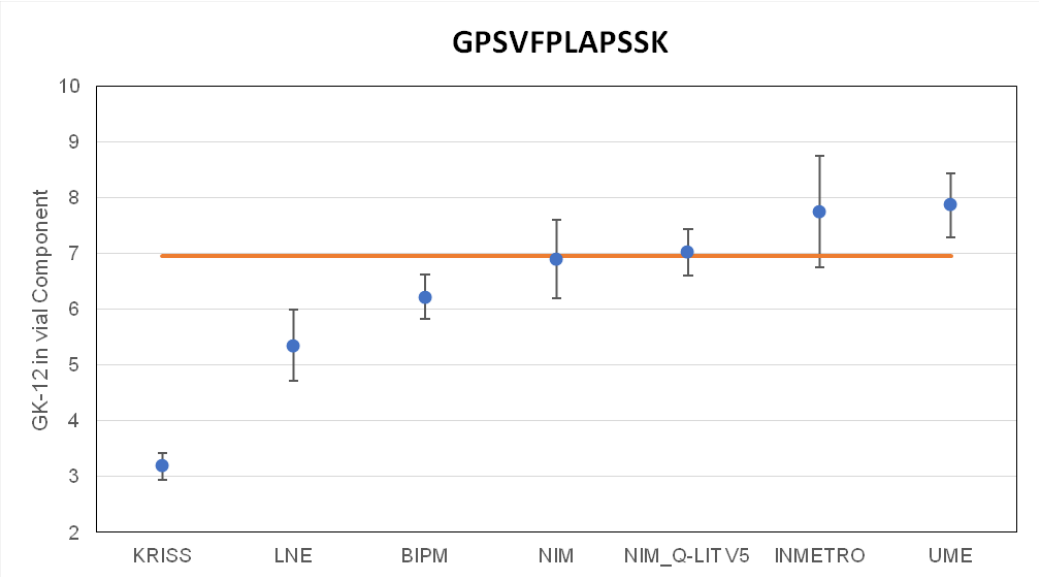

Figure 16: Mass fraction of proteotypic peptide **GPSVFPLAPSSK** in the anti-S IgG mAb material reported by participants in CCQM-P216 - sorted by increasing value plotted with expanded uncertainties (*U*) at a confidence level of approximately95 %. The horizontal lines represent the median of peptide **GPSVFPLAPSSK**.

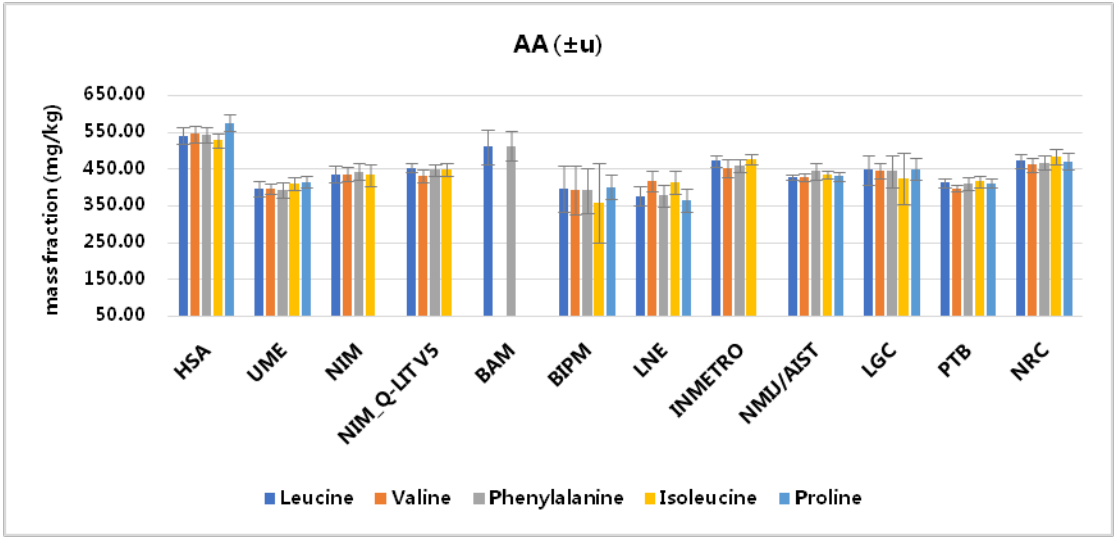

Figure 17: Mass fraction of anti-S IgG mAb - plotted with expanded uncertainties (*U*) at a confidence level of approximately 95 % and converted from the mass fraction of different AAs.

The submitted results in mass fraction of different AAs converted to mass fraction of anti-S IgG mAb using molecular weight of Leu,Val, Phe,Ile, and Pro with 131.17, 117.15, 165.19, 131.17, 115.13 g/mol, respectively. The molecular weight of anti-S IgG mAb is 149176.42 g/mol, and the purity of the material used as 0.9967.

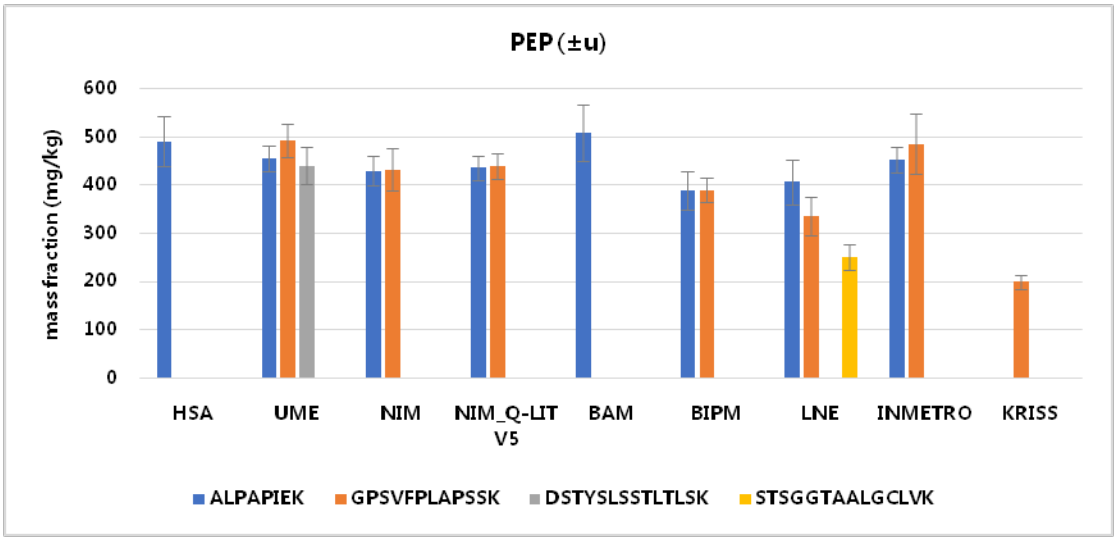

Figure 18: Mass fraction of anti-S IgG mAb - plotted with expanded uncertainties (*U*) at a confidence level of approximately 95 % and converted from the mass fraction of different PEPs.

The submitted results in mass fraction of different PEPs converted to mass fraction of anti-S IgG mAb using molecular weight of ALPAPIEK, GPSVFPLAPSSK, DSTYSLSSTLTLSK, STSGGTAALGCLVK with 838, 1186.35, 1502.61, 1264.44 g/mol, respectively. The molecular weight of anti-S IgG mAb is 149176.42 g/mol, and the purity of the material used as 0.9967.

The reference values (RV) and the differences from reference value (D)

An initial discussion of the results proceeded at the PAWG meetings in April and May 2021. In general, good agreement of mass fraction values submitted by the NMIs/DIs was obtained for both amino acid analyses and peptide analyses. It became apparent that HSA reported significantly higher mass fraction values for target amino acids analysis of all five amino acids Leu, Val, Phe, Ile and Pro compared to the results submitted by all other NMIs/DIs. HSA agreed to withdraw their results from reference value calculations due the apparent issues with the amino acids analysis. HSA investigated further on their amino acid analyses as it is described in more detail in the following chapter. KRISS reported a significant lower mass fraction value for target peptide GPSVFPLAPSSK analysis compared with the results submitted by the other participants. KRISS agreed to withdraw their result from reference value calculations for target peptide GPSVFPLAPSSK analysis due some unknown technical issues.
The statistical model used in the analysis was hierarchical random effects model (normal-normal). The model was fit to data using Markov-chain Monte Carlo sampling in R using rjagsapp ”https://metrology.shinyapps.io/consensus-calculator/”.
All uncertainty bars in the above plots correspond to approximately 95 % confidence.

The reference value (RV) for the mass fraction of **Leucine** was calculated to be 36.10 mg/kg (u = 0.78 mg/kg) with an expanded uncertainty ($U_{95\,\%}$) of 1.55 mg/kg based on 11 laboratory results marked with black. The dark uncertainty contribution (u_t) is 2.15 mg/kg (u^2_t = 5.52). The singles results, reference value (RV) and differences from reference value (D) for the mass fraction of Leucine are presented in Figure 19. The difference from reference value (D) for mass fraction of Leucine is listed in Table 17.

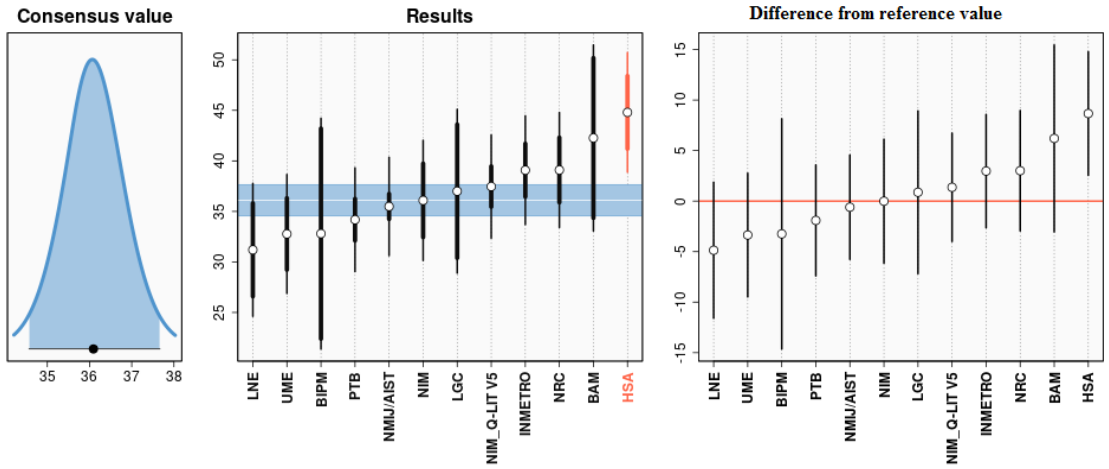

Figure 19: The reference value (RV) and difference from reference value (D) for the mass fraction of **Leucine**

Table 17: Difference from reference value (D) for mass fraction of **Leucine**

Laboratory	D	Uncertainty	U95
LNE	-4.90	3.38	6.73
UME	-3.33	3.05	6.12
BIPM	-3.30	5.79	11.39
PTB	-1.92	2.70	5.49
NMIJ/AIST	-0.61	2.55	5.16
NIM	-0.01	3.07	6.13
LGC	0.90	4.14	8.05
NIM_Q-LIT V5	1.36	2.65	5.36
INMETRO	2.98	2.80	5.60
NRC	3.00	2.95	5.95
BAM	6.17	4.68	9.25
HSA	8.70	3.07	6.11

The reference value (RV) for the mass fraction of **Valine** was calculated to be 38.75 mg/kg (u = 0.75 mg/kg) with an expanded uncertainty ($U_{95\,\%}$) of 1.47 mg/kg based on 10 laboratory results marked with black. The dark uncertainty contribution (u_t) is1.60 mg/kg (u^2_t = 3.22). The singles results, reference value (RV) and differences from reference value (D) for the mass fraction of **Valine** are presented in Figure 20. The difference from reference value (D) for mass fraction of **Valine** is listed in Table 18.

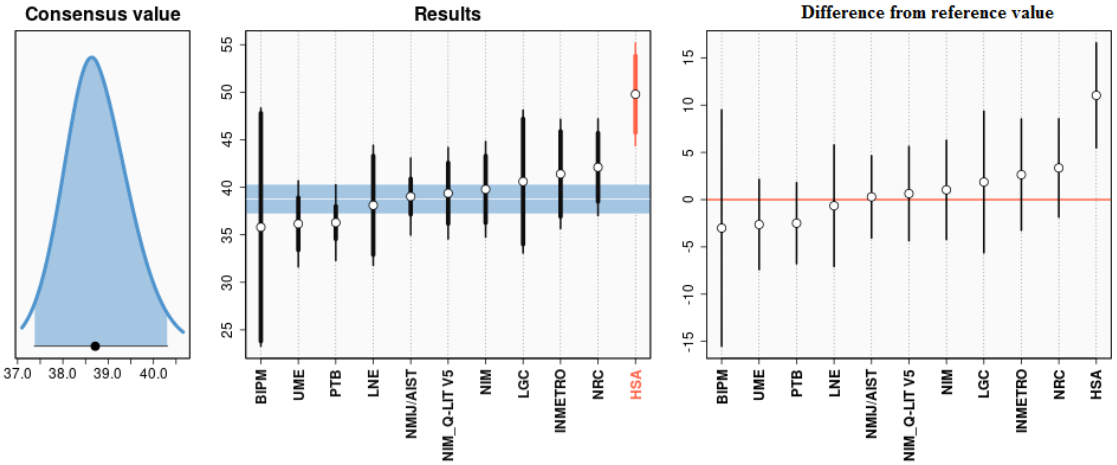

Figure 20: The reference value (RV) and difference from reference value (D) for the mass fraction of **Valine**

Table 18: Difference from reference value (D) for mass fraction of **Valine**

Laboratory	D	Uncertainty	U95
BIPM	-2.95	6.30	12.50
UME	-2.60	2.39	4.77
PTB	-2.47	2.11	4.28
LNE	-0.65	3.25	6.42
NMIJ/AIST	0.27	2.17	4.35
NIM_Q-LIT V5	0.62	2.53	4.98
NIM	1.03	2.65	5.25
LGC	1.85	3.84	7.49
INMETRO	2.65	2.98	5.89
NRC	3.35	2.63	5.20
HSA	11.03	2.81	5.55

The reference value (RV) for the mass fraction of **Phenylalanine** was calculated to be 18.46 mg/kg (u = 0.40 mg/kg) with an expanded uncertainty ($U_{95\,\%}$) of 0.78 mg/kg based on 11 laboratory results marked with black. The dark uncertainty contribution (u_t) is 0.92 mg/kg (u^2_t = 1.09). The singles results, reference value (RV) and differences from reference value (D) for the mass fraction of **Phenylalanine** are presented in Figure 21. The difference from reference value (D) for mass fraction of **Phenylalanine** is listed in Table 19.

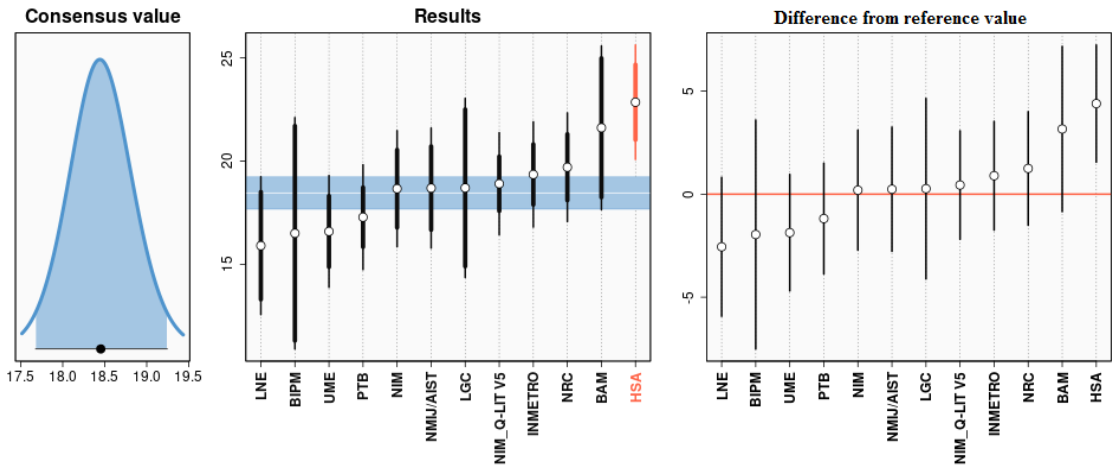

Figure 21: The reference value (RV) and difference from reference value (D) for the mass fraction of **Phenylalanine**

Table 19: Difference from reference value (D) for mass fraction of **Phenylalanine**

Laboratory	D	Uncertainty	U95
LNE	-2.56	1.71	3.37
BIPM	-1.96	2.83	5.55
UME	-1.87	1.41	2.83
PTB	-1.18	1.34	2.69
NIM	0.20	1.46	2.91
NMIJ/AIST	0.23	1.52	3.01
LGC	0.24	2.22	4.38
NIM_Q-LIT V5	0.44	1.31	2.63
INMETRO	0.89	1.32	2.64
NRC	1.24	1.37	2.76
BAM	3.16	2.02	4.01
HSA	4.39	1.43	2.84

The reference value (RV) for the mass fraction of **Isoleucine** was calculated to be 16.20 mg/kg (u = 0.34 mg/kg) with an expanded uncertainty ($U_{95\,\%}$) of 0.67 mg/kg based on 10 laboratory results marked with black. The dark uncertainty contribution (u_t) is 0.79 mg/kg (u^2_t = 0.77). The singles results, reference value (RV) and differences from reference value (D) for the mass fraction of **Isoleucine** are presented in Figure 22. The difference from reference value (D) for mass fraction of **Isoleucine** is listed in Table 20.

4.The reference value and difference from reference value (D)for mass fraction of **Isoleucine**

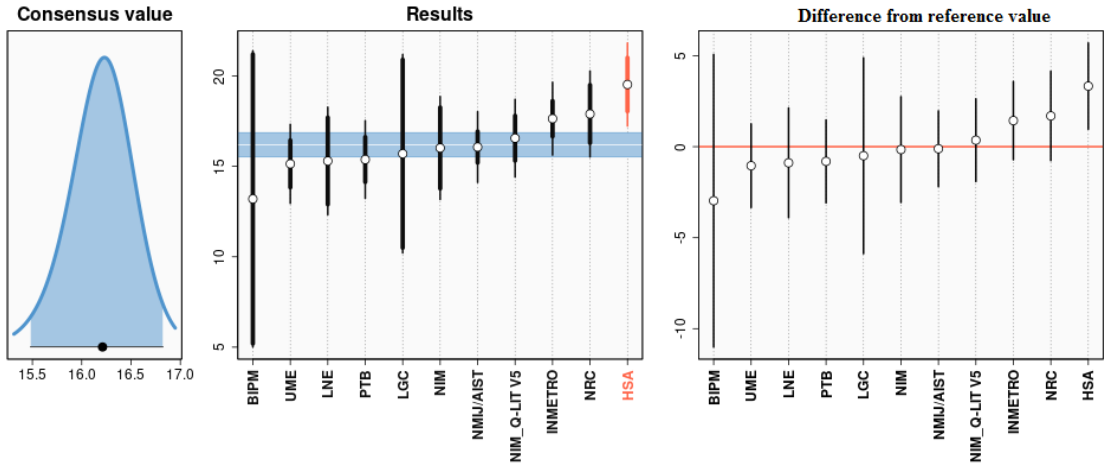

Figure 22: The reference value (RV) and difference from reference value (D) for the mass fraction of **Isoleucine**

Table 20: Difference from reference value (D) for mass fraction of **Isoleucine**

Laboratory	D	Uncertainty	U95
BIPM	-3.00	4.13	8.04
UME	-1.06	1.15	2.30
LNE	-0.90	1.53	3.01
PTB	-0.82	1.14	2.28
LGC	-0.50	2.77	5.38
NIM	-0.18	1.46	2.90
NMIJ/AIST	-0.13	1.03	2.08
NIM_Q-LIT V5	0.36	1.13	2.27
INMETRO	1.44	1.06	2.15
NRC	1.70	1.24	2.45
HSA	3.30	1.20	2.37

The reference value (RV) for the mass fraction of **Proline** was calculated to be 30.61 mg/kg (u = 0.66 mg/kg) with an expanded uncertainty ($U_{95\,\%}$) of 1.30 mg/kg based on 7 laboratory results marked with black. The dark uncertainty contribution (u_t) is 1.10 mg/kg (u^2_t = 1.94). The singles results, reference value (RV) and differences from reference value (D) for the mass fraction of **Proline** are presented in Figure 23. The difference from reference value (D) for mass fraction of **Proline** is listed in Table 21.

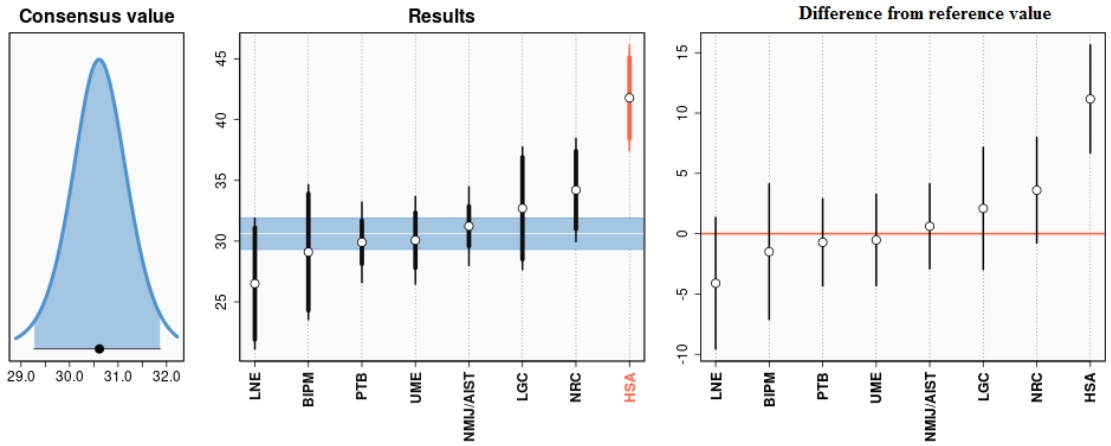

Figure 23: The reference value (RV) and difference from reference value (D) for the mass fraction of **Proline**

Table 21: Difference from reference value (D) for mass fraction of **Proline**

Laboratory	D	Uncertainty	U95
LNE	-4.11	2.77	5.45
BIPM	-1.51	2.85	5.61
PTB	-0.71	1.76	3.61
UME	-0.54	1.89	3.78
NMIJ/AIST	0.62	1.74	3.52
LGC	2.09	2.58	5.06
NRC	3.59	2.22	4.37
HSA	11.18	2.28	4.47

The reference value (RV) for the mass fraction of **ALPAPIEK peptide** was calculated to be 4.99 mg/kg (u = 0.14 mg/kg) with an expanded uncertainty ($U_{95\ \%}$) of 0.28 mg/kg based on 8 laboratory results marked with black. The dark uncertainty contribution (u_t) is 0.16 mg/kg (u^2_t = 0.05). The singles results, reference value (RV) and differences from reference value (D) for the mass fraction of **ALPAPIEK peptide** are presented in Figure 24. The difference from reference value (D) for mass fraction of **ALPAPIEK peptide** is listed in Table 22.

ALPAPIEK

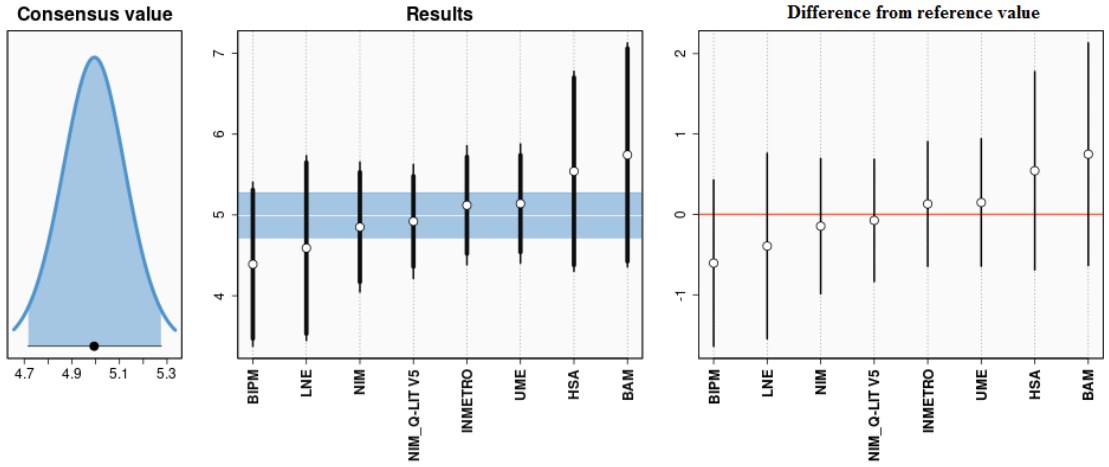

Figure 24: The reference value (RV) and difference from reference value (D) for the mass fraction of **ALPAPIEK peptide**

Table 22: Difference from reference value (D) for mass fraction of **ALPAPIEK peptide**

Laboratory	D	Uncertainty	U95
BIPM	-0.60	0.53	1.03
LNE	-0.40	0.59	1.15
NIM	-0.14	0.43	0.84
NIM_Q-LIT V5	-0.07	0.38	0.76
INMETRO	0.13	0.40	0.78
UME	0.15	0.40	0.79
HSA	0.55	0.63	1.23
BAM	0.75	0.71	1.39

The reference value (RV) for the mass fraction of **GPSVFPLAPSSK peptide** was calculated to be 6.83 mg/kg (u = 0.32 mg/kg) with an expanded uncertainty ($U_{95\ \%}$) of 0.65 mg/kg based on 7 laboratory results marked with black. The dark uncertainty contribution (u_t) is 0.70 mg/kg (u^2_t = 0.67). The singles results, reference value (RV) and differences from reference value (D) for the mass fraction of **GPSVFPLAPSSK peptide** are presented in Figure 25. The difference from reference value (D) for mass fraction of **GPSVFPLAPSSK peptide** is listed in Table 23.

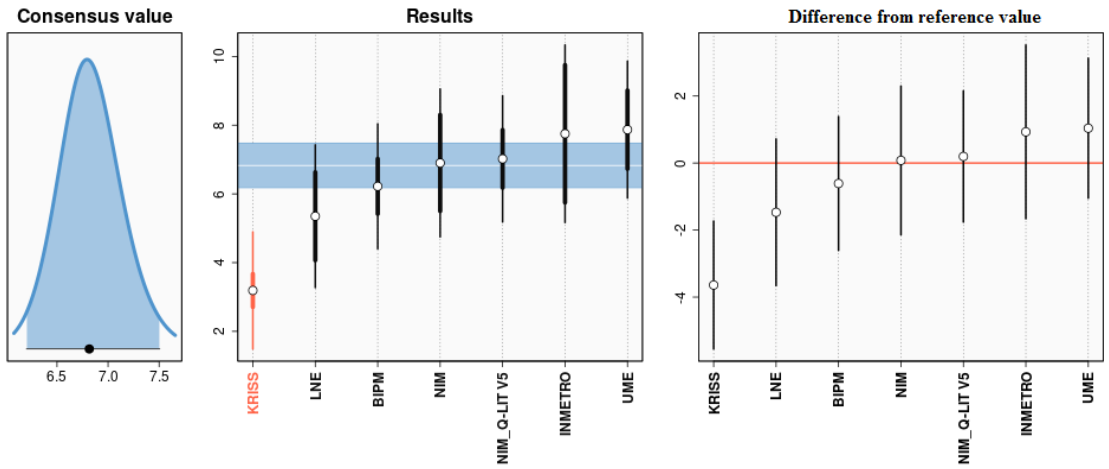

Figure 25: The reference value (RV) and difference from reference value (D) for the mass fraction of **GPSVFPLAPSSK peptide**

Table 23: Difference from reference value (D) for mass fraction of **GPSVFPLAPSSK peptide**

Laboratory	D	Uncertainty	U95
KRISS	-3.64	0.92	1.91
LNE	-1.48	1.09	2.19
BIPM	-0.61	0.97	2.00
NIM	0.07	1.12	2.22
NIM_Q-LIT V5	0.19	0.97	1.96
INMETRO	0.92	1.32	2.59
UME	1.04	1.05	2.09

ADDITIONAL EXPERIMENTS

During the course of the data evaluation HSA investigated and confirmed a technical issue with their working calibration solution for the amino acid analysis. HSA repeated the amino acid analysis with a freshly prepared working calibration solution and provided revised/corrected results for information. The revised data of HSA is listed in Table 9-13 and named as HSA_corrected. Figure 26-30 are depicting all results of CCQM-P216 including the corrected results from HSA. It should be noted both HSA results (original and corrected) have not been used for the reference value calculations.

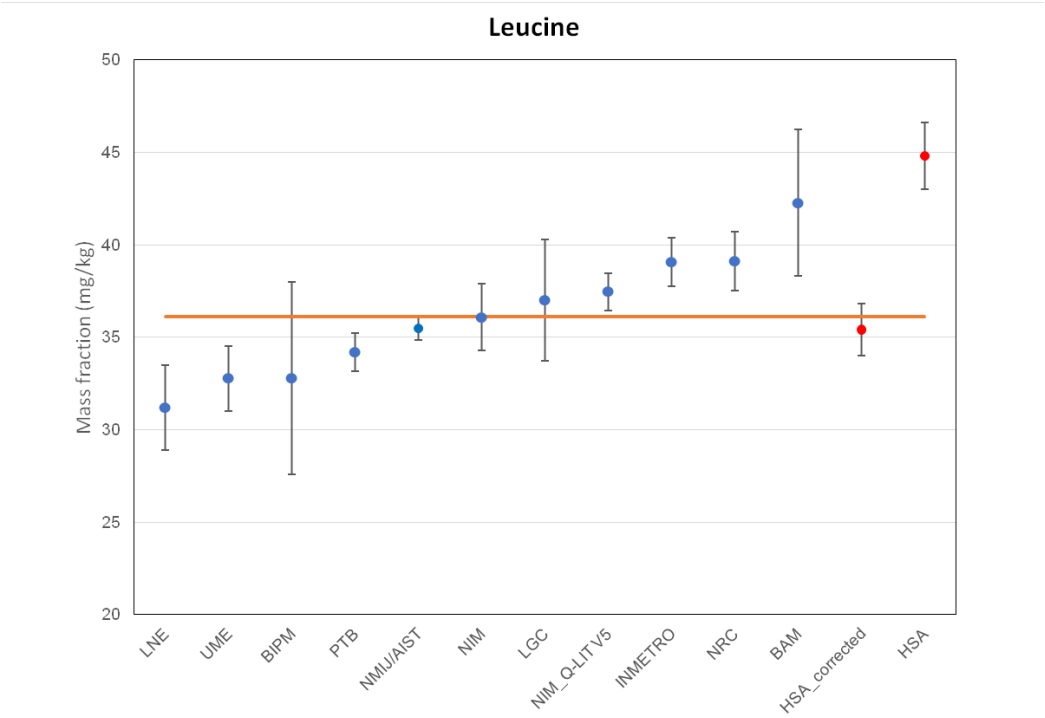

Figure 26: Mass fraction of Leucine in the anti-S IgG mAb material reported by participants in CCQM-P216 with expanded uncertainties (*U*) at a confidence level of approximately 95 %.

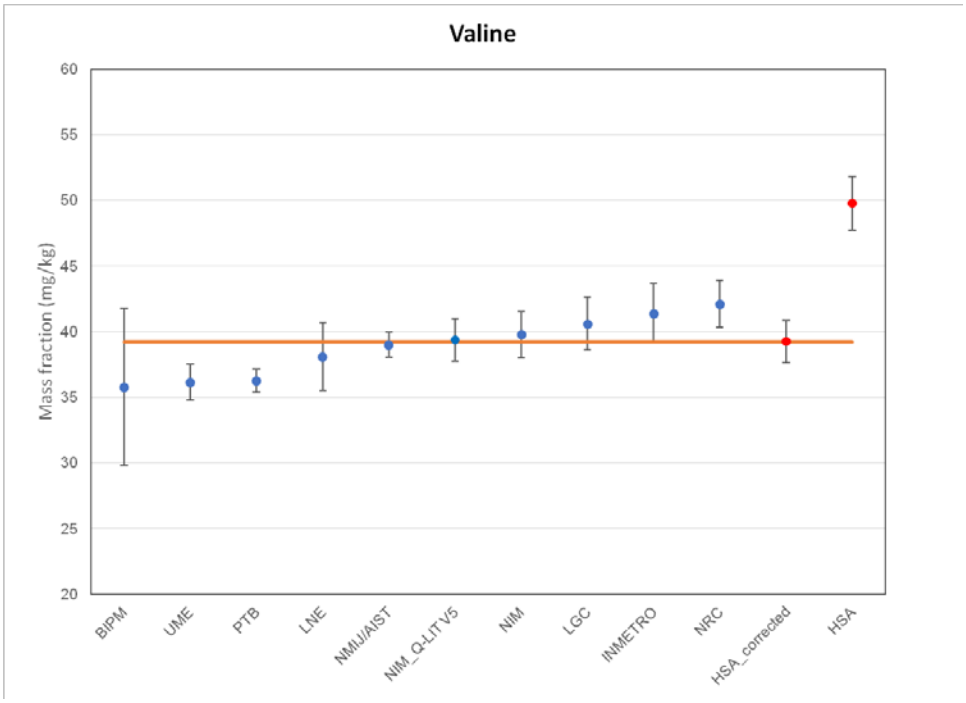

Figure 27: Mass fraction of Valine in the anti-S IgG mAb material reported by participants in CCQM-P216 with expanded uncertainties (*U*) at a confidence level of approximately 95 %.

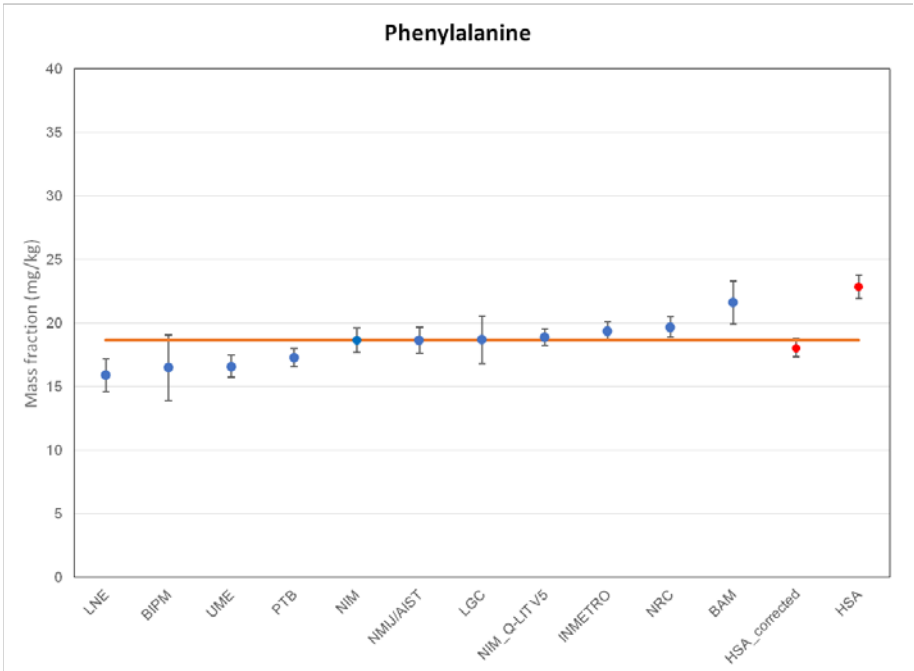

Figure 28: Mass fraction of Phenylanaline in the anti-S IgG mAb material reported by participants in CCQM-P216 with expanded uncertainties (*U*) at a confidence level of approximately 95 %.

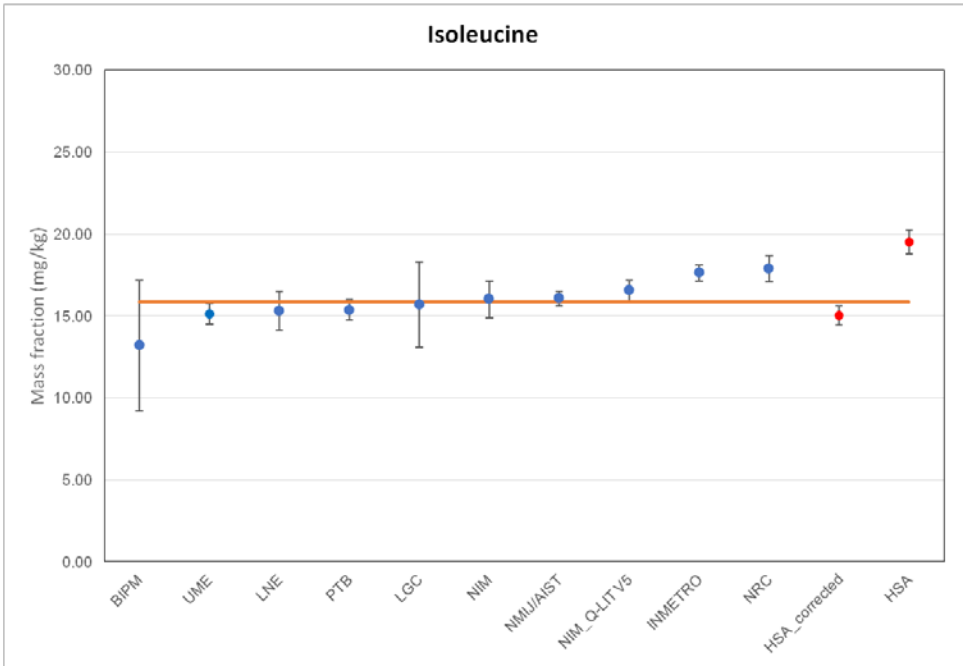

Figure 29: Mass fraction of Isoleucine in the anti-S IgG mAb material reported by participants in CCQM-P216 with expanded uncertainties (*U*) at a confidence level of approximately 95 %.

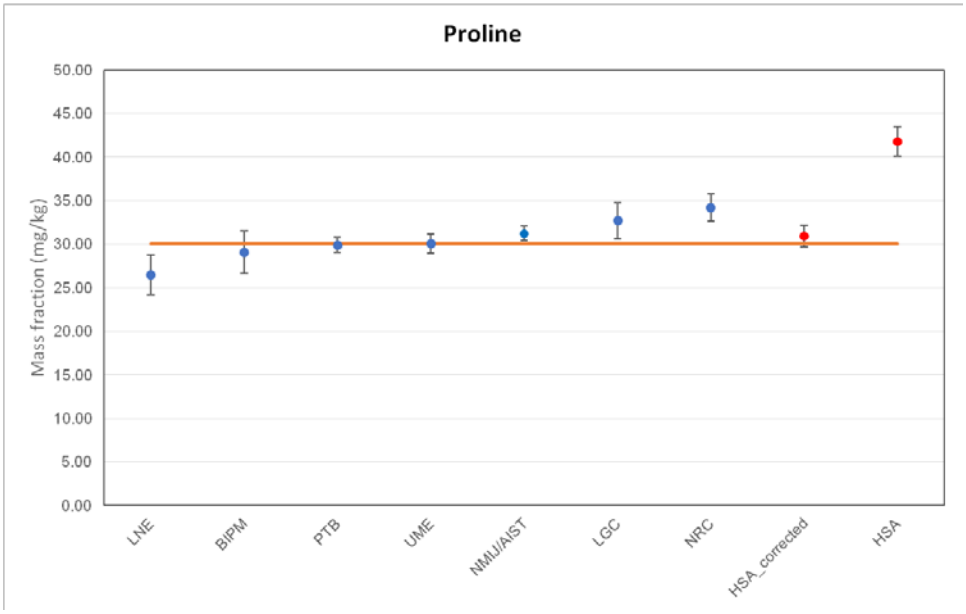

Figure 30: Mass fraction of Proline in the anti-S IgG mAb material reported by participants in CCQM-P216 with expanded uncertainties (*U*) at a confidence level of approximately 95 %.

CONCLUSIONS

The purpose of this pilot study was to develop measurement capabilities for larger proteins within the National Metrology Institute community, using a recombinant humanized IgG monoclonal antibody against Spike glycoprotein of SARS-CoV-2 (Anti-S IgG mAb) in solution. The first phase of the study was designed to employ established methods that had been previously studies by the CCQM Protein Analysis Working Group, involving the digestion of protein down to the peptide or amino acid level.

Agreement between nearly all laboratories was achieved for the amino acid analysis within 2 to 2.5 %, with one participant achieving markedly higher results due to a technical issue found in their procedure; this result was thus excluded from the reference value. The relatively good agreement within a laboratory between different amino acids was not dissimilar to previous results for peptides or small proteins, indicating that factors such as hydrolysis conditions and calibration procedures could be the largest sources of variability.

Not surprisingly due to prior knowledge from previous studies on peptide quantitation, agreement between laboratories for the peptide-based analysis was slightly poorer at 3 to 5 %, with one laboratory's result excluded for the peptide GPSVFPLAPSSK. Again, this level of agreement was not significantly poorer than that achieved in previous studies with smaller or less complex proteins.

ACKNOWLEDGEMENTS

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