CCQM-K115.b

Key Comparison Study on Peptide Purity - Synthetic Oxytocin

Final Report

May 2020

Prepared by:

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

Coordination laboratories:

Ralf D. Josephs*, Ming Li*[#], Adeline Daireaux*, Tiphaine Choteau*, Gustavo Martos*, Steven Westwood*, Robert Wielgosz*, Hongmei Li[#], Sheng Wang[#], Xiaomin Li[#], Naijie Shi[#], Peize Wu[#], Liuxing Feng[#], Ting Huang[#], Tianji Zhang[#], Shuangqing Li[#]
* Bureau International des Poids et Mesures (BIPM)
Sèvres, France
[#] National Institute of Metrology (NIM)
Beijing, China

With contributions from:

Paulo J. Miranda da Silva Iwakami Beltrão, Antonio Marcos Saraiva, Bruno C. Garrido, Sandra M. Naressi Scapin, Wagner Wollinger, Youssef Bacila Sade
Instituto Nacional de Metrologia, Qualidade e Tecnologia (INMETRO)
Xerém, Brazil

Marie-Pier Thibeault, Bradley B. Stocks, Jeremy E. Melanson
Measurement Science and Standards - National Research Council of Canada (NRC)
Ottawa, Canada

Tomoya Kinumi
National Metrology Institute of Japan (NMIJ), National Institute of Advanced Industrial Science and Technology (AIST)
Tsukuba, Japan

Mohd Fazlin Bin Rezali
National Metrology Institute of Malaysia (NMIM)
Bandar Baru Salak Tinggi, Malaysia

Merve Oztug, Müslüm Akgöz
National Metrology Institute of Turkey (TUBITAK UME)
Gebze-Kocaeli, Turkey

Milena Quaglia
LGC Limited (LGC)
Teddington, United Kingdom

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

TABLE OF CONTENT

INTRODUCTION
RATIONALE/PURPOSE
CHARACTERIZATION OF STUDY MATERIAL
CHARACTERIZATION STUDIES
HOMOGENEITY STUDIES
STABILITY STUDIES
SORPTION MEASUREMENTS
SAMPLE DISTRIBUTION
QUANTITIES AND UNITS
REPORTED MASS FRACTIONS OF OXT AND IMPURITIES IN CCQM-K115.B

- Peptide Related Impurity Profile of CCQM-K115.b
- Hydrolysis Efficiency Study

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

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

CONCLUSIONS
HOW FAR THE LIGHT SHINES STATEMENT (HFTLS)
ACKNOWLEDGEMENTS
REFERENCES

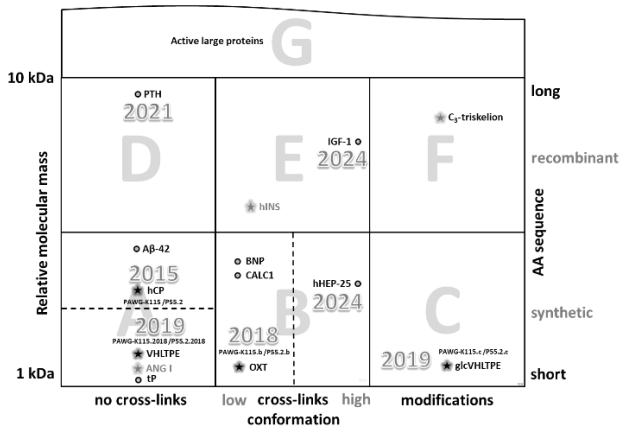
INTRODUCTION

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 such as peptides/proteins.

The provision of Primary Calibration Reference Services has been identified as a core technical competency for NMIs [2]. 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 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 is depicted in Figure 1 [1]. 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 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 [3,4]. The second cycle of peptide purity comparisons, CCQM-K115.b/P55.2.b on oxytocin (OXT) covers the space of quadrant B 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.



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 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) [5]. 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 [6]. These approaches have been successfully applied to a large variety of small molecules [7-11].

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 [12-14]. These approaches have been investigated for example for the routine analysis of human growth hormone and its biomarkers [15-16]. A number of NMIs have been developing higher order measurement procedures for the analysis of purified protein calibrators [17] and serum based matrix materials [16]. 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 [18-19]. 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 [11]. 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 [4] or quantitative nuclear magnetic resonance (qNMR) spectroscopy with a correction for structurally-related peptide impurities (PICqNMR) [20].

The timeline for the CCQM-K115.b study ‘Key Comparison Study on Peptide Purity - Synthetic Oxytocin’ is summarized in Table 1.

Table 1: CCQM-K115.b Timetable

Action	Date
Initial discussion	April and October 2016 PAWG/OAWG meetings
Approval of Study Proposal	April 2017 PAWG meeting
Draft protocol and confirmation	September 2017 PAWG meeting
Sample characterization completed	February 2018
Call for participation	April 1st, 2018
Final date to register	April 30th, 2018
Sample distribution	June 5th, 2018
Date due to coordinator	December 31st, 2018
Justification for 10 months period	2 months for identification of impurities 2 - 3 months to obtain tailor-made impurities 1 months for quantification and calculation
Initial report and discussion of results	April 2019 PAWG/OAWG meeting
Draft A report and discussion	October 2019 PAWG meeting
Draft B report	April 2020 approved by PAWG
Final report to PAWG Chair	May 2020

CHARACTERIZATION OF STUDY MATERIAL

The cyclic nonapeptide hormone oxytocin (OXT) is defined as oxytocin-neurophysin 1 (OT-NPI) (20-28) fragment with the amino acid sequence CYIQNCPLG (1-6 SS) and a relative molecular mass (M_r) of 1007.2 g/mol. The C-terminus has been converted to a primary amide and a disulfide bridge joins the cysteine moieties.

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.b 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

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/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 STUDIES

The BIPM/NIM 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.

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 OXT and OXT(1-8), 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 0.44 %, 1.35 %, 0.62 %, and 2.39 % was adopted as an estimate for the uncertainty contribution due to potential inhomogeneity for OXT, OXT (free acid), OXT + G, and OXT(1-8), respectively. OXT (free acid), OXT + G, and OXT(1-8) represent high (about 7.3 mg/g), medium (about 2.4 mg/g) and low (about 0.3 mg/g) mass fractions level impurities, respectively.

Table 2: Homogeneity results of representative OXT impurities

	Water	OXT	OXT acid High level	OXT + G Medium level	OXT(1-8) Small level
N	20	29	29	29	29
s_{wb} (%)	7.29	1.34	4.16	1.90	7.37
s_{bb} (%)	-[1]	-[1]	1.24	0.22	-[1]
u^*_{bb} (%)	2.82	0.44	1.35	0.62	2.39
$\boldsymbol{u_{bb}}$[2] (%)	**2.86**	**0.44**	**1.35**	**0.62**	**2.39**
F	0.425	0.236	1.264	1.038	0.543
F_{crit}	3.020	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.b OXT candidate material was studied using an LC-UV-hrMS method for the quantitative determination of OXT, OXT (free acid), OXT + G, and OXT(1-8). Acceptable uncertainties due to inhomogeneity were obtained for the pure OXT 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 0.10 mg/g (1.35 %), 0.015 mg/g (0.62 %) and 0.007 mg/g (2.29 %) could be assigned to the inherent impurities of OXT (free acid), OXT + G, and OXT(1-8), respectively. In addition, an uncertainty contribution due to between unit inhomogeneity (u_{bb}) of 3.57 mg/g (0.44

%) for the OXT content was verified by use of UV detection. Therefore, this candidate material is appropriate to serve in the K115.b 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.

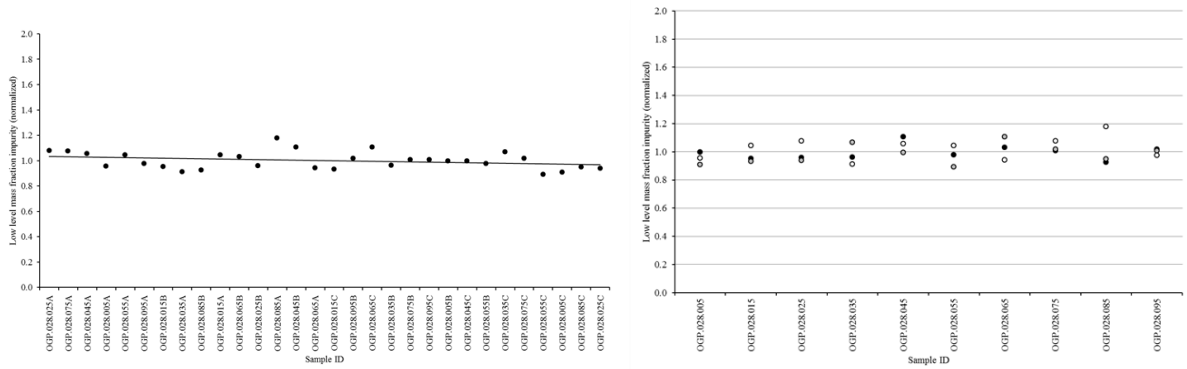


Figure 2: Homogeneity of OXT(1-8) - Low level mass fraction impurity - Injection and filling sequence

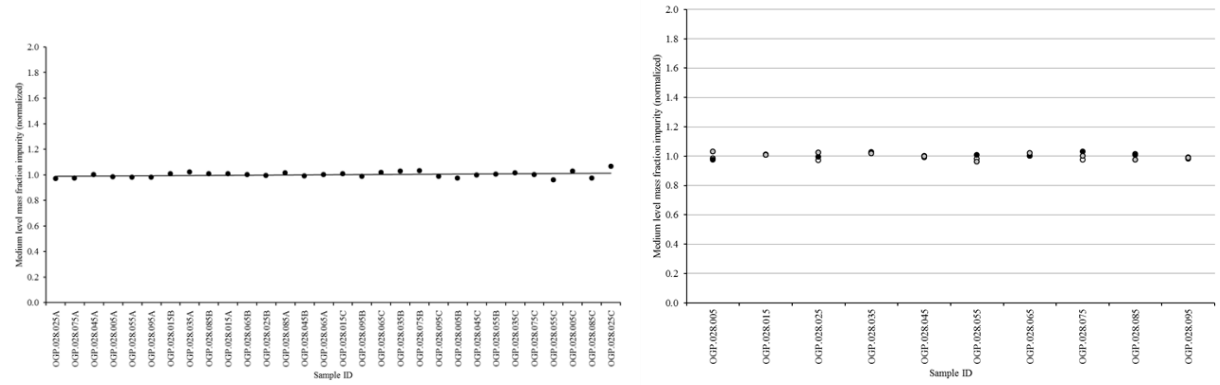


Figure 3: Homogeneity of OXT+G - Medium level mass fraction impurity - Injection and filling sequence

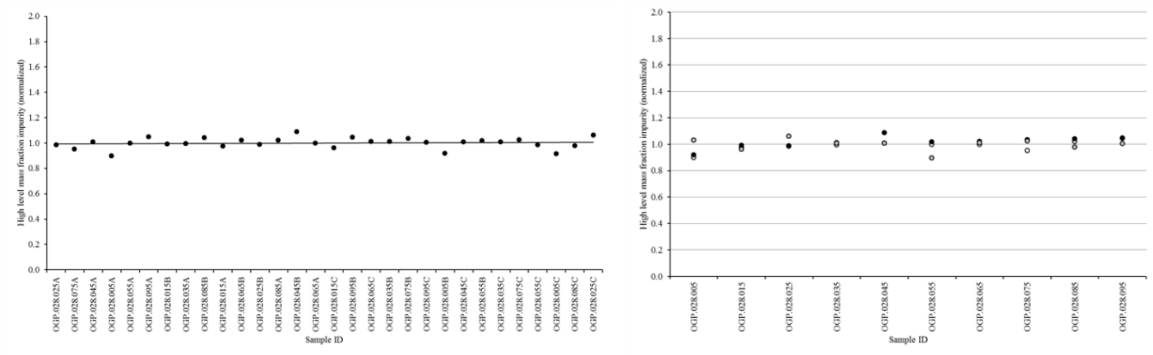


Figure 4: Homogeneity of OXT acid - High level mass fraction impurity - Injection and filling sequence

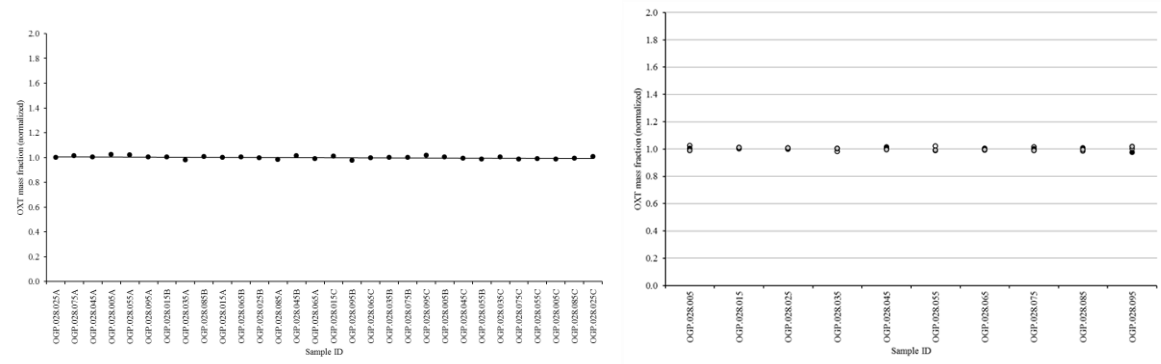


Figure 5: Homogeneity of OXT - Injection and filling sequence

For the homogeneity measurements of the OXT candidate material, 10 vials taken at regular intervals from the filling sequence were analysed in duplicate in randomly stratified order for their water content by coulometric Karl Fischer titration using oven transfer and a minimum sample size of 5 mg per analysis.

The results of the ANOVA are summarized 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. Therefore, the u^*_{bb} of 2.82 % was adopted as an estimate for the uncertainty contribution due to potential inhomogeneity.

Additionally, linear regression functions were calculated for the results due to filling and analysis order. The slopes of the lines were tested for significance on a 95 % confidence level to check for significant trends. No significant trends due to analysis order have been observed (Figure 6). The normalized result due to the analysis and filling sequences are presented in the Figure 6. The first and second replicates are represented by circles and dots respectively.

For the OXT candidate material contained water at a mass fraction of 62.2 mg/g. An absolute uncertainty contribution due to between-unit inhomogeneity (u_{bb}) of 1.8 mg/g was obtained for the water content by use of Karl Fischer Titration method. The material is appropriate to serve in the

CCQM-K115.b study to evaluate water content at mass fraction levels down to 62.2 mg/g provided a suitable sample intake of more than 5 mg is used for analysis of the material.

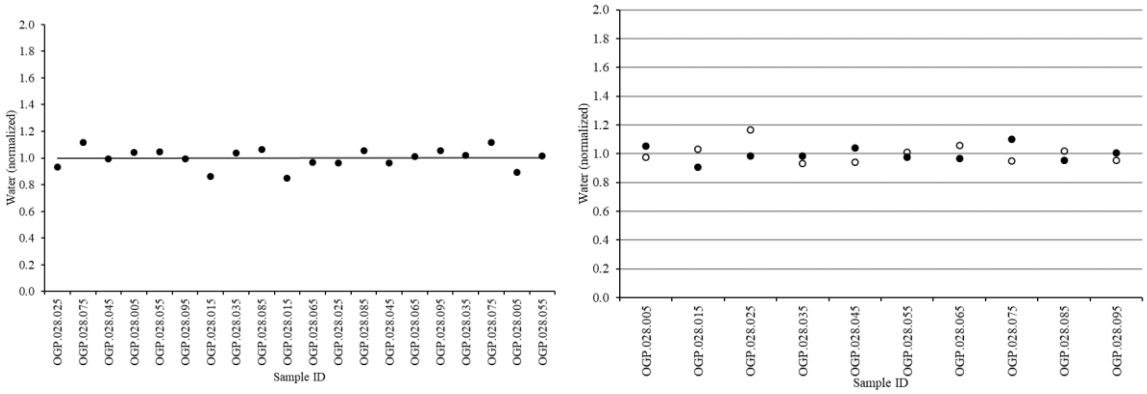


Figure 6: Homogeneity of water in OXT - Injection and filling sequence

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.

Trend analysis of the data obtained by LC-hrMS analysis of the stability test samples under repeatability conditions indicated no significant changes in the relative composition of OXT or of the related peptide impurities over longer time and at elevated temperatures.

The OXT mass fraction of the material was stable on storage at 4 °C, 22 °C and 40 °C over the entire storage study period.

The OXT acid mass fraction of the material, representing high mass fraction level impurities, was stable on storage at 4 °C but did increase significantly after storage beyond 2 weeks at 22 °C. The OXT acid mass fraction did increase significantly over the entire storage study period at 40 °C.

The OXT + G mass fraction of the material, representing medium mass fraction level impurities, was stable on storage at 4 °C, 22 °C and 40 °C over the entire storage study period.

The OXT(1-8) mass fraction of the material, representing low mass fraction level impurities, was stable on storage at 4 °C but did increase significantly after storage beyond 2 weeks at 22 °C. The OXT(1-8) mass fraction did increase significantly over the entire storage study period at 40 °C.

No significant changes in water mass fraction were observed after storage at 4 °C or 22 °C. There was some evidence of loss of water but only after prolonged storage at 40 °C.

The effect of storage temperatures on the mass fractions of OXT, related peptide impurities and water of the comparison material is shown in Figures 7-11.

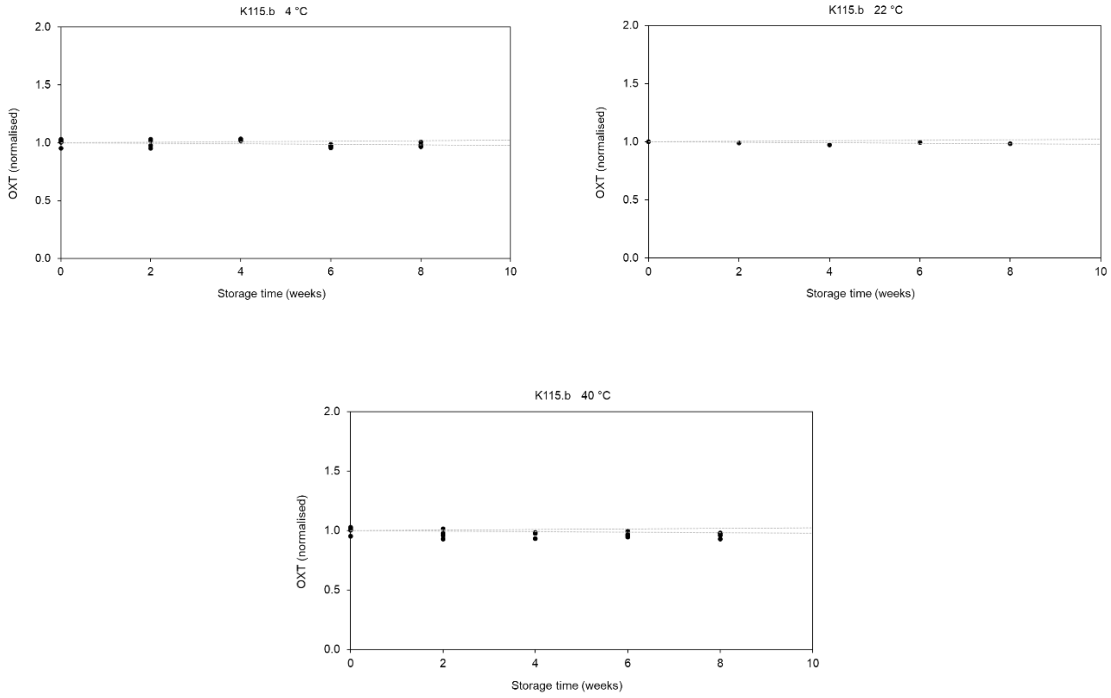


Figure 7: Stability study of OXT

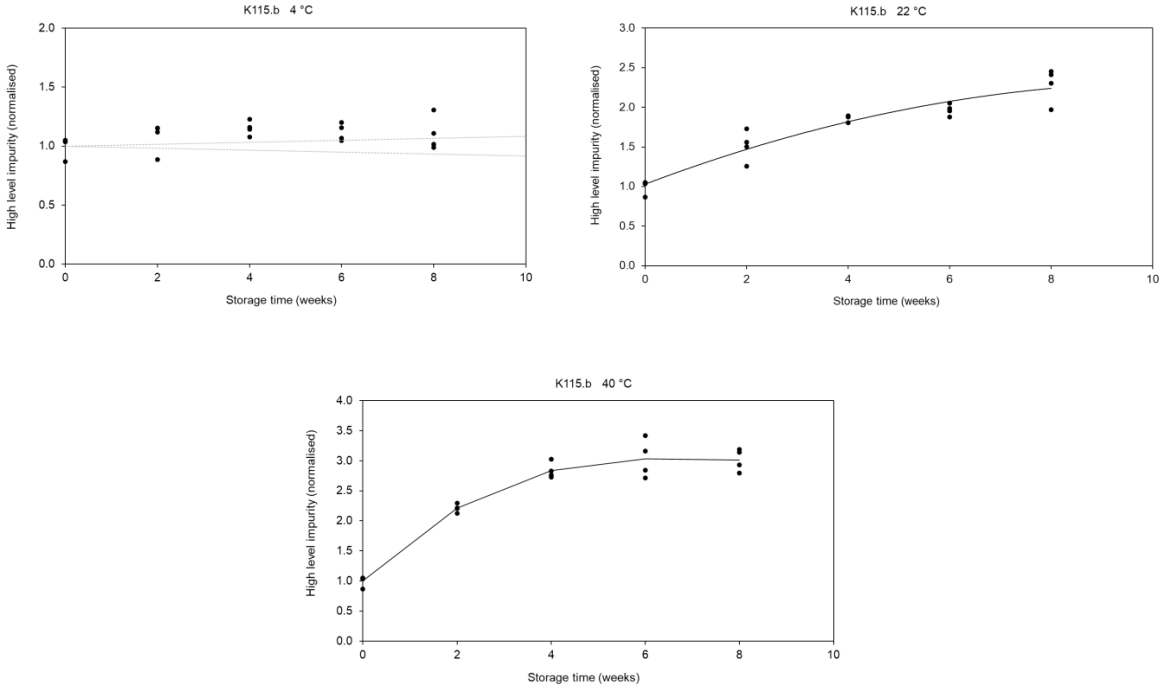


Figure 8: Stability study of OXT acid - High level mass fraction impurity

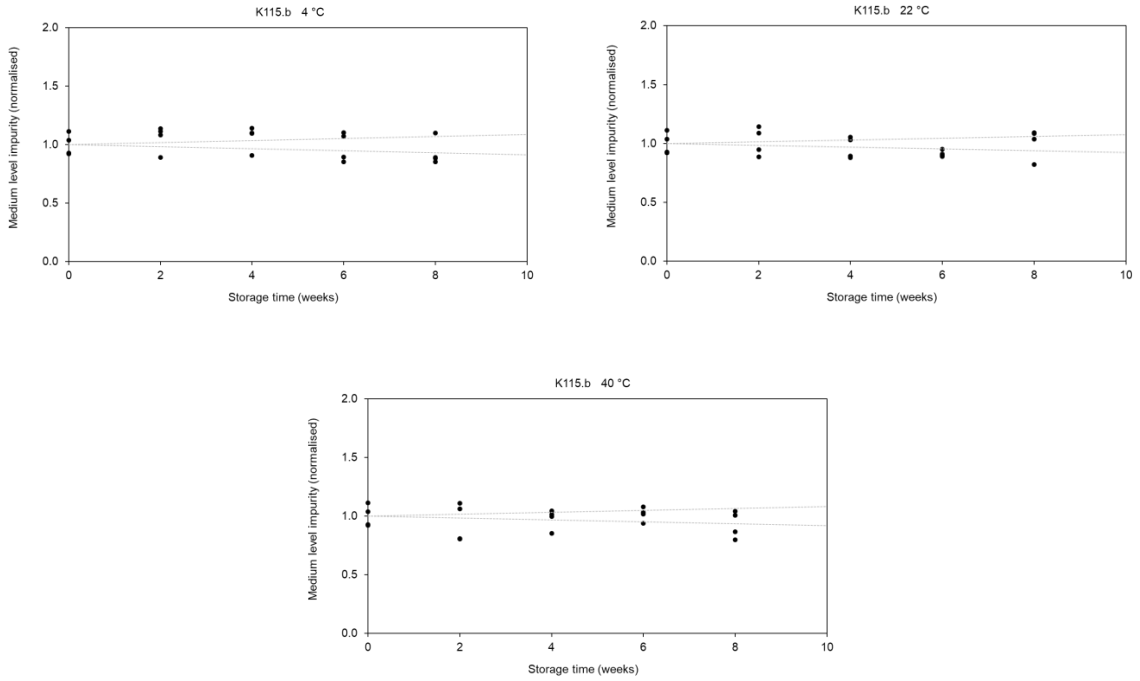


Figure 9: Stability study of OXT + G - Medium level mass fraction impurity

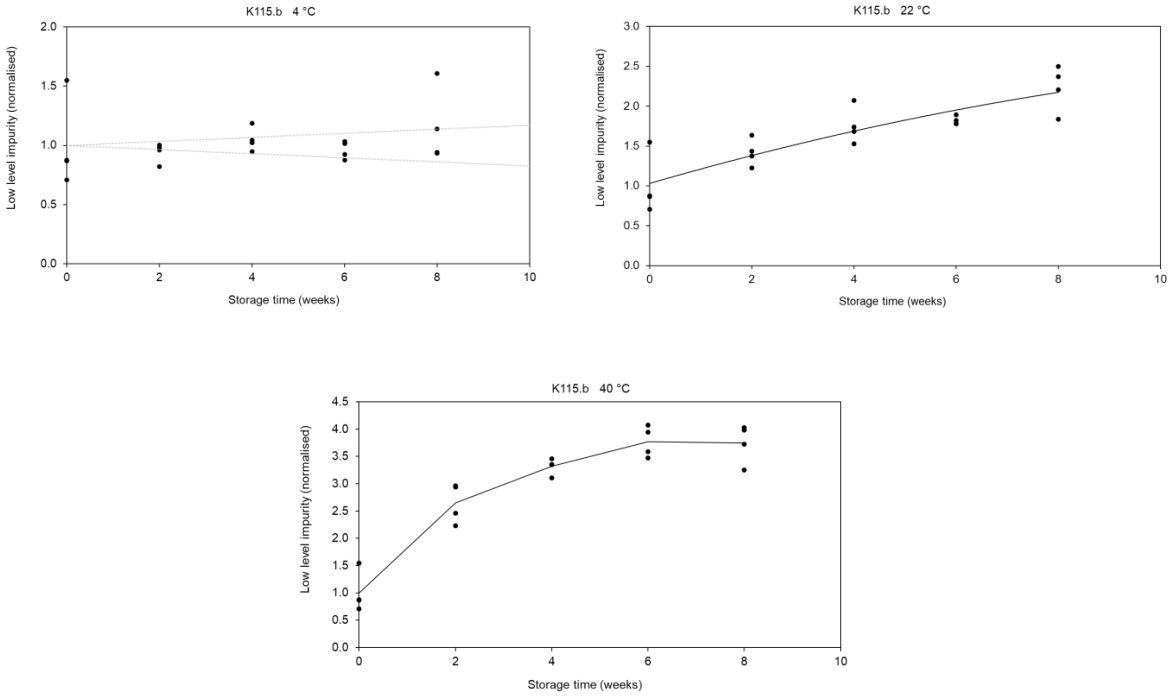


Figure 10: Stability study of OXT(1-8) - Low level mass fraction impurity

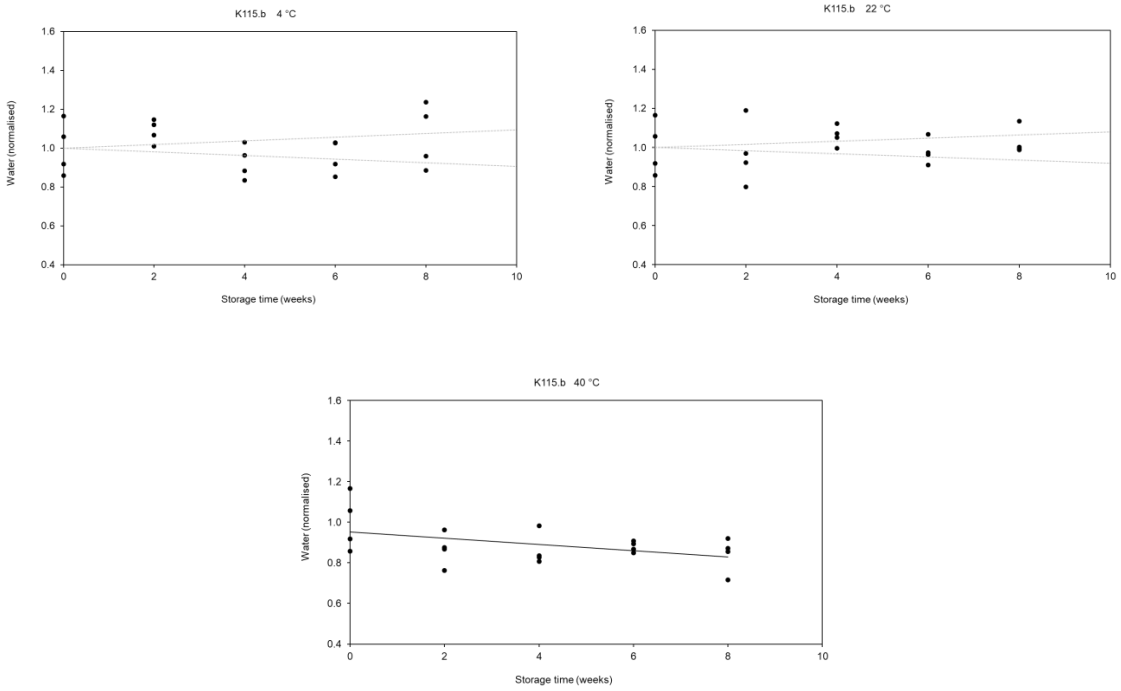


Figure 11: Stability study of water in OXT

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 about 4 °C, provided it was not exposed to temperatures significantly in excess of 22 °C, and for longer term storage at -20 °C.
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 DVS sorption balance indicate that weighings of the CCQM-K115.b 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 12).

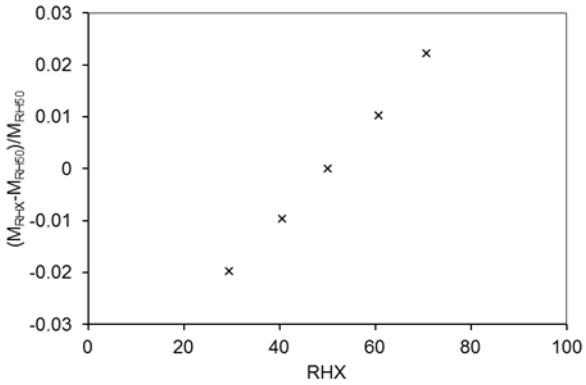


Figure 12: Sorption balance measurements indicating reversible water adsorption/desorption

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 weightings 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 = 0.0007 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

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 transport containers equipped with two temperature indicators (indicating exceeding 5 °C and 20 °C). Participants were asked to return the temperature indicator form acknowledging receipt of the samples and to advise the coordinator if any obvious damage had occurred to the vials during shipping. The coordinator verified that the temperature indicators inside the shipping container had not registered a temperature in excess of 20 °C for more than 2 weeks during the transport process.

QUANTITIES AND UNITS

Participants were required to report the mass fraction of OXT, the major component of the comparison sample. In addition, all participants who used a PICAA or qNMR procedure to determine the OXT content were required 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 OXT 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.

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

REPORTED MASS FRACTIONS OF OXT AND IMPURITIES IN CCQM-K115.B

The values reported by participants for the OXT mass fraction in CCQM-K115.b are given in Table 3 with a summary plot in Figure 13. The values reported by participants for the peptide related impurity (PRI) mass fractions in CCQM-K115.b are given in Table 4 with a summary plot in Figure 14. NMIJ has revised the OXT mass fraction value after the PAWG meeting in April 2019 confirming incorrect identification and quantification of OXT succinimide at the asparagine residue (Asn). The revised NMIJ value is provided in brackets in Table 3 for information. In addition, the value obtained by NMIM cannot be used to establish a KCRV as it lacks correction for peptide related impurities.

Table 3: Results for CCQM-K115.b: OXT mass fractions and uncertainties as received

Participant	Mass fractions (mg/g)			Coverage Factor (k)	Approach
	OXT	u(OXT)	U(OXT)		
BIPM	799.8	7.1	14.2	2	Mass Balance
NIM, China	796.5	3.3	6.5	2	Mass Balance
NMIM, Malaysia	987	4	8	2	qNMR
NRC, Canada	786.6	10.4	20.8	2	PICqNMR
INMETRO, Brazil	781	9	18	2	PICAA/PICqNMR
UME, Turkey	773.09	26.42	52.84	2	PICAA
LGC, United Kingdom	767	16	32	2	PICAA
NMIJ, Japan	766.3	12.6	25.1	2	PICAA
	(773.2)	(12.5)	(24.9)	(2)	(PICAA)

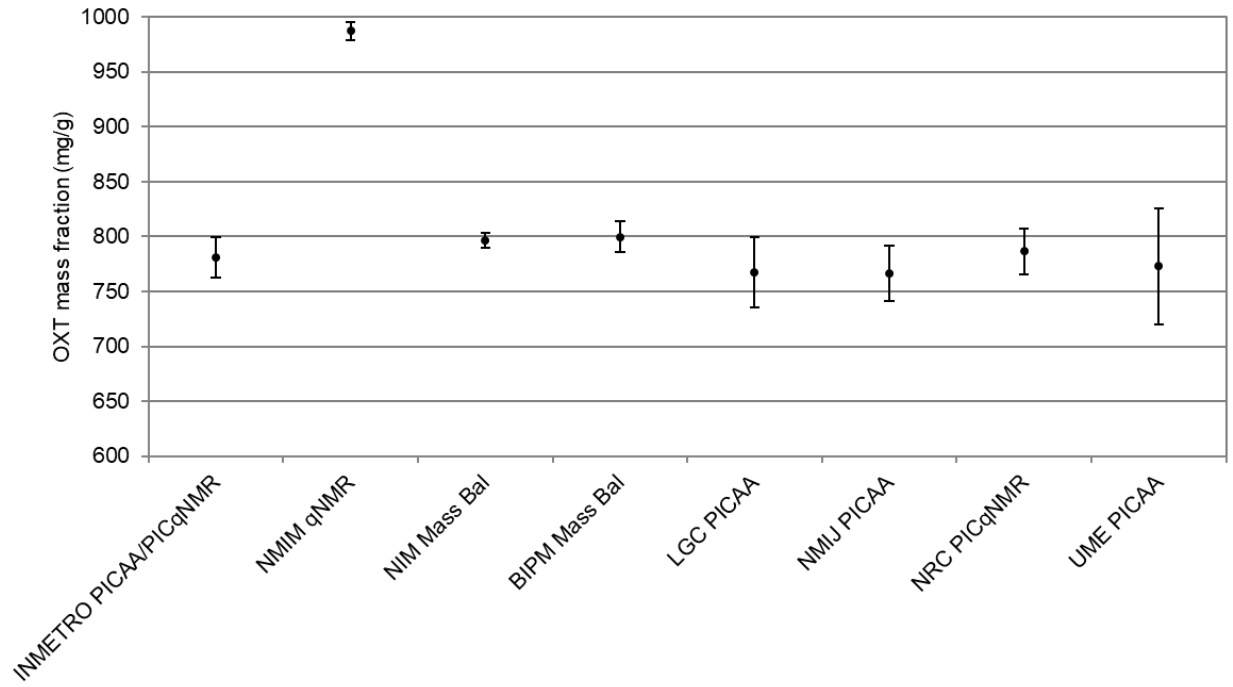


Figure 13: OXT mass fractions reported by participants in CCQM-K115.b - plotted with expanded uncertainties (*U*) at a confidence level of about 95 %

The reported values for the OXT mass fractions in CCQM-K115.b can be divided into three main groups - one group with both the BIPM and NIM using mass balance approaches, a second group using PICAA approaches and a third group using qNMR approaches. The hCP mass fraction values obtained by mass balance approaches show generally smaller uncertainties than the values obtained by PICAA or qNMR approaches.

The OXT mass fraction values obtained by the BIPM and NIM using a mass balance approach do agree within their estimated uncertainties. The related peptide impurity profile obtained by BIPM and NIM are in agreement as well as TFA and water measurements.
TFA impurity mass fraction values of 104.3 ± 0.5 mg/g by ^{19}F-qNMR and 103.5 ± 1.6 mg/g by ion chromatography were submitted by BIPM and NIM, respectively, resulting in a total TFA mass fraction of 103.9 mg/g with a corresponding expanded uncertainty of 1.2 mg/g. The total TFA mass fraction is in agreement with TFA impurity mass fraction values of 106 ± 6 mg/g by ^{19}F-qNMR and 104.3 ± 1.1 mg/g by ion chromatography obtained by NRC and BIPM, respectively. Water impurity mass fraction values of 62.2 ± 13.8 mg/g by KFT and 64.4 ± 5.6 mg/g by KFT were submitted by BIPM and NIM, respectively, resulting in a total water mass fraction of 63.3 mg/g with a corresponding expanded uncertainty of 10.5 mg/g. The total water mass fraction is in agreement with water impurity mass fraction values of 63 ± 32 mg/g by KFT obtained by NRC.

The OXT mass fraction values obtained by the participants using PICAA approaches in many cases agree within their estimated uncertainties. However, UME has assigned a significantly higher value to the peptide related impurity mass fraction mainly due to the unique identification and quantification of some acetyl- and formyl-peptide impurity fragments at very high mass fraction levels of about 14 mg/g as depicted in Table 4. INMETRO has assigned a significantly lower value to the peptide related impurity mass fraction (Table 4) missing some of the peptide related impurities as it becomes clear from INMETROs individual components table that only lists deamidated OXT fragments as impurities. NMIJ has revised the peptide related impurity mass fraction value after the PAWG meeting in April 2019 confirming incorrect identification and quantification of OXT succinimide at Asn at a high mass fraction level of about 6.79 mg/g. The revised NMIJ value is provided in brackets in Table 4 for information. INMETRO, NMIJ and UME have agreed that their values are not used for establishing the $KCRV_{PepImp}$ as their results showed certain technical deficiencies. In addition, NMIM has not provided a mass fraction values for peptide related impurities.

Table 4: Results for CCQM-K115.b: 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)		
BIPM	31.1	0.8	1.7	2	LC-hrMS
NIM, China	32.43	1.52	3.05	2	LC-hrMS
NMIM, Malaysia	-	-	-	-	-
NRC, Canada	28.2	5.5	11.0	2	LC-hrMS
INMETRO, Brazil	27.3	0.3	0.6	2	LC-UV
UME, Turkey	46.7	0.94	1.88	2	LC-hrMS
LGC, United Kingdom	35.2	2.8	5.6	2	LC-hrMS(/MS) and LC-UV
NMIJ, Japan	34.4 (27.6)	2.4 (1.9)	4.7 (3.7)	2 (2)	LC-QTOFMS (LC-QTOFMS)

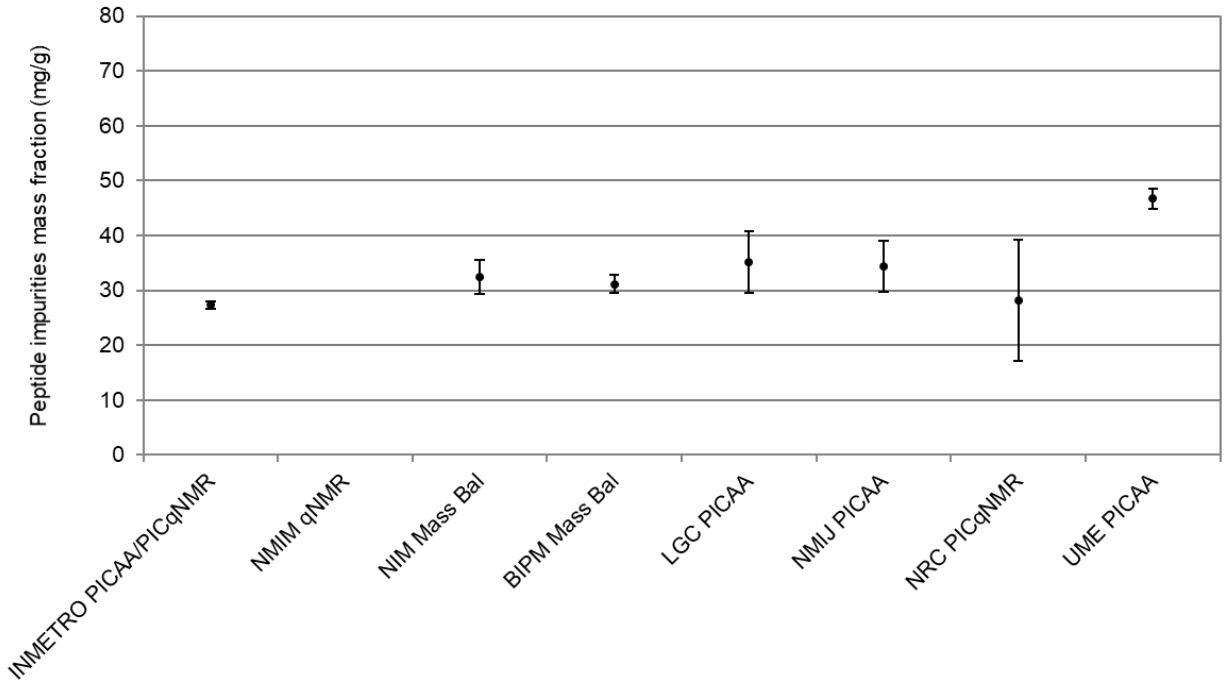


Figure 14: Overall peptide related impurities (PepImp) mass fractions reported by participants in CCQM-K115.b - plotted with expanded uncertainties (*U*) at a confidence level of about 95 %

In general, the CCQM-K115.b/P55.2.b comparison on OXT purity shows much better agreement of participants' results as the first CCQM-K115/P55.2 comparison on hCP for both peptide related impurity and peptide purity determinations. However, there was discussion on possible reasons for the discrepancy between CCQM-K115.b/P55.2.b results after presentation of the results of selected participants at the PAWG meeting in April 2019 and October 2020.

The peptide related impurities (PepImp) identification and quantification (Figure 15) has become much better compared to the CCQM-K115/P55.2 comparison on hCP but is still a weak point. The number of potential impurities is much smaller for OXT compared with hCP as OXT exhibits a much shorter primary sequence. In many cases, the major peptide related impurities have been identified/quantified resulting in mainly coherent estimations of the peptide related impurity mass fractions and consequently in consistent estimations of the mass fraction values for OXT. However, incorrectly identification/quantification of a few impurities resulted in an overestimation of the peptide related impurity mass fractions and consequently in an underestimation of the mass fraction value for OXT. It has been discussed that an overestimation of the peptide related impurity mass fraction values could be caused by in-source fragmentation in LC-MS analysis due to poor chromatographic separation or other sample manipulation.

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. Four participants have quantified the peptide related impurities using a response factor (RF = 1), RF with ionization efficiency correction or a relative response method although three participants have used synthesized

impurity standards to a different degree. NIM used 9 synthesized impurity standards (purities taken into account), BIPM used 12 synthesized impurities standards (purities taken into account) to quantify the individual impurities and closely structurally related impurities and a RF = 1 approach with OXT for other impurities and NMIJ used 2 synthesized impurities standards and has quantified others with RF = 1.
Four participants have used the PICAA approach in CCQM-K115.b. LGC has used microwave assisted hydrolysis. INMETRO, NMIJ 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 hydrolysis efficiency performance of all PICAA results has been calculated and 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.b

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 15 shows more details on the peptide related impurities of the CCQM-K115.b or -P55.2.b 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 eight as there are eight 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 hCP+A, OXT isomers and deaOXT. 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, issues with the data set for peptide impurities of INMETRO, NMIJ and UME were identified and discussed during the PAWG meeting in April 2019.
UME is the only laboratory that had identified and quantified acetyl- and formyl-peptide impurity fragments at very high mass fraction levels of about 14 mg/g. Related peptide impurities of that large mass fraction levels should have been identified and quantified by the majority of the participants. NRC, NIM and BIPM have agreed during the PAWG meeting in October 2019 to re-assess the data from UME and their own data concerning the presence of acetyl- and formyl-peptide impurity fragments. In summary, it was found that there is the likelihood of acetyl-PLG being present at negligible mass fraction levels of about 0.08 mg/g based on the assessment of the acetyl-formyl-PLG adduct considering charge states, response factors and lower MW. UME reported a mass fraction of 13.99 mg/g for acetyl-PLG based on the *m/z* 400.256. The *m/z* 400.256

does not correspond to acetyl-PLG. Mass fraction and monoisotopic mass originally reported by UME are not in agreement. UME had agreed that their peptide impurity value is not been used for establishing the peptide impurity KCRV.

NMIJ is the only laboratory that has identified and quantified OXT succinimide at Asn at a high mass fraction level of about 6.79 mg/g that has not been identified and quantified by any other laboratory as discussed during the PAWG in April 2019 meeting. NMIJ had agreed that their peptide impurity value is not been used for establishing the peptide impurity KCRV. NMIJ has repeated the measurements and reported during the PAWG October 2019 that the previously assigned succinimide at Asn impurity is an artefact produced during the ionization process of the LC-MS measurements. An artefact corrected peptide impurity value with a mass fraction of 27.6 mg/g and corresponding expanded uncertainty of 3.7 mg/g (k = 2) was reported NMIJ. Consequently, a corrected OXT purity value with a mass fraction of 773.2 and corresponding expanded uncertainty of 24.9 mg/g (k = 2) was communicated.

It has been perceived that both the peptide impurity value of INMETRO (27.3 mg/g) and its corresponding expanded uncertainty (0.6 mg/g) are lower than the values and uncertainties provided by the other NMIs/DIs. INMETRO has only reported dea4OXT and dea5OXT as peptide impurities. The major impurity OXTacid and several other impurities as [Cya1, Cya6]OXT and OXT+O and OXT+G10 were not identified and quantified. INMETRO had agreed that their peptide impurity value is not been used for establishing the peptide impurity KCRV.

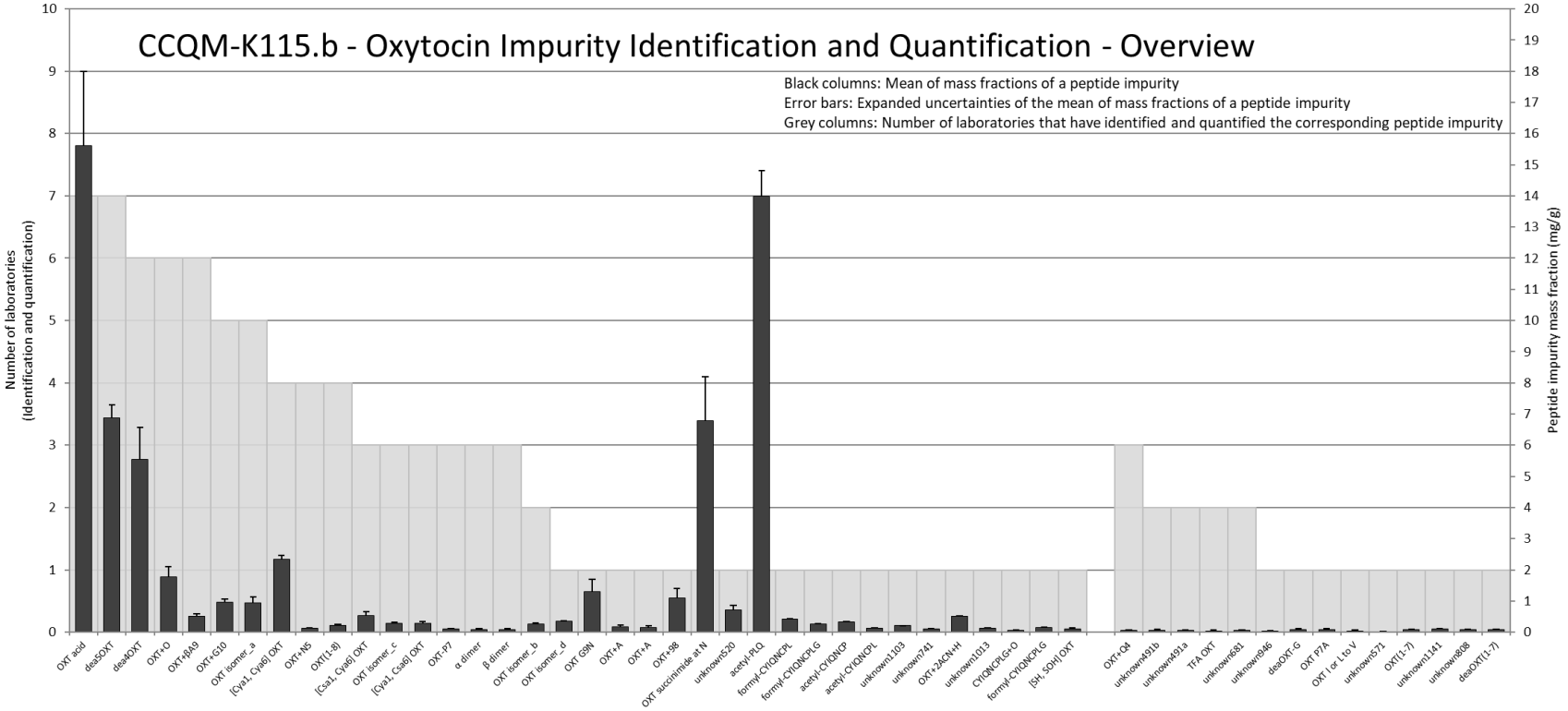


Figure 15: OXT impurity identification and quantification - Overview (deaOXT: deamidated OXT)

Hydrolysis Efficiency Study

The BIPM has calculated the hydrolysis efficiencies of the PICAA methods used in the CCQM-PAWG-K115.b/P55.2.b studies.
Figure 16 and Table 5 are providing overviews of the total hydrolysis efficiencies (5 labs in total) according to the hydrolysis methods employed (microwave-assisted vapor phase hydrolysis by 2 labs and classical gas/liquid phase hydrolysis by 3 labs) and the amino acid that was analyzed (Leucine, Isoleucine and Proline). Leucine, Proline and Valine have been selected because they are the most frequently analyzed amino acids in the K115.b/P55.2.b studies on OXT and the BIPM has a complete set of peptide related impurities and uncertainties for them ready for verification purposes.
In general, the hydrolysis efficiencies are very high (>95 %). No significant differences are observed for hydrolysis efficiencies between both hydrolysis methods (microwave-assisted hydrolysis or classical gas/liquid phase hydrolysis) employed or amino acids (Leucine, Proline and Valine) selected for analysis. Hydrolysis efficiencies for Isoleucine are slightly smaller but not significantly different from the others.

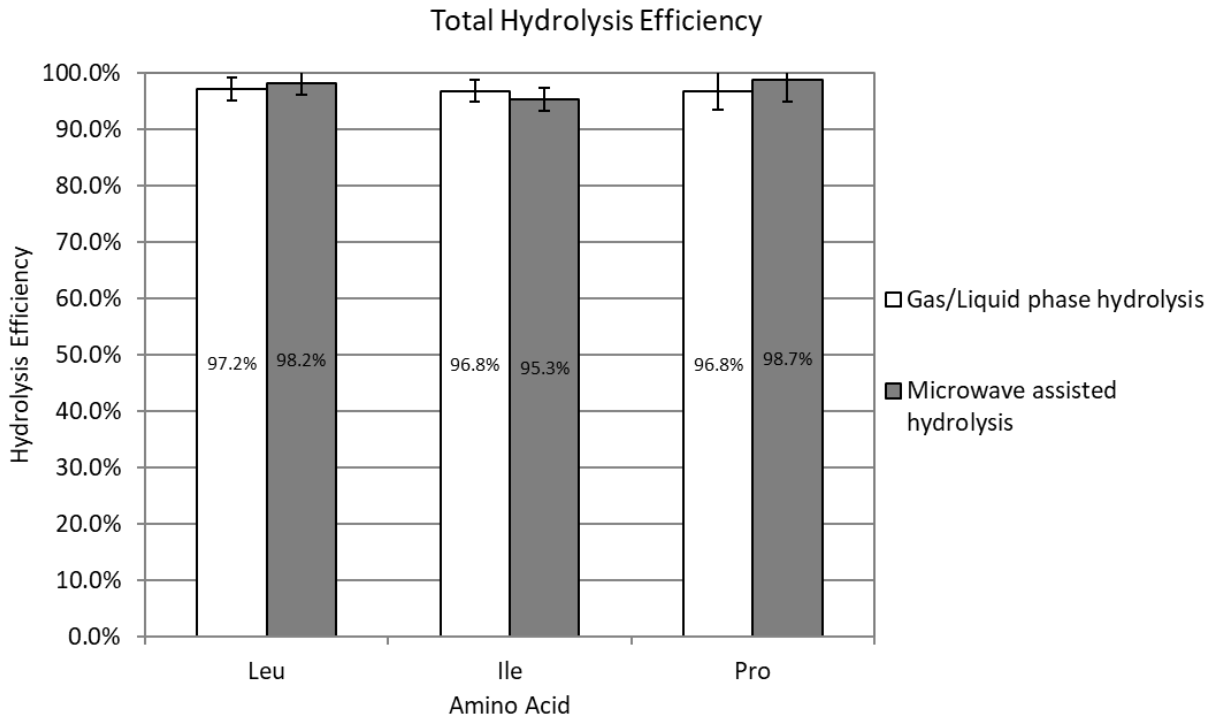


Figure 16: Overview of the total hydrolysis efficiency for Leucine, Isoleucine and Proline in OXT by gas/liquid phase hydrolysis and microwave-assisted hydrolysis (Error bars are standard deviations)

Table 5: Total hydrolysis efficiency means and standard deviations for Leucine, Isoleucine and Proline in OXT for both gas/liquid phase hydrolysis and microwave-assisted hydrolysis methods

	Total Hydrolysis Efficiency			
	Gas/Liquid phase hydrolysis		Microwave-assisted hydrolysis	
	Mean (%)	SD (%)	Mean (%)	SD (%)
Leucine	97.2	2.0	98.2	2.1
Isoleucine	96.8	2.0	95.3	2.0
Proline	96.8	3.4	98.7	3.8

In Figure 17 and Table 6 hydrolysis efficiencies are shown in more detail broken down to NMIs, hydrolysis methods used and amino acids analysed. The error bars are expanded uncertainties that are composed of the contribution of the AAA values of the NMIs and the contributions of the TFA, other anions, cations and water measurements of the BIPM required to obtain the target value. The uncertainties are asymmetric but at negligible decimal place. The estimation of the target value based on mass balance measurements permits hydrolysis efficiencies of slightly more than 100 %.

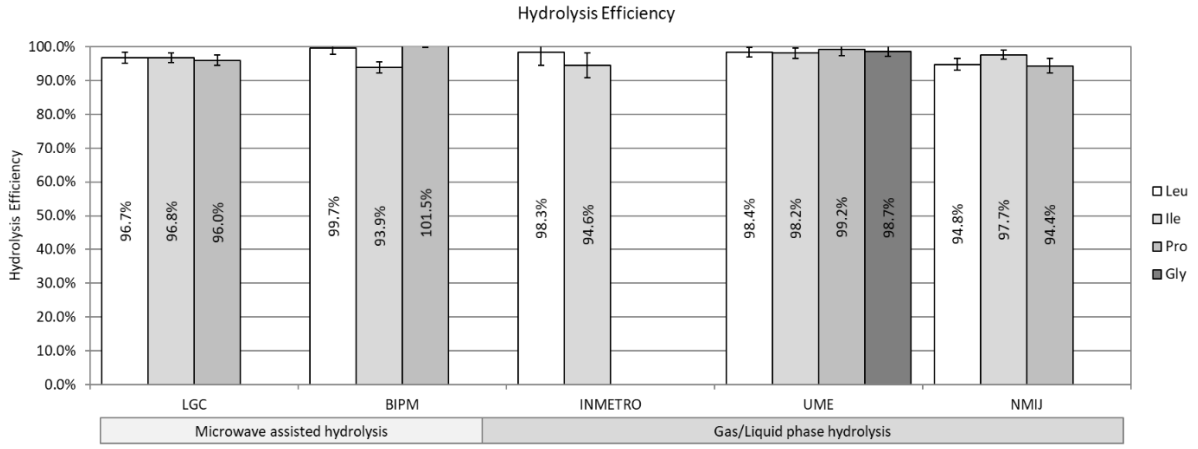


Figure 17: Overview of the hydrolysis efficiency for Leucine, Isoleucine, Proline and Glycine in OXT obtained by the NMIs by gas/liquid phase hydrolysis and microwave-assisted hydrolysis (Error bars are expanded uncertainties)

Table 6: Hydrolysis efficiency means and corresponding expanded uncertainties of NMIs for Leucine, Isoleucine and Proline in OXT for both gas/liquid phase hydrolysis and microwave-assisted hydrolysis methods (Expanded uncertainties (*U*) are composed of the contribution of the AAA values of the NMIs and the contributions of the TFA, anions, cations and water measurements of the BIPM required to obtain the target value)

	Hydrolysis Efficiency												
	Leucine			Isoleucine			Proline			Glycine			
	Mean (%)	U- (%)	U+ (%)	Mean (%)	U- (%)	U+ (%)	Mean (%)	U- (%)	U+ (%)	Mean (%)	U- (%)	U+ (%)	Hydrolysis Method
LGC	96.7	1.7	1.6	96.8	1.4	1.4	96.0	1.6	1.6				Microwave-assisted
BIPM	99.7	1.8	1.8	93.9	1.6	1.6	101.5	1.6	1.6				Microwave-assisted
INMETRO	98.3	3.9	3.9	94.6	3.7	3.7				98.7	1.6	1.6	Gas/Liquid phase
UME	98.4	1.4	1.4	98.2	1.5	1.5	99.2	1.9	1.9				Gas/Liquid phase
NMIJ	94.8	1.7	1.7	97.7	1.4	1.3	94.4	2.1	2.1				Gas/Liquid phase
Mean of means (%)	97.6			96.2			96.6			98.7			
SD (%)	1.9			2.1			3.2						
Maximum (%)	99.7			98.2			101.5						
Minimum (%)	94.8			93.9			94.4						

It can be summarized that:

- the hydrolysis even of peptides containing a disulfide bridge is 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.b/P55.2.b;
- in general, an excellent comparability of hydrolysis efficiencies with small variances was obtained;
- the accurate identification and quantification of peptide related impurities has a larger impact on the individual results of the OXT purity (CCQM-K115.b/P55.2) determinations than the hydrolysis efficiency (methods used or amino acid analysed).

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

The values used to establish the Key Comparison Reference Values (KCRV) for CCQM-K115.b are summarized in Table 3 and Table 4 for the OXT mass fraction and the peptide related impurity mass fractions, respectively. All participants in CCQM-K115.b were required to give estimates for the mass fraction of the sub-class of peptide related impurities they quantified to obtain their final OXT mass fraction estimate. The coordinator has calculated the overall KCRV for OXT mass fraction and separate KCRV for the peptide related impurities 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.b

The $KCRV_{PepImp}$ for the mass fraction of peptide impurities is based on the assumption that only the Largest Consistent Subset (LCS) of results is taken for the calculation of the $KCRV_{PepImp}$. Only peptide related impurities mass fractions that have been identified and quantified by BIPM, LGC, NIM and NRC have been used to establish the $KCRV_{PepImp}$ and the corresponding standard uncertainty ($u(KCRV_{PepImp})$) based on the weighted mean (WM).
The mass fraction value obtained by NMIJ has been excluded from the calculation as NMIJ had confirmed incorrect identification and quantification of OXT succinimide at Asn. The mass fraction value obtained by UME has not been considered as a significantly higher value has been assigned to the peptide related impurity value mainly due to the unique identification and quantification of some acetyl- and formyl-peptide impurity fragments that have not been confirmed at that high level by any other participant. The mass fraction value obtained by INMETRO has not been considered as a significantly lower value has been assigned to the peptide related impurity value by most likely missing some of the peptide related impurities. INMETRO, NMIJ and UME have agreed that their values are not used for establishing the $KCRV_{PepImp}$.

$KCRV_{PepImp}$ = 31.6 mg/g
$u(KCRV_{PepImp})$ = 0.7 mg/g

The results reported by participants with their corresponding standard uncertainties (k = 1) plotted against the $KCRV_{PepImp}$ are shown in Figure 18. Figure 19 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).

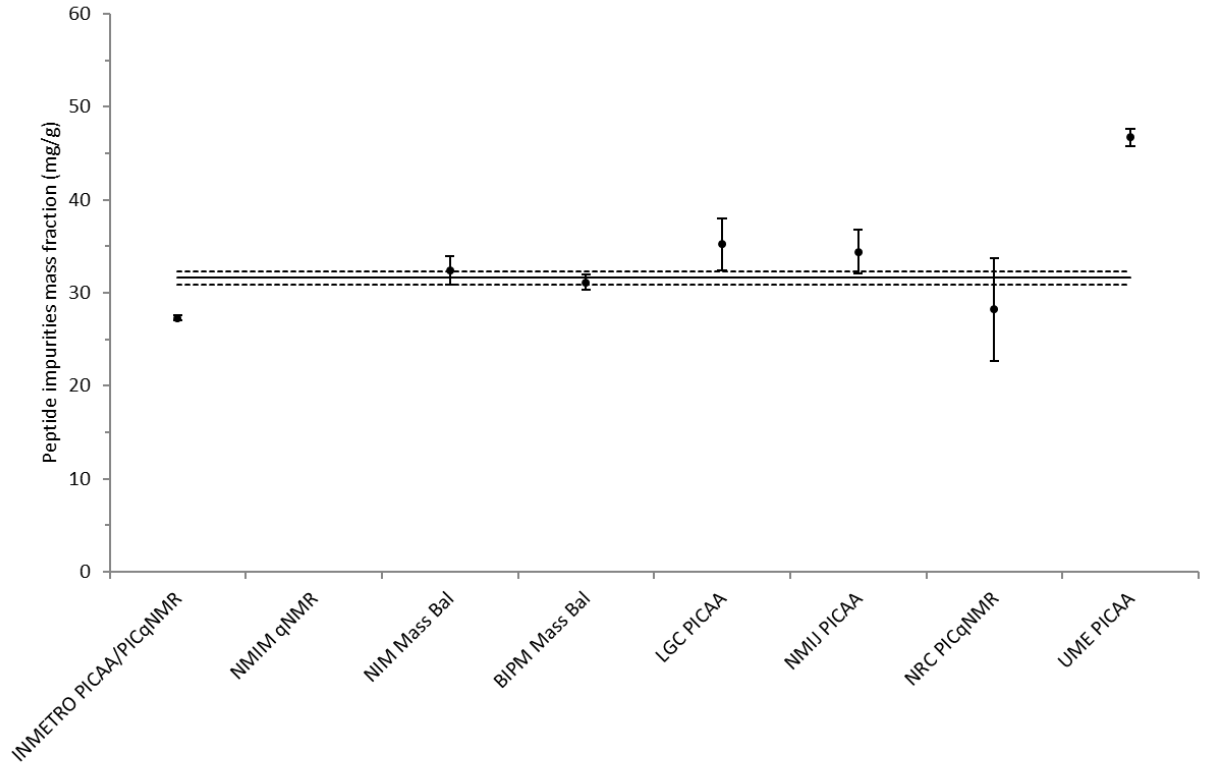


Figure 18: Estimates of total related peptide impurities in CCQM-K115.b plotted with their reported standard uncertainties ($\pm\, u_c$, $k = 1$). The $KCRV_{PepImp}$ (solid line) is 31.6 mg/g. Dashed lines show the $u(KCRV_{PepImp})$ ($k = 1$) of the $KCRV_{PepImp}$.

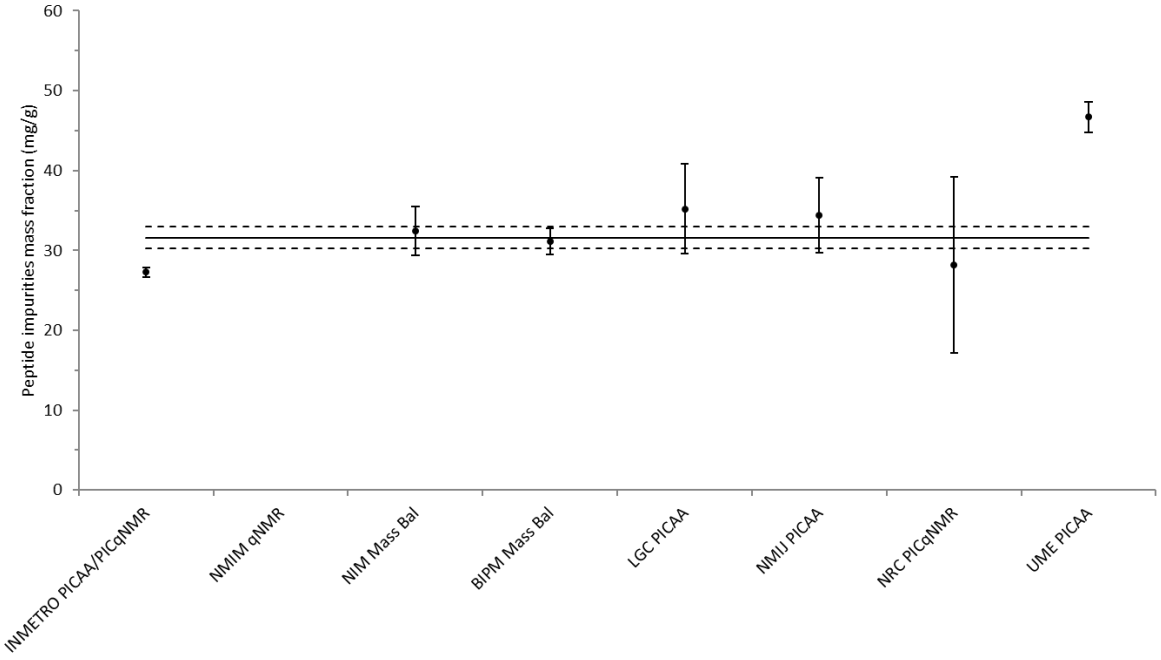


Figure 19: Mass fraction estimates by participants of total related peptide impurities in CCQM-K115.b with their reported expanded uncertainties (± *U*, *k* = 2). The $KCRV_{PepImp}$ for CCQM-K115.b (solid line) is 31.6 mg/g. The calculated expanded uncertainty of the $KCRV_{PepImp}$ is 1.4 mg/g. Dashed lines show the $U(KCRV_{PepImp})$ (*k* = 2) 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:

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

Figure 20 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.

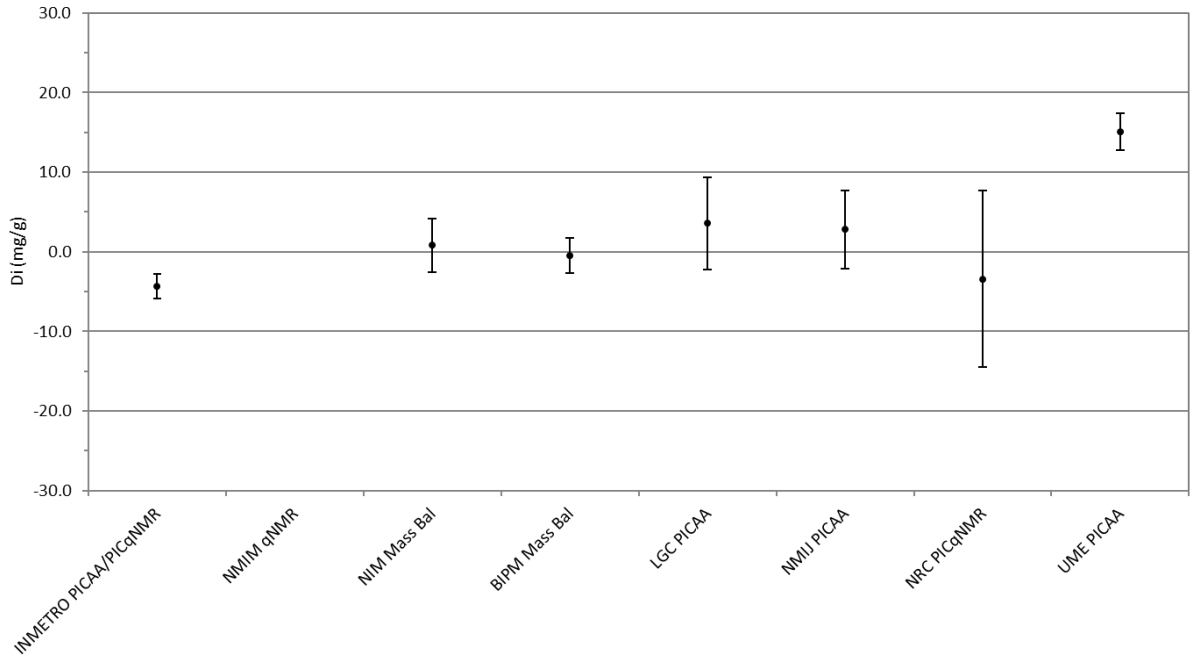


Figure 20: Degree of equivalence with the $KCRV_{PepImp}$ for total related peptide impurities for each participant. Points are plotted with the associated expanded uncertainty in the degree of equivalence corresponding to a confidence level of about 95 %.

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 PICAA/PICqNMR	-4.3	1.5
NMIM qNMR	-	-
NIM Mass Balance	0.8	3.4
BIPM Mass Balance	-0.5	2.2
LGC PICAA	3.6	5.8
NMIJ PICAA	2.8	4.9
NRC PICqNMR	-3.4	11.1
UME PICAA	15.1	2.3

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

The $KCRV_{OXT}$ for the mass fraction of OXT is based on the assumption that nearly all results are directly taken for the calculation of the $KCRV_{OXT}$. Only peptide related impurities mass fractions that have been identified and quantified by BIPM, INMETRO, LGC, NIM, NMIJ, NRC and UME have been used to establish the $KCRV_{OXT}$ and the corresponding standard uncertainty ($u(KCRV_{OXT})$) based on the DerSimonian-Laird variance-weighted mean (DSL) [21-22]. The OXT mass fraction obtained by NMIM has been excluded as the qNMR result was not corrected for related peptide impurities. The DSL-mean takes into account the uncertainties of the LCS while introducing sufficient excess variance to allow for their observed dispersion. The DSL approach to obtain the $KCRV_{OXT}$ has been accepted by all participating NMIs/DIs although NIM would have preferred use a mass balance calculation that would have taken into account the $KCRV_{PepImp}$ for the peptide related impurities, the TFA mass fraction values, water and other minor counter ions from the NIM and BIPM.

Figure 21 shows the participant results with their reported standard uncertainties plotted against the $KCRV_{OXT}$ of 787.2 mg/g for OXT in CCQM-K115.b (solid line) and its corresponding standard uncertainty of 5.3 mg/g (k = 1). Figure 22 shows the same results with their expanded uncertainties and the $KCRV_{OXT}$ with the corresponding expanded uncertainty of 12.9 mg/g (k = 2.45) at a confidence level of about 95 % (dashed lines).

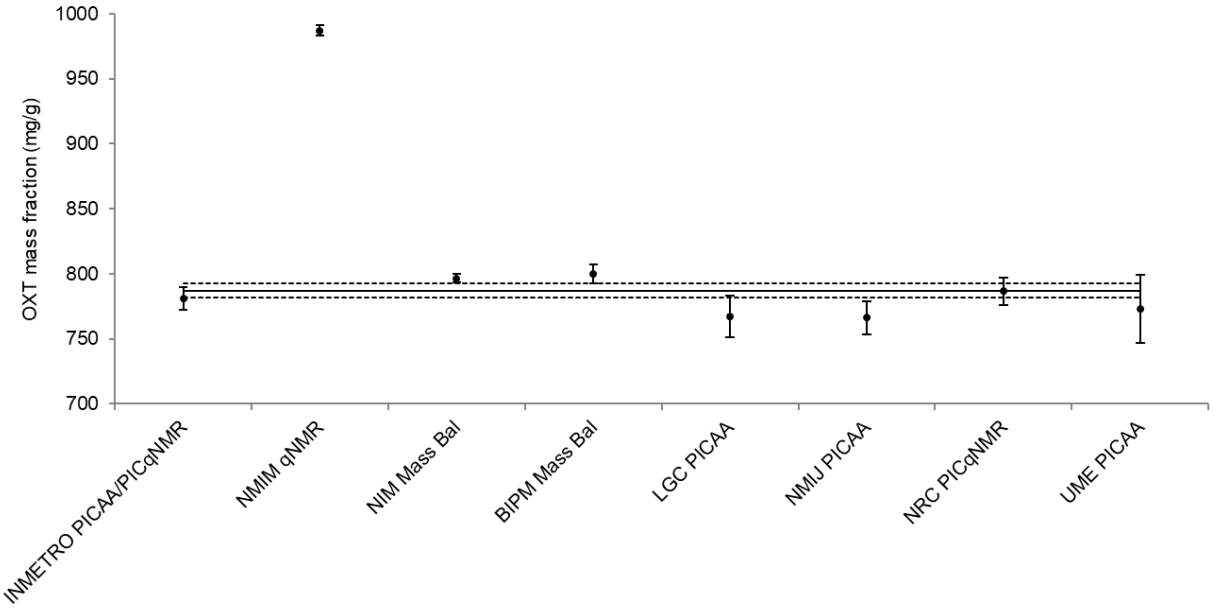


Figure 21: Mass fraction estimates by participants for OXT in CCQM-K115.b with their reported combined standard uncertainties (± u_c, k = 1). The $KCRV_{OXT}$ for CCQM-K115.b (solid line) is 787.2 mg/g. The calculated combined standard uncertainty of the $KCRV_{OXT}$ is ±5.3 mg/g. Dashed lines show the $u(KCRV_{OXT})$ (k = 1) of the $KCRV_{OXT}$.

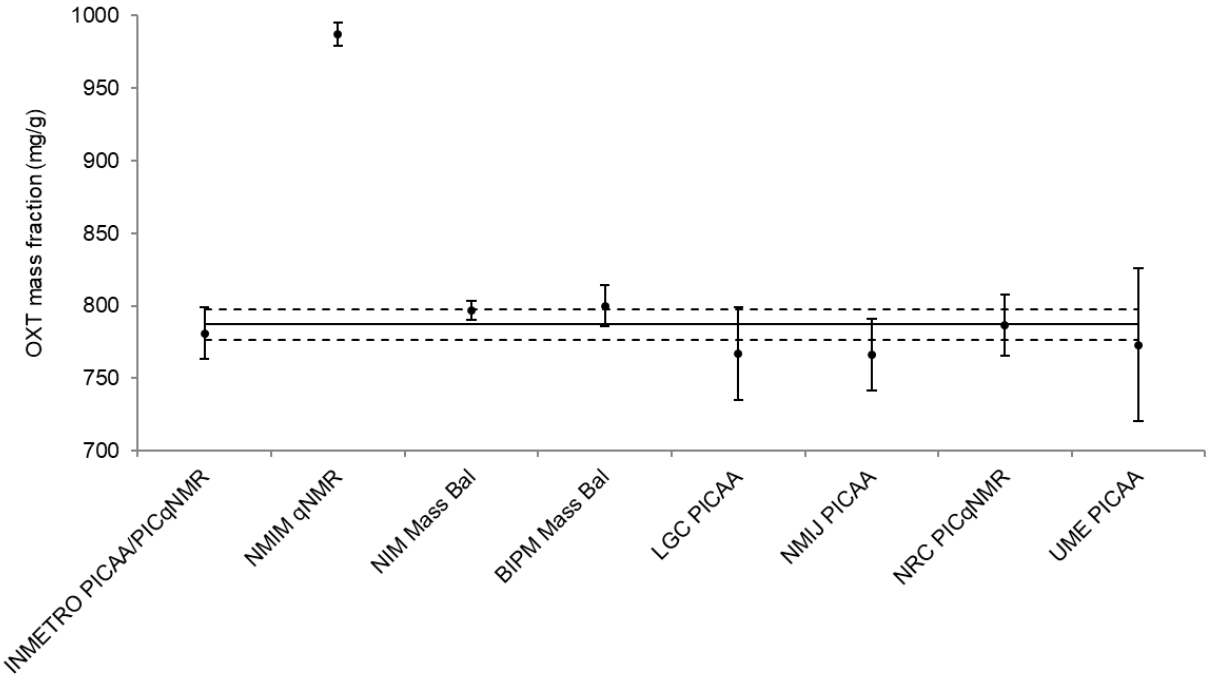


Figure 22: Mass fraction estimates by participants for OXT in CCQM-K115.b with their reported expanded uncertainties (± *U*, *k* = 2). The $KCRV_{OXT}$ for CCQM-K115.b (solid line) is 787.2 mg/g. The calculated expanded uncertainty of the $KCRV_{OXT}$ is ±12.9 mg/g. Dashed lines show the *U*($KCRV_{OXT}$) (*k* = 2.45) of the $KCRV_{OXT}$.

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

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

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

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

Figure 23 indicates the degree of equivalence (D_i) of each key comparison participant's result with the $KCRV_{OXT}$ for OXT. The corresponding values are listed in Table 8.

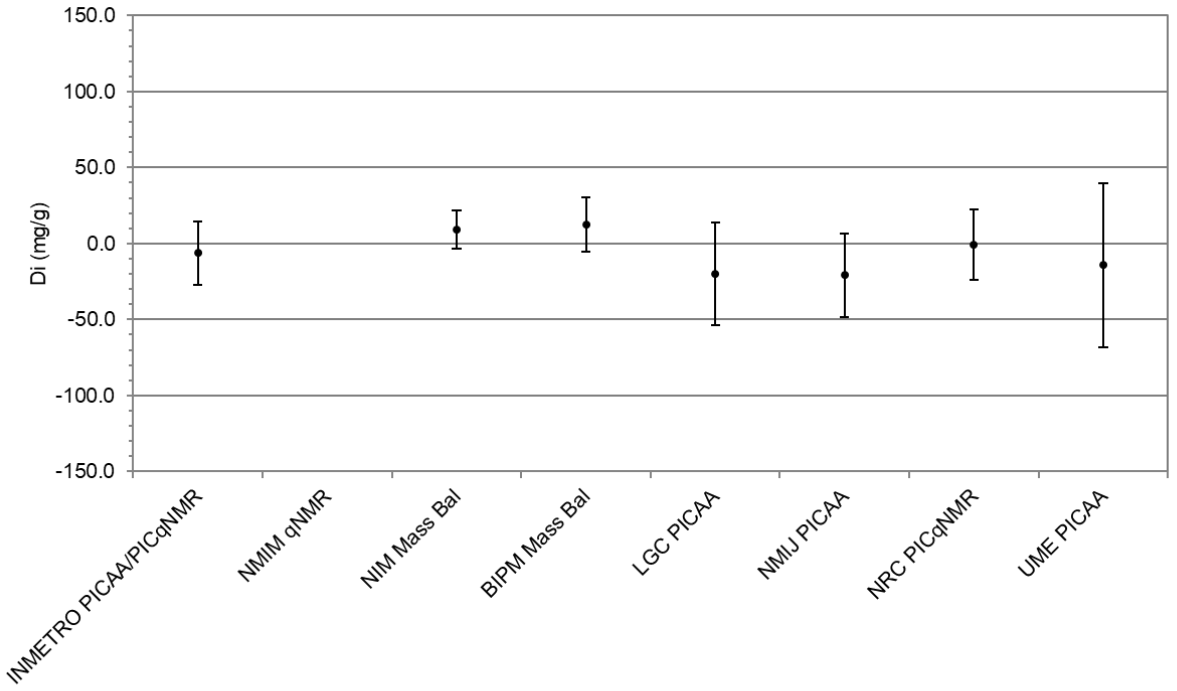


Figure 23: Degree of equivalence with the $KCRV_{OXT}$ for OXT for each participant. Points are plotted with the associated expanded uncertainty in the degree of equivalence corresponding to a confidence level of about 95 %.

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 $KCRV_{OXT}$ for OXT

	D_i	$U(D_i)$
INMETRO PICAA/PICqNMR	-6.2	20.9
NMIM qNMR	199.8	13.3
NIM Mass Balance	9.3	12.5
BIPM Mass Balance	12.6	17.7
LGC PICAA	-20.2	33.7
NMIJ PICAA	-20.9	27.3
NRC PICqNMR	-0.6	23.3
UME PICAA	-14.1	53.9

CONCLUSIONS

OXT 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 participants used a PICAA or PICqNMR 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 OXT 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 OXT purity. This allows participants to demonstrate the efficacy of their implementation of the approaches used to determine the OXT 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 OXT mass fraction assignment.

Different methods had been investigated to obtain a $KCRV_{PepImp}$ for the mass fraction of peptide impurities. INMETRO, NMIJ and UME have agreed that their values are not used for establishing the $KCRV_{PepImp}$ as their results showed certain technical deficiencies. The assessment of the BIPM, LGC, NIM and NRC is based on the assumption that only the Largest Consistent Subset (LCS) of results is taken for the calculation of the $KCRV_{PepImp}$ by use of the weighted mean. The LCS is the remaining set of related peptide impurity results obtained by the BIPM, LGC, NIM and NRC that shows the largest number of overlaps. Consequently, the $KCRV_{PepImp}$ of 31.6 mg/g is associated with a small corresponding expanded uncertainty of ±1.4 mg/g (k =2) 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 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.

Different methods had also been investigated to obtain a $KCRV_{OXT}$ for the OXT mass fraction. OXT mass fraction results submitted by all NMIs/DIs without the NMIM result are taken directly into account for the calculation of the $KCRV_{OXT}$. NMIM result has been excluded as the result was not corrected for related peptide impurities. The approach selected to obtain a $KCRV_{OXT}$ 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 $U(KCRV_{OXT})$.

The $KCRV_{OXT}$ for CCQM-K115.b is 787.2 mg/g with a corresponding expanded uncertainty of the $KCRV_{OXT}$ of ±12.9 mg/g. All OXT mass fraction results except the result of NMIM are in agreement with the $KCRV_{OXT}$. It should be pointed out that the mass balance approaches show smaller uncertainties than PICAA or PICqNMR approaches. Mass balance approaches seem to produce slightly higher OXT mass fractions while PICAA approaches deliver slightly lower OXT mass fractions.

HOW FAR THE LIGHT SHINES STATEMENT (HFTLS)

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

- Chemically synthesized peptides of known sequence, with one cross-link, up to 5 kDa. 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.

Oxytocin (OXT) has been proposed as the comparison material, since:

- it will allow the generic capabilities listed above to be demonstrated for peptides with one cross-link and up to 5 kDa molecular mass;
- it can be obtained in sufficiently large quantities required for the comparison;
- it will directly support NMI services and certified reference materials currently being provided by NMIs;
- it is an important medication to facilitate childbirth. It is an analyte for which methods have been developed in clinical chemistry as it plays a role in social bonding, sexual reproduction in both sexes, and during and after childbirth.

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] R. D. Josephs, G. Martos, M. Li, L. Wu, J. E. Melanson, M. Quaglia, P. J. Beltrao, D. Prevoo-Franzsen, A. Boeuf, V. Delatour, M. Oztug, J.-S. Jeong, S.-R. Park: Establishment of measurement traceability for peptide and protein quantification through rigorous purity assessment – A review, Metrologia 56 (2019) 4, 044006 (29pp).

[2] 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

[3] R. D. Josephs, M. Li, D. Song, S. Westwood, N. Stoppacher, A. Daireaux, T. Choteau, R. I. 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: Final Report on key comparison CCQM-K115: Peptide Purity - Synthetic Human C-Peptide, Metrologia 54 (2017) Tech. Suppl., 1A, 08007.

[4] R. D. Josephs, M. Li, D. Song, A. Daireaux, T. Choteau, N. Stoppacher, S. Westwood, R. I. Wielgosz, H. Li, J. E. Melanson, M. Akgöz, A. C. Gören, I. Ün, M. Quaglia, J. Warren: Final Report on pilot study CCQM-P55.2: Peptide Purity - Synthetic Human C-Peptide, Metrologia 54 (2017) Tech. Suppl., 1A, 08011.

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

[6] 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.

[7] 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.

[8] 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.

[9] 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.

[10] 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.

[11] 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.

[12] 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.

[13] 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.

[14] 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.

[15] 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.

[16] 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.

[17] 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.

[18] 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.

[19] R. D. Josephs, N. Stoppacher, A. Daireaux, T. Choteau, K. A. Lippa, K. W. Phinney, S. Westwood, R. I. Wielgosz: State-of-the-art and Trends for the SI Traceable Value Assignment of the Purity of Peptides Using the Model Compound Angiotensin I, Trends Anal. Chem. 101 (2018) 108-119.

[20] J. E. Melanson, M. P. Thibeault, B. B. Stocks, D. M. Leek, G. McRae, J. Meija: Purity assignment for peptide certified reference materials by combining qNMR and LC-MS/MS amino acid analysis results: application to angiotensin II, Anal. Bioanal. Chem. 410 (2018) 6719–6731.

[21] R. DerSimonian, N. Laird: Meta-analysis in clinical trials, Control. Clin. Trials 7 (1986) 177–188.

[22] Calculated using the DerSimonian-Laird estimator of the 'NIST Consensus Builder' online app (https://consensus.nist.gov).