Pilot study on peptide purity: synthetic human C-peptide

Josephs, R. D.; Li, M.; Song, D.; Daireaux, A.; Choteau, T.; Stoppacher, N.; Westwood, S.; Wielgosz, R.; Xiao, P.; Liu, Y.; Gao, X.; Zhang, C.; Zhang, T.; Mi, W.; Quan, C.; Huang, T.; Li, H.; Melanson, J. E.; Ün, I.; Gören, A. C.; Quaglia, M.; Warren, J.

Publisher's version / Version de l'éditeur:
https://doi.org/10.1088/0026-1394/54/1A/08011
Metrologia, 54, Technical Suppl. 1A, pp. 08011-08011, 2017-01-01

CCQM-P55.2

Pilot Study on Peptide Purity - Synthetic Human C-Peptide

Final Report

January 2017

Prepared by:

Ralf D. Josephs
Bureau International des Poids et Mesures (BIPM)
Sèvres, France

Coordination laboratories:

Ralf D. Josephs*, Ming Li*[#], Dewei Song*[#], Adeline Daireaux*, Tiphaine Choteau*, Norbert Stoppacher*, Steven Westwood*, Robert Wielgosz*, Peng Xiao[#], Yu Liu[#], Xing Gao[#], Chunli Zhang[#], Tianji Zhang[#], Wei Mi[#], Can Quan[#], Ting Huang[#], Hongmei Li[#]
* Bureau International des Poids et Mesures (BIPM)
Sèvres, France
[#] National Institute of Metrology (NIM)
Beijing, China

With contributions from:

Jeremy E. Melanson
Measurement Science and Standards - National Research Council of Canada (NRC)
Ottawa, Canada

Ilker Ün, Ahmet Ceyhan Gören
National Metrology Institute of Turkey (TUBITAK UME)
Gebze-Kocaeli, Turkey

Milena Quaglia, John Warren
LGC Limited (LGC)
Teddington, United Kingdom

Coordinating laboratory contact: Ralf D. Josephs (ralf.josephs@bipm.org)

TABLE OF CONTENT

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). 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 such as peptides/proteins.

The provision of Primary Calibration Reference Services has been identified as a core technical competency for NMIs [1]. NMIs 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/P55.2 comparison [2]. With the aim of leveraging the work required for the CCQM-K115 comparison and thereby minimising the workload for NMIs and simultaneously focussing on a material directly relevant to existing CMC claims, human C-peptide (hCP) was proposed as the most appropriate choice for a study material for a first CCQM key comparison (CCQM-K115) and parallel pilot study (CCQM-P55.2) looking at competencies to perform peptide purity mass fraction assignment.

The CCQM-K115 and -P55.2 comparison for hCP (marked as black star) and other peptides of current interest to NMIs (marked in black) are marked in the model for the classification of peptides for primary structure purity determinations (Figure 1). CCQM-K115 and -P55.2 covers the space of quadrant A for short (1 kDa to 5 kDa), non-cross-linked synthetic peptides [3].

Peptide	AAs	kDa	SSs
OXT	9	1	1
ANG I	10	1.2	0
hHEP-25	25	2.8	4
hCP	31	3	0
CALC1	32	3.4	1
BNP	32	3.5	1
Aβ-42	42	4.5	0
hINS	51	5.8	3
IGF-1	70	7.6	3
PTH	84	9.4	0

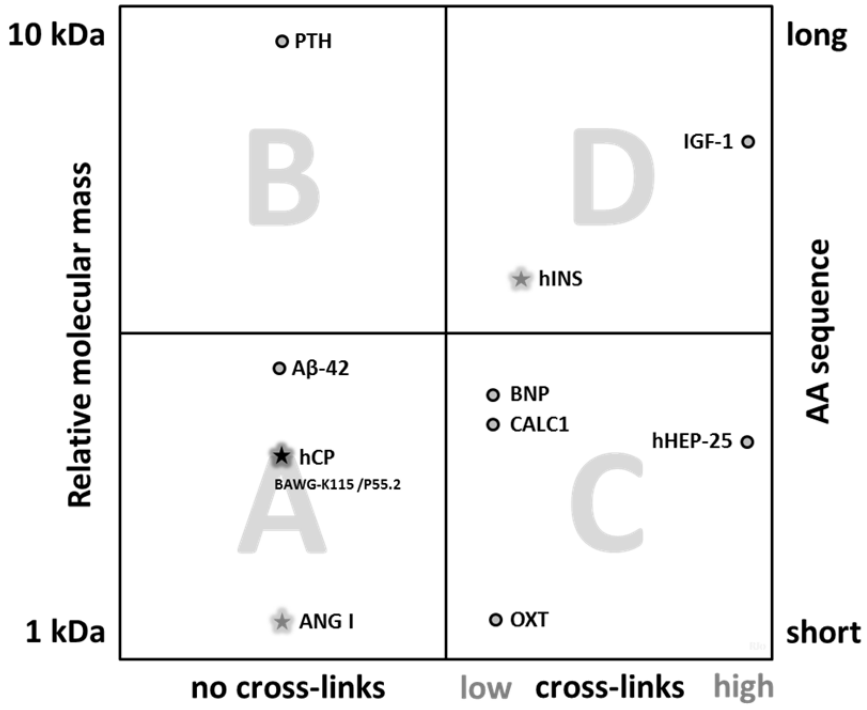

Figure 1: Model for the classification of peptides for primary structure purity determinations

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 National Measurement Institutes (NMIs) 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) [4]. NMIs 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, 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 [5]. These approaches have been successfully applied to a large variety of small molecules [6-10].

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 [11-13]. These approaches have been investigated for example for the routine analysis of human growth hormone and its biomarkers [14-15]. A number of NMIs have been developing higher order measurement procedures for the analysis of purified protein calibrators [16] and serum based matrix materials [15]. 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.

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 [17-18]. 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 recently assessed in the CCQM-K55.c comparison in the frame of the OAWG [10]. 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 [19].

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 spectroscopy (qNMR).

The timeline for the CCQM-P55.2 study ‘Pilot Study on Peptide Purity - Synthetic Human C-Peptide’ parallel to CCQM-K115 is summarized in Table 1.

Table 1: CCQM-P55.2 Timetable

Action	Date
Initial discussion	April 2012 BAWG/OAWG meetings
Approval of Study Proposal	April 2013 BAWG meeting
Draft protocol and confirmation	April 2014 BAWG meeting
Sample characterization completed	August 2014
Call for participation	October 1st, 2014
Final date to register	October 31st, 2014
Sample distribution	November 2014 (following BAWG meeting)
Date due to coordinator	September 1st, 2015
Justification for 10 months period	3 months for identification of impurities 3 - 4 months to obtain tailor-made impurities 3 months for quantification and calculation
Initial report and discussion of results	November 2015 PAWG meeting
Draft A report and discussion	April 2016 PAWG/OAWG meeting
Draft B report	October 2016
Final report to PAWG Chair	January 2017

CHARACTERIZATION OF STUDY MATERIAL

Human C-peptide is defined as human proinsulin [57-87] fragment with the amino acid sequence EAEDLQVGQVELGGGPGAGSLQPLALEGSLQ [relative molecular mass (M_r) 3020.3]. The study material was prepared by the BIPM/NIM by characterization of a commercially sourced sample of synthetic human C-peptide. The methods used to investigate, assign and confirm the quantitative composition of the CCQM-K115 and -P55.2 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 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

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

- IC for common elements and counter ions (acetate, chloride, formate, nitrate, oxalate, phosphate, sulfate, trifluoroacetate (TFA), ammonium, calcium, magnesium, potassium, sodium)
- Microanalysis (% C, H, N content) as a consistency check for the assigned values

The BIPM/NIM have

- investigated the levels of within and between vial homogeneity of the main component and all 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 K115/P55.2 candidate material vials were evaluated for impurity profile, homogeneity, stability and water adsorption/desorption by the BIPM/NIM. The mass fraction of the hCP in the comparison material was assessed by the BIPM to be about 800 mg/g while the homogeneity and stability of the hCP and peptide related impurities were shown to be suitable for the purpose of the comparison. Sorption balance measurements indicated that weighings of the CCQM-K115/P55.2 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 hCP mass fraction and of the methods used to investigate, assign and confirm the composition of the CCQM-K115/P55.2 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 Draft B Report [20].

SAMPLE DISTRIBUTION

One unit of the study sample, each containing a minimum of 25 mg of material, was distributed to each participant by express mail service in insulated and cooled boxes equipped with an electronic temperature data logger. Participants were asked to return the temperature data logger form acknowledging receipt of the samples and to advise the coordinator if any obvious damage had occurred to the vials during shipping. The coordinator verified that the temperature data logger inside the shipping container had not registered a temperature in excess of 22 °C during the transport process.
With the exception of AECOM, USA all other registered participants in the CCQM-P55.2 comparison provided a result for their sample.

QUANTITIES AND UNITS

Participants were required to report the mass fraction of hCP, the major component of the comparison sample. In addition, all participants who used a PICAA or qNMR procedure to determine the hCP content were asked to report the combined mass fraction assignment and corresponding uncertainty for total related peptide impurities.
In addition, the BIPM and NIM, China who employed a mass balance (summation of impurities) procedure to determine the hCP content 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 Draft B Report [20].

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

REPORTED MASS FRACTIONS OF HCP AND IMPURITIES IN CCQM-P55.2

The values reported by participants for the hCP mass fraction in CCQM-P55.2 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 are given in Table 3 with a summary plot in Figure 3.

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

Participant	Mass fractions (mg/g)			Coverage Factor (k)	Approach
	hCP	u(hCP)	U(hCP)		
NIM, China	766.95	9.51	19.02	2	PICAA
NIM, China	778.6	19.3	38.5	2	qNMR*
LGC, United Kingdom	806.8	9.1	40.2	4.3	qNMR
BIPM	814.79	+28.16/-28.19	+56.32/-56.37	2	PICAA
NRC, Canada	827.12	8.49	17.0	2	qNMR
BIPM	827.91	+7.61/-7.62	+15.21/-15.23	2	PICCHN
UME, Turkey	853.154	4.030	8.060	2	qNMR
AECOM, USA	-	-	-	-	-

*hCP mass fraction value assignment has been performed by use of an in-house high purity hCP.

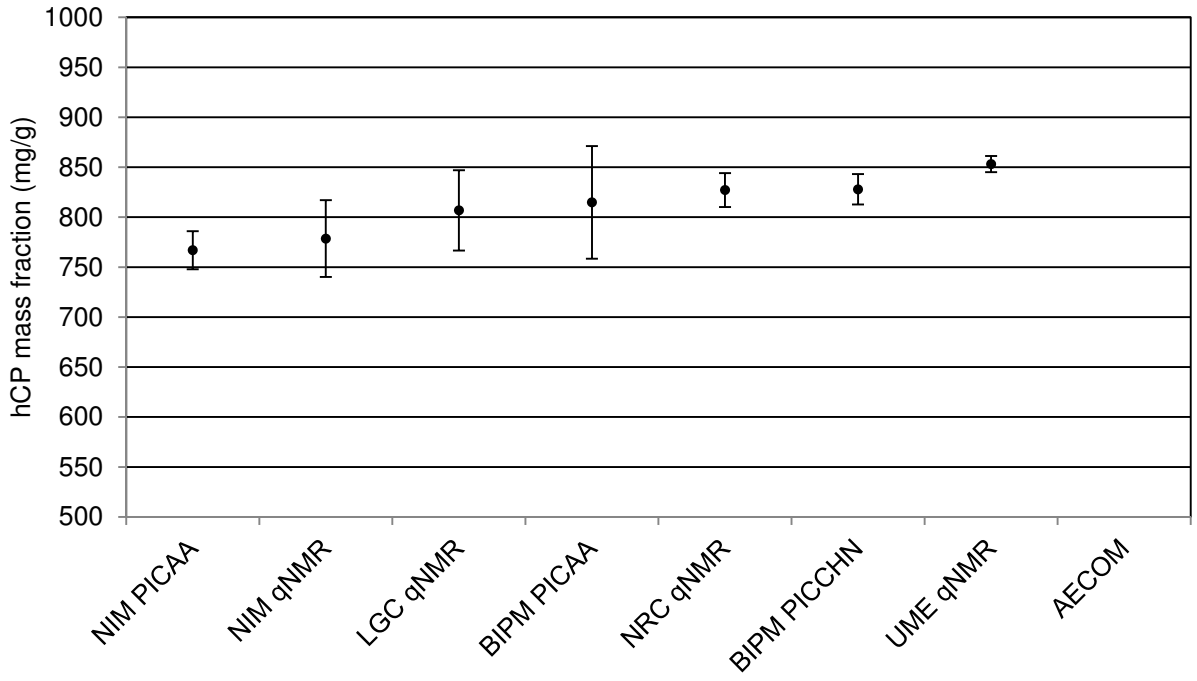

Figure 2: hCP mass fractions reported by participants in CCQM-P55.2 - plotted with expanded uncertainties (U) at a confidence level of about 95 %

The reported values for the hCP mass fractions in CCQM-P55.2 can be divided into two main groups. One group with both the BIPM and NIM using peptide impurity corrected amino acid analysis (PICAA). A second group with four participants has employed quantitative nuclear magnetic resonance spectroscopy (qNMR) approaches. The BIPM was the only participant applying a peptide impurity corrected elemental analysis (PICCHN) approach.

In general, the hCP mass fraction values obtained for CCQM-P55.2 show a similar spread of results as the hCP mass fraction values obtained for CCQM-K115. The hCP mass fraction values obtained by mass balance approaches in CCQM-K115 show generally smaller uncertainties than the values obtained by the alternative approaches (PICAA, qNMR and PICCHN) employed in CCQM-P55.2.

The NIM PICAA mass fraction value for hCP is lower as the NIM has assigned higher values to the peptide related impurity mass fractions (Table 3). The related peptide impurity profile obtained by NIM for PICAA is significantly different from related peptide impurity profiles obtained by the other participants.

The hCP mass fraction values obtained by the participants using a qNMR approaches in many cases agree within their estimated uncertainties. Both NRC and UME have assigned a higher hCP mass fraction value because lower values have been assigned to the peptide related impurity mass fractions (Table 3). The NIM qNMR mass fraction value for hCP has been obtained through value assignment by use of an in-house high purity hCP material and correction for peptides containing aspartic acid.

Table 3: Results for CCQM-P55.2: 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 PICAA, China	102.58	3.86	7.71	2	LC-hrMS LC-MS/MS
NIM qNMR, China	-	-	-	-	-*
LGC qNMR, UK	60.3	2.1	4.1	2	LC-UV
BIPM PICAA/PICCHN	83.26	+1.51/-1.48	+3.02/-2.96	2	LC-hrMS
NRC qNMR, Canada	36.86	7.76	15.53	2	LC-hrMS
UME qNMR, Turkey	31.1	2.0	4.0	2	LC-tofMS
AECOM, USA	-	-	-	-	-

*hCP mass fraction value assignment has been performed by use of an in-house high purity hCP.

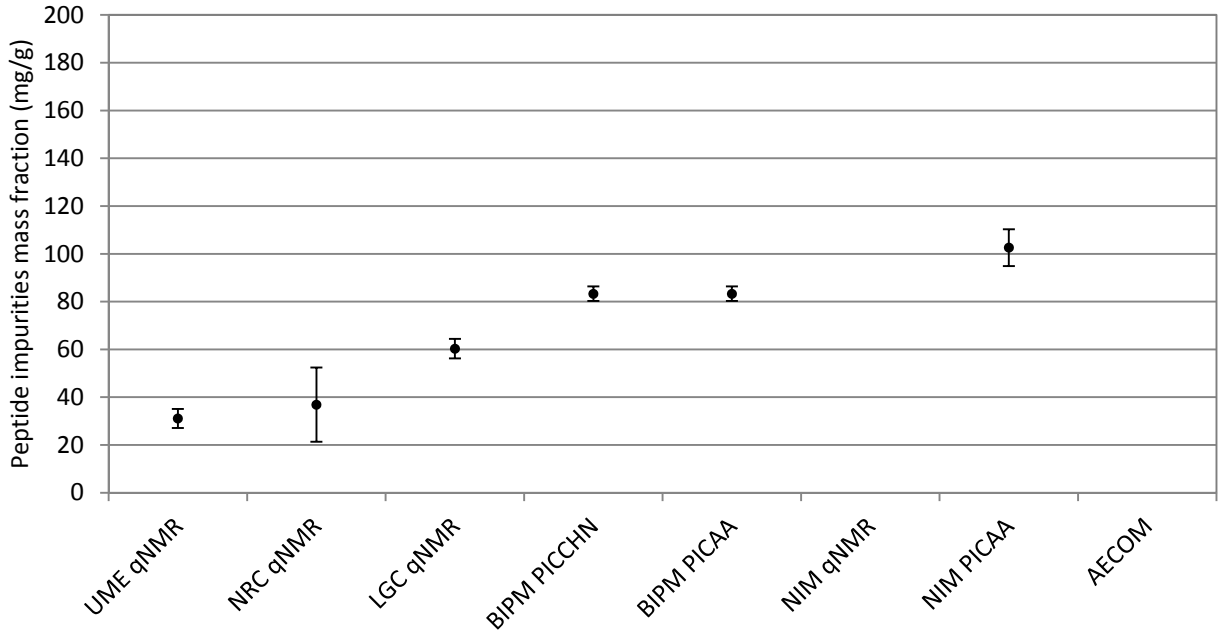

Figure 3: Overall peptide related impurities (PepImp) mass fractions reported by participants in CCQM-P55.2 - plotted with expanded uncertainties (U) at a confidence level of about 95 %

In general, there was agreement at the PAWG meeting discussions in October 2015 that the study benchmarks the real situation for services in the field of peptide purity determination. However, there was considerable discussion on possible reasons for the discrepancy between results after presentation of the results of the individual participants for CCQM-K115/P55.2.

The major shortcoming is the peptide related impurities (PepImp) identification and quantification as described in detail in the CCQM-K115 Draft B Report [20]. In many cases, only a very small number of impurities have been identified/quantified resulting in an underestimation of the peptide related impurity mass fractions and consequently in an overestimation of the mass fraction value for hCP. *Vice versa* identification/quantification of a large number of impurities results in an overestimation of the peptide related impurity mass fractions and consequently in an underestimation of the mass fraction value for hCP. It has been discussed that an overestimation of the peptide related impurity mass fraction values could be caused by insource fragmentation in LC-MS analysis due to poor chromatographic separation or other sample manipulation e.g. impurity pre-enrichment. It has also been pointed out that the use of synthesized impurity standards has a positive impact on the quantification of the peptide related impurity mass fractions. It was highlighted that the use of the response factor (RF = 1) approach could lead to an overestimation of the peptide related impurity mass fractions.
It was discussed that the impact of the hydrolysis methods employed for PICAA on the quantification of the hCP mass fraction is not clear. In addition, previous pilot studies on peptide hydrolysis have shown discrepancies in hydrolysis efficiencies for a certain peptide [19]. BIPM and NIM have used the PICAA approach in CCQM-P55.2 as both have applied a mass balance approach in CCQM-K115. BIPM has used microwave assisted vapor phase hydrolysis while NIM has applied liquid phase hydrolysis. However, both participants that have used PICAA have performed an efficiency correction for the hydrolysis methods.
It should be noted that the NIM qNMR approach doesn't provide separate information on the peptide related impurities because the hCP mass fraction value assignment has been performed by use of an in-house high purity hCP material and correction for peptides containing aspartic acid.
In addition, the NRC has independently determined trifluoroacetic acid by ^{19}F-qNMR analysis with a mass fraction and corresponding expanded uncertainty of 59.684 ± 0.973 mg/g. The LGC has identified traces of methyl *tertiary*-butyl ether with a mass fraction of less than 0.1 mg/g.

To attempt to resolve the issues additional work was requested in the following areas:

- Revision of the TFA impurity mass fraction by the BIPM;
- Breaking down the peptide related impurities values and to establish a means to visualize identification and quantification issues for the peptide related impurities;
- Calculation of the hydrolysis efficiency performance through a recalculation of all PICAA results with one consistent peptide related impurity data set.

The PAWG has decided in April 2016 to use the TFA mass fraction and corresponding combined uncertainty of 57.1 ± 1.9 mg/g obtained by the NIM by ion chromatography for the calculation of the key comparison reference value for the mass fraction of hCP. The TFA mass fraction value obtained by the NIM in CCQM-K115 has been confirmed by the NRC through qNMR in CCQM-P55.2 [20].

Peptide Related Impurity Profile of CCQM-K115/P55.2

In addition, 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 4 shows more details on the peptide related impurities of the CCQM-K115 or -P55.2 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 nine as there are nine 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 hCP+G, hCP+A, hCP+Q, hCP isomers and deahCP. 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, there is an obvious issue with the data set for peptide impurities of NIM as was already indicated during the PAWG meeting in October 2015. NIM the only laboratory that has identified and quantified the peptide impurities hCP+A at 1, hCP+Q at 1 and hCP(11-31) at very high mass fraction levels of about 22.5 mg/g, 7.92 mg/g and 6.56 mg/g, respectively. Related peptide impurities of that large mass fraction levels should have been identified and quantified by the majority of the participants as for acetyl-hCP(6-31). In addition, the same laboratory has not identified and quantified the peptide impurities dea9hCP, dea6hCP and phCP that have been identified and quantified by the majority of laboratories.

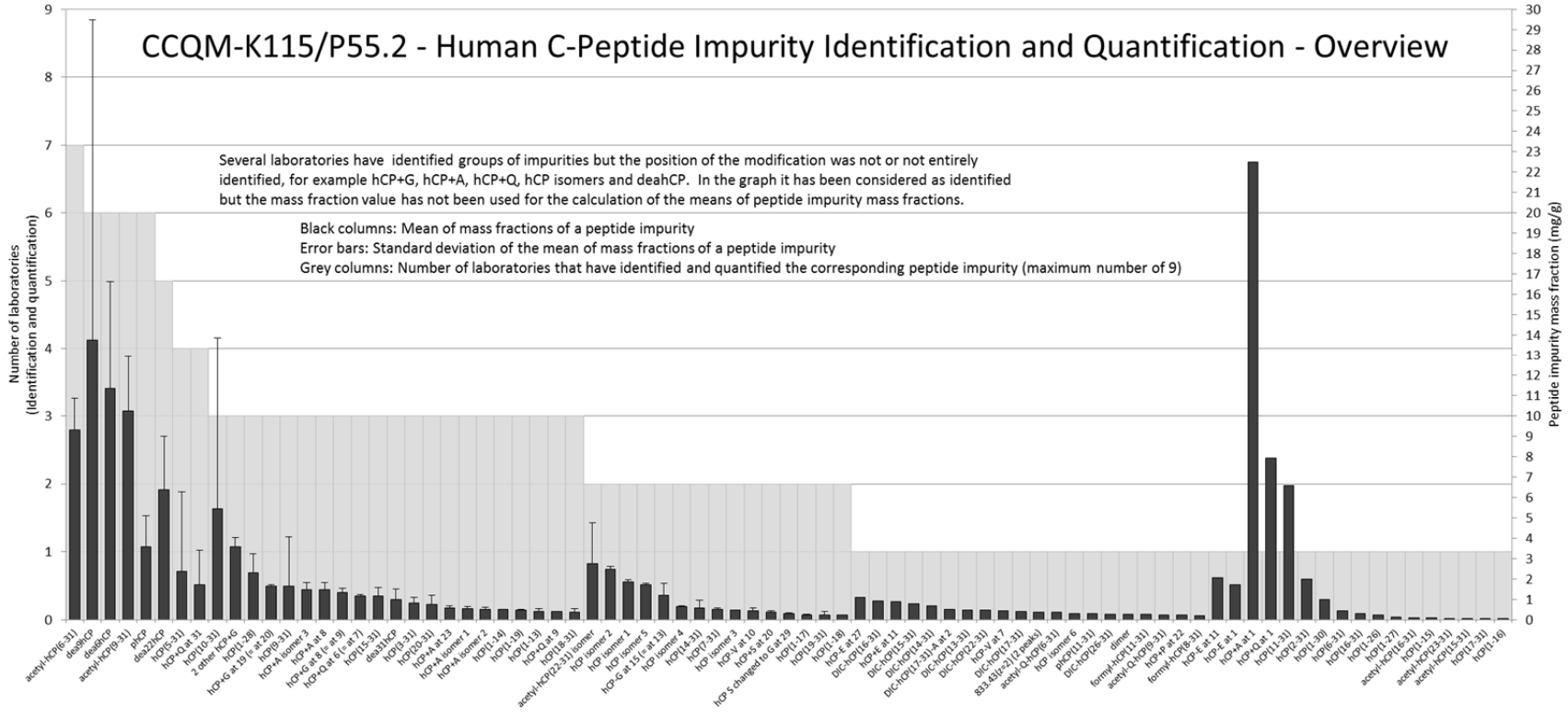

Figure 4: hCP impurity identification and quantification - Overview
(deahCP: deamidated hCP, phCP: pyroglutamylated hCP and DIC-hCP: N,N‘-Diisopropylcarbodiimide-hCP)

Hydrolysis Efficiency Study

The BIPM has calculated the hydrolysis efficiencies of the PICAA methods used in the CCQM-PAWG-K115/P55.2 studies as discussed during the PAWG meeting in October 2015. Details are provided in the CCQM-K115 Draft B Report [20]. It can be summarized that:

- the hydrolysis even of a large, 31 amino acids containing, peptides is very efficient (nearly complete) independent of the method used or amino acid analysed. However, small biases need to be corrected and/or need to be considered in the calculation of the uncertainties as it was done by the participants in CCQM-K115/P55.2;
- in general, an excellent comparability of hydrolysis efficiencies with small variances was obtained. Variances of the microwave-assisted method are slightly larger;
- the accurate identification and quantification of peptide related impurities has a larger impact on the individual results of the hCP purity (CCQM-K115/P55.2) determinations than the hydrolysis efficiency (methods used or amino acid analysed).

REFERENCE VALUES FOR CCQM-P55.2

It was agreed by the CCQM-K115/P55.2 participants that the comparison coordinator should propose 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 hCP. The values and approaches used to establish the reference values (equal key comparison reference values, KCRVs) for CCQM-K115/P55.2 are described in detail in the Draft B Report on CCQM-K115 [20].

Impurity Profile and Reference Value for the Mass Fraction of Peptide Related Impurities in CCQM-P55.2

The reference value ($KCRV_{PepImp}$) for the mass fraction of peptide related impurities is based on the assumption that only the set of results obtained by the BIPM is taken for the calculation of the $KCRV_{PepImp}$ as outlined in the Draft B Report on CCQM-K115 [20].

$KCRV_{PepImp}$ = 83.3 mg/g
$u+(KCRV_{PepImp})$ = 1.5 mg/g
$u-(KCRV_{PepImp})$ = 1.5 mg/g

The results reported by CCQM-P55.2 participants with their corresponding standard uncertainties (k = 1) plotted against the $KCRV_{PepImp}$ are presented in Figure 5. Figure 6 shows the same results with their expanded uncertainties and the $KCRV_{PepImp}$ with the corresponding expanded uncertainty at a confidence level of about 95 % (dashed lines).
It should be noted that the NIM qNMR approach doesn't provide separate information on the peptide related impurities as the hCP mass fraction value assignment has been performed by use of an in-house high purity hCP material and correction for peptides containing aspartic acid.

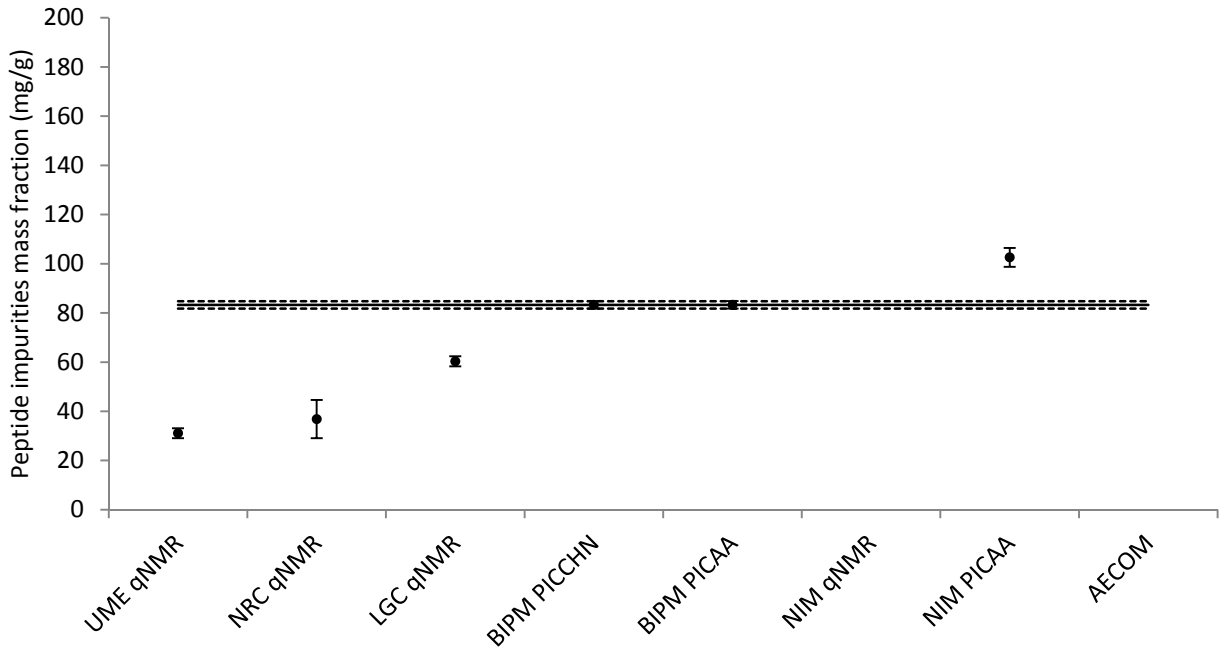

Figure 5: Estimates of total related peptide impurities in CCQM-P55.2 plotted with their reported standard uncertainties (± u_c, k = 1). The $KCRV_{PepImp}$ (solid line) is 83.3 mg/g. Dashed lines show the $u(KCRV_{PepImp})$ (k = 1).

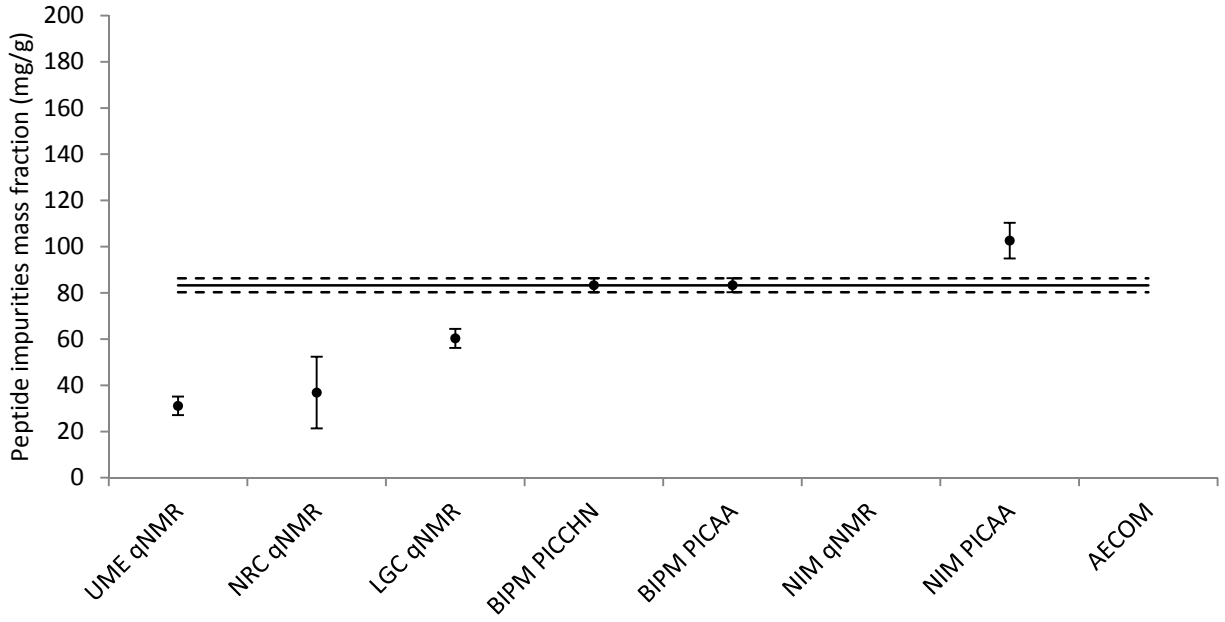

Figure 6: Mass fraction estimates by participants of total related peptide impurities in CCQM-P55.2 with their reported expanded uncertainties (± U, k = 2). The $KCRV_{PepImp}$ (solid line) is 83.3 mg/g. The calculated expanded uncertainty of the $KCRV_{PepImp}$ is +3.0/-3.0 mg/g. Dashed lines show the $U(KCRV_{PepImp})$ (k = 2).

The degree of equivalence of a participant's result w_i 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:

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

Figure 7 indicates the degree of equivalence (D_i) of each CCQM-P55.2 participant's result with the $KCRV_{PepImp}$ for related peptide impurities. The corresponding values are listed in Table 4.

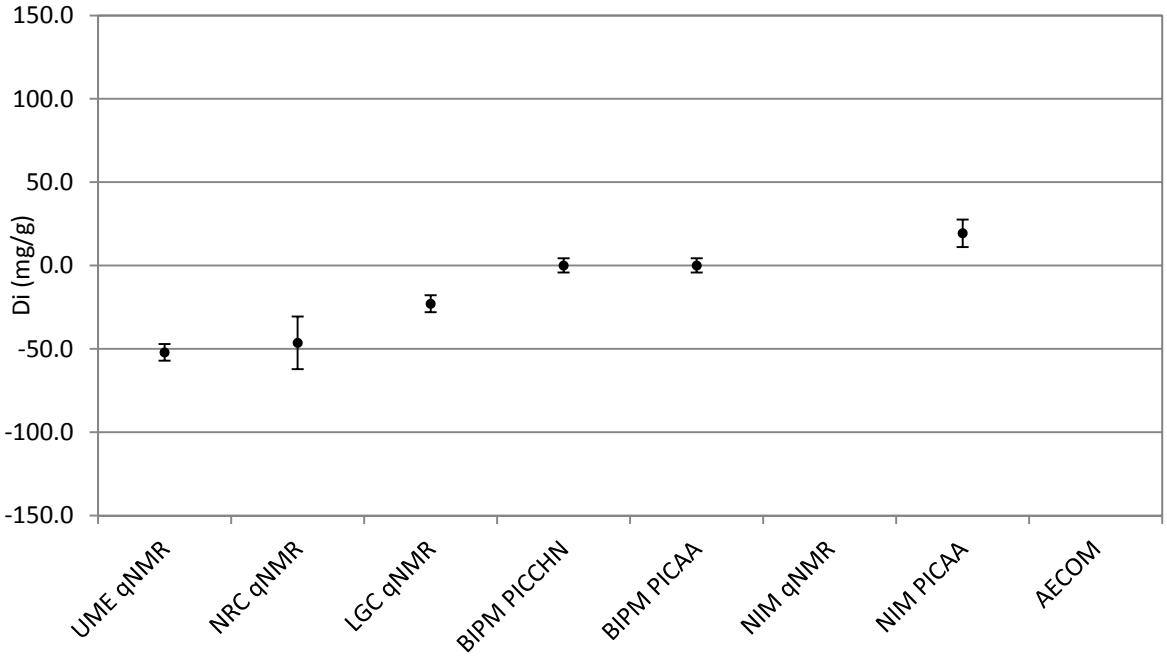

Figure 7: Degree of equivalence with the $KCRV_{PepImp}$ for total related peptide impurities for each CCQM-P55.2 participant. Points are plotted with the associated expanded uncertainty in the degree of equivalence corresponding to a confidence level of about 95 %.

Table 4: Degrees of equivalence D_i and expanded uncertainties $U(D_i)$ at a confidence level of about 95 % in mg/g for the $KCRV_{PepImp}$ for total related peptide impurities for CCQM-P55.2

	D_i	$U+(D_i)$	$U-(D_i)$
UME qNMR	-52.2	5.0	5.0
NRC qNMR	-46.4	15.8	15.8
LGC qNMR	-23.0	5.1	5.1
BIPM PICCHN	0	4.3	4.2
BIPM PICAA	0	4.3	4.2
NIM qNMR	-	-	-
NIM PICAA	19.3	8.3	8.3

Reference Value for the Mass Fraction of hCP in CCQM-P55.2

The reference value ($KCRV_{hCP}$) for the mass fraction of hCP is based on a mass balance calculation that takes into account the most exhaustive and elaborate BIPM set of results for the peptide related impurities $KCRV_{PepImp}$, the revised TFA mass fraction value from the NIM, water and other minor counter ions as described in the Final Report on CCQM-K115 [20].
The input values for impurities used for the calculation of $KCRV_{hCP}$ and the corresponding combined standard uncertainty in CCQM-K115 are given in Table 5.

Table 5: Input values for impurities used for the calculation of $KCRV_{hCP}$ and corresponding combined standard uncertainty in CCQM-K115

	w (mg/g)	n	u_w (mg/g)
Peptide related impurities ($KCRV_{PepImp}$)	83.3	large	+1.5/-1.5
Water	50.6	large	2.7
TFA	59.68	large	0.48
Cations	2.90	2	0.36
Anions	1.72	2	0.21
Volatile organics	-	4	-
$KCRV_{hCP}$	801.8		+3.1/-3.1

Figure 8 shows the CCQM-P55.2 participant results with their reported standard uncertainties plotted against the $KCRV_{hCP}$ (solid line) and its corresponding standard uncertainty (k = 1). Figure 9 shows the same results with their expanded uncertainties and the $KCRV_{hCP}$ with the corresponding expanded uncertainty at a confidence level of about 95 % (dashed lines).

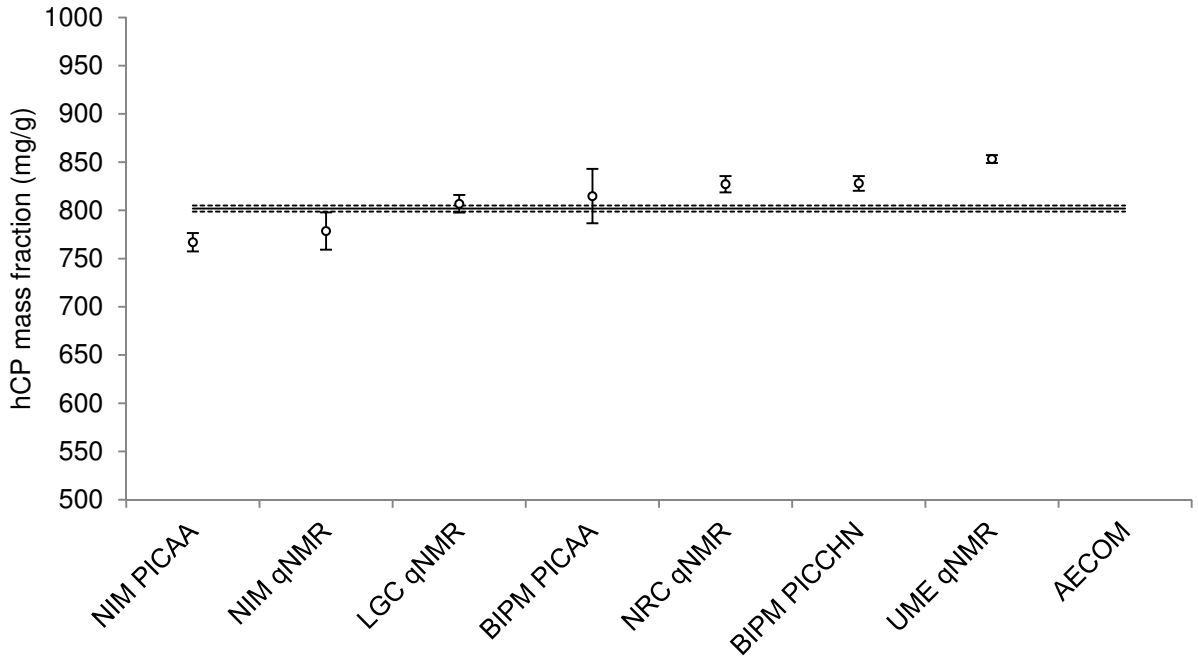

Figure 8: Mass fraction estimates by participants for hCP in CCQM-P55.2 with their reported combined standard uncertainties (± u_c, k = 1). The $KCRV_{hCP}$ (solid line) is 801.8 mg/g. The calculated combined standard uncertainty of the $KCRV_{hCP}$ is +3.1/-3.1 mg/g. Dashed lines show the u($KCRV_{hCP}$) (k = 1).

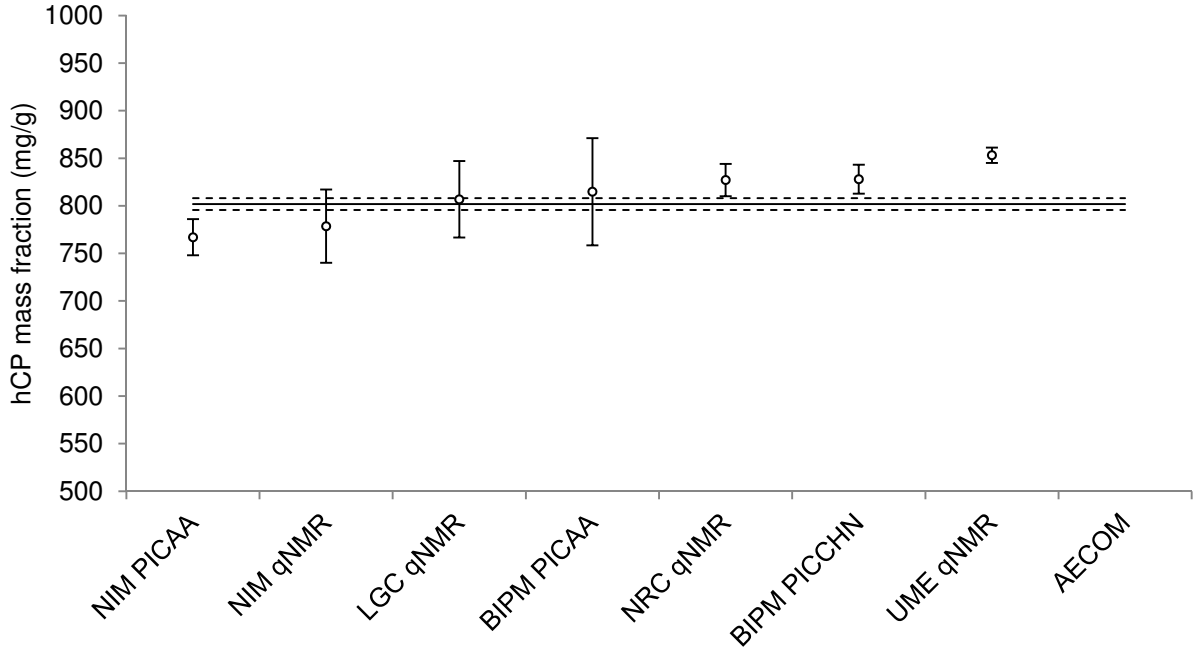

Figure 9: Mass fraction estimates by participants for hCP in CCQM-P55.2 with their reported expanded uncertainties (± U, k = 2). The $KCRV_{hCP}$ (solid line) is 801.8 mg/g. The calculated expanded uncertainty of the $KCRV_{hCP}$ is +6.2/-6.2 mg/g. Dashed lines show the U($KCRV_{hCP}$) (k = 2).

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

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

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

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

Figure 10 indicates the degree of equivalence (D_i) of each CCQM-P55.2 participant's result with the $KCRV_{hCP}$ for hCP. The corresponding values are listed in Table 6.

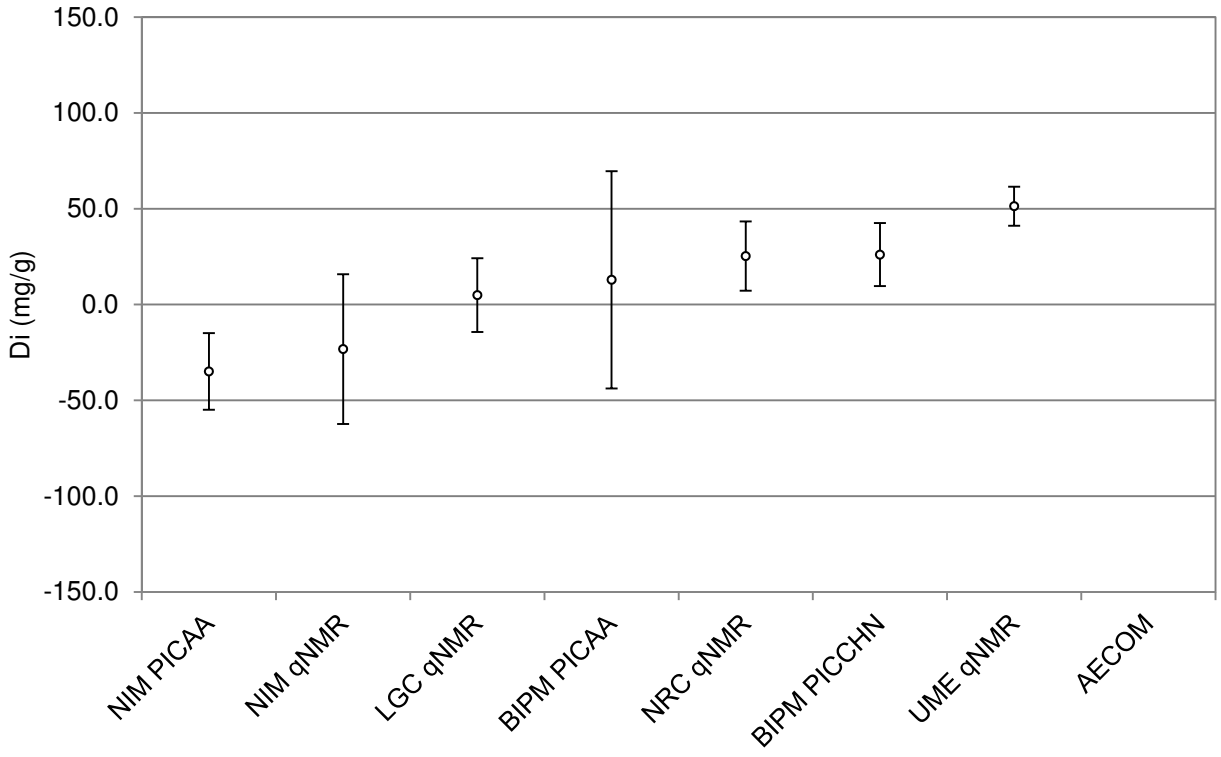

Figure 10: Degree of equivalence with the $KCRV_{hCP}$ for hCP for each CCQM-P55.2 participant. Points are plotted with the associated expanded uncertainty in the degree of equivalence corresponding to a confidence level of about 95 %.

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 with the $KCRV_{hCP}$ for hCP

	D_i	$U+(D_i)$	$U-(D_i)$
NIM PICAA	-34.9	20.0	20.0
NIM qNMR	-23.2	39.1	39.1
LGC qNMR	5.0	19.2	19.2
BIPM PICAA	12.9	56.7	56.7
NRC qNMR	25.3	18.1	18.1
BIPM PICCHN	26.1	16.4	16.5
UME qNMR	51.3	10.2	10.2

CONCLUSIONS

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

The majority of CCQM-P55.2 participants used a qNMR approach. Other participants provided results obtained by PICAA and PICCHN. It was decided to assign reference values based on the KCRVs for both the hCP 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 hCP purity. This allows participants to demonstrate the efficacy of their implementation of the approaches used to determine the hCP 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 and the hydrolysis efficiency 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 hCP mass fraction assignment.

The assessment of the mass fraction of peptide impurities is based on the assumption that only the set of results obtained by the BIPM is taken for the calculation of the $KCRV_{PepImp}$. The BIPM set of results is the most exhaustive and elaborate set of related peptide impurities that shows the largest number of overlaps with the results obtained by other participants. This approach has the advantage that it minimizes the potential overestimation of the proposed $KCRV_{PepImp}$ as many authentic standards have been used for the quantification of the peptide related impurities with the larger mass fraction values rather than an approach solely based on a response factor of 1. Consequently, the $KCRV_{PepImp}$ is associated with small uncertainties providing a more realistic basis of evaluation for the capabilities of the participants to identify/quantify peptide related impurities.
Inspection of the degree of equivalence plots for CCQM-P55.2 for the mass fraction of peptide impurities and additional information obtained from the peptide related impurity profile indicates that in many cases only a very small number of impurities have been identified and quantified resulting in an underestimation of the peptide related impurity mass fractions. One case of overestimation of the peptide related impurity mass fraction value could be caused by poor chromatographic separation and subsequent in-source fragmentation in mass spectrometry.

The approach to obtain a $KCRV_{hCP}$ for the mass fraction of hCP is based on a mass balance calculation that takes into account the most exhaustive and elaborate BIPM set of results for the peptide related impurities $KCRV_{PepImp}$, the revised TFA mass fraction from the NIM, water and

other minor counter ions. Differences in the quality of the results obtained for both peptides related impurity mass fractions and hCP mass fractions are better weighted and reflected in smaller uncertainties. The reference value ($KCRV_{hCP}$) for CCQM-P55.2 is 801.8 mg/g with a corresponding expanded uncertainty of +6.2/-6.2 mg/g.
In general, mass balance approaches show smaller uncertainties than the approaches used in CCQM-P55.2. Inspection of the degree of equivalence plots for CCQM-P55.2 for the mass fraction of hCP shows that three results agree with the reference value. The mass fraction of hCP has been overestimated by the qNMR approaches of both UME and NRC because of an underestimation of the peptide related impurity mass fractions. The NIM PICAA approach had underestimated the mass fraction of hCP as a result of an overestimation of the peptide related impurity mass fractions.

HOW FAR THE LIGHT SHINES STATEMENT (HFTLS)

Successful participation in the CCQM-P55.2 comparison will support CMCs for:

- 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.

The HFTLS statement is applicable to chemically synthesized linear peptides of known sequence, without cross-links, up to 5 kDa. Additional evidence is required to support claims related to peptides that are larger than 5 kDa, or include cross-links, or have been produced using a recombinant process.

In addition, the comparison will support traceability statements of CMCs for peptide/protein quantification which are dependent on pure peptide reference materials or methods for their value assignment for peptides meeting the above criteria.
The hCP has been proposed as the comparison material, since:

- it allows the generic capabilities listed above to be demonstrated for linear peptides without cross links and up to 5 kDa molecular mass;
- it could be obtained in sufficiently large quantities required for the comparison;
- it directly supports NMI services and certified reference materials currently being provided by NMIs [21];
- it is an important analyte for which reference methods have been developed in laboratory medicine [22-25].

ACKNOWLEDGEMENTS

The study coordinators thank all of the participating laboratories for providing all the requested information and excellent collaboration during the course of these studies.

REFERENCES

[1] J. Marriott, G. O'Connor, H. Parkes: Final Report - Study of Measurement Service and Comparison Needs for an International Measurement Infrastructure for the Biosciences and Biotechnology: Input for the BIPM Work Programme, Rapport BIPM-2011/02 (2011) http://www.bipm.org/utils/common/pdf/rapportBIPM/2011/02.pdf

[2] R. D. Josephs, M. Li, H. Li, R.I. Wielgosz: CCQM-K115/P55.2: Key Comparison on Peptide Purity: Protocol Proposal (NIM and BIPM) BAWG/xx-xx and OAWG/13-43 and (2013) BIPM webpage, CCQM BAWG/OAWG working documents.

[3] R. D. Josephs, N. Stoppacher, M. Li: Input for BAWG Drafting Group for CON 3: Routes to Macromolecular / Biomolecular Purity - Concept Paper on SI Value Assignment of Purity: Model for the classification of peptides for primary structure purity determinations, circulated for BAWG 13, Pretoria (2013) 1-10. (http://www.bipm.org/wg/CCQM/BAWG/Restricted/November_2013/BAWG_13_49_RJ_Con003.pdf)

[4] L. Mackay: CCQM OAWG Strategy Document for Rolling Programme Development, OAWG/13-02 (2013) BIPM webpage, CCQM OAWG working documents.

[5] S. Westwood, T. Choteau, A. Daireaux, R. D. Josephs, R. I. Wielgosz: Mass Balance Method for the SI Value Assignment of the Purity of Organic Compounds, Anal. Chem. 85 (2013) 3118-3126.

[6] S. Westwood, R. D. Josephs, T. Choteau, A. Daireaux, C. Mesquida, R. I. Wielgosz, A. Rosso, M. Ruiz de Arechavaleta, S. Davies, H. Wang, E. C. Pires do Rego, J. Marques Rodrigues, E. de Freitas Guimarães, M. V. Barreto Sousa, T. M. Monteiro, L. Alves das Neves Valente, F. G. Marques Violante, R. R. Ribeiro Almeida, M. C. Baptista Quaresma, R. Nogueira, A. Windust, X. Dai, X. Li, W. Zhang, M. Li, M. Shao, C. Wei, S.-K. Wong, J. Cabillic, F. Gantois, R. Philipp, D. Pfeifer, S. Hein, U.-A. Klyk-Seitz, K. Ishikawa, E. Castro, N. Gonzalez, A. Krylov, T. Tang Lin, L. Tong Kooi, M. Fernandes-Whaley, D. Prévoo, M. Archer, R. Visser, N. Nlhapo, B.J. de Vos, S. Ahn, P. Pookrod, K. Wiangnon, N. Sudsiri, K. Muaksang, C. Cherdchu, A. C. Gören, M. Bilsel, T. LeGoff, D. Bearden, M. Bedner, D. Duewer, D. Hancock, B. Lang, K. Lippa, M. Schantz, J. Sieber: Final report on key comparison CCQM-K55.b (aldrin): An international comparison of mass fraction purity assignment of aldrin, Metrologia 49 (2012) Tech. Suppl., 1A, 08014.

[7] S. Westwood, R. D. Josephs, A. Daireaux, R. I. Wielgosz, S. Davies, H. Wang, J. Rodrigues, W. Wollinger, A. Windust, M. Kang, S. Fuhai, R. Philipp, P. Kuhlich, S.-K. Wong, Y. Shimizu, M. Pérez, M. Avila, M. Fernandes-Whaley, D. Prevoo, B.J. de Vos, R. Visser, M. Archer, T. LeGoff, S. Wood, D. Bearden, M. Bedner, A. Boroujerdi, D. Duewer, D. Hancock, B. Lang, B. Porter, M. Schantz, J. Sieber, E. White, S. A. Wise: Final report on key comparison CCQM-K55.a (estradiol): An international comparison of mass fraction purity assignment of estradiol, Metrologia 49 (2012) Tech. Suppl., 1A, 08009.

[8] S. Westwood, R. D. Josephs, T. Choteau, C. Mesquida, A. Daireaux, R. Wielgosz, S. Davies, A. Windust, M. Kang, H. Ting, K. Kato, E. Frias, M. Pérez, P. Apps, M. Fernandes-Whaley, K. Wiangnon, N. Ruangrittinon, S. Wood, T. LeGoff, D. Duewer, M. Schantz, L. Siekmann, J. Esker: An international comparison of mass fraction purity assignment of digoxin: CCQM Pilot Study CCQM-P20.f (Digoxin), Metrologia 48 (2011) Tech. Suppl., 08013.

[9] S. Westwood, R. D. Josephs, A. Daireaux, R. Wielgosz, S. Davies, M. Kang, H. Ting, R. Phillip, F. Malz, Y. Shimizu, E. Frias, M. Pérez, P. Apps, M. Fernandes-Whaley, B. De Vos, K. Wiangnon, N. Ruangrittinon, S. Wood, D. Duewer, M. Schantz, M. Bedner, D. Hancock, J. Esker: An international comparison of mass fraction purity assignment of theophylline: CCQM Pilot Study CCQM-P20.e (Theophylline), Metrologia 46 (2009) 1A, 08019.

[10] S. Westwood, R. D. Josephs, T. Choteau, A. Daireaux, R. Wielgosz, S. Davies, M. Moad, B. Chan, A. Muñoz, P. Conneely, M. Ricci, E. C. Pires do Rego, B. C. Garrido, F. G. M. Violante, A. Windust, X. Dai, T. Huang, W. Zhang, F. Su, C. Quan, H. Wang, M.-F. Lo, W.-F. Wong, F. Gantois, B. Lalere, U. Dorgerloh, M. Koch, U.-A. Klyk-Seitz, D. Pfeifer, R. Philipp, C. Piechotta, S. Recknagel, R. Rothe, T. Yamazaki, O. B. Zakaria, E. Castro, M. Balderas, N. González, C. Salazar, L. Regalado, E. Valle, L. Rodríguez, L. Á. Laguna, P. Ramírez, M. Avila, J. Ibarra, L. Valle, M. Pérez, M. Arce, Y. Mitani, L. Konopelko, A. Krylov, E. Lopushanskaya, T. T. Lin, Q. Liu, L. T. Kooi, M. Fernandes-Whaley, D. Prevoo-Franzsen, N. Nhlapo, R. Visser, B. Kim, H. Lee, P. Kankaew, P. Pookrod, N. Sudsiri, K. Shearman, A. C. Gören, G. Bilsel, H. Yilmaz, M. Bilsel, M. Çergel, F. G. Çoskun, E. Uysal, S. Gündüz, I. Ün, J. Warren, D. W. Bearden, M. Bedner, D. L. Duewer, B. E. Lang, K. A. Lippa, M. M. Schantz, J. R. Sieber: Final report on key comparison CCQM-K55.c (L-(+)-Valine): Characterization of organic substances for chemical purity, Metrologia 51 (2014) Tech. Suppl., 1A, 08010.

[11] C. Pritchard, F. A. Torma, C. Hopley, M. Quaglia, G. O'Connor: Investigating microwave hydrolysis for the traceable quantification of peptide standards using gas chromatography-mass spectrometry, Anal. Biochem. 412 (2011) 1, 40-46.

[12] C. Pritchard, M. Quaglia, C. Mussell, W. I. Burkitt, H. Parkes, G. O'Connor: Fully traceable absolute protein quantification of somatropin that allows independent comparison of somatropin standards, Clin. Chem. 55 (2009) 11, 1984-1990.

[13] W. I. Burkitt, C. Pritchard, C. Arsene, A. Henrion, D. Bunk, G. O'Connor: Toward Systeme International d'Unite-traceable protein quantification: From amino acids to proteins, Anal. Biochem. 376 (2008) 2, 242-251.

[14] C. G. Arsene, R. Ohlendorf, W. Burkitt, C. Pritchard, A. Henrion, G. O'Connor, D. M. Bunk, B.Guettler: Protein Quantification by Isotope Dilution Mass Spectrometry of Proteolytic Fragments: Cleavage Rate and Accuracy, Anal. Chem. 80 (2008) 11, 4154-4160.

[15] C. G. Arsene, A. Henrion, N. Diekmann, J. Manolopoulou, M. Bidlingmaier: Quantification of growth hormone in serum by isotope dilution mass spectrometry, Anal. Biochem. 401 (2010) 2, 228-235.

[16] A. Munoz, R. Kral, H. Schimmel: Quantification of protein calibrants by amino acid analysis using isotope dilution mass spectrometry, Anal. Biochem. 408 (2011) 124-131.

[17] N. Stoppacher, R. D. Josephs, A. Daireaux, S. Westwood, R. I. Wielgosz: Impurity identification and determination for the peptide hormone angiotensin I by liquid chromatography – high resolution tandem mass spectrometry and the metrological impact on value assignments by amino acid analysis, Anal. Bioanal. Chem. 405 (2013) 8039–8051.

[18] R. D. Josephs, N. Stoppacher, A. Daireaux, T. Choteau, I. McEwan, K. Lippa, K. Phinney, S. Westwood, R. Wielgosz: A Comparison of Approaches for the SI Value Assignment of the Purity of Peptides Using the Model Compound Angiotensin I (2017) [in preparation].

[19] D. Bunk: P55.1 Scope of applicability BAWG/12-54 and Hydrolysis investigation study BAWG/12-59 (2012) BIPM webpage, CCQM BAWG working documents.

[20] R. D. Josephs, M. Li, D. Song, S. Westwood, N. Stoppacher, A. Daireaux, T. Choteau, R. Wielgosz, P. Xiao, Y. Liu, X. Gao, C. Zhang, T. Zhang, W. Mi, C. Quan, T. Huang, H. Li, R. Flatschart, R. Borges Oliveira, J. E. Melanson, R. Ohlendorf, A. Henrion, T. Kinumi, L. Wong, Q. Liu, M. Oztug Senal, B. Vatansever, I. Ün, A. C. Gören, M. Akgöz, M. Quaglia, J. Warren: CCQM-K115 Key Comparison Study on Peptide Purity - Synthetic Human C-Peptide, Metrologia XX (2017) Tech. Suppl., 1A, 00000. [in press].

[21] T. Kinumi, M. Goto, S. Eyama, M. Kato, T. Kasama, A. Takatsu: Development of SI-traceable C-peptide certified reference material NMIJ CRM 6901-a using isotope-dilution mass spectrometry-based amino acid analyses, Anal. Bioanal. Chem. 404 (2012) 13-21.

[22] R. R. Little, C. L. Rohlfing, A. L. Tennill, R. W. Madsen, K. S. Polonsky, G. L. Myers, C. J. Greenbaum, J. P. Palmer, E. Rogatsky, D. T. Stein: Standardization of C-Peptide Measurements, Clin. Chem. 54 (2008) 1023-1026.

[23] C. Fierens, D. Stockl, D. Baetens, A. P. De Leenheer, L. M. Thienpont: Application of a C-peptide electrospray ionization-isotope dilution-liquid chromatography tandem mass spectrometry measurement procedure for the evaluation of five C-peptide immunoassays for urine, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 792 (2003) 249-259.

[24] D. Rodrıguez-Cabaleiro, D. Stockl, L. M. Thienpont: Improvement of sample pretreatment prior to analysis of C-peptide in serum by isotope dilution-liquid chromatography-tandem mass spectrometry, Rapid Commun. Mass Spectrom. 19 (2005) 3600-3602.

[25] D. Rodríguez Cabaleiro, D. Stöckl, J. M. Kaufman, T. Fiers, L. M. Thienpont, Feasibility of Standardization of Serum C-Peptide Immunoassays with Isotope-Dilution Liquid Chromatography–Tandem Mass Spectrometry, Clin. Chem. 52 (2006) 1193-1196.