CCQM-P55.2.2018

Pilot Study on Peptide Purity - Hexapeptide of HbA0

Final Report

April 2022

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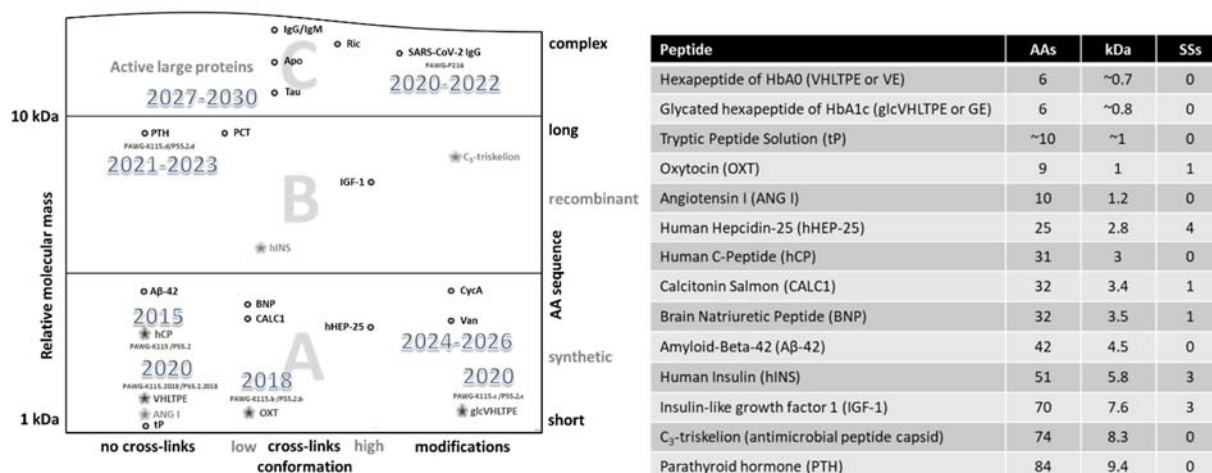
Figure 1: Model for the classification of peptides for primary structure purity determinations

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

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 present CCQM-P55.2.2018 study 'Pilot Study on Peptide Purity - Hexapeptide of HbA0' (VE) is the first repeated study of the CCQM-P55.2 series to cover 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 5 kDa. The timeline for the CCQM-P55.2.2018 pilot study is summarized in Table 1.

Table 1: CCQM-P55.2.2018 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 hexapeptide of HbA0 (VE) in the material is to be determined. VE is defined as hemoglobin subunit beta [2-7] fragment with the amino acid sequence VHLTPE and a relative molecular mass (M_r) of about 694.7 g/mol.

The study material was prepared by the BIPM/HSA by characterization of a commercially sourced sample of synthetic VE. The methods used to investigate, assign and confirm the quantitative composition of the CCQM-K115.2018 and CCQM-P55.2.2018 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/ inorganics 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 AND STABILITY STUDIES AND SORPTION MEASUREMENTS

The batch of CCQM-K115.2018 and CCQM-P55.2.2018 candidate material vials were evaluated for impurity profile, homogeneity, stability and water adsorption/desorption by the BIPM/HSA. The mass fraction of the VE in the comparison material was assessed by the BIPM to be about 625 mg/g while the homogeneity and stability of the VE and peptide related impurities were shown to be suitable for the purpose of the comparison. Dynamic vapor sorption balance measurements indicated that weighings of the CCQM-K115.2018 and CCQM-P55.2.2018 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. A full summary of the results for VE mass fraction and of the methods used to investigate, assign and confirm the composition of the CCQMK115.2018 and CCQM-P55.2.2018 candidate material and to demonstrate the fitness for purpose of the homogeneity, stability and reversible water adsorption/desorption of the material are given in detail in the CCQM-K115.2018 Final Report [24].

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 CCQM-P55.2.2018 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 CCQM-P55.2.2018 participants received the samples within one week from the time the samples were shipped out. The data logger reports to all participants except TUBITAK UME showed that the samples had not been exposed to temperature above 8 °C during the transport process. The data logger reports to TUBITAK UME showed that the samples had been exposed to temperature above 8 °C for about one day (highest temperature reached: 15.3 °C). 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. It should be noted that CENAM, Mexico has also been supplied with samples to participate in CCQM-P55.2.2018. However, CENAM was not able to finalize the study and submit results because of constraints due to the Coronavirus pandemic.

QUANTITIES AND UNITS

Participants were required to report the mass fraction of VE, the major component of the comparison sample. In addition, all participants who used a PICAA or qNMR procedure to determine the VE 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 who employed a mass balance (summation of impurities) procedure to determine the VE 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. Details are provided in the CCQM-K115.2018 Final Report [24].

Participants were encouraged to also provide mass fraction estimates for the main impurity components they identified in the comparison sample.

REPORTED MASS FRACTIONS OF VE AND IMPURITIES IN CCQM-P55.2.2018

The values reported by participants for the VE mass fraction in CCQM-P55.2.2018 are given in Table 2 with a summary plot in Figure 2. The values reported by participants for the peptide related impurity (PepImp) mass fractions in CCQM-P55.2.2018 are given in Table 3 with a summary plot in Figure 3.

The reported values for the VE mass fractions in CCQM-P55.2.2018 can be divided into two groups. Three participants have employed PICAA approaches and two participants have used PICqNMR approaches.

Table 2: Results for CCQM-P55.2.2018: VE mass fractions and uncertainties as received

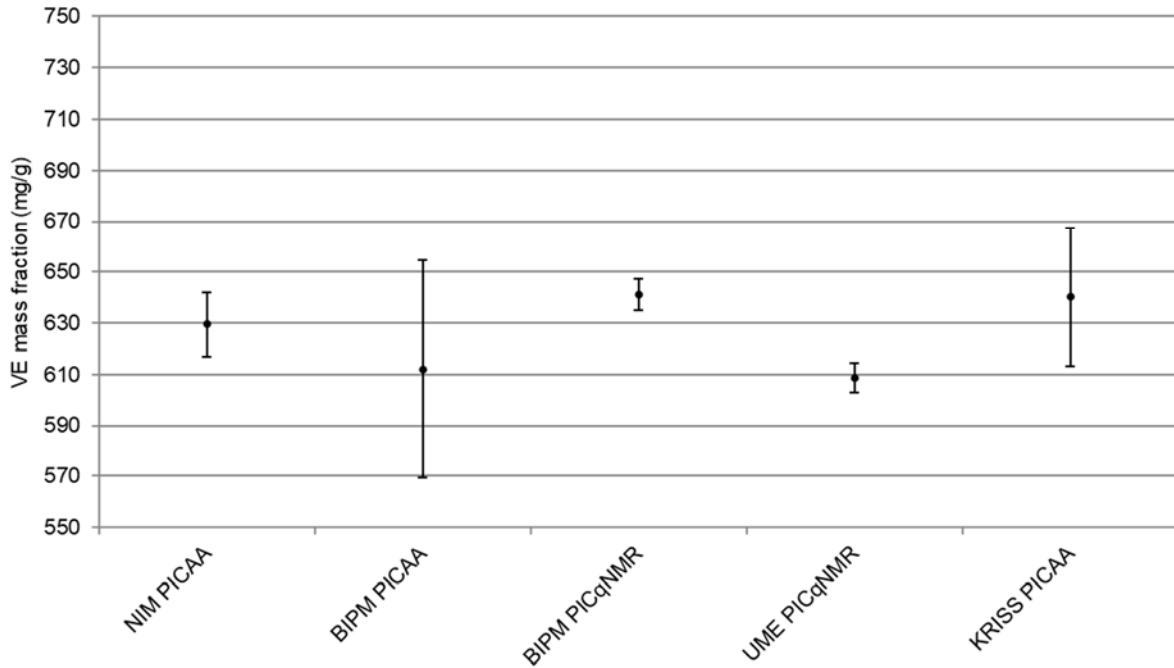| Participant	Mass fractions (mg/g)			Coverage Factor (k)	Approach
	VE	u(VE)	U(VE)		
NIM, China	629.5	6.2	12.4	2	PICAA
BIPM	611.9	21.3	42.6	2	PICAA
BIPM	641.2	3.1	6.2	2	PICqNMR
UME, Turkey	608.7	2.9	5.8	2	PICqNMR
KRISS, Korea (Rep. of)	640.39	11.73	27.04	2.306	PICAA

Figure 2: VE mass fractions reported by participants in CCQM-P55.2.2018 - plotted with expanded uncertainties (U) at a confidence level of about 95 %

Table 3: Results for CCQM-P55.2.2018: Overall peptide related impurities (PepImp) mass fractions and uncertainties as received

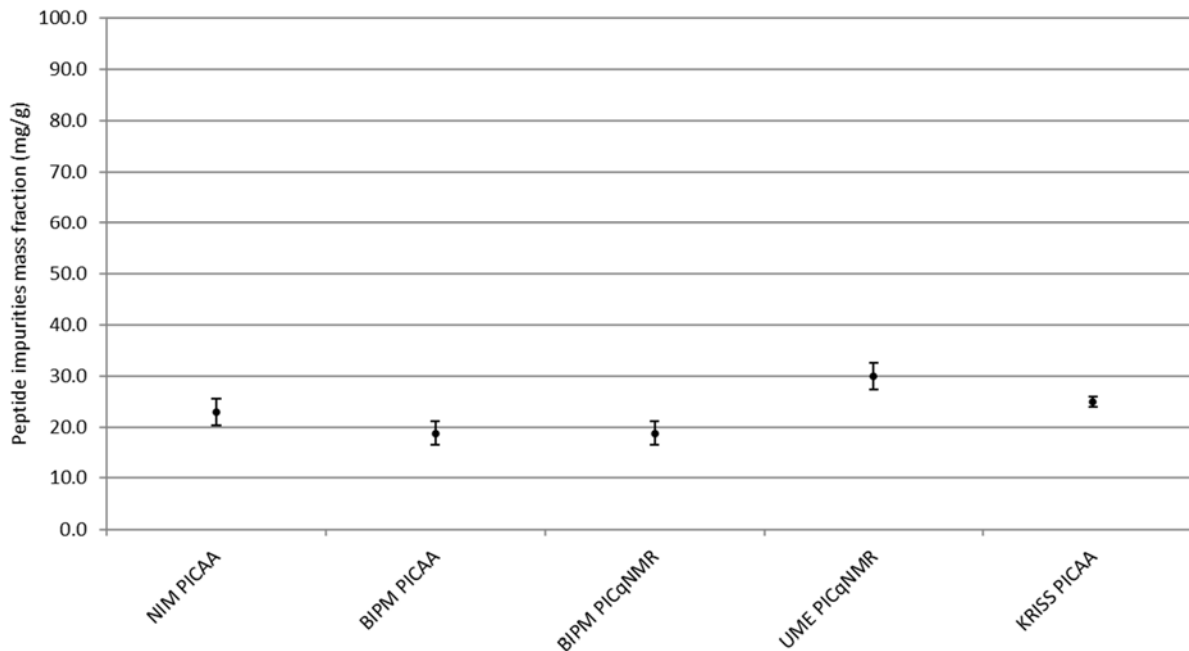| Participant	Mass fractions (mg/g)			Coverage Factor (k)	Approach
	PepImp	u(PepImp)	U(PepImp)		
NIM, China	23	1.3	2.6	2	HPLC-MS/MS
BIPM	18.83	1.17	2.34	2	LC-hrMS
BIPM	18.83	1.17	2.34	2	LC-hrMS
UME, Turkey	30	1.3	2.6	2	LC-hrMS
KRISS, Korea (Rep. of)	24.92	0.46	1.05	2.306	nanoLC-MS/MS

Figure 3: Overall peptide related impurities (PepImp) mass fractions reported by participants in CCQM-P55.2.2018 - plotted with expanded uncertainties (U) at a confidence level of about 95 %

In general, the CCQM-K115.2018 and CCQM-P55.2.2018 comparison on VE purity shows less agreement of participants' results as the previous CCQM-K115/CCQM-P55.2 series comparisons on hCP and OXT for peptide purity determinations. The peptide related impurity (PepImp) determinations showed a superior level of agreement as for hCP and inferior level of agreement as for OXT. However, there was discussion on possible reasons for the discrepancy between CCQM-K115.2018/CCQM-P55.2.2018 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 3) is still a weak point as for both comparison on hCP and OXT as described in detail in the CCQM-K115.2018 Final Report [24]. The number of potential impurities is much smaller for VE compared with both hCP and OXT as VE exhibits a shorter primary sequence. All laboratories have identified/quantified the larger peptide related impurity VE+Me resulting in mainly coherent estimations of the peptide related impurity mass fractions. However, the major peptide impurity, VE depsipeptide, has only been correctly identified and quantified by the NRC as described in detail in the CCQM-K115.2018 Final Report [24]. Hence the pilot study participants have underestimated the sum of peptide related impurity mass fractions. A few key comparison participants, for example BIPM, LGC, HSA and LNE, have observed an additional broad peak but it was not identified as VE depsipeptide. It has been discussed if that peak could relate to the VE depsipeptide if certain solvent conditions are maintained in LC-MS analysis as the VE 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 [24].

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. Three laboratories have used synthesized impurity standards to quantify the major impurity VE. Two participants have quantified the peptide related impurities using a response factor (RF = 1) method. 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.

The BIPM and UME have used the PICqNMR approached in CCQM-P55.2.2018. All other participants have used the PICAA approach. BIPM has used microwave assisted hydrolysis. KRISS and NIM 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-K115.2018

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 5 shows more details on the peptide related impurities of the CCQM-K115.2018 or CCQM-P55.2.2018 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 PICqNMR 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 VHLTPE(OMe).

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.

Three large peptide related impurities [1Ψ2, C(NH2)=N]VHLTPE (or equivalent impurities with a -0.98 mass shift related to VE as uniquely identifiable), VHLTPE(OMe) and VHLTPEE have been identified and quantified by all five pilot study laboratories. However, the major peptide impurity, VE depsipeptide, has only been correctly identified and quantified by the NRC via ^{1}H-NMR in the parallel key comparison CCQM-K115.2018 [24]. 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 VE 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 4. The formed VE depsipeptide exists as a mixture of *cis-trans* isomers in solution [25-27].
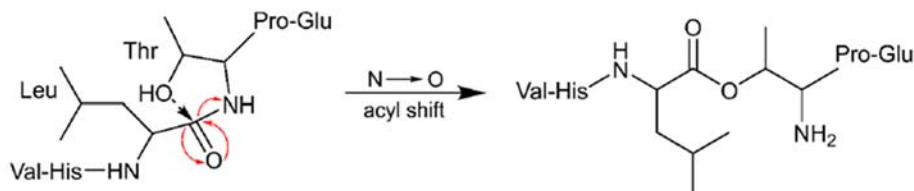

Figure 4:   N to O acyl shift exemplarily depicted for non-glycated hexapeptide (VE)

The NRC has identified and quantified both *cis-* and *trans*-isomers of the VE depsipeptide via ^{1}H-NMR at mass fraction levels of 5.9 ± 3.6 mg/g ($k = 2$) and 14.2 ± 8.6 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 as the BIPM in the pilot study. The BIPM has agreed during the PAWG meeting in November 2020 to re-assess their own data concerning the presence of VE depsipeptide impurity fragments. In summary, the 2D COSY spectrum obtained on a VE sample in D_2O was re-analyzed and the VE 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 VE samples in deuteromethanol. The combined VE depsipeptide mass fraction was 15.2 ± 0.4 mg/g based on the integration of the signal due to the threonine γ-CH$_3$ protons. The VE depsipeptide mass fraction assignments of the BIPM are in agreement and confirming the findings of the NRC.

The identification and quantification of the VE depsipeptide by use of LC-(hr)MS(/MS) techniques have proved to be difficult. Initially, the VE depsipeptide impurity was missed or misinterpreted by all participants using LC-(hr)MS(/MS). It was confirmed that the VE depsipeptide is only present in freshly prepared aqueous solution of the VE material. Aqueous solutions are acidic (about pH 4) due to the high TFA content of the VE material. The VE depsipeptide peak decreased and disappeared completely after a few days (< 4 days) when the VE sample is prepared in an acidic aqueous solution (pH 4). The VE depsipeptide peak disappeared instantly when VE materials were dissolved in alkaline buffer (pH 9). The HSA investigated HPLC behavior of the VE depsipeptide using pure VE depsipeptide standard material. It was found that when pH ~ 6.0 mobile phase (20 mM ammonium acetate) was used, VE depsipeptide appeared as a broad peak right after VE peak. The same broad peak was observed in the comparison sample, which confirmed that VE depsipeptide had been accounted for as part of the total unknown impurities in HSA's report. It was also found that when pH ~2.8 mobile phase (0.1 % formic acid) was used, VE depsipeptide appeared as a broad peak at a shorter retention time than the VE peak, which was

consistent with what was observed by BIPM, LGC and LNE. In addition, HSA also provided that the VE depsipeptide transformation in alkaline or weak acid solution (pH > 4) is irreversible (no depsipeptide production upon re-acidification to pH ~ 2.5). These findings imply that the VE 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 VE 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 a quantification mismatch (0.45 mg/g instead of 9.12 mg/g) for Ac-HLTPE. Details on the VE depsipeptide issue are provided in the CCQM-K115.2018 Final Report [24].
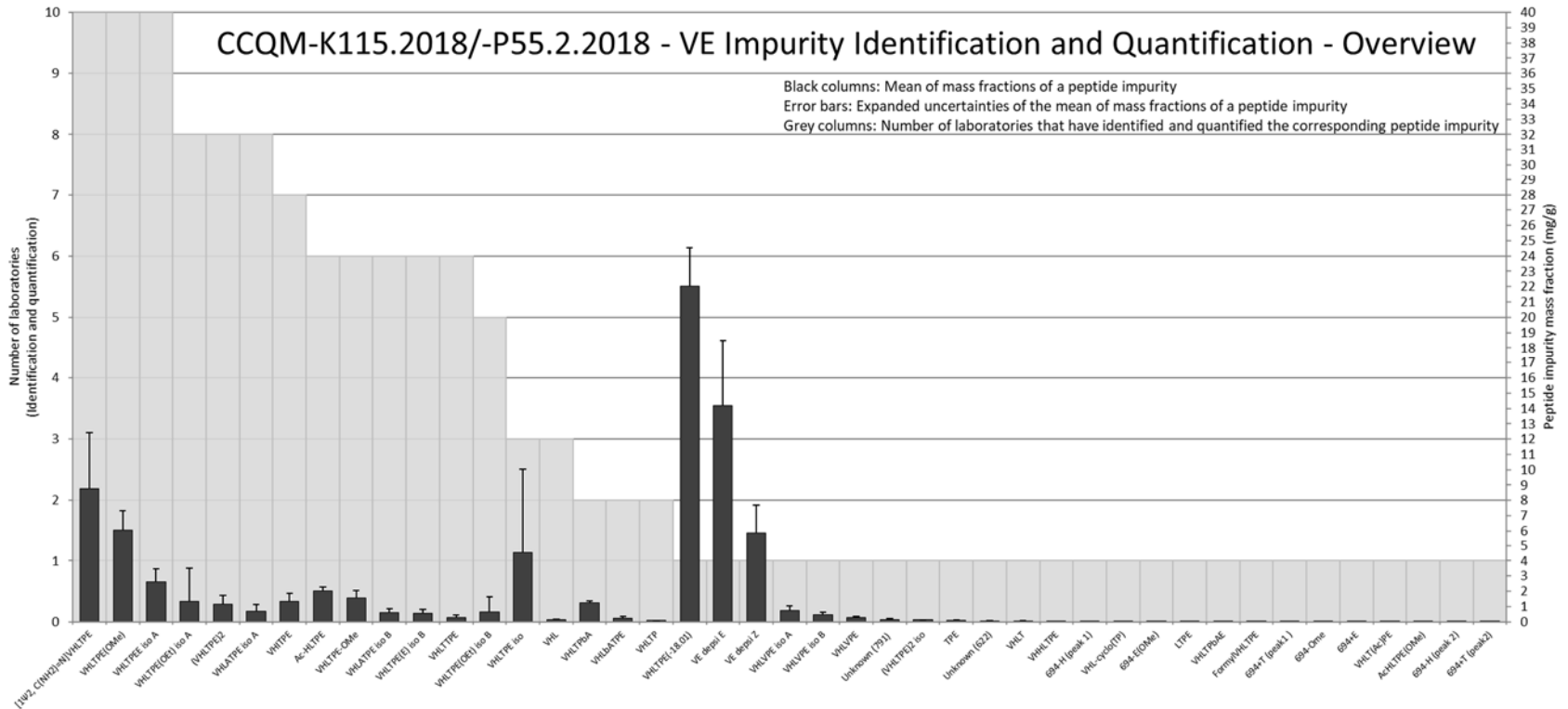
Figure 5: VE impurity identification and quantification - Overview

REFERENCE VALUES (RVS) FOR CCQM-P55.2.2018

It was agreed by the CCQM-K115.2018 and CCQM-P55.2.2018 participants that the comparison coordinator should establish an individual reference value for the mass fraction of the peptide related impurities (PepImp) present in the comparison material and assign an overall reference value for the mass fraction of VE. The key comparison reference values (KCRVs) of the parallel CCQM-K115.2018 have been adopted as reference values (RVs) of CCQM-P55.2.2018. The approaches to establish the KCRVs for VE and its impurity are described in detail in the Final Report on CCQM-K115.2018 [24].

Impurity Profile and Reference Value (RV) for Mass Fraction of Peptide Related Impurities in CCQM-P55.2.2018

The reference value ($RV_{PepImp} = KCRV_{PepImp}$) for the mass fraction of peptide impurities is based on the assumption that only the most consistent set of results of the parallel CCQM-K115.2018 key comparison is taken for the calculation of the RV_{PepImp}. The sum of the combined *cis/trans* VE depsipeptide impurities (only identified/quantified by NRC and confirmed by BIPM and HSA) and the means of the mass fractions of peptide related impurities that have been identified by at least two participants according to Figure 5 (impurities starting on the left until VHTLP inclusive) have been used to establish the RV_{PepImp}. The corresponding standard uncertainty ($u(RV_{PepImp})$) of the RV_{PepImp} is the combined uncertainty of the individual uncertainties provided by the participants for the individual peptide impurities that have been considered. Peptide related impurities that have not been confirmed by at least one other participant are not considered.

Figure 6 shows the participant results with their reported standard uncertainties plotted against the RV_{PepImp} of 53.0 mg/g for peptide impurities in CCQM-K115.2018 (solid line) and its corresponding standard uncertainty of 8.6 mg/g ($k = 1$). A corresponding expanded uncertainty of 17.3 mg/g ($k = 2$) at a confidence level of about 95 % was calculated. The level of disagreement with the RV_{PepImp} indicates that all pilot study participants have underestimated the sum of peptide related impurity mass fractions because all of them have omitted to identify and quantify the major related peptide impurity, VE depsipeptide.
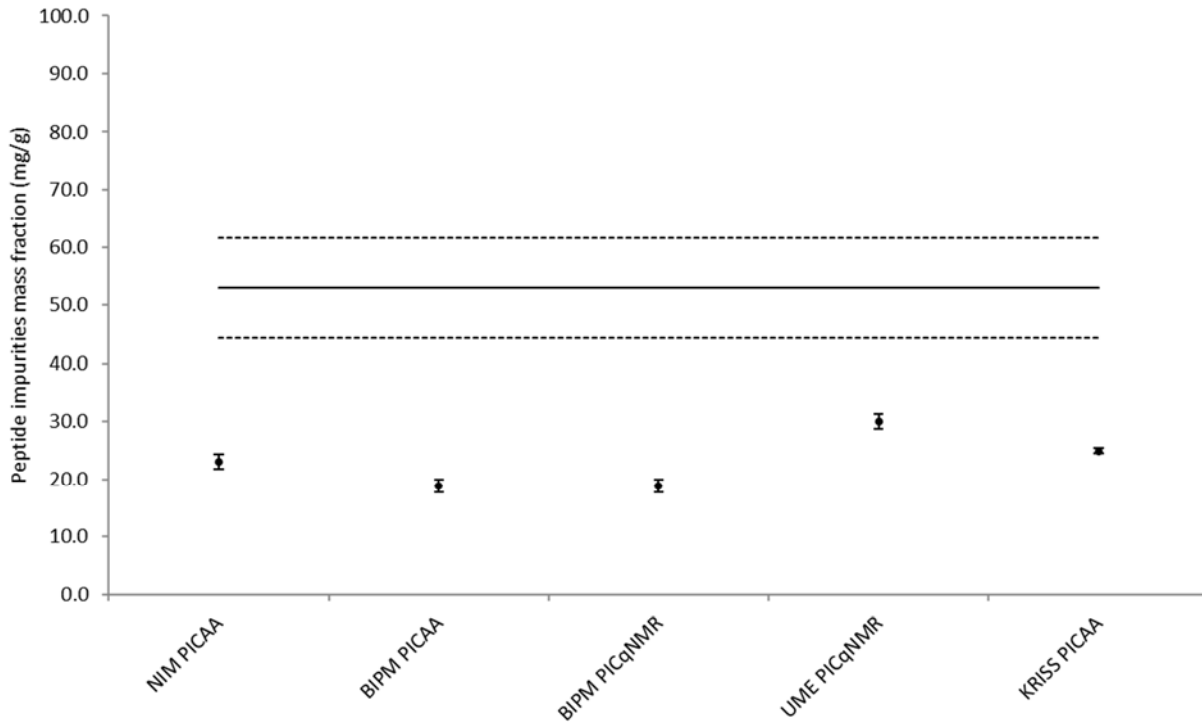
Figure 6: Estimates of total related peptide impurities in CCQM-P55.2.2018 plotted with their reported standard uncertainties ($\pm u_c$, $k = 1$). The RV_{PepImp} (solid line) is 53.0 mg/g. Dashed lines show the $u(RV_{PepImp})$ ($k = 1$) of the RV_{PepImp}.

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

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

The expanded uncertainty U_i at a confidence level of about 95 % associated with the D_i was calculated as [28]:

$$U_{95\%}(D_i) = 2 \cdot \sqrt{u(w_i)^2 + u(RV_{PepImp})^2}$$

Figure 7 indicates the degree of equivalence (D_i) of each pilot study participant's result with the RV_{PepImp} for related peptide impurities. The corresponding values are listed in Table 4. The level of disagreement with the RV_{PepImp} indicates that all pilot study participants have underestimated the sum of peptide related impurity mass fractions because all of them have omitted to identify and quantify the major related peptide impurity, VE depsipeptide.
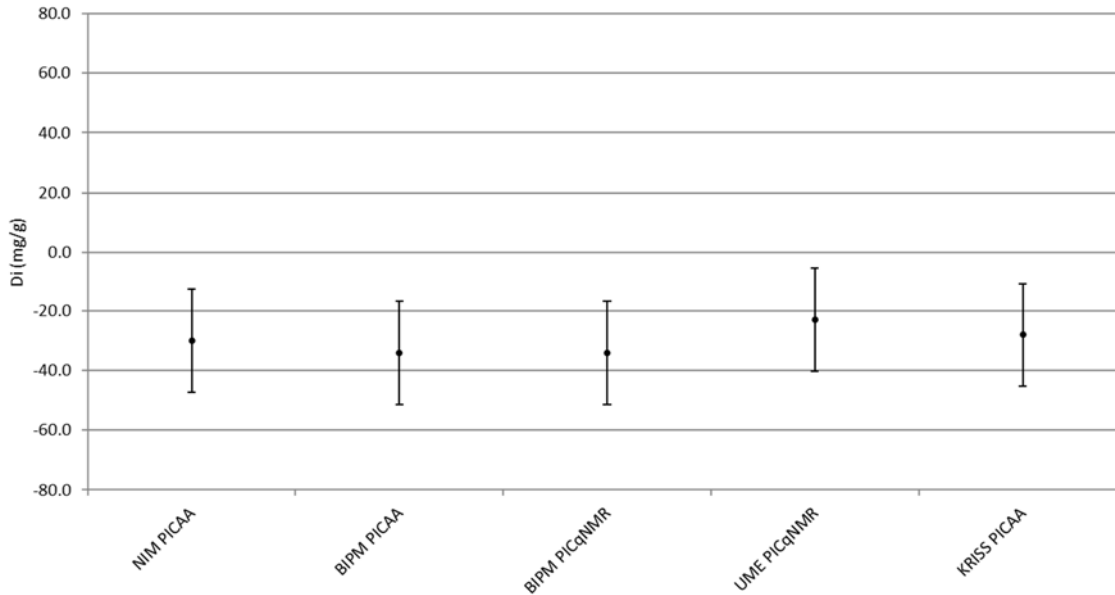
Figure 7: Degree of equivalence for the CCQM-P55.2.2018 with the RV$_{PepImp}$ for total related peptide impurities for each participant. Points are plotted with the associated expanded uncertainty in the degree of equivalence corresponding to a confidence level of about 95 %.

Table 4: Degrees of equivalence D_i and expanded uncertainties $U(D_i)$ at a confidence level of about 95 % in mg/g for CCQM-P55.2.2018 with the RV$_{PepImp}$ for total related peptide impurities

	D_i	$U(D_i)$
NIM PICAA	-30.0	17.5
BIPM PICAA	-34.1	17.4
BIPM PICqNMR	-34.1	17.4
UME PICqNMR	-23.0	17.5
KRISS PICAA	-28.0	17.3

Reference Value (RV) for the Mass Fraction of VE in CCQM-P55.2.2018

The reference value (RV_{VE} = $KCRV_{VE}$) for the mass fraction of VE is based on a mass balance calculation that takes into account the most consistent set of results for the peptide related impurities RV_{PepImp}, TFA mass fraction and the water mass fraction [9]. The input values, data evaluation, measurement equation to assign the RV_{VE} of VE and its corresponding standard uncertainty ($u(RV_{VE})$) is described in detail in the Final Report on CCQM-K115.2018 [24] and values have been summarized in Table 5.

Table 5: Input values for impurities used for the calculation of RV_{VE} and corresponding combined standard uncertainty in CCQM-P55.2.2018

	w (mg/g)	n	u_w (mg/g)
Peptide related impurities ($KCRV_{PepImp}$)	53.0	large	8.6
Water	47.5	large	4.1
TFA	286.7	large	2.3
$KCRV_{VE}$	**613**		**10**

Figure 8 shows the participant results with their reported standard uncertainties plotted against the RV_{VE} of 613 mg/g for VE in CCQM-K115.2018 (solid line) and its corresponding standard uncertainty of 10 mg/g ($k = 1$). A corresponding expanded uncertainty of 20 mg/g ($k = 2$) at a confidence level of about 95 % was calculated.
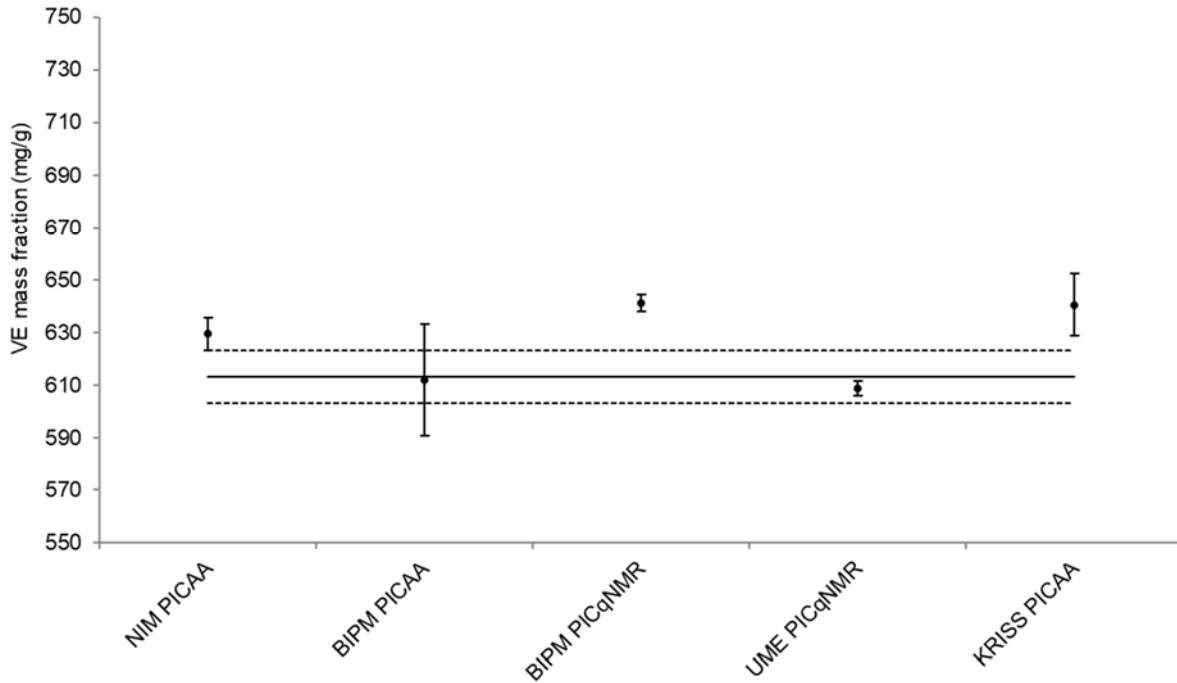
Figure 8: Mass fraction estimates by participants for VE in CCQM-P55.2.2018 with their reported combined standard uncertainties ($\pm u_c$, $k = 1$). The RV$_{VE}$ for CCQM-K115.2018 (solid line) is 613 mg/g. The calculated combined standard uncertainty of the RV$_{VE}$ is $\pm$ 10 mg/g. Dashed lines show the u(RV$_{VE}$) ($k = 1$) of the RV$_{VE}$.

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

$$D_i = w_i - RV_{VE}$$

The expanded uncertainty U$_i$ at a confidence level of about 95 % associated with the D_i was calculated as [28]:

$$U_{95\,\%}(D_i) = 2 \cdot \sqrt{u(w_i)^2 + u(RV_{VE})^2}$$

Figure 9 indicates the degree of equivalence (D_i) of each pilot study participant's result with the RV$_{VE}$ for VE. The corresponding values are listed in Table 6. The VE purity values of the pilot study participants using PICAA agree with the RV$_{PepImp}$ whereas the value of the BIPM using PICqNMR disagrees with the as the RV$_{PepImp}$.
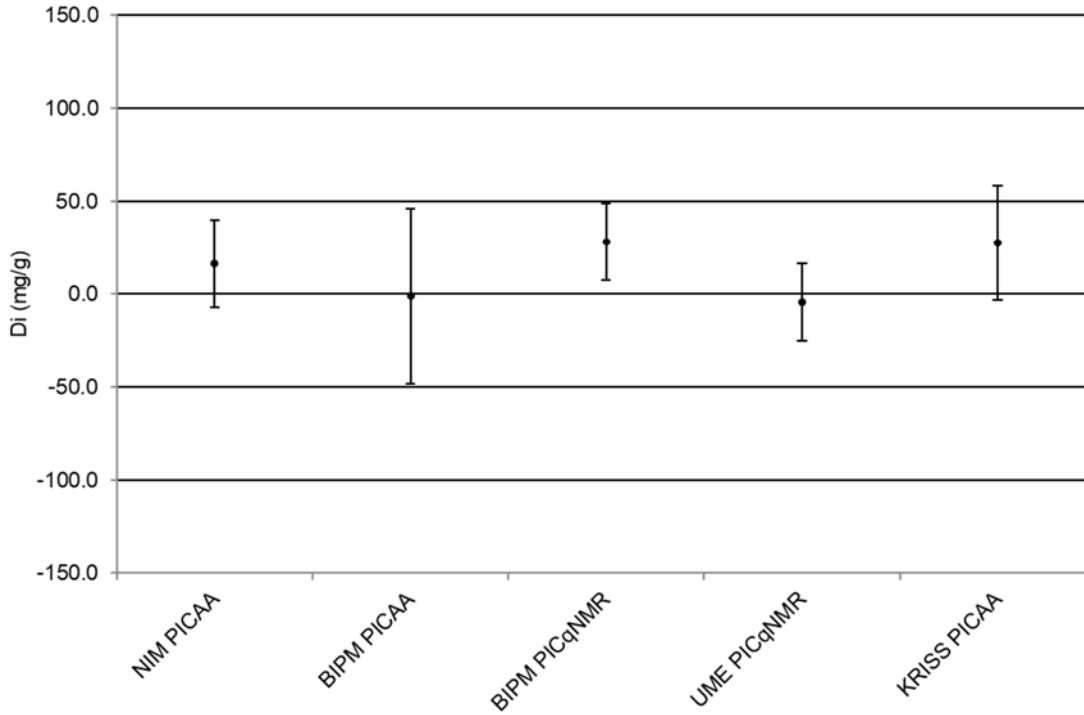
Figure 9: Degree of equivalence for the CCQM-P55.2.2018 with the RV_{VE} for VE for each participant. Points are plotted with the associated expanded uncertainty in the degree of equivalence corresponding to a confidence level of about 95 %.

Table 6: Degrees of equivalence D_i and expanded uncertainties $U(D_i)$ at a confidence level of about 95 % in mg/g for CCQM-P55.2.2018 with the RV_{VE} for VE

	D_i	$U(D_i)$
NIM PICAA	16.5	23.5
BIPM PICAA	-1.1	47.1
BIPM PICqNMR	28.2	20.9
UME PICqNMR	-4.3	20.8
KRISS PICAA	27.4	30.8

CONCLUSIONS

VE was selected to be representative of chemically synthesized linear peptides of known sequence, without cross-links, up to 5 kDa and without modification. 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 VE 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 VE purity. This allows participants to demonstrate the efficacy of their implementation of the approaches used to determine the VE 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 VE mass fraction assignment.

The key comparison reference values (KCRVs) of CCQM K115.2018 have been adopted as reference values (RVs) of CCQM-P55.2.2018. The approaches to establish the KCRVs for VE and its impurity are described in detail in the Final Report on CCQM-K115.2018 [24].

The RV_{PepImp} for the mass fraction of peptide impurities is based on the assumption that only the most consistent set of results of the parallel CCQM-K115.2018 key comparison [24] is taken for the calculation of the RV_{PepImp}. The sum of the combined *cis/trans* VE depsipeptide impurities (only identified/quantified by NRC and confirmed by BIPM and HSA) and mass fractions of peptide related impurities that have been identified by at least two participants have been used to establish the RV_{PepImp}. The corresponding standard uncertainty ($u(RV_{PepImp})$) of the RV_{PepImp} is the combined uncertainty of the individual uncertainties provided by the participants for the individual peptide impurities that have been considered. Consequently, the RV_{PepImp} of 53.0 mg/g is associated with a relatively large corresponding expanded uncertainty of ± 17.3 mg/g ($k = 2$) providing a more realistic basis of evaluation for the capabilities of the participants to identify/quantify peptide related impurities. Anyway, none of the VE related peptide impurity mass fraction results for CCQM-P55.2.2018 are in agreement with the RV_{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 all cases the major related peptide impurity, VE depsipeptide, has not been identified and quantified.

The RV_{VE} for the mass fraction of VE is based on a mass balance calculation that takes into account the most consistent set of results for the peptide related impurities RV_{PepImp}, TFA mass fraction and the water mass fraction obtained from the parallel CCQM-K115.2018 key comparison.

The RV_{VE} is 613 mg/g with a corresponding expanded uncertainty of the RV_{VE} of ± 20 mg/g ($k = 2$). Inspection of the degree of equivalence plots for CCQM-P55.2.2018 for the mass fraction of VE shows that all results obtained by PICAA agree with the RV_{VE} while the result obtained by the BIPM with PICqNMR disagrees with the RV_{VE}.

The VE material is not sufficient 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)

The pilot study CCQM-P55.2.2018 cannot be used to support CMC claims as the pilot study has its parallel key comparison CCQM-K115.2018. However, successful participation in the CCQM-K115.2018 comparison will support CMCs for:

- chemically synthesized peptides of known sequence, without cross-links, up to 5 kDa and without modifications. 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 CCQM-K115.2018 key 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.

The hexapeptide of HbA0 (VHLTPE or VE) has been proposed as the comparison material, since:

- it will allow the generic capabilities listed above to be demonstrated for non-modified 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 [29];
- Hemoglobin A1c (HbA1c) is an important analyte for which reference methods have been developed in laboratory medicine [30-35] where VE is the signature peptide for the quantification of HbA0.

ACKNOWLEDGEMENTS

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