CCQM-K115.c

Key Comparison Study on Peptide Purity - Glycated Hexapeptide of HbA1c

Final Report

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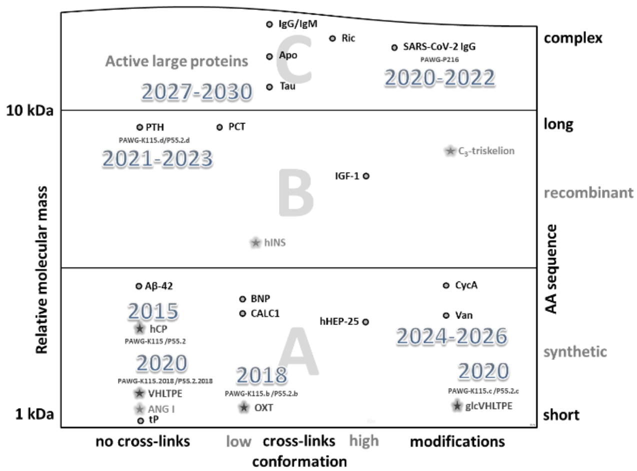
INTRODUCTION

Comparability of (bio)chemical measurements is a prerequisite of any measurement undertaken in support of legislative purposes. For most chemical analysis this can be achieved by ensuring that measurement results are traceable to a known reference such as the base units of the Système International d'Unités (SI) [1]. By maintaining such a link, results can be compared over time and space enabling informed decisions to be made and improving our overall knowledge of a subject area. The importance of traceable measurement results can be inferred by its requirement in quality standards (ISO 17025) and in the formation of specialized committees as the Joint Committee on Traceability in Laboratory Medicine (JCTLM). However, whilst the required metrological tools, such as higher order reference measurements procedures, pure substance and matrix certified reference materials, are established for small well defined molecules difficulties still remain in the provision of such standards in the area of larger biomolecules notably peptides/proteins.

The provision of Primary Calibration Reference Services has been identified as a core technical competency for National Metrology Institutes (NMIs) and Designated Institutes (DIs) [2]. NMIs/DIs providing measurement services in peptide/protein analysis are expected to participate in a limited number of comparisons that are intended to test and demonstrate their capabilities in this area.

Primary Calibration Reference Services refers to a technical capability for composition assignment, usually as the mass fraction content, of a peptide/protein in the form of high purity solids or standard solutions thereof.

The assignment of the mass fraction content of high purity materials is the subject of the CCQM-K115 comparison series. A model to classify peptides in terms of their, relative molecular mass, the amount of cross-linking, and modifications has been developed and upgraded as it is depicted in Figure 1 [1,3]. With the aim of leveraging the work required for the CCQM-K115 comparison and thereby minimising the workload for NMIs/DIs and simultaneously focussing on a material directly relevant to existing CMC claims, human C-peptide (hCP) was the most appropriate choice for a study material for a first CCQM key comparison and parallel pilot study looking at competencies to perform peptide purity mass fraction assignment. hCP covers the space of quadrant A of the model as it allowed generic capabilities to be demonstrated for linear peptides without cross-links and of up to 31 amino acids in length [4,5]. The second cycle of peptide purity comparisons, CCQM-K115.b/P55.2.b on oxytocin (OXT) covered the space of quadrant A for short (1 kDa to 5 kDa), cross-linked and non-modified synthetic peptides as OXT is a cyclic peptide possessing nine amino acid residues and a disulfide bond. OXT is a chemically synthesized peptide hormone [6,7].



Peptide	AAs	kDa	SSs
Hexapeptide of HbA0 (VHLTPE or VE)	6	~0.7	0
Glycated hexapeptide of HbA1c (glcVHLTPE or GE)	6	~0.8	0
Tryptic Peptide Solution (tP)	~10	~1	0
Oxytocin (OXT)	9	1	1
Angiotensin I (ANG I)	10	1.2	0
Human Hepcidin-25 (hHEP-25)	25	2.8	4
Human C-Peptide (hCP)	31	3	0
Calcitonin Salmon (CALC1)	32	3.4	1
Brain Natriuretic Peptide (BNP)	32	3.5	1
Amyloid-Beta-42 (Aβ-42)	42	4.5	0
Human Insulin (hINS)	51	5.8	3
Insulin-like growth factor 1 (IGF-1)	70	7.6	3
C_3-triskelion (antimicrobial peptide capsid)	74	8.3	0
Parathyroid hormone (PTH)	84	9.4	0

Figure 1: Model for the classification of peptides for primary structure purity determinations

RATIONALE/PURPOSE

The approach taken for small molecules relies on Primary Calibrators, often in the form of a synthetic standard of known purity. The provision of Primary Calibration Reference Services has been identified as a core technical competency for NMIs/DIs in the strategy developed for the planning of ongoing Key Comparisons of the Organic Analysis Working Group (OAWG) within the Comité Consultatif pour la Quantité de Matière (CCQM) [8]. NMIs/DIs providing measurement services in organic analysis are expected to participate in a limited number of Track A comparisons that are intended to test and demonstrate their capabilities in this area. Primary Calibration Reference Services refers to a technical capability for composition assignment, usually as the mass fraction content, of organic compound(s) such as pure substances or solutions. The procedure adopted by most NMIs/DIs, for the provision of primary pure substance calibrators relies on a mass balance approach. This can be determined either by approaches that measure the mass fraction or mole fraction of the main component directly, or by indirect approaches that identify and estimate the mass fraction of the individual impurities and/or distinct classes of impurities present in the material and, by subtraction, provide a measure for the main component of the material [9]. These approaches have been successfully applied to a large variety of small molecules [10-14].
The quantification of larger molecules is complicated by the fact that they can exhibit higher order structures, and that characterization of the primary structure of the molecule maybe insufficient to correlate the amount of the molecule to its biological activity. Nevertheless, the quantification of the primary structure purity of a larger molecule is the first step in establishing a primary calibrator material for that molecule, where the quantity of interest is the mass fraction of the large molecule. The current discussion is limited to the measurement of the primary structure mass fraction of the molecule within a material.

Another complication for the provision of traceable peptide/protein measurements is that pure peptides/proteins can usually not be obtained in sufficiently large quantities. This has resulted in the harmonisation of many large molecule measurements by the provision of accepted practices, methods and/or standards. However, the increased use of targeted hydrolysis based digestion and peptide quantification strategies has enabled the determination of protein amounts via prototypic peptides [15-17]. These approaches have been investigated for example for the routine analysis of human growth hormone and its biomarkers [18-19]. A number of NMIs/DIs have been developing higher order measurement procedures for the analysis of purified protein calibrators [20] and serum based matrix materials [19]. These approaches show great promise for the standardisation of priority protein measurands. However, the mass fractions value assignment of proteins requires proteotypic peptides of known purity [1].

The purity of proteotypic peptides and peptides that show direct bioactivity by themselves can be assessed by use of the full mass balance approach. However, a full mass balance approach could require unviably large quantities of peptide material. A simpler alternative to the full mass balance approach is a peptide impurity corrected amino acid (PICAA) analysis, requiring quantification of constituent amino acids following hydrolysis of the material and correction for amino acids originating from impurities [4-7, 21-22]. It requires identification and quantification of peptide impurities for the most accurate results.

Traceability of the amino acid analysis results is to pure amino acid certified reference materials (CRMs). Few pure amino acid CRMs are commercially available. Alternatively, traceability could be established through in-house or NMI purity capabilities for amino acids. NMI capabilities to determine the purity of L-valine, were assessed in the CCQM-K55.c comparison in the frame of the OAWG [12]. In addition, amino acid analysis and peptide hydrolysis capabilities for the mass concentration assignment of peptide solutions are evaluated in the series of CCQM-P55 comparisons in the framework of the former BAWG using peptide materials of unknown purity [1].

The application of other approaches for the assessment of peptide purity that require only minor quantities of peptide material is conceivable, for example elemental analysis (CHN/O) with a correction for nitrogen originating from impurities or quantitative nuclear magnetic resonance (qNMR) spectroscopy with a correction for structurally-related peptide impurities (PICqNMR) [1, 4, 23].

The timeline for the CCQM-K115.c study ‘Key Comparison Study on Peptide Purity - Glycated Hexapeptide of HbA1c’ is summarized in Table 1.

Table 1: CCQM-K115.c Timetable

Action	Date
Initial discussion	October 2016 and April 2017 PAWG meetings
Approval of Study Proposal	September 2017 PAWG meeting
Draft protocol and confirmation	April 2018 PAWG meeting
Sample characterization completed	January 2019
Call for participation	April 1st, 2019
Final date to register	April 30th, 2019
Sample distribution	June to July 2019
Date due to coordinator	September 18th, 2020
Justification for 14 months period	Shifted several times because of the coronavirus pandemic
Initial report and discussion of results	November 2020 PAWG meeting
Discussion and reference value established	April 2021 PAWG meeting
Draft B report	March 2022 approved by PAWG
Final report to PAWG Chair	April 2022

CHARACTERIZATION OF STUDY MATERIAL

The mass fraction of the glycated hexapeptide of HbA1c (GE) in the material is to be determined. GE is defined as glycated hemoglobin subunit beta [2-7] fragment with the amino acid sequence glcVHLTPE and a relative molecular mass (M_r) of about 856.6 g/mol. The N-terminus valine of the hemoglobin β-chain has been converted to a stable adduct of glucose (1-deoxyfructosyl).
The study material was prepared by the BIPM/HSA by characterization of a commercially sourced sample of synthetic GE. The methods used to investigate, assign and confirm the quantitative composition of the CCQM-K115.c candidate material by the BIPM are summarized below.

CHARACTERIZATION STUDIES

Peptide related impurity content was evaluated by
- LC-hrMS/MS

Water content was evaluated by
- Coulometric Karl Fischer titration (KFT) with oven transfer of water from the sample
- Thermogravimetric analysis (TGA) as a consistency check for the assigned value
- Microanalysis (% C, H, N content) as a consistency check for assigned value
- Sorption balance measurements

Residual solvent content was evaluated by
- GC-MS by direct injection
- ^{1}H-NMR
- Thermogravimetric analysis as a consistency check for the assigned value
- Microanalysis (% C, H, N content) as a consistency check for the assigned value

Non-volatile organic/ inorganic content by
- ^{19}F-NMR
- IC for common elements and counter ions (acetate, chloride, formate, nitrate, oxalate, phosphate, sulfate, trifluoroacetate (TFA), ammonium, calcium, magnesium, potassium, sodium) as a consistency check for the assigned values
- Microanalysis (% C, H, N content) as a consistency check for the assigned values

The BIPM/HSA have
- investigated the levels of within and between vial homogeneity of the main component and selected significant minor components;
- identified a minimum sample size which reduces to an acceptable level the effect of between-bottle inhomogeneity of both the main component and the minor components;
- completed isochronous stability studies of both the main component and the minor components to confirm that the material is sufficiently stable within the proposed time scale of the study if stored at low temperature (4 °C to -20 °C);
- determined appropriate conditions for its storage (4 °C to -20 °C), transport (cooled and temperature controlled) and handling;
- studied the impact of the relative humidity and temperature on the water content and provide a correction function for the gravimetric preparation of the comparison sample.

HOMOGENEITY STUDIES

The BIPM/HSA have investigated the levels of within and between vial homogeneity of the main component and selected significant minor components, and have identified a minimum sample size which reduces to an acceptable level the effect of between bottle inhomogeneity of both the main component and the minor components [24].

The results of the ANOVA are summarised in Table 2. No differences in the within- and between-sample variances could be detected by the F-tests at the 95 % confidence level. The material could be regarded as homogeneous. For GE, VE, GE dimer and $(Glc)_2VE$, the s_{bb} could not be calculated due to the fact that for all $MS_{between}$ was smaller than MS_{within}. The u^*_{bb} of 1.62 %, 1.04 %, 2.87 %, and 1.60 % was adopted as an estimate for the uncertainty contribution due to potential inhomogeneity for GE, VE, GE dimer and $(Glc)_2VE$, respectively. VE, GE dimer and $(Glc)_2VE$ represent high (about 34 mg/g), medium (about 2.0 mg/g) and low (about 1.2 mg/g) mass fractions level impurities, respectively.

Table 2: Homogeneity results of representative GE and selected GE impurities

	GE	VE High level	GE dimer Medium level	$(Glc)_2VE$ Low level
N	29	29	29	29
s_{wb} (%)	5.01	3.21	8.84	4.94
s_{bb} (%)	-[(1)]	-[(1)]	-[(1)]	-[(1)]
u^*_{bb} (%)	1.62	1.04	2.87	1.60
$\boldsymbol{u_{bb}}$[(2)] (%)	**1.62**	**1.04**	**2.87**	**1.60**
F	0.810	0.515	0.834	0.324
F_{crit}	2.393	2.393	2.393	2.393

(1) Not calculable because $MS_{between}$ < MS_{within}

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

Linear regression functions were calculated for the results according to analysis order. The slopes of the lines were tested for significance on a 95 % confidence level to check for significant trends. No significant trend was observed for the injection sequences. The normalized result due to the analysis and filling sequences are presented in the Figures 2-5. The first, second and third replicates are represented by circles, grey filled circles and dots respectively.

The homogeneity of the pure K115.c GE candidate material was studied using an LC-UV-hrMS method for the quantitative determination of GE, VE, GE dimer and $(Glc)_2VE$. Acceptable uncertainties due to inhomogeneity were obtained for the pure GE material by use of the LC-hrMS method under repeatability conditions applying mass spectrometric detection for the main component and inherent related impurities. Absolute uncertainties due to between unit inhomogeneity of 0.35 mg/g (1.04 %), 0.057 mg/g (2.87 %) and 0.019 mg/g (1.60 %) could be assigned to the inherent impurities of VE, GE dimer and $(Glc)_2VE$, respectively. In addition, an uncertainty contribution due to between unit inhomogeneity (u_{bb}) of 10.2 mg/g (1.62 %) for the GE content was verified by use of UV detection. Therefore, this candidate material is appropriate to serve in the K115.c study to evaluate mass fraction range of inherent impurities, provided a suitable sample intake of more than 2.5 mg is used for analysis of the material.

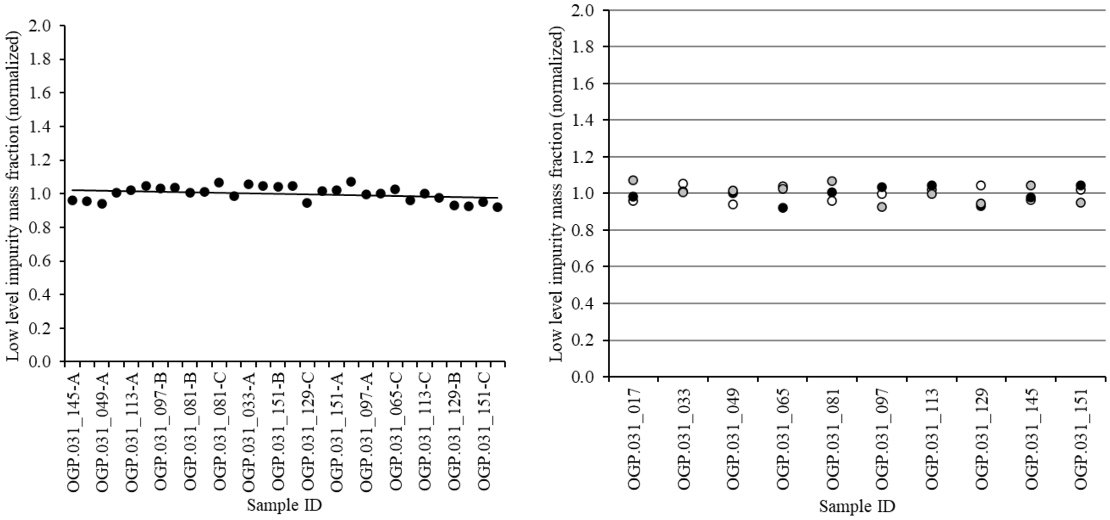


Figure 2: Homogeneity of $(Glc)_2VE$ - Low level mass fraction impurity - Injection and filling sequence

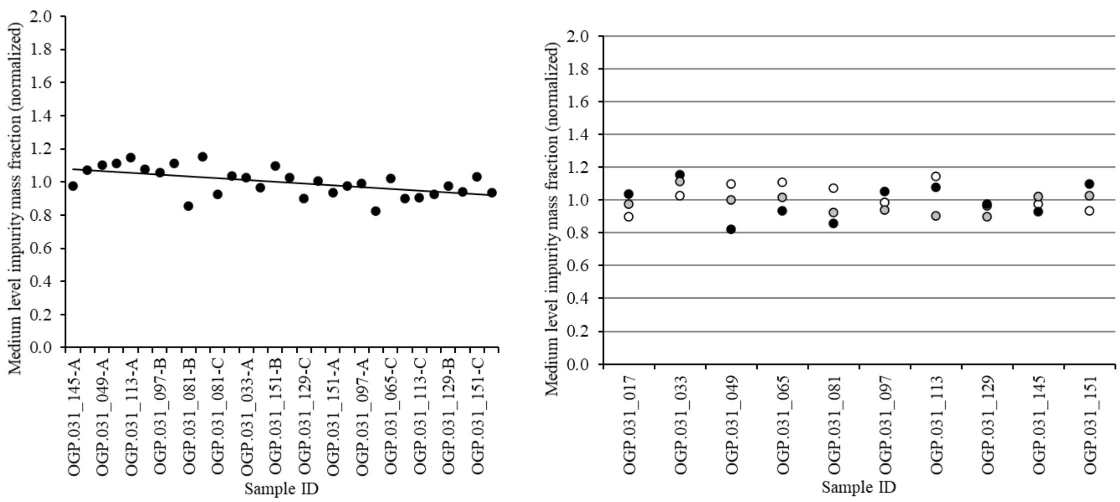


Figure 3: Homogeneity of GE dimer - Medium level mass fraction impurity - Injection and filling sequence

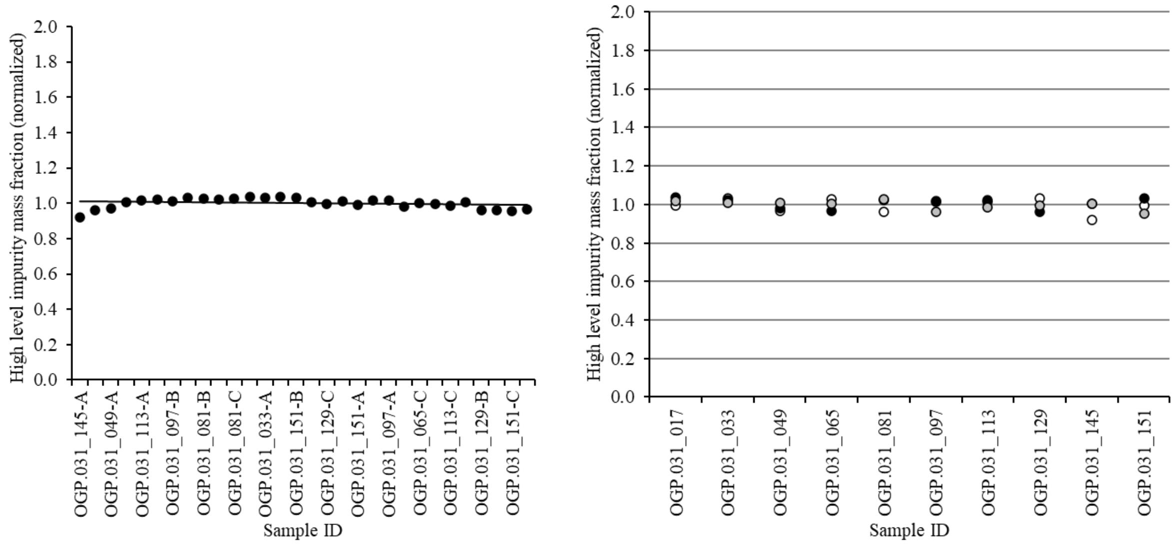


Figure 4: Homogeneity of VE - High level mass fraction impurity - Injection and filling sequence

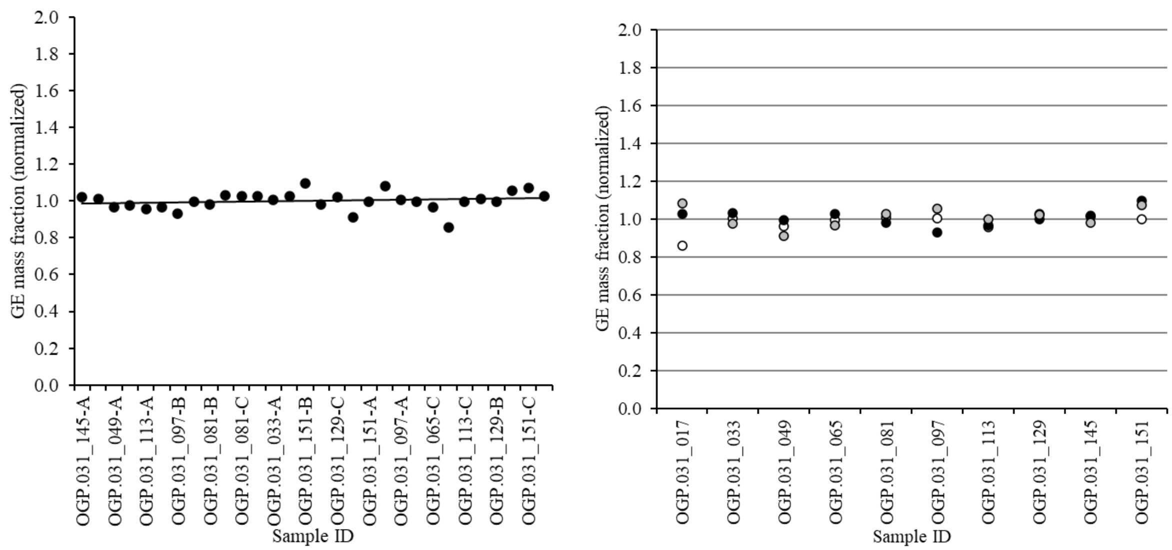


Figure 5: Homogeneity of GE - Injection and filling sequence

STABILITY STUDIES

Isochronous stability studies were performed using a reference storage temperature of -20 °C and test temperatures of 4 °C, 22 °C and 40 °C. A set of units from the production batch were stored at each selected temperature over 8 weeks, with units transferred to reference temperature storage at 2 week intervals. Vials that had been stored at 40 °C were not analyzed due to their degradation appearance (brown color and caramelized aspect).

Trend analysis of the data obtained by LC-UV-hrMS analysis of the stability test samples under repeatability conditions indicated no significant changes in the relative composition of GE or of the related peptide impurities over longer time and at low temperatures.
The GE mass fraction of the material was stable on storage at 4 °C and 22 °C over the entire storage study period. The peptide related impurity VE and $(Glc)_2$VE mass fraction of the material, representing high mass and low mass fraction level impurities, respectively, were also both stable on storage at 4 °C and 22 °C over the entire storage study period.
The GE dimer mass fraction of the material, representing medium mass fraction level impurities, was stable on storage but did increase significantly after storage beyond 2 weeks at 4 °C. The GE dimer mass fraction did increase significantly over the entire storage study period at 22 °C.
The effect of storage temperatures on the mass fractions of GE and related peptide impurities of the comparison material is shown in Figures 6-9.

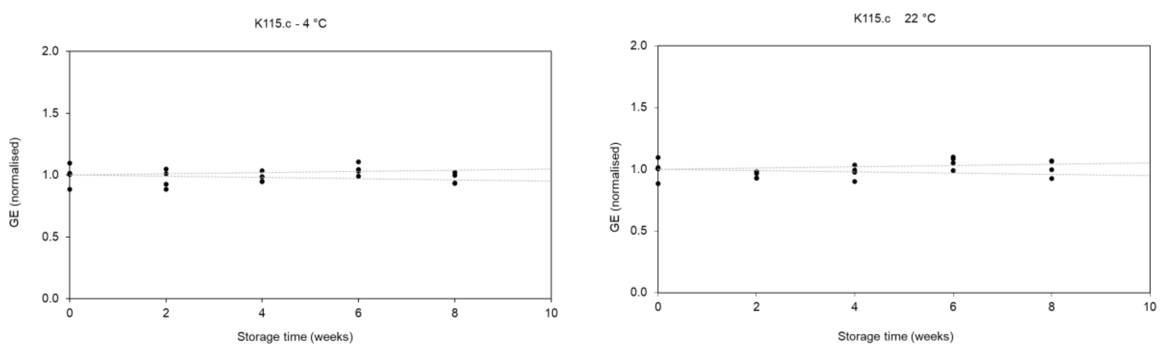


Figure 6: Stability study of GE

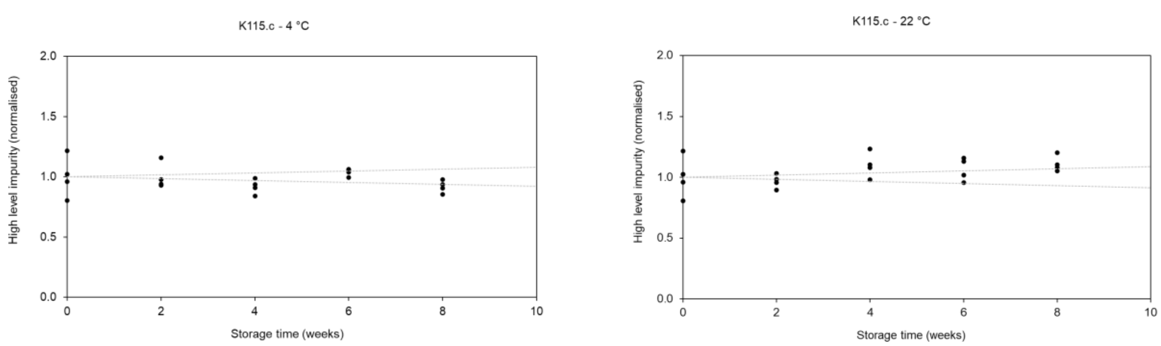


Figure 7: Stability study of VE - High level mass fraction impurity

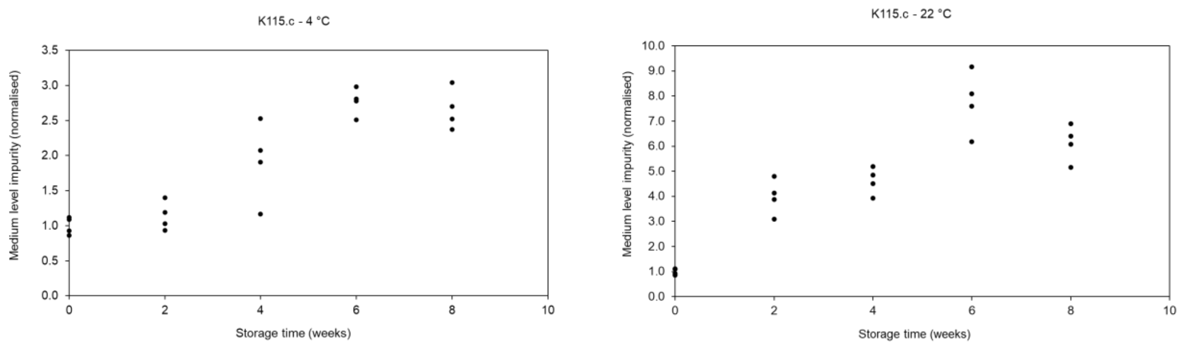


Figure 8: Stability study of GE dimer - Medium level mass fraction impurity

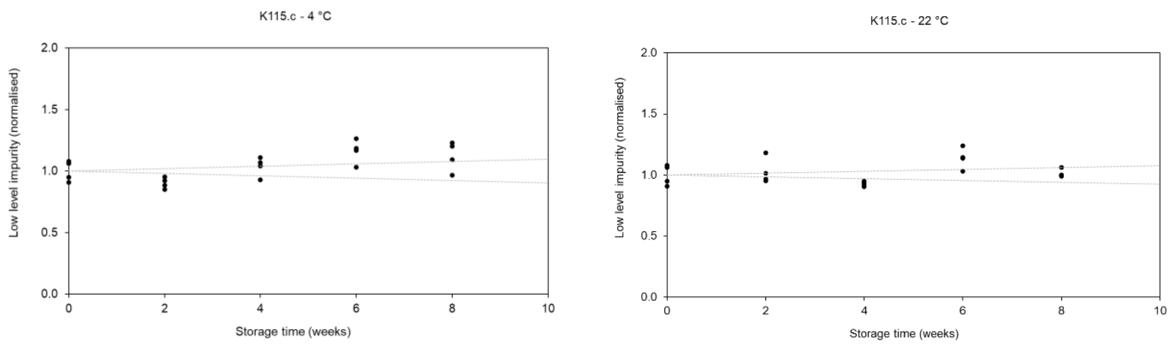


Figure 9: Stability study of $(Glc)_2VE$ - Low level mass fraction impurity

On the basis of these studies, it was concluded that for the purposes of the comparison the material was suitably stable for short-term cooled transport at low temperatures, provided it was not exposed to temperatures significantly in excess of 4 °C for more than 2 weeks, and for longer term storage at -20 °C.

The vials were shipped by courier using insulated shipping containers under -20 °C. The internal temperatures were recorded by data loggers.

To minimize the potential for changes in the material composition, participants were instructed to store the material in the freezer at -20 °C.

SORPTION MEASUREMENTS

Additional measurements performed on a dynamic vapor sorption (DVS) balance indicate that weighings of the CCQM-K115.c comparison material need to be performed under controlled conditions of temperature and relative humidity (RH) as the water content of the comparison material changes reversibly as a function of the RH (Figure 10).

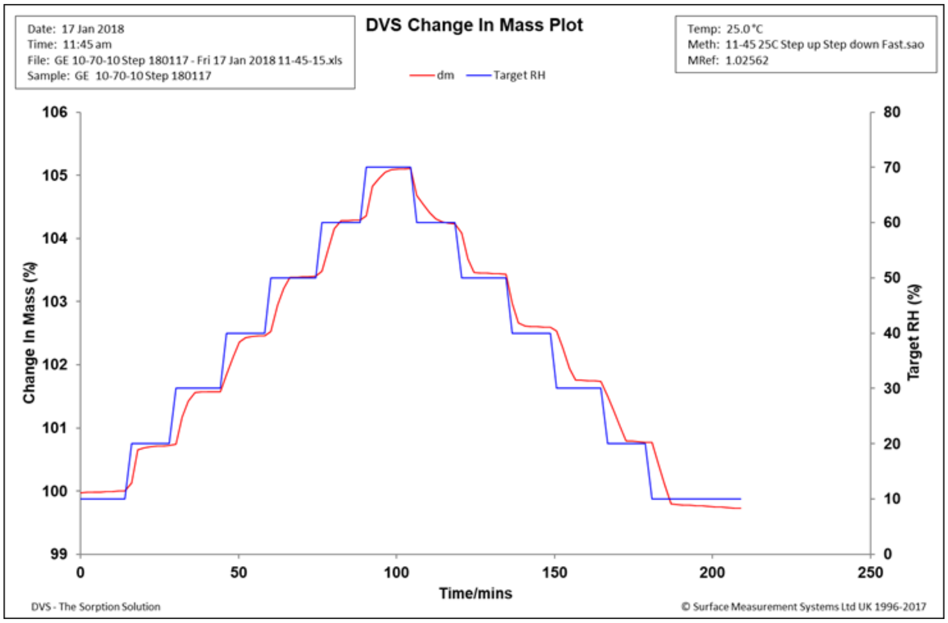


Figure 10: Sorption balance measurements indicating reversible water adsorption/desorption. Influence of RH (blue, % RH) on relative mass of GE (red, % change).

The temperature at which weighings are performed had to be measured and reported and had to be maintained between 20 °C and 30 °C. The relative humidity (RHX) at which weighings of the powdered material were performed has been recorded. The RH range over which the material can be weighed is between 30 % and 70 %. After opening of the vial, the comparison material needs to equilibrate at constant RHX for a minimum of 60 min before starting the weighing process. The mass of sample (M_{RHX}) measured at the relative humidity (RHX) shall be corrected to the mass of sample (M_{RH50}) at a RH of 50 % using the numerical equation:

$$M_{RH50} = M_{RHX}/(1+ F\cdot(RHX-50))$$

where F = 0.0008 and u(F) = 0.0001

RHX is the numerical value of the measured relative humidity expressed in %.

(Note: Relative humidity measurements with a standard uncertainty of 2 % and temperature measurements with a standard uncertainty of 0.2 °C will be sufficient to achieve the required accuracy for this correction)

SAMPLE DISTRIBUTION

Samples were distributed by HSA to all participants and the co-coordinating institutes (BIPM and NIM) in June and July 2019.
Two units of the study sample, each containing a minimum of 25 mg of materials, were distributed to each participant by express mail service in insulated and cooled transport containers equipped with a temperature data logger to record the temperature throughout the transport process. Participants were asked to return the sample receipt form and the data logger report acknowledging receipt of the samples and to advise the coordinators if any obvious damage had occurred during the shipping. All participants except INMETRO received the samples within one week from the time the samples were shipped out. The data logger reports to all participants except NRC and TUBITAK UME showed that the samples had not been exposed to temperature above 8 °C during the transport process. The data logger reports to NRC and TUBITAK UME showed that the samples had been exposed to temperature above 8 °C for about one day (highest temperature reached: 15.3 °C for samples to NRC and 13.2 °C for samples to TUBITAK UME). As the time above 8 °C was very short and the temperature did not even reach room temperature, the coordinators concluded that the samples were still appropriate for study. The samples to INMETRO were held at Brazilian custom for very long time and were finally destroyed. INMETRO arranged a subcontract to collect the replacement samples in January 2020.
As co-coordinating institutes would use mass balance method to determine the purity of the material, 22 units of the study samples, each containing a minimum of 25 mg of material, were distributed to each co-coordinating institute (BIPM and NIM) in insulated and cooled transport containers. Temperature was monitored by the courier and ice pack top-up was requested to ensure the temperature to be maintained below 8 °C during the transport process. Both co-coordinating institutes received the samples within three days from the time the samples were shipped out.

QUANTITIES AND UNITS

Participants were required to report the mass fraction of GE, the major component of the comparison sample. In addition, all participants who used a PICAA or qNMR procedure to determine the GE mass fraction were required to report the combined mass fraction assignment and corresponding uncertainty for total related peptide impurities.
In addition, the BIPM, HSA, and NIM, China who employed a mass balance (summation of impurities) procedure to determine the GE mass fraction were required to report the combined mass fraction assignment and corresponding uncertainty for the sub-classes of total related peptide impurities, water, total residual organic solvent / volatile organic compounds (VOCs) and total non-volatile organics & inorganics.
Participants were encouraged to also provide mass fraction estimates for the main impurity components they identified in the comparison sample.

REPORTED MASS FRACTIONS OF GE AND IMPURITIES IN CCQM-K115.C

The values reported by participants for the GE mass fraction in CCQM-K115.c are given in Table 3 with a summary plot in Figure 11. The values reported by participants for the peptide related impurity (PepImp) mass fractions in CCQM-K115.c are given in Table 6 with a summary plot in Figure 12.

The reported values for the GE mass fractions in CCQM-K115.c can be divided into two main groups - one group with both the BIPM and NIM using mass balance approaches and a second group using PICAA approaches. NRC has used qNMR and HSA has reported the average of three approaches (mass balance, PICAA and IDMS).

Table 3: Results for CCQM-K115.c: GE mass fractions and uncertainties as received

Participant	Mass fractions (mg/g)			Coverage Factor (*k*)	Approach
	GE	*u*(GE)	*U*(GE)		
INMETRO, Brazil	597.6	15.4	30.8	2	PICAA
LNE, France	618.444	17.624	35.248	2	PICAA
NIM, China	683.41	7.13	14.26	2	Mass balance
BIPM	630.9	14.5	29.0	2	Mass balance
LGC, United Kingdom	584.8	6.4	20.4	3.2	PICAA/ PICqNMR
NMIJ, Japan	625.2	6.3	12.6	2	PICAA
NRC, Canada	643.8	14.9	29.8	2	PICqNMR
PTB, Germany	660.1	7.5	15	2	PICAA
UME, Turkey	603.5	18.4	36.8	2	PICAA
HSA, Singapore	628.9	9.5	19.1	2	Mass balance/ PICAA/ IDMS

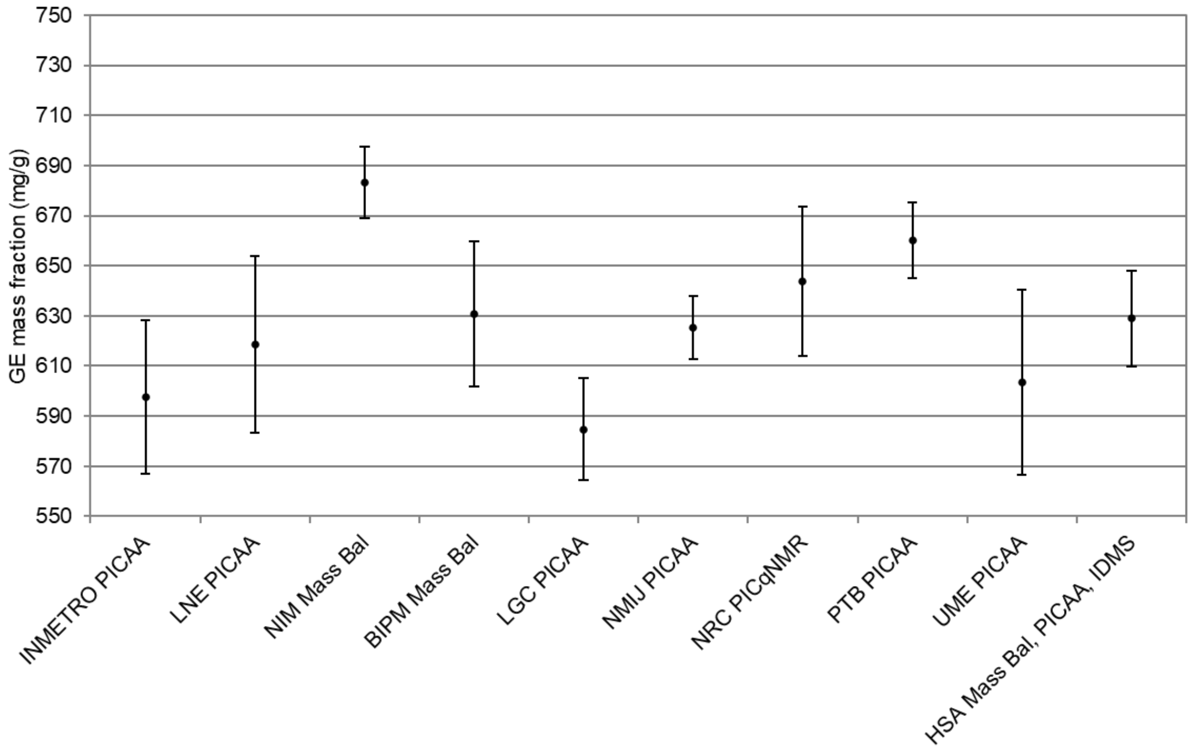


Figure 11: GE mass fractions reported by participants in CCQM-K115.c - plotted with expanded uncertainties (*U*) at a confidence level of about 95 %

The GE mass fraction values obtained by the BIPM and NIM using a mass balance approach do not agree within their estimated uncertainties. The related peptide impurity profile obtained by BIPM and NIM are in agreement.

TFA impurity mass fraction values are listed in Table 4. TFA impurity mass fraction values obtained by ^{19}F-qNMR were submitted by BIPM, HSA and NRC. In addition, both BIPM and NIM have submitted TFA impurity mass fraction values based by ion chromatography. ^{19}F-qNMR TFA impurity mass fraction values obtained by HSA and NRC are in agreement while the BIPM has obtained a slightly larger value. The TFA impurity mass fraction value from NIM obtained by ion chromatography is significantly lower than the values obtained by ^{19}F-qNMR. NIM has revised the ion chromatography mass fraction value after the PAWG meeting in April 2021 confirming issues with the TFA calibration CRM for ion chromatography. A revised NIM value is provided in brackets in Table 4 for information. A total TFA mass fraction of 248.6 mg/g with a corresponding expanded uncertainty of 22.9 mg/g ($k = 4.3$) could be calculated taking into consideration the TFA impurity mass fraction values obtained by ^{19}F-qNMR. The total TFA mass fraction is in agreement with the revised TFA impurity mass fraction value from NIM and the information value from BIPM both obtained by ion chromatography.

Water impurity mass fraction values obtained by KFT were submitted by BIPM, HSA and NIM (Table 5). All water impurity mass fraction values are in agreement, resulting in a total water mass fraction of about 56.1 mg/g with a corresponding expanded uncertainty of 9.8 mg/g (k = 4.3).

Table 4: TFA mass fractions and uncertainties

Participant	Mass fractions (mg/g)			Coverage Factor (k)	Approach
	TFA	u(TFA)	U(TFA)		
HSA, Singapore	247.3	2.0	4.0	2	qNMR
NRC, Canada	239.7	3.1	6.2	2	qNMR
BIPM	258.4	2.6	5.2	2	qNMR
BIPM	270*	18*	36*	2*	IC*
NIM, China	219.36 (251.97)	5.96 (2.9)	11.93 (5.8)	2 (2)	IC (IC)

* not traceable to the SI provided for information.

Table 5: Water mass fractions and uncertainties

Participant	Mass fractions (mg/g)			Coverage Factor (k)	Approach
	Water	u(Water)	U(Water)		
HSA, Singapore	59.35	6.42	12.84	2	KFT
BIPM	65.6	14.5	29.0	2	KFT
NIM, China	54.7	3.3	6.5	2	KFT

The GE mass fraction values obtained by the participants in many cases agree within their estimated uncertainties. However, LGC has assigned a significantly higher value to the peptide related impurity mass fraction mainly due to the quantification of the major impurity VE at very high mass fraction levels of about 52.6 mg/g as depicted in Table 6. Additional standard addition experiments were carried out by the LGC and confirmed the reported mass fraction of VE in the vial. NRC has also assigned a significantly higher value to the peptide related impurity mass fraction (Table 6). NRC was the only participant that clearly identified and quantified the second largest impurity GE depsipeptide as it becomes clear from NRCs individual components table that lists the *cis-trans* GE depsipeptide isomers as impurities.

Table 6: Results for CCQM-K115.c: Overall peptide related impurities (PepImp) mass fractions and uncertainties as received

Participant	Mass fractions (mg/g)			Coverage Factor (*k*)	Approach
	PepImp	*u*(PepImp)	*U*(PepImp)		
INMETRO, Brazil	40.6	8.1	22.7	2.8	LC-UV-hrMS
LNE, France	51.530	8.042	16.083	2	LC-hrMS
NIM, China	40.25	2.105	4.21	2	UHPLC-MS/MS and UHPLC-hrMS
BIPM	36.5	1.8	3.6	2	LC-hrMS
LGC, United Kingdom	81.0	6.1	12.2	2	UHPLC-MS/MS and UHPLC-hrMS
NMIJ, Japan	40.3	2.7	5.4	2	LC-hrMS
NRC, Canada	56.6	11.1	22.2	2	LC-hrMS and qNMR
PTB, Germany	33.7	1.3	2.5	2	LC-MS/MS
UME, Turkey	48.5	1.8	3.5	2	LC-MS/MS
HSA, Singapore	42.30	8	16	2	LC-UV and LC-MS/MS

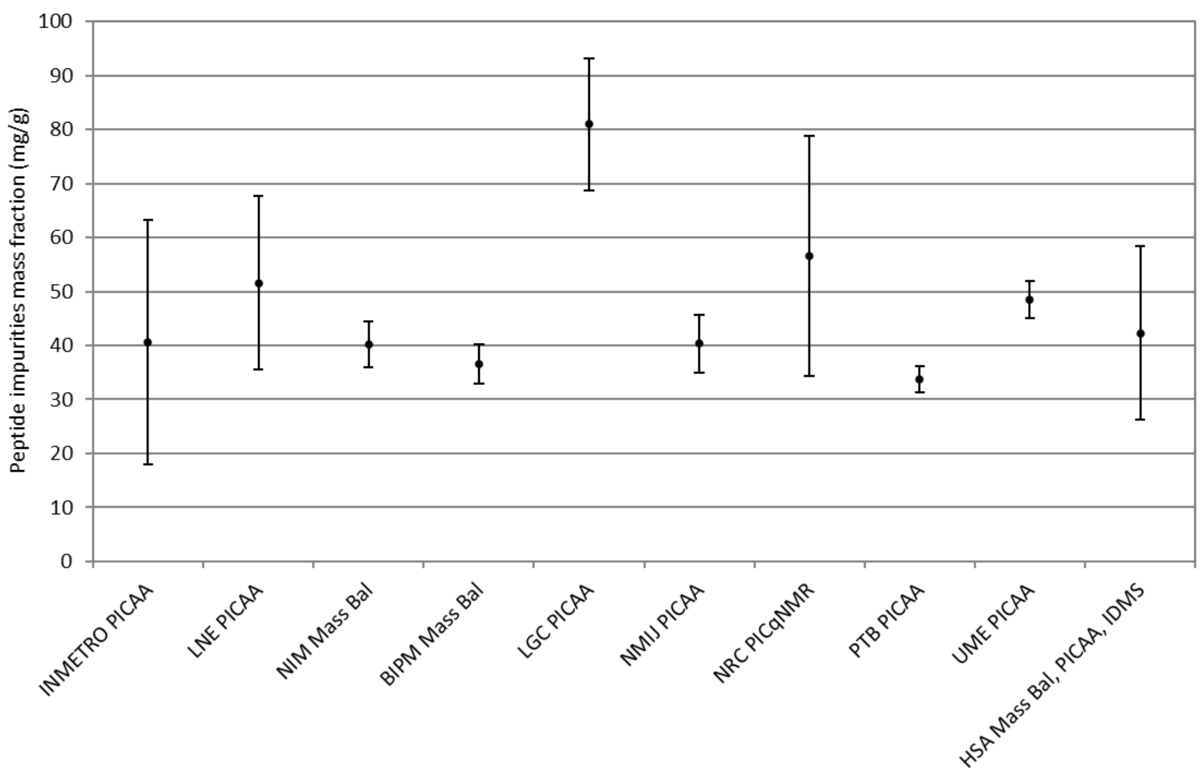


Figure 12: Overall peptide related impurities (PepImp) mass fractions reported by participants in CCQM-K115.c - plotted with expanded uncertainties (*U*) at a confidence level of about 95 %

In general, the CCQM-K115.c/P55.2.c comparison on GE purity shows less agreement of participants' results as the previous CCQM-K115/P55.2 series comparisons on hCP and OXT for peptide purity determinations. The peptide related impurity (PepImp) determinations showed the same level of agreement as for OXT. However, there was discussion on possible reasons for the discrepancy between CCQM-K115.c/P55.2.c results after presentation of the results of participants at the PAWG meeting in November 2020 and April 2021.

The peptide related impurities identification and quantification (Figure 12) is still a weak point as for both comparison on hCP and OXT. The number of potential impurities is much smaller for GE compared with both hCP and OXT as GE exhibits a shorter primary sequence. All ten laboratories have identified/quantified the dominating major peptide related impurity VE resulting in mainly coherent estimations of the peptide related impurity mass fractions. However, the second largest peptide impurity, GE depsipeptide, has only been correctly identified and quantified by the NRC. Hence most of the other participants have slightly underestimated the sum of peptide related impurity mass fractions. A few participants, for example BIPM, LGC, HSA and LNE, have observed an additional broad peak but it was not identified as GE depsipeptide. It has been discussed if that peak could relate to the GE depsipeptide if certain solvent conditions are maintained in LC-MS analysis as the GE depsipeptide is only stable at low pH conditions for a few days. The depsipeptide issue is discussed in detail in the section Peptide Related Impurity Profile of CCQM-K115.c.

It has been pointed out that the use of synthesized impurity standards has a positive impact on the quantification of the peptide related impurity mass fractions. Five laboratories have used synthesized impurity standards to quantify the major impurity VE. Nine participants have quantified the peptide related impurities using a response factor (RF = 1), RF with correction factor or a relative response method although four participants have used synthesized impurity standards to a different degree. NIM used 13 synthesized impurity standards (purities taken into account), BIPM used 4 synthesized impurities standards (purities taken into account) to quantify the individual impurities and closely structurally related impurities and NMIJ, LNE and PTB used 1 synthesized impurities standard and has quantified others with RF = 1.

NIM and BIPM have used the mass balance approach in CCQM-K115.c. HSA has used a combination of mass balance, PICAA and direct IDMS. NRC has used PICqNMR. LGC has used a combination of PICqNMR and PICAA. Five participants have used the PICAA approach. LGC has used microwave assisted hydrolysis. HSA, INMETRO, LNE, NMIJ, PTB and UME have employed gas/liquid phase hydrolysis. However, all participants that have used PICAA have performed an efficiency correction for the hydrolysis methods. The peptide related impurities values have been broken down to establish a means to visualize identification and quantification issues for the peptide related impurities.

Peptide Related Impurity Profile of CCQM-K115.c

The BIPM has broken down the peptide related impurities values to establish a means to visualize identification and quantification issues for the peptide related impurities. Figure 14 shows more details on the peptide related impurities of the CCQM-K115.c or -P55.2.c studies. The graph shows the peptide impurities that have been identified, the mean of the corresponding mass fractions, the corresponding standard deviations and the corresponding number of laboratories that have identified and quantified that impurity. The maximum possible number of identifications is ten as there are ten theoretical independent data sets due to the fact that some laboratories have used the same peptide impurity data set twice for example to correct both PICAA and qNMR results.
Please note that several laboratories have identified groups of impurities but the position of the modification was not or not entirely identified, for example GE dimer isomers and GE(OMe). In the graph it has been considered as identified but the mass fraction value has not been used for the calculation of the means of peptide impurity mass fractions.
In general, the identification and quantification of peptide impurities is quite coherent among laboratories. However, certain issues were discussed during the PAWG meetings in November 2020 and April 2021.
The dominating major peptide related impurity VE has been identified and quantified by all ten laboratories. The sum of peptide related impurity mass fractions consists of about 75 % of VE. However, the second largest peptide impurity, GE depsipeptide, has only been correctly identified and quantified by the NRC via ^{1}H-NMR. The structures of peptides containing β-hydroxy amino acids, i.e. serine and threonine can alter as a result of an N- to O- acyl shift. In the process the amide linkage of the peptide backbone due to the component is cleaved and replaced by an ester bond at the β-hydroxyl group. In the case of the GE peptide, N- to O- acyl shift can potentially occur at the leucine-threonine junction via a stable five-membered ring cyclic intermediate as exemplarily depicted for the non-glycated hexapeptide (VE) in Figure 13. The formed GE depsipeptide exists as a mixture of *cis-trans* isomers in solution [25-27].

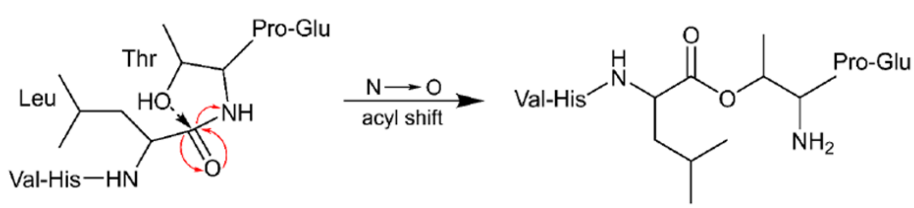


Figure 13: N to O acyl shift exemplarily depicted for non-glycated hexapeptide (VE)

The NRC has identified and quantified both *cis*- and *trans*-isomers of the GE depsipeptide via ^{1}H-NMR at mass fraction levels of 1.7 ± 1.0 mg/g (k = 2) and 9.5 ± 5.8 mg/g (k = 2), respectively. Related peptide impurities of that large mass fraction levels should have been identified and quantified by other participants using ^{1}H-NMR. The BIPM has agreed during the PAWG meeting in November 2020 to re-assess their own data concerning the presence of GE depsipeptide impurity fragments. In summary, the 2D COSY spectrum obtained on a GE sample in D_2O was re-analyzed and the GE depsipeptide isomers were identified. Given that the quantification signals were based on histidine protons, the purity values should have been corrected for the amount of depsipeptides. The approximate depsipeptide mass fractions were calculated in the GE samples in deuteromethanol. The combined GE depsispeptide mass fraction was 14.7 ± 7.0 mg/g based on the integration of the signal due to the threonine γ-CH_3 protons. The GE depsipeptide mass fraction assignments of the BIPM are in agreement and confirming the findings of the NRC.

The identification and quantification of the GE depsipeptide by use of LC-(hr)MS(/MS) techniques have proved to be difficult. Initially, the GE depsipeptide impurity was missed or misinterpreted by all participants using by LC-(hr)MS(/MS). Several participants, notably the BIPM, LGC and LNE, have observed a very broad peak eluting at shorter retention times than the main GE peak but it was not identified as GE depsipeptide. The BIPM and HSA have agreed during the PAWG meeting in November 2020 to re-assess their LC-(hr)MS data. Retrospective analysis of mass spectrometry data and subsequent investigations led to the conclusion that the presence of the GE depsipeptide was wrongly ignored. A GE isomer eluting before the main GE peak was quantified but eventually disqualified as an artefact because of inconsistent abundance in GE samples. The MS spectra showed that the broad peak was isobaric with GE but presented some characteristic water loss ions. The inconsistent peak area assignments could be attributed to the instability of depsipeptides and its pH dependency, as evidenced in subsequent experiments performed. It was confirmed that the GE depsipetide is only present in freshly prepared aqueous solution of the GE material. Aqueous solutions are acidic (about pH 4) due to the high TFA content of the GE material. The GE depsipeptide peak decreased and disappear completely after a few days (< 4 days) when the GE sample is prepared in an acidic aqueous solution (pH 4). The GE depsipeptide peak disappeared instantly when GE materials were dissolved in alkaline buffer (pH 9). In addition, HSA proved, using the example of VE depsipeptide, that the depsipeptide transformation in alkaline or week acid solution (pH > 4) is irreversible (no depsipeptide production upon re-acidification to pH ~ 2.5). These findings imply that the GE depsipeptide was already present in the solid material. It should be noted that the instability of depsipeptide impurities could impact measurements for clinical purposes if the LC-MS methods used are employed under alkaline conditions.

Furthermore, it has been decided during the discussions within the CCQM PAWG in April 2021 that the GE depsipeptide structural isomer would be counted as impurity whereas the stereoisomers *cis/trans*, also present in the material, would not be counted as separate impurities.

UME has also re-assessed their data and in retrospect reported an identification mismatch of Glc-AVHLTPEE to Glc-VHLTAPE iso A.

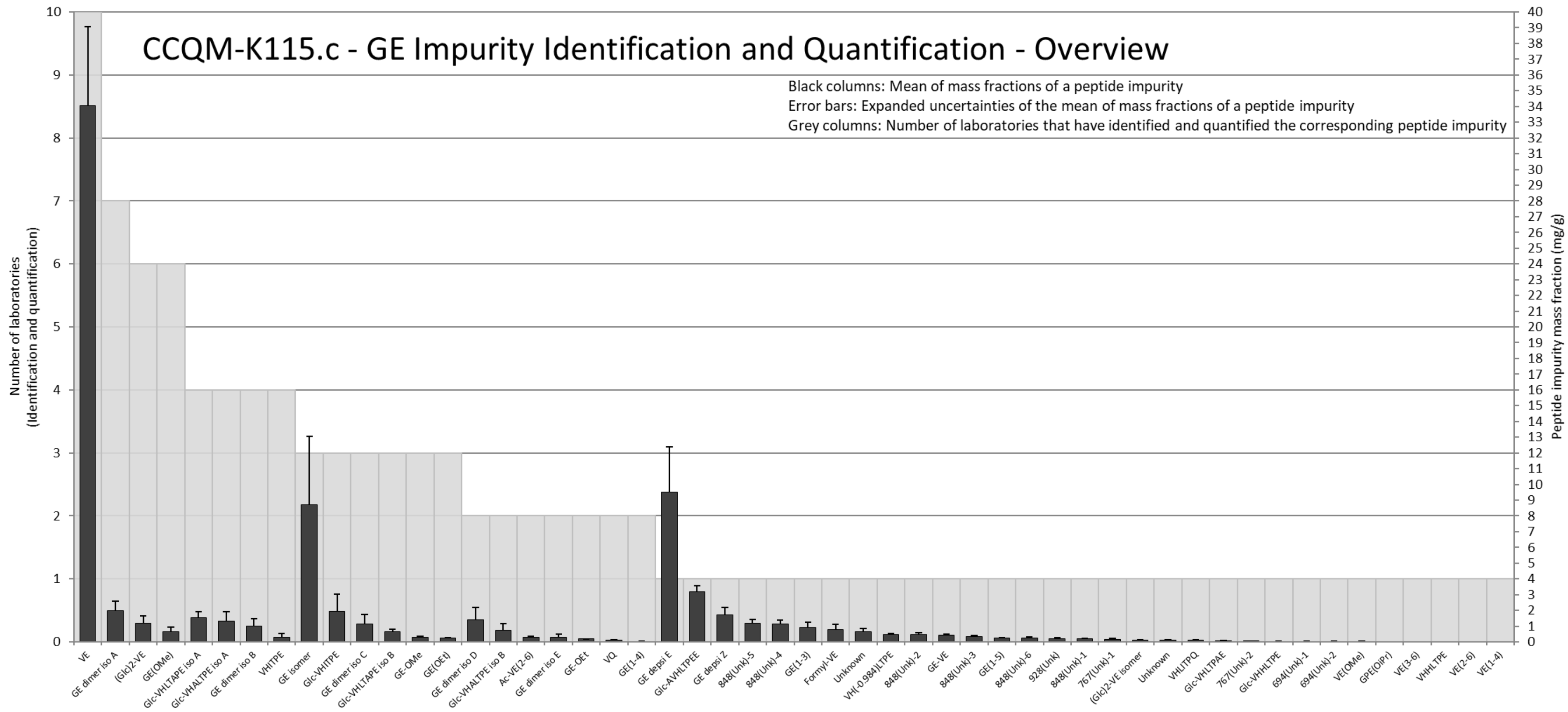


Figure 14: GE impurity identification and quantification - Overview

KEY COMPARISON REFERENCE VALUES (KCRVS) FOR CCQM-K115.C

The values used to establish the Key Comparison Reference Values (KCRV) for CCQM-K115.c are summarized in Table 3 and Table 6 for the GE mass fraction and the peptide related impurity mass fractions, respectively. All participants in CCQM-K115.c were required to give estimates for the mass fraction of the sub-class of peptide related impurities they quantified to obtain their final GE mass fraction estimate. The coordinator has calculated the overall KCRV for GE mass fraction and separate KCRV for the total peptide related impurities content as the peptide related impurity profile and quantification is of utmost importance.

Impurity Profile and Key Comparison Reference Value (KCRV) for Mass Fraction of Peptide Related Impurities in CCQM-K115.c

The $KCRV_{PepImp}$ for the mass fraction of peptide impurities is based on the assumption that all results are directly taken for the calculation of the $KCRV_{PepImp}$. The $KCRV_{PepImp}$ and the corresponding standard uncertainty (u($KCRV_{PepImp}$)) was establish based on the DerSimonian-Laird variance-weighted mean (DSL) [28-29]. The DSL-mean takes into account the uncertainties while introducing sufficient excess variance (λ) to allow for their observed dispersion. The DSL approach to obtain the $KCRV_{PepImp}$ has been accepted by all participating NMIs/DIs as none of the results could be excluded for technical shortcomings.
Figure 15 shows the participant results with their reported standard uncertainties plotted against the $KCRV_{PepImp}$ of 45.4 mg/g for peptide impurities in CCQM-K115.c (solid line) and its corresponding standard uncertainty of 4.2 mg/g (k = 1). The excess variance is derived from the dark uncertainty (τ) that of 8.4 mg/g ($\tau^2 = \lambda$). A corresponding expanded uncertainty of 9.5 mg/g (k = 2.26) at a confidence level of about 95 % was calculated.

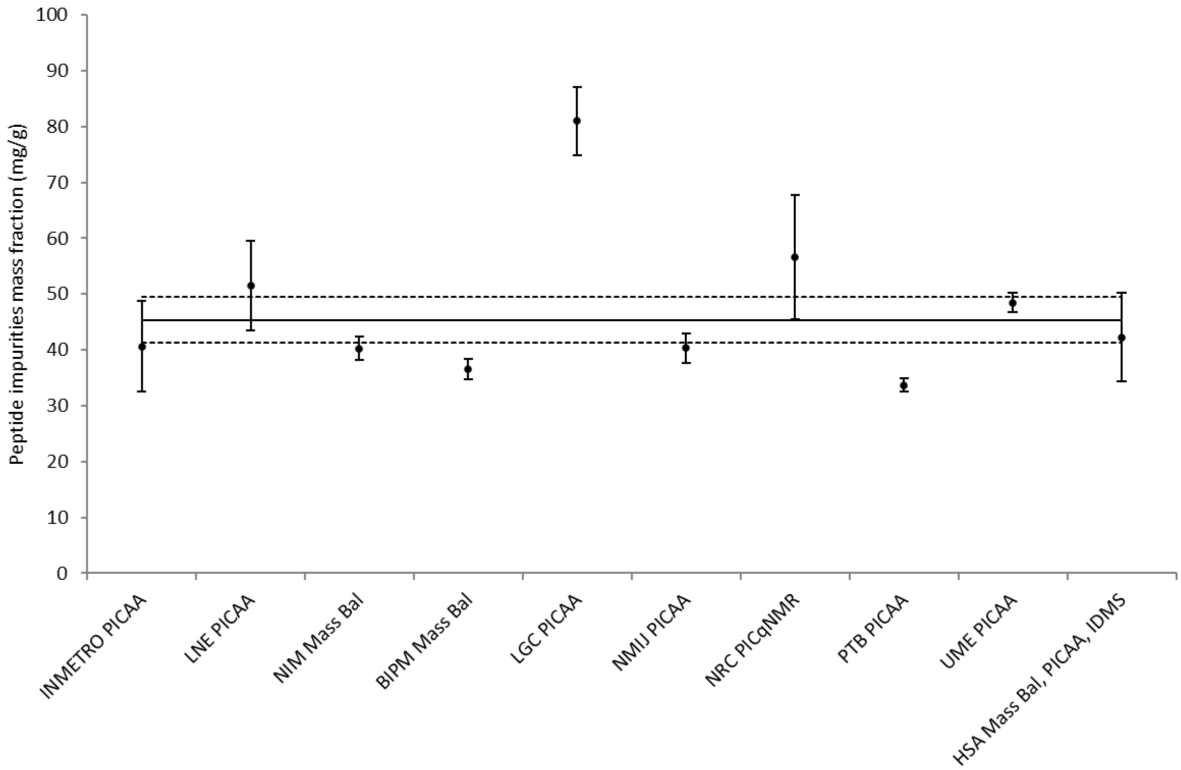


Figure 15: Estimates of total related peptide impurities in CCQM-K115.c plotted with their reported standard uncertainties ($\pm u_c$, $k = 1$). The $KCRV_{PepImp}$ (solid line) is 45.4 mg/g. Dashed lines show the $u(KCRV_{PepImp})$ ($k = 1$) of the $KCRV_{PepImp}$.

The degree of equivalence of a participant's result with the $KCRV_{PepImp}$ (D_i) is given by:

$$D_i = w_i - KCRV_{PepImp}$$

The expanded uncertainty U_i at a confidence level of about 95 % associated with the D_i was calculated as [30]:

$$U_{95\,\%}(D_i) = 2 \cdot \sqrt{u(w_i)^2 + \lambda - \ u\left(KCRV_{PepImp}\right)^2}$$

Figure 16 indicates the degree of equivalence (D_i) of each key comparison participant's result with the $KCRV_{PepImp}$ for related peptide impurities. The corresponding values are listed in Table 7.

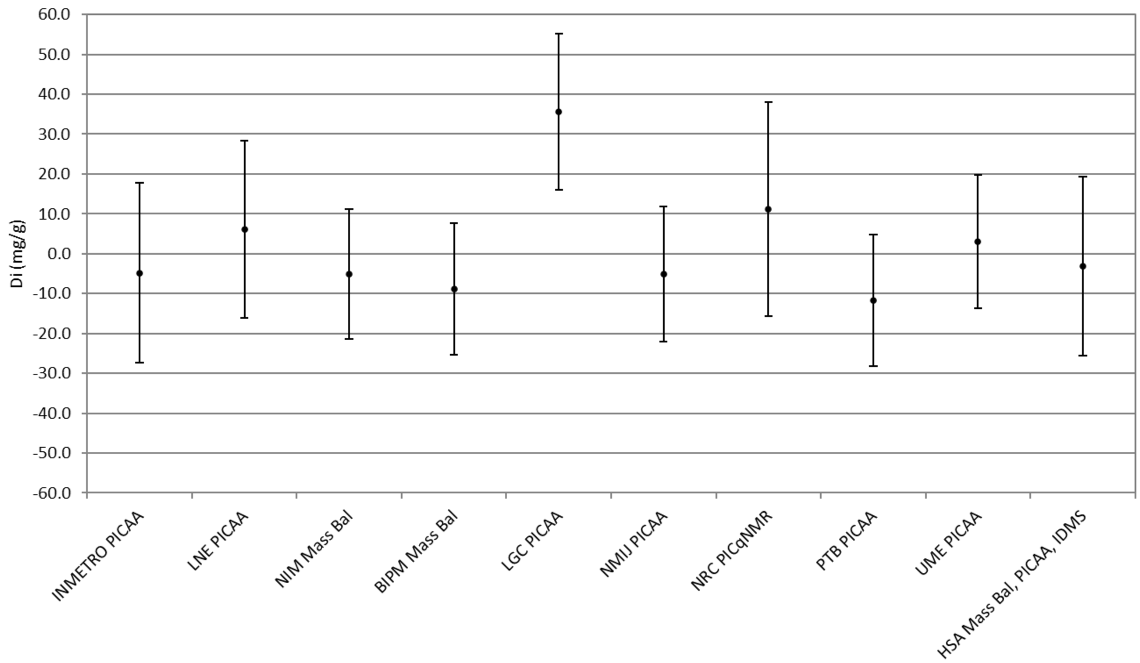


Figure 16: Degree of equivalence with the $KCRV_{PepImp}$ for total related peptide impurities for each participant. Points are plotted with the associated expanded uncertainty in the degree of equivalence corresponding to a confidence level of about 95 %.

Table 7: Degrees of equivalence D_i and expanded uncertainties $U(D_i)$ at a confidence level of about 95 % in mg/g for the $KCRV_{PepImp}$ for total related peptide impurities

	D_i	$U(D_i)$
INMETRO, Brazil	-4.8	22.6
LNE, France	6.1	22.2
NIM, China	-5.1	16.3
BIPM	-8.9	16.5
LGC, United Kingdom	35.6	19.5
NMIJ, Japan	-5.1	17.0
NRC, Canada	11.2	26.8
PTB, Germany	-11.7	16.6
UME, Turkey	3.1	16.7
HSA, Singapore	-3.1	22.5

Key Comparison Reference Value (KCRV) for the Mass Fraction of GE in CCQM-K115.c

The $KCRV_{GE}$ for the mass fraction of GE is based on the assumption that all results are directly taken for the calculation of the $KCRV_{GE}$. The $KCRV_{GE}$ and the corresponding standard uncertainty ($u(KCRV_{GE})$) was established based on the DerSimonian-Laird variance-weighted mean (DSL) [28-29]. The DSL-mean takes into account the uncertainties while introducing sufficient excess variance (λ) to allow for their observed dispersion. The DSL approach to obtain the $KCRV_{GE}$ has been accepted by all participating NMIs/DIs as none of the results could be excluded for technical shortcomings.

Figure 17 shows the participant results with their reported standard uncertainties plotted against the $KCRV_{GE}$ of 628 mg/g for GE in CCQM-K115.c (solid line) and its corresponding standard uncertainty of 12 mg/g (k = 1). The excess variance is derived from the dark uncertainty (τ) that of 34.9 mg/g ($\tau^2 = \lambda$). A corresponding expanded uncertainty of 27 mg/g (k = 2.26) at a confidence level of about 95 % was calculated.

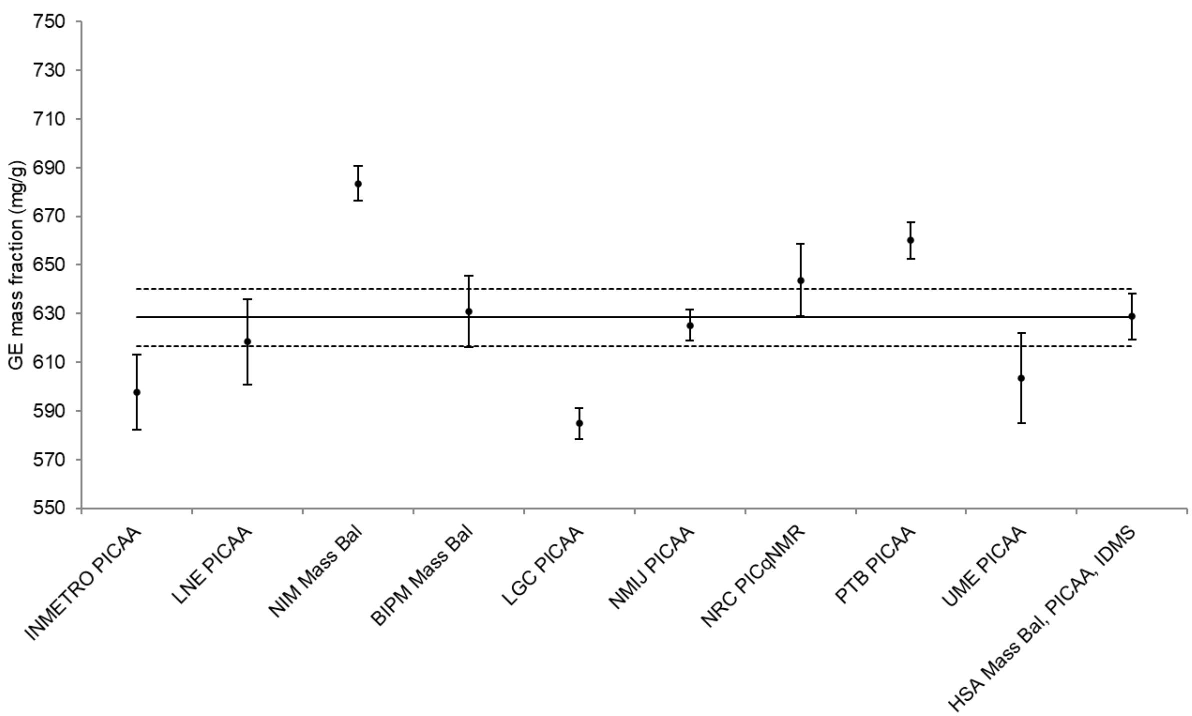


Figure 17: Mass fraction estimates by participants for GE in CCQM-K115.c with their reported combined standard uncertainties ($\pm u_c$, k = 1). The $KCRV_{GE}$ for CCQM-K115.c (solid line) is 628 mg/g. The calculated combined standard uncertainty of the $KCRV_{GE}$ is ±12 mg/g. Dashed lines show the $u(KCRV_{GE})$ (k = 1) of the $KCRV_{GE}$.

The degree of equivalence of a participant's result with the $KCRV_{GE}$ (D_i) is given by:

$$D_i = w_i - KCRV_{GE}$$

The expanded uncertainty U_i at a confidence level of about 95 % associated with the D_i was calculated as [30]:

$$U_{95\,\%}(D_i) = 2 \cdot \sqrt{u(w_i)^2 + \lambda - u(KCRV_{GE})^2}$$

Figure 18 indicates the degree of equivalence (D_i) of each key comparison participant's result with the $KCRV_{GE}$ for GE. The corresponding values are listed in Table 8.

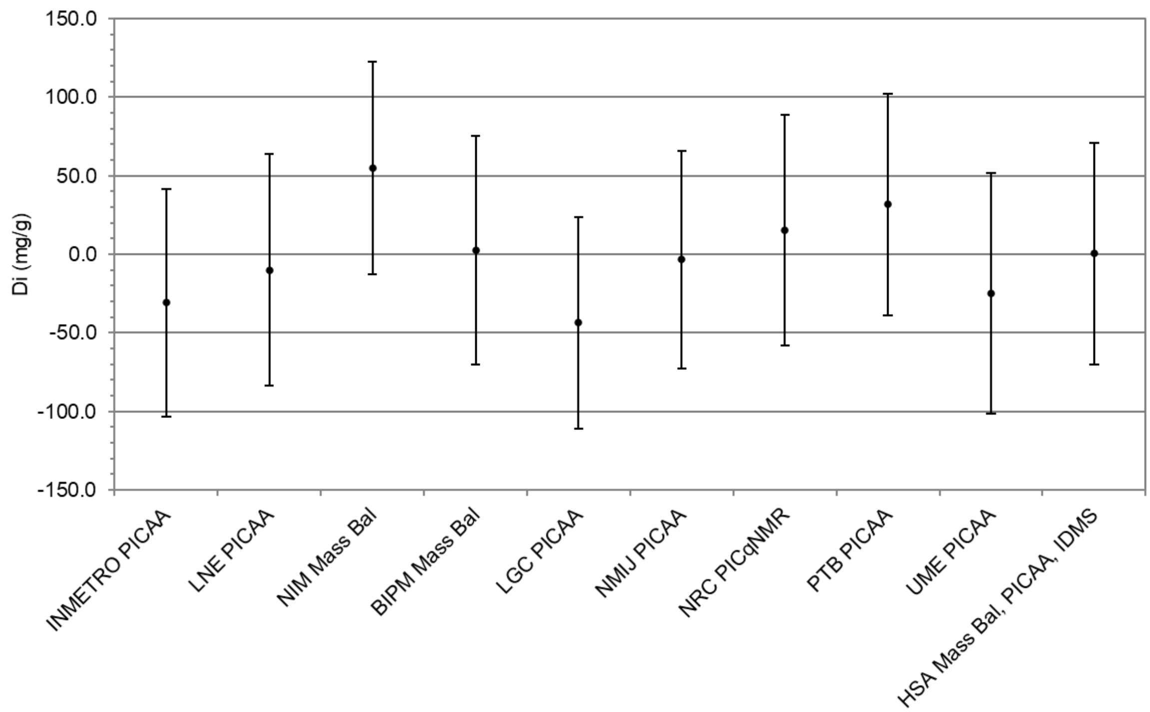


Figure 18: Degree of equivalence with the $KCRV_{GE}$ for GE for each participant. Points are plotted with the associated expanded uncertainty in the degree of equivalence corresponding to a confidence level of about 95 %.

Table 8: Degrees of equivalence D_i and expanded uncertainties $U(D_i)$ at a confidence level of about 95 % in mg/g for the $KCRV_{GE}$ for GE

	D_i	$U(D_i)$
INMETRO, Brazil	-30.8	72.4
LNE, France	-10.0	73.6
NIM, China	55.0	67.9
BIPM	2.5	72.9
LGC, United Kingdom	-43.6	67.2
NMIJ, Japan	-3.2	69.3
NRC, Canada	15.4	73.4
PTB, Germany	31.7	70.6
UME, Turkey	-24.9	76.4
HSA, Singapore	0.5	70.3

CONCLUSIONS

GE was selected to be representative of chemically synthesized linear peptides of known sequence, without cross-links, up to 5 kDa and modification (mono-glycation). It was anticipated to provide an analytical measurement challenge representative for the value-assignment of compounds of broadly similar structural characteristics.

The majority of participants used a PICAA approach as the amount of material that has been provided to each participant (25 mg) is insufficient to perform a full mass balance based characterization of the material by a participating laboratory. The coordinators, both the BIPM and the NIM, were the laboratories to use the mass balance approach as they had more material available.

It was decided to propose KCRVs for both the GE mass fraction and the mass fraction of the peptide related impurities as indispensable contributor regardless of the use of PICAA, mass balance or any other approach to determine the GE purity. This allows participants to demonstrate the efficacy of their implementation of the approaches used to determine the GE mass fraction. In particular, it allows participants to demonstrate the efficacy of their implementation of peptide related impurity identification and quantification.

More detailed studies on the identification/quantification of peptide related impurities revealed that the integrity of the impurity profile of the related peptide impurities obtained by the participant is crucial for the impact on accuracy of the GE mass fraction assignment.

Different methods had been investigated to obtain a $KCRV_{PepImp}$ for the mass fraction of peptide impurities. GE related peptide impurity mass fraction results submitted by all NMIs/DIs are taken directly into account for the calculation of the $KCRV_{PepImp}$. The approach selected to obtain a $KCRV_{PepImp}$ is based on random-effects meta-analysis (DerSimonian-Laird (DSL) variance-weighted mean). The DSL-mean takes into account the uncertainties of the results while introducing sufficient excess variance to allow for their observed dispersion resulting in a larger expanded uncertainty $U(KCRV_{PepImp})$. Consequently, the $KCRV_{PepImp}$ of 45.4 mg/g is associated with a relatively large corresponding expanded uncertainty of ± 9.5 mg/g (k = 2.26) providing a more realistic basis of evaluation for the capabilities of the participants to identify/quantify peptide related impurities. All GE related peptide impurity mass fraction results except the result of LGC are in agreement with the $KCRV_{PepImp}$. Inspection of the degree of equivalence plots for the mass fraction of peptide impurities and additional information obtained from the peptide related impurity profile indicates that in many cases the major related peptide impurities have been identified and quantified with the exception of the GE depsipeptide impurity. The GE depsipeptide impurity was initially and uniquely identified and quantified by the NRC by the use of ^{1}H-NMR.

Different methods had also been investigated to obtain a $KCRV_{GE}$ for the GE mass fraction. GE mass fraction results submitted by all NMIs/DIs are taken directly into account for the calculation of the $KCRV_{GE}$. The approach selected to obtain a $KCRV_{GE}$ is also based on random-effects meta-analysis (DSL) as the DSL-mean takes into account the uncertainties of the results while introducing sufficient excess variance to allow for their observed dispersion resulting in a larger expanded uncertainty $U(KCRV_{GE})$. The $KCRV_{GE}$ for CCQM-K115.c is 628 mg/g with a corresponding expanded uncertainty of the $KCRV_{GE}$ of ± 27 mg/g (k = 2.26).

The GE material is not sufficiently pure and the corresponding expanded uncertainty is too large to serve as a calibrator to directly support a comparison on the HbA1c quantification in biological samples by IDMS.

HOW FAR THE LIGHT SHINES STATEMENT (HFTLS)

Successful participation in the CCQM-K115.c comparison will support CMCs for:

- chemically synthesized peptides of known sequence, without cross-links, up to 5 kDa and modification (mono-glycation). Additional evidence is required to support claims related to peptides that contain more than 5 kDa, or have been produced using a recombinant process;
- pure peptide primary reference materials value assigned for the mass fraction of the main component peptide within the material;
- methods for the value assignment of the mass fraction of the main component peptide within the material;
- the identification and quantification of minor component peptide impurities within the material.

In addition, the comparison will support traceability statements of CMCs for peptide and protein quantification which are dependent on pure peptide reference materials or methods for their value assignment for peptides meeting the above criteria.

Glycated hexapeptide of HbA1c (glcVHLTPE or GE) has been proposed as the comparison material, since:

- it will allow the generic capabilities listed above to be demonstrated for modified (mono-glycated) peptides without cross-links and up to 5 kDa molecular mass [1];
- it can be obtained in sufficiently large quantities required for the comparison;
- it will directly support NMI/DI services and certified reference materials currently being provided by NMIs/DIs [31];
- Hemoglobin A1c (HbA1c) is an important analyte for which reference methods have been developed in laboratory medicine [32-37] where GE is the signature peptide for the quantification of HbA1c.

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