CCQM-K148.b
Polar analyte in solid organic material:
Mass fraction of oxytetracycline in solid organic material

Key Comparison
Track A

Draft B Report
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Gustavo Martos, Steven Westwood, Magali Bedu, Tiphaine Choteau, Ralf Josephs and Robert Wielgosz

Bureau International des Poids et Mesures (BIPM)

Sèvres, France

Mine Bilsel, Gokhan Aktas, Hatice Altuntas, Gonca Coşkun, Tugba Dıspınar Gezer, Simay Gündüz, Alper Isleyen, Sukran Akkus Ozen, İlker Ün and Hasibe Yılmaz.

National Metrology Institute of Turkey (TUBITAK UME)

Gebze-Kocaeli, Turkey

With contributions from:

Stephen R. Davies, Mahiuddin Alamgir, Benjamin Chan, Michael Moawad, Emma Kent, Chantelle Nguyen.
National Measurement Institute Australia (NMIA)
North Ryde, Australia

Eliane Cristina Pires do Rego, Bruno Carius Garrido, Jane Luiza Nogueira Fernandes, Wagner Wollinger, Thiago Oliveira Araújo, Ana Catalina Osorio and Marcelo Dominguez de Almeida.
Instituto Nacional de Metrologia, Qualidade e Tecnologia (INMETRO)
Duque de Caxias, Brazil

Marie-Pier Thibeault, Isabelle Rajotte, Jennifer Bates, Sabrina D. Giddings, Elliott J. Wright, Krista Thomas, Pearse McCarron, Andreas Brinkmann.
National Research Council Canada, Metrology
Ottawa, Canada

Fuhai Su, Qinghe Zhang, Qi Wei, Wei Zhang, Lianhua Shi, Weihua Wang, Ting Huang, Yaxin Han, Wenxin Li.
National Institute of Metrology (China) (NIM)
Beijing, China

Jasmine Po-kwan LAU, Chun-wai TSE, Wai-hong FUNG.
Government Laboratory of Hong Kong SAR (GLHK)
Kowloon, Hong Kong SAR, China

Klas Meyer.
Bundesanstalt für Materialforschung und -prüfung. (BAM)
Berlin, Germany

Panagiota Giannikopoulou, Ilias Kakoulidis
Chemical Metrology Laboratory (EXHM/GCSL-EIM)
Athens, Greece

Yoshitaka Shimizu, Migaku Kawaguchi, Sakae Eyama, Toshihiro Suzuki, Yoshiyasu Yamauchi, Taichi Yamazaki, Satoe Nakamura, Xinnu Bao, Yuko Kitamaki, Megumi Kato, Nobuyasu Itoh.
National Metrology Institute of Japan (NMIJ)
Tsukuba, Japan

Anatoliy I. Krylov, Ludmila V. Eliseeva, Anna Yu. Ivanova, Elena M. Lopushanskaya, Alena Yu. Mikheeva, Marina Yu. Palagina, Sergey G. Kharitonov
D.I. Mendeleyev Scientific and Research Institute for Metrology (VNIIM)
St Petersburg, Russia

Teo Tang Lin, Cheow Pui Sze, Lu Ting, Gui Ee Mei, Wang Juan, Benny Tong, Sim Lay Peng, Leung Ho Wah, Richard Shin.
Health Sciences Authority (HSA)
Singapore

Désirée Prevoo-Franzsen, Nontete Nhlapo, Caitlin Swiegelaar, Maria Fernandes-Whaley, Thabang Chiloane, Laura Quinn, Mare Linsky, Dominique Marajh.
National Metrology Institute of South Africa (NMISA)
Pretoria, South Africa

Sunyoung Lee, Song-Yee Baek, Ha-jeong Kwon, Jae Hyun Lee, Kihwan Choi.
Korea Research Institute of Standards and Science (KRISS)
Daejeon, South Korea

Sornkrit Marbumrung, Ponhatai Kankaew, Pornnipa Jongmesuk, Cheerapa Boonyakong, Patumporn Rodruangtham, Nittaya Sudsiri, Chomchai Suksai and Sarayut Watchasit.
National Institute of Metrology (Thailand) (NIMT)
Bangkok, Thailand

Cailean Clarkson, Eli Achtar, Sumiksha Rai.
LGC Limited (LGC)
Teddington, United Kingdom

Michael A. Nelson, Jerome Mulloor, Brian E. Lang, Aaron A. Urbas.
National Institute of Standards and Technology (NIST)
Gaithersburg, MD, USA

Ferial Tadjine, Joachim Polzer.
Federal office of consumer protection and food safety (BVL)
Berlin, Germany

Chiara Portesi, Consolato Schiavone.
Istituto Nazionale di Ricerca Metrologica (INRiM)
Torino, Italy

Shima Hashim, Haznita Rose Bahari, Nor Fariha Muhamad Zin, Siti Nor Fadhilah Sulaiman
Department of Chemistry Malaysia (KIMIA)
Selangor, Malaysia

SUMMARY

The CCQM-K148.b comparison, undertaken with a parallel pilot study CCQM-P187.b, was coordinated by the BIPM and UME on behalf of the Consultative Committee for Amount of Substance: Metrology in Chemistry and Biology (CCQM) Working Group on Organic Analysis (OAWG). It was undertaken for National Measurement Institutes (NMIs) and Designated Institutes (DIs) which provide measurement services in organic analysis under the CIPM Mutual Recognition Arrangement (MRA) and was designated a Track A comparison within the OAWG implementation of the CCQM Strategy for Programme Development 2021-2030.[1]

The ability to assign the mass fraction content of the primary component in a solid organic material that an NMI makes available as a pure substance Reference Material or that is used by an NMI in-house as a Primary Reference Material is a critical technical competency for the provision of SI-traceable quantitative measurement results in organic analysis. The purity property value assigned to the Primary Reference Material in a measurement hierarchy anchors the calibration chain for all results linked to that material.

Participation in the series of Track A purity comparisons organized by the OAWG allows an NMI/DI to demonstrate that their procedure for the assignment of a purity property value and its associated uncertainty are fit for purpose for their intended application. Evidence of successful participation in formal, relevant international comparisons is required under the CIPM Mutual Recognition Arrangement (MRA) to support calibration and measurement capability (CMC) claims made by NMIs and DIs.

Nineteen NMIs in addition to the BIPM, submitted results in CCQM-K148.b (one laboratory submitted results to the pilot study). Participants were required to assign the mass fraction of oxytetracycline free base (OTC), standardized to the value expected at 50% relative humidity, present in a solid material containing the oxytetracycline hydrochloride salt as the principal component.

Eight participants assigned their final value for the comparison through the combination of values obtained by independent mass balance and qNMR methods. Seven participants reported a result from a mass balance method only and five reported a result by qNMR only.

Successful participation in CCQM-K148.b demonstrates capabilities for assigning the mass fraction of organic compounds with molar mass in the range of 75 g/mol to 500 g/mol, having high polarity (pKow > -2), including compounds presenting significant hygroscopicity, in an organic solid material.

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INTRODUCTION

Evidence of successful participation in formal, relevant international comparisons is required to establish measurement capability claims (CMCs) made by NMIs and Designated Institutes (DIs) with active programmes in organic analysis. In April 2019, the Consultative Committee for Amount of Substance: Metrology in Chemistry and Biology (CCQM) approved the Key Comparison (KC) CCQM-K148.b on high polarity pure organics. CCQM-K148.b was designed to assess participants' capabilities for the mass fraction value assignment of high purity organic substances containing a polar analyte ($pK_{OW} > -2$) having a molar mass in the range 75-500 g/mol as the primary component. It is a component of the overall OAWG strategy of Track A key comparisons that serve to underpin and benchmark NMI capabilities for the provision of primary calibration services for organic analysis.

Oxytetracycline's (OTC's) physical properties meet the model requirements of the OAWG. It is a member of the tetracyclines group of broad-spectrum antibiotic compounds, widely used in veterinary medicine, that have a common basic structure. Because of concerns with the potential health risk to the consumer of long-term exposure to low levels of these compounds, monitoring programs for the presence of tetracycline residues in the environment and in food of animal origin including meat, fish, milk, eggs and honey are in place in many countries.[2] These activities, which improve food safety and reduce the potential for technical trade barriers in this area, need to be supported by a sound reference measurement infrastructure for tetracycline analysis.

This comparison compliments CCQM-K148.a, completed in 2018, which examined the measurement of a non-polar organic analyte present as the primary component in a high-purity organic material. In addition, the current CCQM-K148.b comparison material poses a genuine challenge due to its highly hygroscopic nature. The comparison protocol distributed to participants included specific instructions on handling and reporting of purity values at standardized conditions of relative humidity.

The following sections of this report document the timeline of CCQM-K148.b, the measurands, study material, participants, results, and the measurement capability claims that participation in CCQM-K148.b can support. The Appendices reproduce the official communication materials and summaries of information about the results provided by the participants.

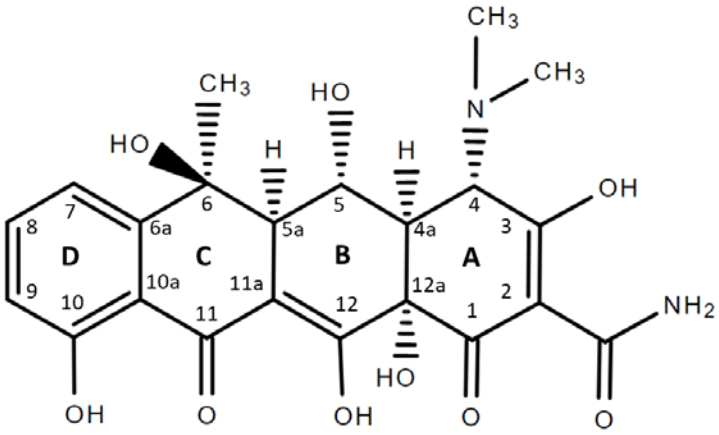
TIMELINE

Table 1. *Comparison timeline*

Date	Action
April 2019	Proposed to CCQM
June 2022	Draft protocol presented to OAWG as potential Track A Key Comparison
October 2022	OAWG authorized CCQM-K148.b as a Track A Key Comparison; protocol approved
October 2022	Call for participation to OAWG members
October 2022 - March 2023	Study samples shipped to participants. The range in shipping times reflects delays from shipping and customs.
15 June 2023	Results due to coordinating laboratory
August 2023	Draft A.1 report sent to participants
October 2023	Draft A report presented to OAWG
November 2024	Draft B report distributed to OAWG
TBD	Final report approved by OAWG

MEASURAND

The comparison requires the assignment of the mass fraction, reported in mg/g, of oxytetracycline free base (OTC) in a unit of the oxytetracycline hydrochloride (OTC.HCl) comparison material under standardized conditions of relative humidity. Figure 1 below displays the molecular structure of the free base (4*S* epimer).



Oxytetracycline (OTC)

Molar mass = 460.43 g/mol; pK_{OW} ~ 0.5

Fig. 1: *Structure and conventional numbering of oxytetracycline*

STUDY MATERIALS

The comparison material was produced by TÜBITAK-UME. A bulk source material of OTC.HCl in the form of a fine yellow crystalline powder was homogenized in a 3D mixer and kept in a vacuumed container until filling to minimize moisture uptake. About 0.5 g of the material were filled into each vial of the comparison batch using an automatic filling machine.

Each participant received as a minimum two vials of the comparison material, each containing a minimum of 500 mg of OTC.HCl. Participants who planned to use multiple independent methods to contribute to their final property value assignment (e.g. a mass balance procedure and a separate qNMR procedure) were allowed to request an additional vial. The recommended minimum sample amount for analysis was at least one vial. The comparison samples were provided in amber glass vials sealed with PTFE-lined screw-caps. Measurement results were to be reported on the material as received without additional treatment but taking into account the hygroscopicity correction described in the comparison protocol.

Homogeneity Assessment of Study Material

The homogeneity of the batch was tested using an LC-UV method for the content of OTC and the main structurally related impurities. An oven-transfer, coulometric Karl Fischer titration was used for determination of water content and ion chromatography for chloride ion content. The uncertainty contribution due to inhomogeneity of the assigned values was evaluated by ANOVA. Ten vials were selected at regular intervals from the filling sequence to ensure that the results would indicate any trend in the filling process. Each vial was analyzed in a random order to ensure any trends in the bottling process were separated from possible trends resulting from the analytical sequence.

The results obtained indicated no statistically significant difference in the within- and between-vial levels of the mass fraction of each component in the material. The upper limit for the uncertainty contribution due to inhomogeneity in all cases was sufficiently small as to be unlikely to influence the effective comparison of participant results. A summary of the observed within- and between-sample variability for the major components is shown in Table 2.

Table 2. *Homogeneity assessment for the main component OTC, the main related structure impurity (coded as "Imp A"), water and chloride in the comparison material.*

ANOVA Estimate	**OTC**	**Imp A**	**H_2O**	**Cl^-**
Between-unit CV (%)	0.36%	0.77%	0.64%	0.87%
Within-unit CV (%)	0.83%	1.10%	1.03%	1.44%
Upper limit of relative uncertainty contribution due to inhomogeneity	0.27%	0.43%	0.37%	0.47%

Probability of falsely rejecting the hypothesis that all samples have the same concentration	< 5%	< 5%	< 5%	< 5%

A plot of the normalized mass fraction for each analyte obtained for the homogeneity assessment is plotted by filling sequence in Figure 2. The normalized values of repeat measurements from three aliquots taken from each individual vial are plotted.

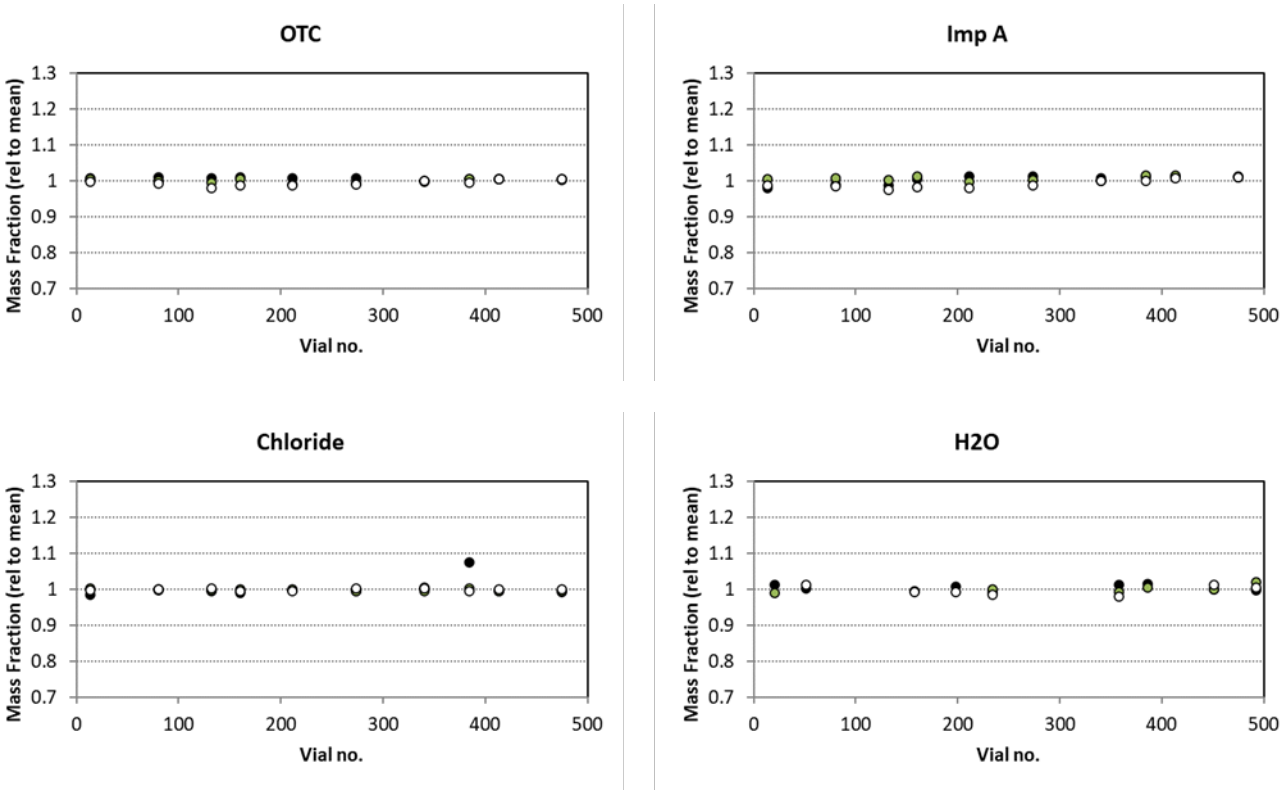


Fig. 2. *Homogeneity evaluation for OTC, the major related structure impurity (coded "Imp A"), water and chloride in the comparison material.*

Stability Assessment of Study Material

An isochronous stability study was undertaken for OTC, related structure impurities, water and chloride on storage at 4 °C, 22 °C and 40 °C in the dark. The analytical methods used were the same as in the homogeneity study. The material is sufficiently stable, within the proposed time scale of the comparison, when stored at 4 °C or 22 °C. OTC and some impurities were not stable at 40 °C. Precautions were taken to monitor if the comparison material was exposed to temperatures above 25 °C during shipment and if this occurred replacement material was provided.

The mass fractions of OTC and chloride relative to the mean value of reference samples stored at -20 °C are shown in Figure 3 for samples stored at 4 °C and 22 °C during the stability study period. The plot displays the normalized results of duplicate analysis of samples prepared from two units of CCQM-K148.b. The upper and lower dashed lines indicate the uncertainty of the regression line, which reflects the analytical method variance in the absence of a significant instability trend.

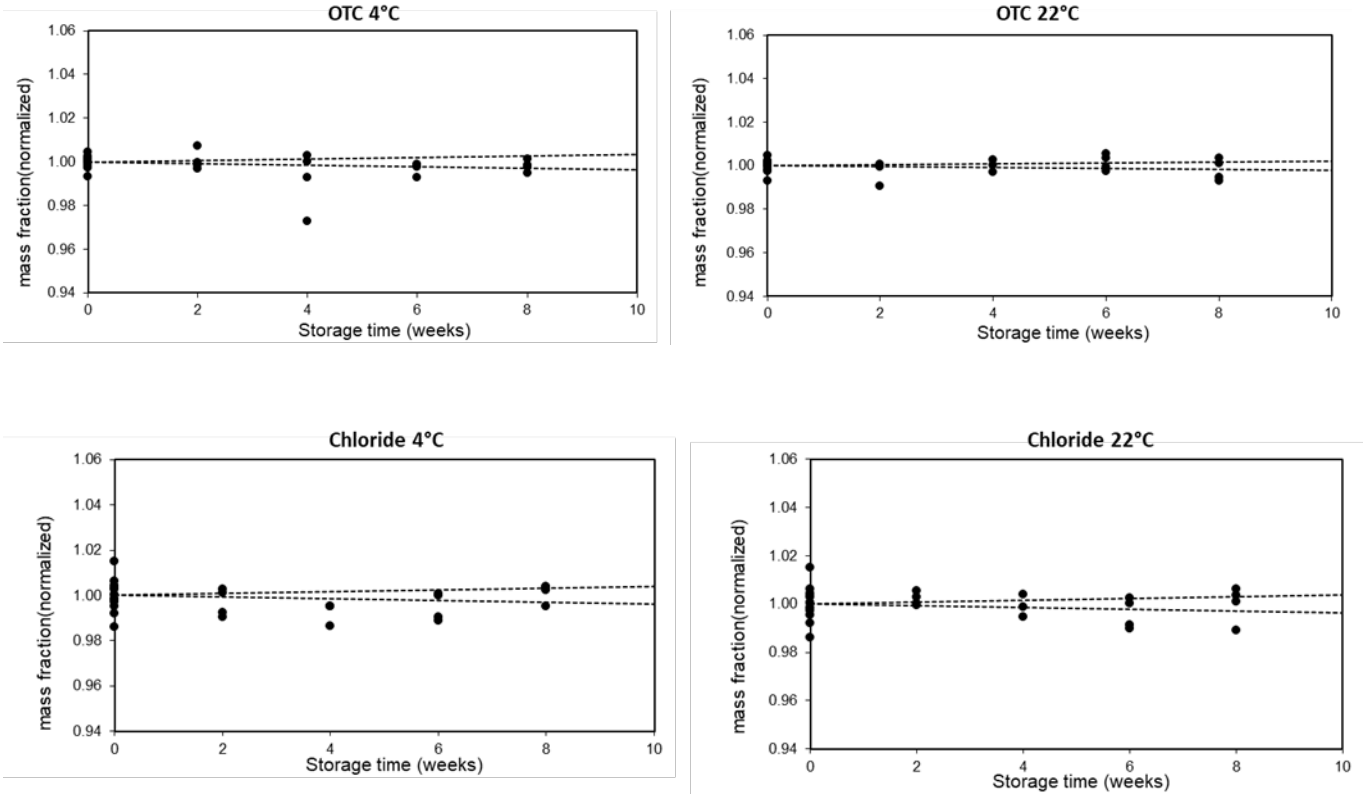


Fig. 3. *Stability evaluation of OTC and chloride content in samples stored at 4 °C and 22 °C for 8 weeks.*

PARTICIPANTS, INSTRUCTIONS AND SAMPLE DISTRIBUTION

The call for participation was distributed in October 2022 with the intent to distribute samples in November 2022, receive results in March 2023 (subsequently postponed to May and eventually June 2023), and discuss results at the online OAWG meeting in October 2023. See Table 1 for study timeline. Appendix A reproduces the call for participation and study protocol.

Twenty institutes including the BIPM registered to participate in the key comparison and one institute, NMLPhil, registered to participate in the parallel pilot study CCQM-P187.b (Table 3). The results of the pilot study are not discussed in this report.

Table 3. *Institutions Registered for CCQM-K148.b*

NMI or DI	Code	Country	Contact
Bureau International des Poids et Mesures	BIPM	France	Gustavo Martos, Steven Westwood
Bundesanstalt für Materialforschung und -prüfung	BAM	Germany	Klas Meyer
German Federal Office of Consumer Protection and Food Safety	BVL	Germany	Ferial Tadjine, Joachim Polzer
EXHM/GCSL-EIM	EXHM	Greece	Elias Kakoulides
Government Laboratory, Hong Kong, China	GLHK	Hong Kong, China	Wai-hong FUNG, Chun-wai TSE, Jasmine Po-kwan LAU
Health Sciences Authority, Chemical Metrology Laboratory	HSA	Singapore	Pui Sze Cheow, Tang Lin Teo
National Institute of Metrology, Quality and Technology	INMETRO	Brazil	Eliane Cristina Pires do Rego, Wagner Wollinger
Department of Chemistry Malaysia	KIMIA	Malaysia	SHIMA HASHIM
Korea Research Institute of Standards and Science	KRISS	Korea	Sunyoung Lee, Ki Hwan Choi
NML, LGC, HS&I, Purity & Calibration	LGC	United Kingdom	Cailean Clarkson
National Institute of Metrology, China	NIM China	China	Fuhai SU, Qinghe ZHANG
NIST / Material Measurement Laboratory	NIST	United States of America	Michael Nelson
National Measurement Institute, Australia	NMIA	Australia	Stephen Davies
National Metrology Institute of Japan	NMIJ	Japan	Yoshitaka Shimizu
National Metrology Institute of South Africa	NMISA	South Africa	Désirée Prevoo-Franzsen

National Research Council Canada	NRC	Canada	Jennifer Bates
TUBITAK Ulusal Metroloji Enstitusu (UME)	UME	Turkey	Mine Bilsel
D.I. Mendeleev All-Russian Institute for Metrology	VNIIM	Russia	Anatoliy Krylov, Alena Mikheeva
National Institute of Metrology	NIMT	Thailand	Sornkrit Marbumrung, Ponhatai Kankaew
Istituto Nazionale di Ricerca Metrologica	INRIM	Italy	Chiara Portesi

Two or three units of the comparison material were shipped by the coordinating laboratory to each participant. The number of vials provided depended on whether the participants used a single purity assignment method or the combination of multiple approaches. Participants returned a form acknowledging receipt of the samples, advising the comparison coordinator if any obvious damage had occurred to the vials during shipping, and indicating whether a monitoring strip inside the container indicated exposure to a temperature in excess of 25 °C during the shipping process. Problems were reported in shipment of the comparison material due to exposure to excessive temperature by HSA, GLHK, NIMT and KIMIA. One participant, KRISS, requested additional samples due to the malfunctioning of their refrigerator, which resulted in the initial samples being exposed to temperatures above 25°C. Replacement units were shipped to all the participants concerned.

Participants were required to report their estimate of the mass fraction of OTC as the free base present in the material in mg/g, standardized to the value expected at 50% RH. The result should be based on combined values obtained by the measurement of multiple aliquots from at least one of the vials supplied. Participants were also required to verify the accuracy of their relative humidity measurements and those who used a mass balance procedure were required to report the combined mass fraction assignment (estimated if measured at RH = 50%) and associated uncertainty for the each of the contributing sub-classes of impurity: total related structure organic impurities, water, chloride, residual solvent and total non-volatiles/inorganics content.

A copy of the text in the format of the Excel spreadsheet provided to participants to submit their results is reproduced in Appendix C.

RESULTS

Participants were requested to report a single estimate of the mass fraction (in mg/g) of OTC in the comparison material, standardized to the value expected at 50% RH. In addition to the quantitative results, participants were instructed to describe their analytical methods, approach to

uncertainty estimation, and the Core Competencies they felt were demonstrated in this study. Appendices B, C, and D reproduce the registration, reporting and core competency forms, respectively.

Participants using a mass balance procedure were required to report the combined mass fraction assignment and associated uncertainty for the assigned sub-classes of impurity: total related structure organic impurities, water, chloride, residual solvent and total non-volatiles/inorganics content. In addition, participants were encouraged but not required to identify and provide mass fraction estimates for all significant individual impurity components quantified in the comparison sample.

CCQM-K148.b results were received from all 20 institutions that received samples. Eight participants assigned their final value for the comparison through the combination of values obtained by independent mass balance and qNMR methods. Seven participants reported a result from a mass balance method only and five reported a result from qNMR only.

Calibration Materials Used by Participants

Participants established the metrological traceability of their results using certified reference materials (CRMs) with stated traceability and/or commercially available high purity materials for which they determined the purity. Table 4 lists the CRMs that were reported by the participants that performed the value assignment of the main component using qNMR methods.

Table 4. *CRMs and high-purity materials used as source of traceability for OTC qNMR measurements in CCQM-K148.b.*

CRM	Provider	Used by	In-house purity assignment of CRM
QNMR010 (Maleic acid)	NMIA	HSA, NMIA	
Tracesure 135-17951 (Maleic acid)	Wako	BIPM	BIPM (qNMR)
Maleic acid CRM	Inmetro	Inmetro	
TraceCert Maleic acid	Merck	BAM, LGC, INRIM, EXHM	BAM, LGC (qNMR), EXHM (qNMR)
HRM-1012A (Acesulfame potassium)	HSA	HSA	
CRM 4601 (3,5-Bis(trifluoro methyl) benzoic acid)	NMIJ	HSA, NMIJ, GLHK, BAM	
NIST PS1 (Benzoic acid)	NIST	HSA, NRC, LGC, UME, KRISS, INRIM	

TraceCERT 1,2,4,5-Tetrachloro-3-nitrobenzene	Merck	NMISA, Inmetro, NIMT	NMISA, Inmetro (qNMR)
MRC 8784.0001 (Dimethyl terephthalate)	INMETRO	NRC	
Dimethyl terephthalate	NIST	NIST	NIST (qNMR)
CRM GBW 06120 (Ethylparaben)	NIM-China	NIM-China	
Tecnazene	NIST	NIST	NIST (qNMR)

Traceability of qNMR measurements was achieved through the use of appropriate standard materials, either produced or value assigned in-house by NMIs/DIs having demonstrated relevant capabilities in previous CCQM Track A Key comparisons. However, NIMT and INRIM directly used the certified values of commercial standards from Merck.

Participants using a mass balance approach employed a variety of CRMs, commercial standards and other materials value-assigned in-house as calibrators for the different techniques used to quantify all the impurity sub-classes: total related structure organic impurities, water, chloride, residual solvent and total non-volatiles/inorganics.

Participant Results for OTC content in CCQM-K148.b

The different approaches used by participants for the mass fraction assignment of OTC were as follows:

- Mass balance as the sole method: NMISA*, EXHM*, KIMIA, BVL, VNIIM, NMIA*, and KRISS* (*Used qNMR as confirmation method only).
- qNMR uncorrected by independent impurity measurements: BAM, LGC, NIST, INRIM
- qNMR corrected by independent impurity measurements: NRC
- Combination of mass balance and qNMR (uncorrected by independent impurity measurements): HSA, NMIJ, NIM China, GLHK, UME and NIMT.
- Combination of mass balance and qNMR (corrected by independent impurity measurements): BIPM, INMETRO

In addition to the laboratories using the mass balance approach, NIST and LGC reported water content values.

Table 5. *CCQM-K148.b results for the mass fraction assignment of OTC and the individual reported values from mass balance (MB) and qNMR methods employed by participants. *Used qNMR value for confirmation purposes only.*

NMI	CCQM.K148.b (mg/g)	u(w) (mg/g)	U_{95}(w) (mg/g)	MB (mg/g)	qNMR (mg/g)
HSA	777.1	6.9	13.8	786.3	767.9
NMISA*	780	6.2	15	780	796
NRC	787	13	26		787
BIPM	789.3	3.1	6.2	788.8	790.1
NMIJ	791.1	3.5	7	795.9	786.4
NMIA*	792	7	14	792	797
NIM-C	792.6	4.9	9.8	796.65	788.6
GLHK	796.5	4.3	8.6	800.9	793.7
INMETRO	796.7	3.3	6.6	799.4	794.1
EXHM*	797.50	4.67	9.35	797.50	799.97
BAM	798.9	0.8	1.6		798.9
LGC	805.6	2.3	4.7		805.6
NIST	806	2.5	5		806
UME	816.5	13	26.1	817.5	815.5
KRISS*	819.4	2.5	5	819.4	812.2
KIMIA	827.12	5.48	10.96	827.12	
BVL	833.33	5.14	10.28	835.46	
VNIIM	844.5	2.7	5.4	844.5	
NIMT	845.8	22.78	45.6	846.56	845
INRIM	861.7	3.07	6.14		861.7

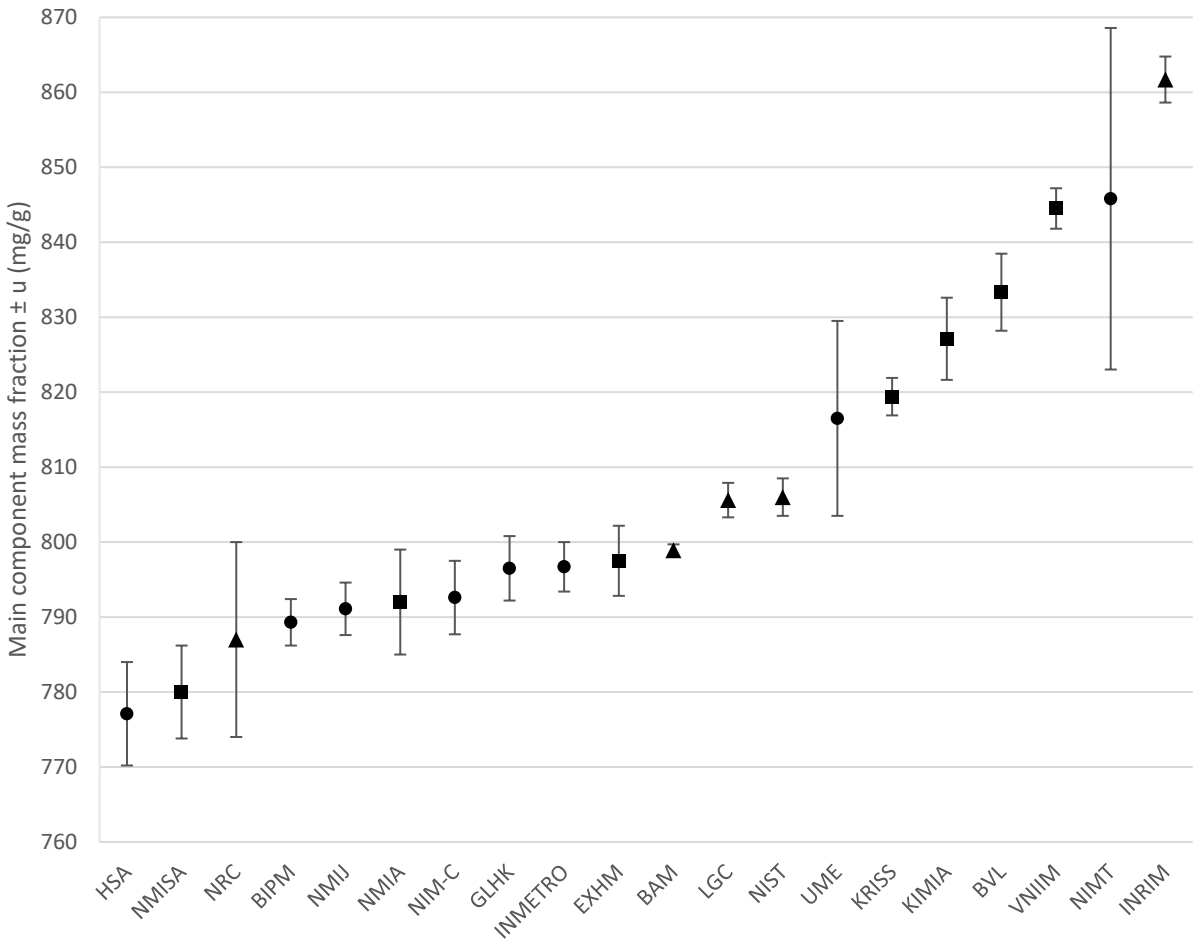


Fig. 4. *CCQM-K148.b reported results for the mass fraction assignment of OTC. The squares, triangles and circles indicate the assignment methods mass balance, qNMR or the combination of both, respectively.*

Overview of main impurity subclasses

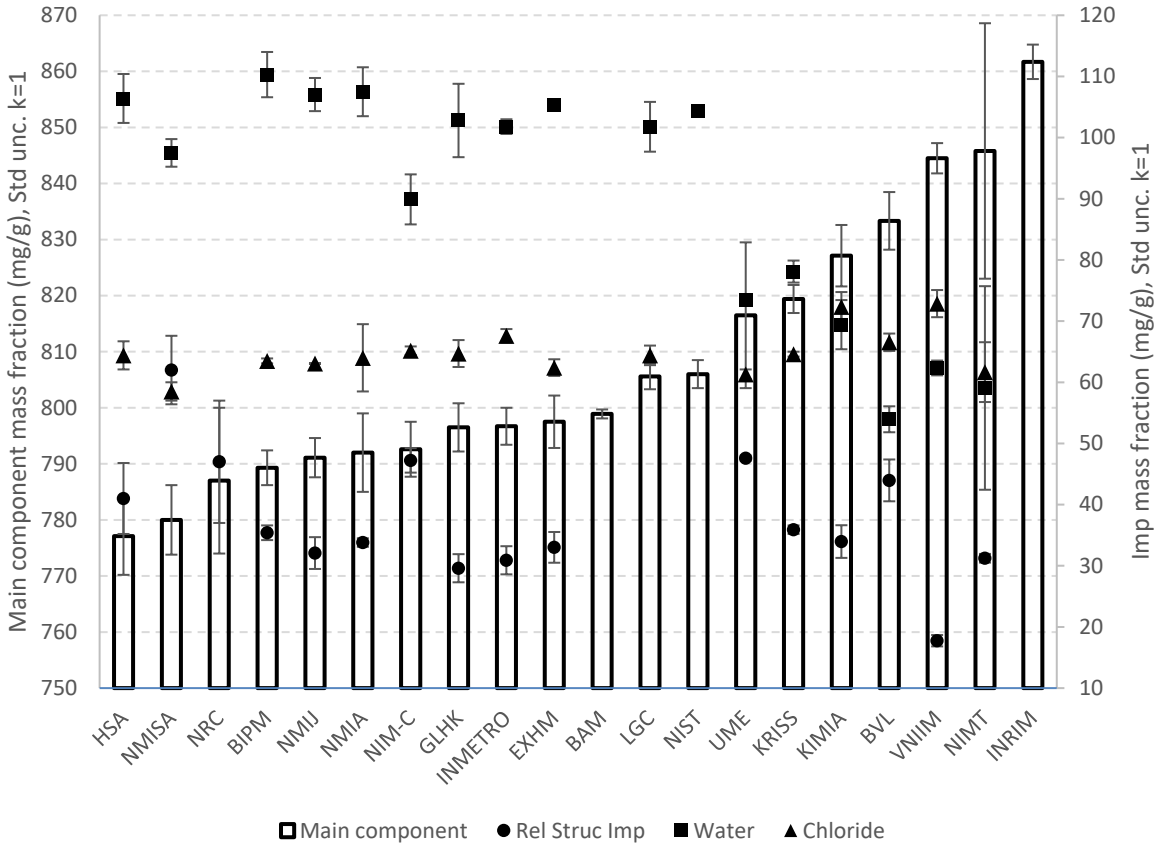


Fig. 5. *CCQM-K148.b results for the mass fraction assignment of OTC and the major impurity subclasses.*

The sections below summarize the results for each impurity class. A summary of the analytical methods used per participant is given in Appendix E.

Related Structure Impurity content

Methods based on LC-UV were the predominant approach used to analyze the material for related structure impurity content. Other methods used included LC-CAD and LC-MS for impurity identity determination or confirmation. Several participants reported instability of impurities under the studied conditions, which included different solvents for sample dissolution.

Table 6. *CCQM-K148.b results for the mass fraction assignment of structurally related organic impurities.*

NMI	w (mg/g)	u(w) (mg/g)
HSA	41	5.8
NMISA	62	5.6
NRC	47	10
BIPM	35.4	1.2
NMIJ	32.08	2.6
NMIA	33.8	0.7
NIM	47.23	2
GLHK	29.6	2.3
INMETRO	30.9	2.3
EXHM	33.01	2.51
UME	47.6	0.4
KRISS	35.9	0.7
KIMIA	33.96	2.67
BVL	43.96	3.42
VNIIM	17.75	0.93
NIMT	31.23	0.71

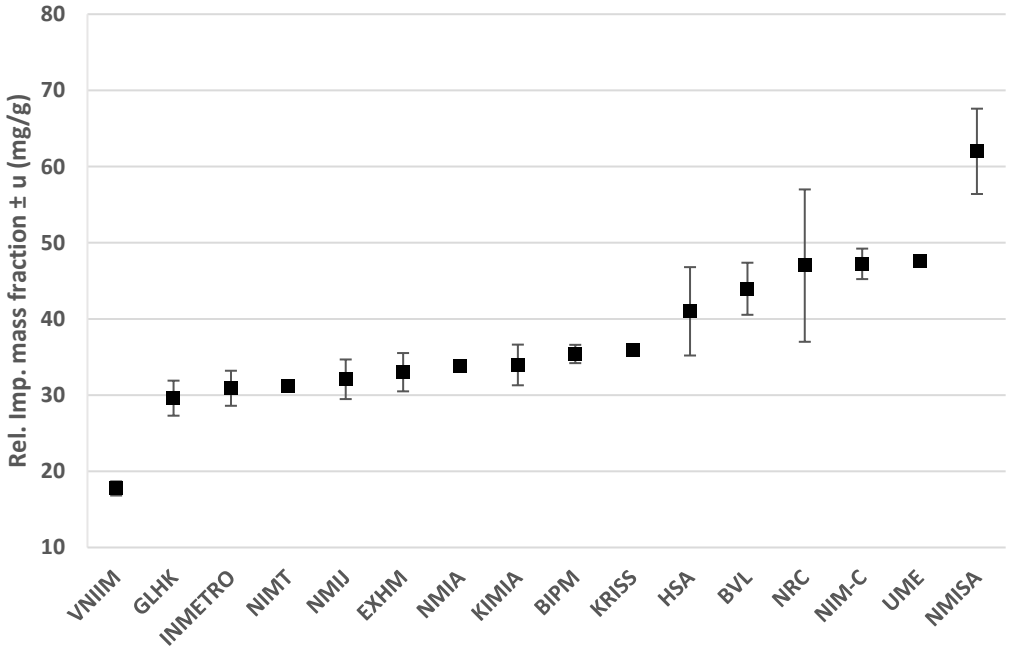


Fig. 6. *CCQM-K148.b reported results for the mass fraction assignment of structurally related organic impurities.*

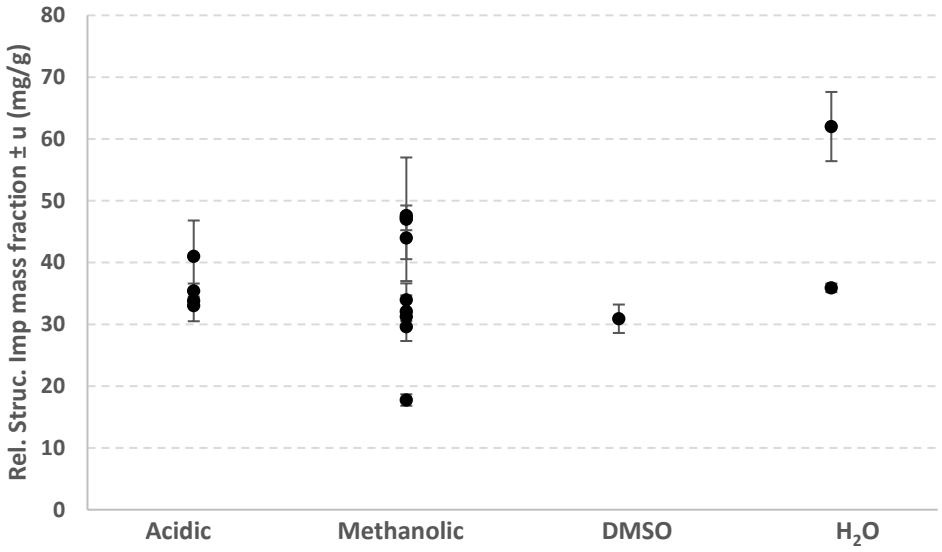


Fig. 7. *Mass fraction values of structurally related organic impurities as a function of solvent used for material dissolution. "Acidic" includes participants using 10 mM HCl (aq), 100 mM HCl (aq) or 0.1% H_3PO_4 (aq):ACN (90:10 v:v); "Methanolic" includes pure CH_3OH and 15% CH_3OH (aq, v:v); "DMSO" stands for $(CH_3)_2SO$.*

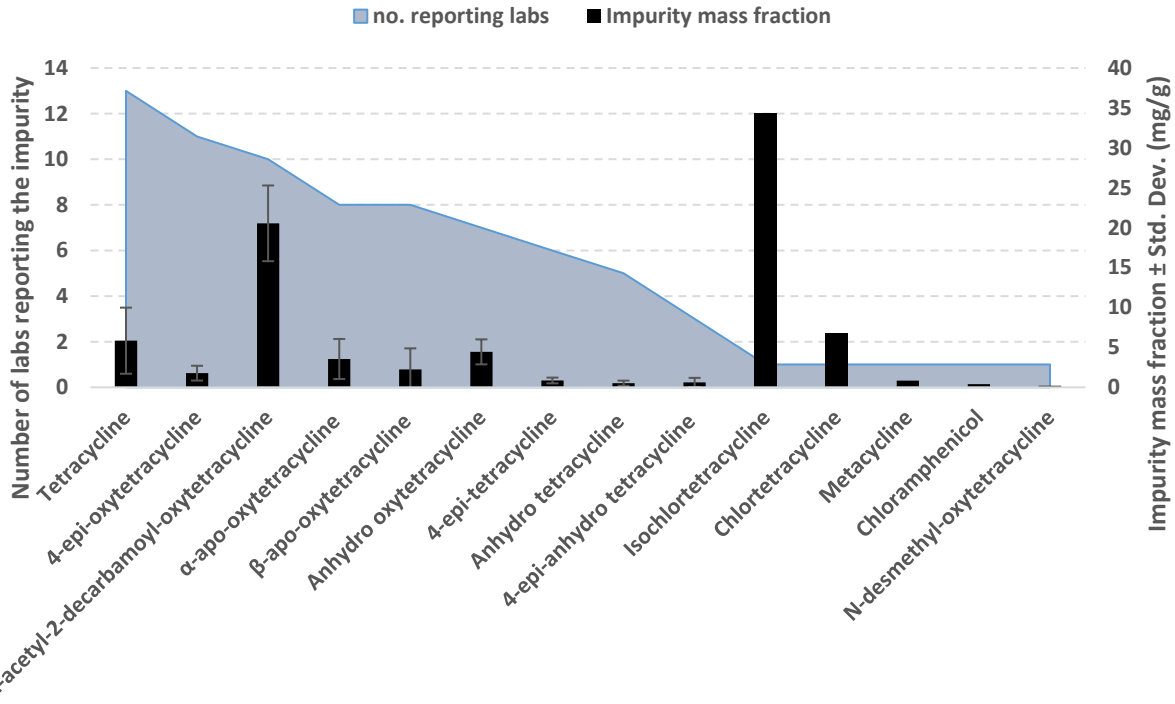


Fig. 8. *Mass fraction values of reported related structure impurities in CCQM-K148.b material ranked by the number of laboratories that identified each impurity.*

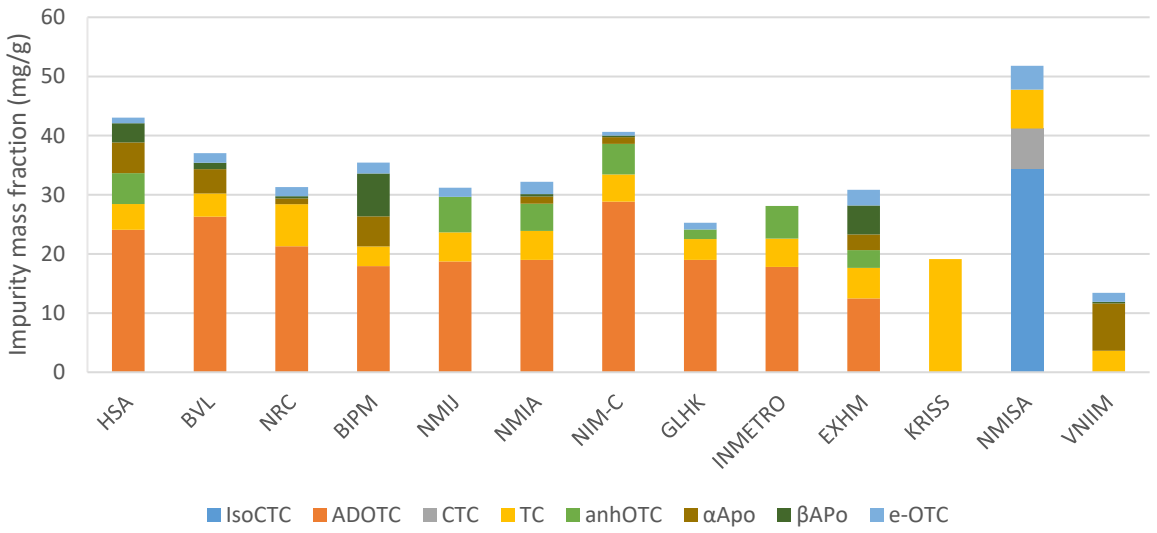


Fig. 9. *Impurity quantification profile displaying the eight most abundant impurities identified by participants. Reported quantified impurities for which identity was not fully established are not represented. See Fig. 8 for the full impurity names.*

Water content

All participants used coulometric Karl Fischer titration, either after introduction of the sample directly into the titration cell or through transfer of the water content into the titration cell from an oven-heated aliquot of the comparison material using a flow of dry gas. A few participants used TGA as confirmatory technique.

Table 7. *CCQM-K148.b results for the water content assignment at standardized conditions of 50% RH and values obtained under laboratory's conditions of relative humidity. n.r.: not reported.*

NMI	w (mg/g) (50% RH)	u(w) (mg/g)	w (mg/g) (Lab RH)	Lab RH (%)
HSA	106.4	4	106.8	46-57
NMISA	97.5	2.26	95.9	45
BIPM	110.3	3.7	110.3	51.6
NMIJ	107.04	2.71	106.95	49-50
NMIA	107.5	4	107.3	54
NIM-C	89.9	4.09	89	47.2
GLHK	102.8	6	*n.r.*	49-52

INMETRO	101.8	1.2	104.6	58
EXHM	105.33	0.66	100.28	44
LGC	101.77	4.07	101.44	49
NIST	104.4	0.8	105.7	53-54
UME	73.4	0.5	74.8	54
KRISS	78.1	1.8	74.8	40-43
KIMIA	69.42	4.02	69.59	57
BVL	53.95	2.12	51.85	44
VNIIM	62.34	1.21	62.34	50
NIMT	59.07	16.64	62.16	59

To report values estimated at standardized conditions of 50% RH (Figure 10), participants were asked to correct their mass fraction assignments using the equation provided in the comparison protocol (Appendix A). Overall, the relative magnitude of the correction for the water content assignment applied by the participants was smaller than 5%, which led to very small differences between the values assigned at laboratories' RHs and the reported ones at 50% RH (Table 7).

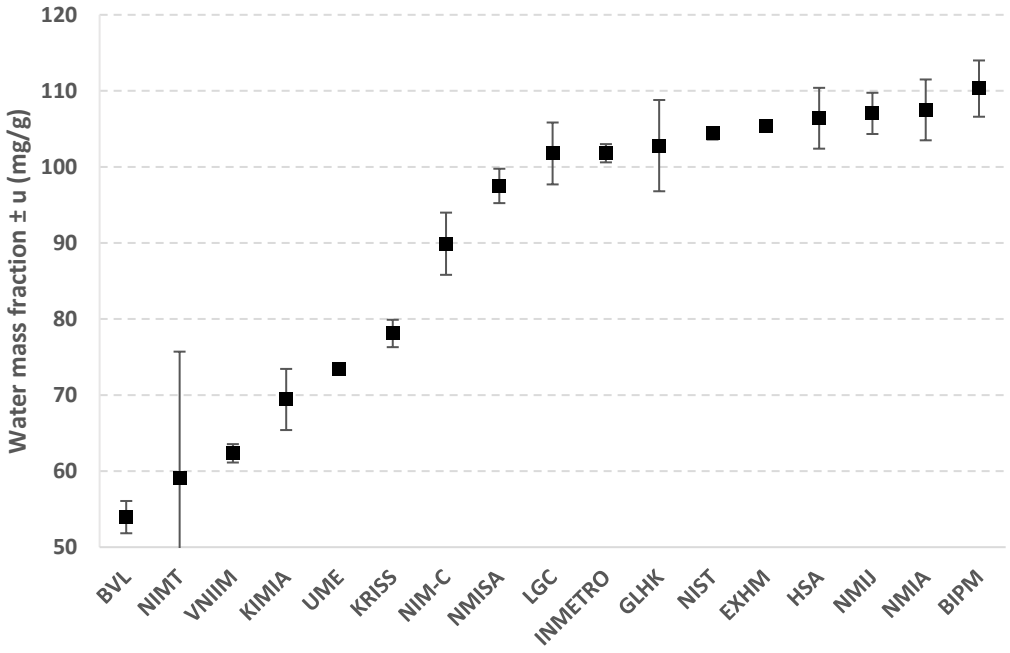


Fig. 10. *CCQM-K148.b reported results for the mass fraction assignment of water content at 50% RH.*

Chloride content

Ion chromatography was predominantly used to analyze the material for chloride ion impurity content. Other methods used included ICP-MS, X-ray fluorescence and CE-UV. A summary of the methods and conditions used per participant is given in Appendix E.

Table 8. *CCQM-K148.b results for the chloride content assignment.*

NMI	w (mg/g)	u(w) (mg/g)
HSA	64.4	2.3
NMISA	58.5	1.5
BIPM	63.5	0.4
NMIJ	63.07	0.07
NMIA	64	5.5
NIM-C	65.16	0.7
GLHK	64.7	2.2
INMETRO	67.6	1.1
EXHM	62.4	1.36
LGC	64.4	1.6
UME	61.3	0.8
KRISS	64.6	0.4
KIMIA	72.33	2.42
BVL	66.55	1.42
VNIIM	72.86	2.22
NIMT	61.66	4.89

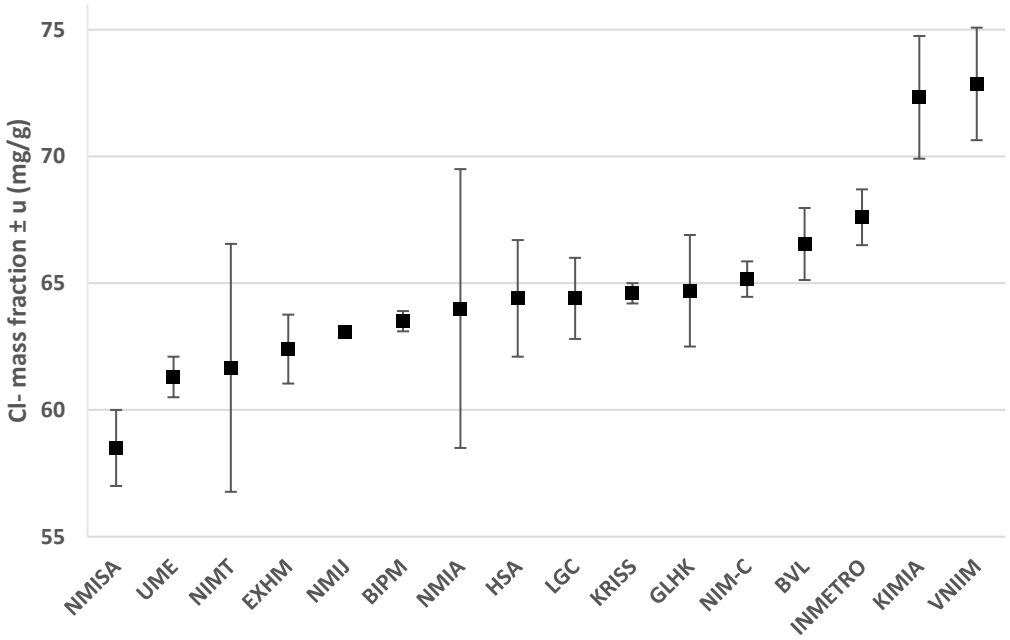


Fig. 11. *CQM-K148.b reported results for the mass fraction assignment of chloride content.*

Volatile organics content

Fifteen participants provided information on the volatile organics content of CCQM-K148.b material. Five participants reported no evidence for the presence of residual solvent above their method detection limits. The results reported by participants with their associated standard uncertainties (k = 1) are listed in Table 9.

Only two participants reported levels above 1 mg/g of this class of impurity. An overview of methods used by each participant to assign and verify total VOC content is provided in Appendix E.

Table 9. *CCQM-K148.b results for the mass fraction assignment of volatile organic content.*

NMI	w (mg/g)	u(w) (mg/g)
HSA	0.024	0.66
NMISA	0.47	0.087
BIPM	0	0.1
NMIJ	0	0.35
NMIA	0	0
NIM-C	0.89	0.02
GLHK	0.021	1
INMETRO	0.2290	0.0094
EXHM	0	0.01
UME	0.17	0.001
KRISS	0.1	1.6
KIMIA	1.8	1.2
BVL	5.958	2.867
VNIIM	0.56	0.007
NIMT	0	1.44

Non-volatiles / inorganics content

Fourteen participants provided information on the non-volatiles / inorganic content of CCQM-K148.b material (Table 10). Three participants (BIPM, INMETRO and EXHM) included chloride ion within this impurity class so, for comparison purposes, the values excluding chloride were calculated in the last column of Table 10. Only three participants reported levels above 1 mg/g for this class of impurity. However, it is noted that hydrogen ion content, if considered an inorganic impurity present in equimolar amounts to chloride, would represent between 1.7 and 2 mg/g according to chloride results reported by participants.

An overview of methods used by each participant to assign and verify non-volatiles / inorganic content is provided in Appendix E.

Table 10. *CCQM-K148.b results for the mass fraction assignment of non-volatiles / inorganics content.*

NMI	w (mg/g)	u(w) (mg/g)	w (mg/g) - {Cl^-}
HSA	0	1.44	0
NMISA	<1	0.005	<1
BIPM*	65.5	0.5	2
NMIJ	0.16	0.1	0.16
NMIA	0	1.2	0
NIM-C	0.18	0.009	0.18
GLHK	0.017	1	0.017
INMETRO*	67.6	1.1	0
EXHM*	64.16	1.40	1.76
LGC	0.078	0.019	0.078
KRISS	0.1	0.7	0.1
KIMIA	0.25	1.44	0.25
VNIIM	< 0.04	0.02	< 0.04
BVL	0.00	<0.01	0.00
NIMT	5.42	0.41	5.42

* Reported total inorganics including chloride content

KEY COMPARISON REFERENCE VALUES (KCRV)

The key comparison reference value for OTC mass fraction in the material was calculated using the mass balance approach, which required estimating KCRVs of each impurity subclass in the material. Therefore, KCRVs were estimated for the mass fraction of water, chloride, total structurally related impurities (SRI), inorganics and volatile organic compounds considering the results from the selected laboratories indicated in Table 11.

According to the technical discussions held, participants who reported significantly lower water contents than the bulk possibly did not allow sufficient time for samples to reach equilibrium with ambient humidity. Their values would reflect different degrees of water absorption at the time of sample weighing. Hence, participants that did not agree with the KCRV for water content were excluded from the calculation of the reference values for the other impurity subclasses. Other reasons for not including a particular result from a participant in the KCRV calculation of an impurity measurand are indicated in the notes of table 11.

Table 11. *Selection of participants reported values for the KCRV calculation of the different impurity measurands. Green and red colors indicate included and excluded for the KCRV calculation, respectively. Grey colors indicate that the participant did not provide the value of the corresponding measurand. Notes: 1) Bias in water content determination; 2) Major related impurity outlier; 3) Inorganic content or its uncertainty provided as a range; 4) Inorganic content other than HCl not reported; 5) Volatile content uncertainty reported as zero with no significant figures.*

	Participant																			
Measurand	HSA	NMISA[2,3]	NRC	BIPM[4]	NMIJ	NMIA[5]	NIM[1]	GLHK	INMETRO[4]	EXHM[4]	BAM	LGC	NIST	UME[1]	KRISS[1]	KIMIA[1]	BVL[1,3]	VNIIM[1,3]	NIMT[1]	INRIM
H_2O																				
Cl^-																				
SRI																				
Volatiles																				
Inorganics																				

Figures 12 and 13 display the participants results against the KCRVs for the impurity subclasses, calculated according to the OAWG guidelines and recent publications about the KCRV estimation.[3,4] The Hierarchical Bayes random effects model (HB-REM), implemented using the NIST Consensus Builder[5] and assuming Gaussian participants effects, was used for the estimation of the KCRVs for water, chloride, volatile and inorganic contents (Appendix H). This model was considered the most appropriate for technically valid, small datasets.

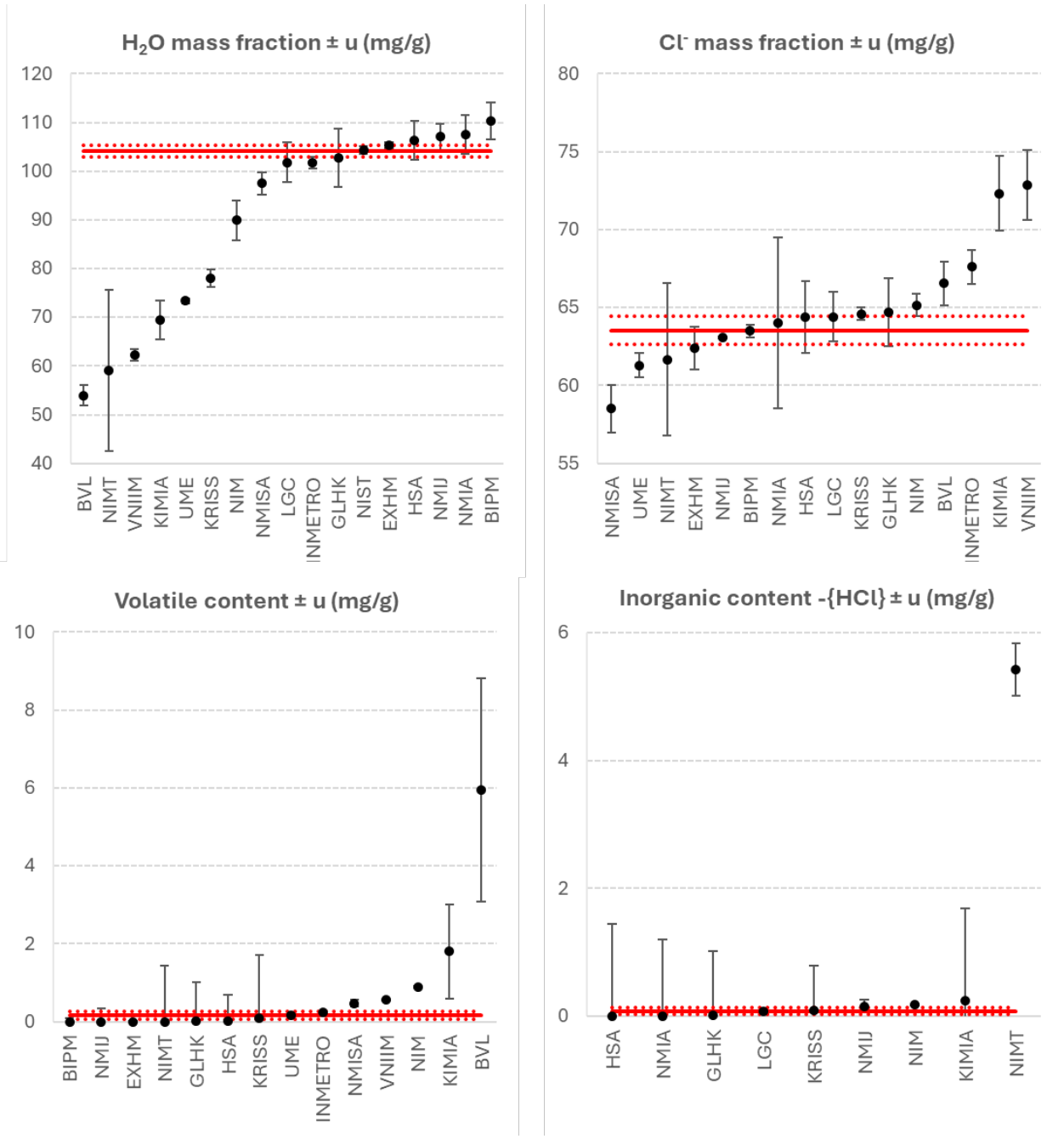


Fig. 12. *CCQM-K148.b reported mass fraction values for water, chloride, volatiles and inorganic substances other than HCl. The solid and dotted, red lines indicate the KCRV and its standard uncertainty, respectively, calculated applying the HB-REM on the selected datasets shown in table 11. Error bars are reported standard uncertainties. The KCRV numerical values are represented in table 12.*

Significant dispersion was observed for the structurally related impurity content (Figure 13). Three related structure impurities presented a particular measurement challenge: anhydro-oxytetracycline (AOTC), α-apo-oxytetracycline (α-apo-OTC) and β-apo-oxytetracycline (β-apo-

OTC). These compounds are isomers with elemental formula $C_{22}H_{22}N_2O_8$ and molar mass 442.4 g/mol. According to literature[6,7] and the information shared by some participants, AOTC degrades rapidly into the α- and β-apo-OTCs upon dissolution. In addition, some NMR signals likely related to the major impurity ADOTC could not be fully explained by a follow-up investigation subgroup of participating laboratories (detailed report can be found in Annex J). For these reasons, a conservative approach that assumed the total SRI content to lie with equal probability between the limits of the selected dataset (rectangular probability distribution) was used. In consequence, the KCRV for SRI content was estimated as the average of the highest and lowest values of the distribution.

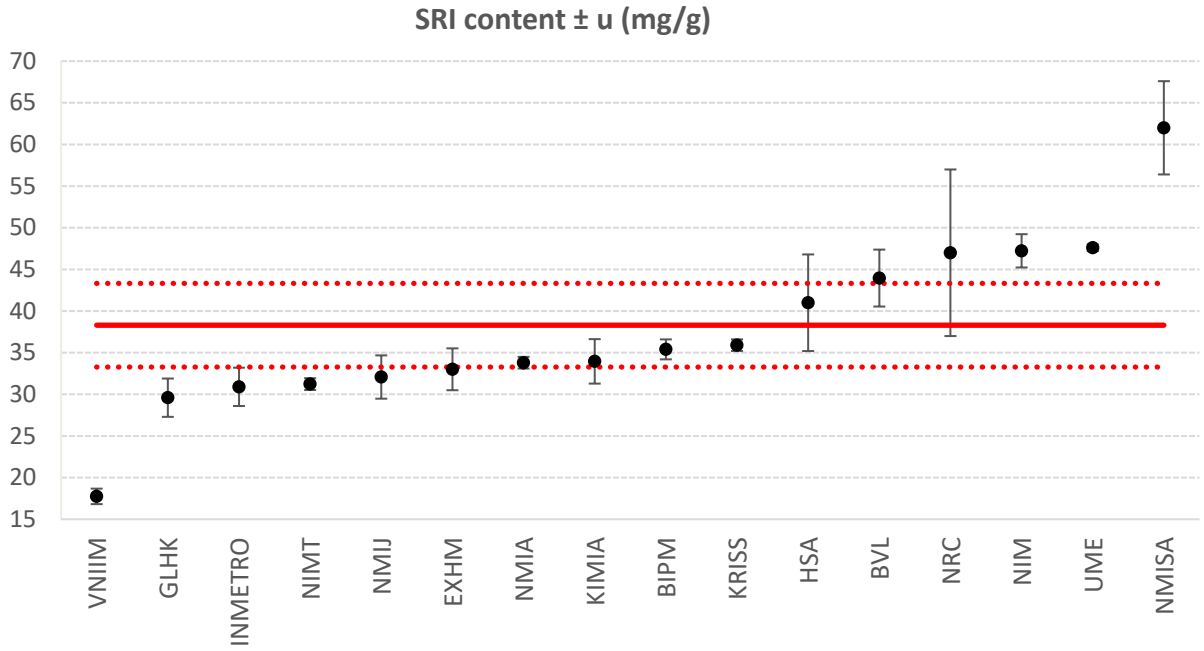


Fig. 13. *CCQM-K148.b reported mass fraction values for structurally related impurity content. The solid and dotted, red lines indicate the KCRV and its standard uncertainty, respectively, calculated assuming a rectangular probability distribution bounded by the highest and lowest values from the selected dataset shown in table 11. Error bars are reported standard uncertainties. The KCRV numerical value is represented in table 12.*

Table 12 summarizes the reference values for each impurity type and the mass balance (MB) KCRV for the main component OTC calculated by total impurity content subtraction from 1000 mg/g. The hydrogen cation content (from HCl) was calculated assuming equimolarity with the chloride content. The participants' results against the KCRV are plotted in Figure 14.

Table 12. *Calculation of the mass balance KCRV for the OTC free base mass fraction based on individual estimates of all impurity types in the comparison material.*

Impurity	RV (mg/g)	u (mg/g)	Estimate
H_2O	104.1	1.2	HB-REM
Cl^-	63.5	0.9	HB-REM
H^+	1.81	0.03	calculated from Cl^-
SRI	38.3	5.0	Rect. Distr.
Inorg-{HCl}	0.09	0.05	HB-REM
Volatiles	0.16	0.10	HB-REM
MB KCRV:	792.0	5.2	1000-Σi

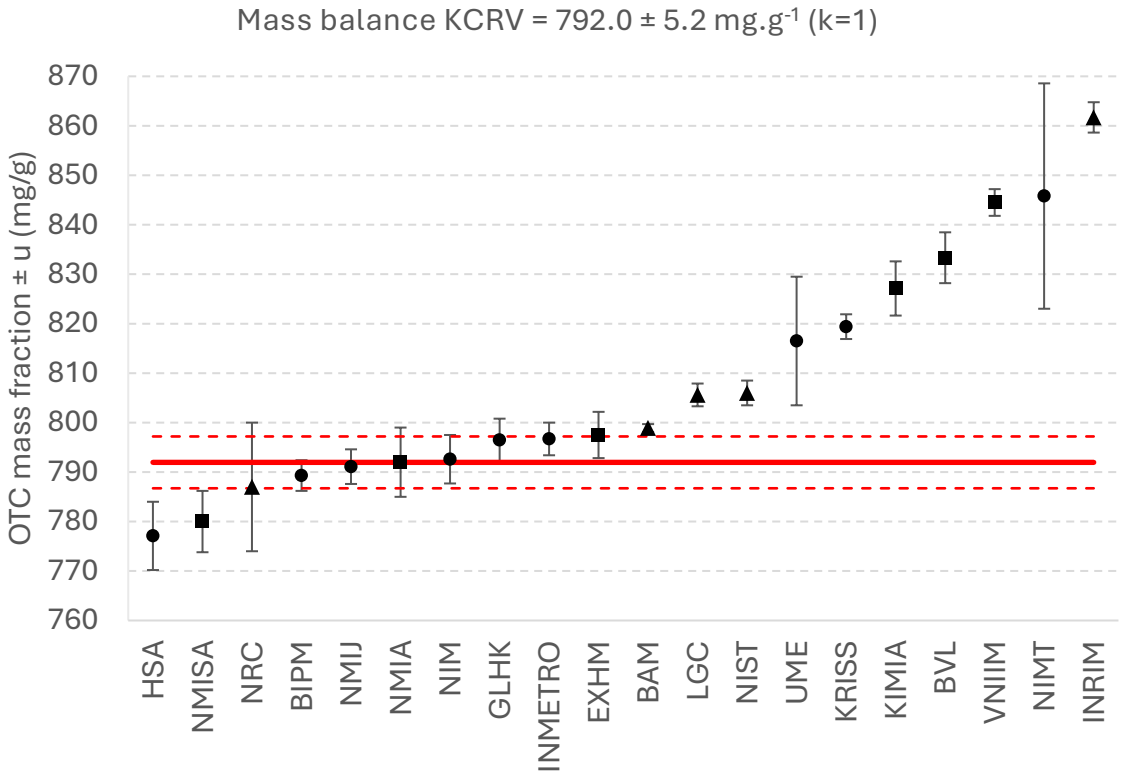


Fig. 14. *Participants reported values for the mass fraction of oxytetracycline free base in the CCQM-K148.b material against the MB KCRV plotted as a horizontal red line with its standard uncertainty interval as dotted red lines. The squares, triangles and circles indicate the assignment methods mass balance, qNMR or the combination of both, respectively. Error bars are reported standard uncertainties.*

A qNMR value based on the qNMR results from participants using this methodology for the OTC mass fraction assignment (Table 5) was calculated using the HB-REM with Gaussian participants effects (Appendix H). Results from UME, NIM, KRISS, NIMT and INTI were not used for the

consensus value estimation. The latter recognized an error post-submission whereas for the others an insufficient sample equilibration bias was suspected based on their water content results (Figure 12). The qNMR-based estimate was consistent with the OTC mass balance KCRV (Figure 15).

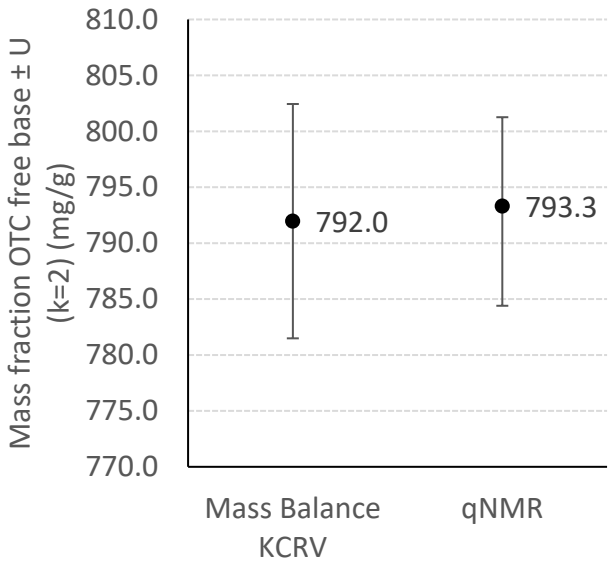


Fig. 15. *Comparison of the mass balance KCRV with a consensus qNMR value estimated from selected qNMR participants results. Error bars are expanded uncertainties corresponding approximately to a 95% confidence level.*

DEGREES OF EQUIVALENCE (DoE)

The degrees of equivalence were calculated for participants' reported mass fraction values of the main component OTC and of the three major impurities: water, chloride and structurally related impurities (Figure 16 and Table 13). They were based on the KCRVs and associated uncertainties of the corresponding measurand (Table 12). A participant result is compatible with the KCRV when the DoE U_{95} (expanded uncertainty at a 95% level of confidence) of the result exceeds the absolute value of the DoE.

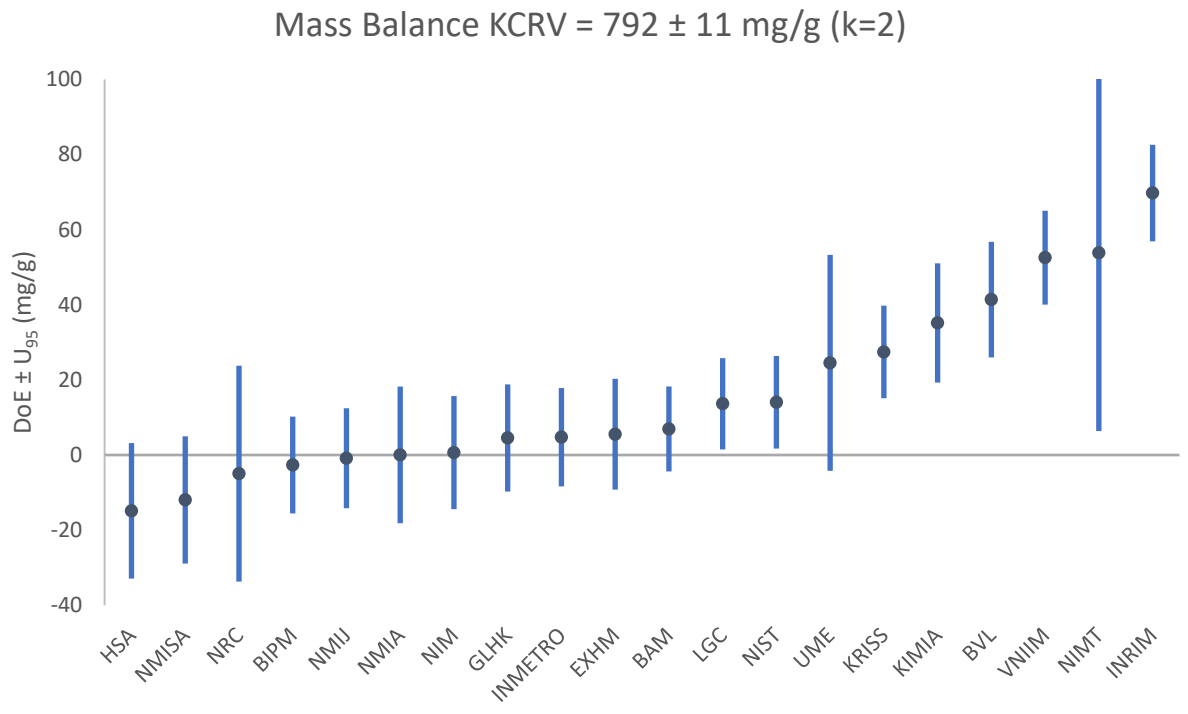


Fig. 16. *Degrees of equivalence and expanded uncertainties of CCQM-K148.b results for the main component OTC mass fraction assignment.*

Table 13. *Degrees of equivalence and expanded uncertainties of CCQM-K148.b results for the mass fraction assignment of OTC and the three major impurity subclasses. Results that agree or disagree with the corresponding KCRV are indicated in green or red, respectively. The grey color indicates that the measurand was not reported by the participant.*

Measurand →	OTC		H_2O		Cl^-		SRI	
Participant ↓	DoE (mg/g)	DoE U_{95} (mg/g)	DoE (mg/g)	DoE U_{95} (mg/g)	DoE (mg/g)	DoE U_{95} (mg/g)	DoE (mg/g)	DoE U_{95} (mg/g)
HSA	-14.9	17.3	2.3	9.8	0.9	6.6	2.7	15.3
NMISA	-12.0	16.2	-6.6	7.7	-5.0	5.7	23.7	15.0
NRC	-5.0	28.0					8.7	22.4
BIPM	-2.7	12.2	6.2	9.6	0.0	5.0	-2.9	10.3
NMIJ	-0.9	12.6	2.9	8.1	-0.5	4.9	-6.2	11.3
NMIA	0.0	17.5	3.4	9.9	0.5	11.6	-4.5	10.1
NIM	0.6	14.3	-14.2	10.0	1.6	5.1	8.9	10.8
GLHK	4.5	13.6	-1.3	13.3	1.2	6.5	-8.7	11.0
INMETRO	4.7	12.4	-2.3	6.7	4.1	5.3	-7.4	11.0
EXHM	5.5	14.0	1.2	6.5	-1.1	5.5	-5.3	11.2
BAM	6.9	10.6						
LGC	13.6	11.4	-2.4	10.1	0.9	5.8		
NIST	14.0	11.6	0.3	6.6				
UME	24.5	28.0	-30.7	6.2	-2.2	5.2	9.3	10.1
KRISS	27.4	11.6	-26.0	7.0	1.1	5.0	-2.4	10.1
KIMIA	35.2	15.2	-34.7	9.9	8.8	6.7	-4.3	11.4
BVL	41.4	14.7	-50.2	7.5	3.0	5.5	5.7	12.2
VNIIM	52.5	11.8	-41.8	6.6	9.3	6.3	-20.6	10.2
NIMT	53.8	46.7	-45.1	33.5	-1.9	10.6	-7.1	10.1
INRIM	69.7	12.1						

USE OF CCQM-K148.b IN SUPPORT OF CALIBRATION AND MEASUREMENT CAPABILITY (CMC) CLAIMS

How Far the Light Shines

Successful participation in CCQM-K148.b demonstrates the measurement capabilities in determining the mass fraction of organic compounds, with molar mass in the range of 75 g/mol to 500 g/mol, having high polarity (pKow > -2), including compounds presenting significant hygroscopicity, in an organic solid material.

Depending on the characterization procedure applied, the participants demonstrated capabilities for organic purity assignment by a mass balance or qNMR approach or by the combination of results obtained using both methods.

In addition to the capability for purity assignment of the primary component, successful participation in CCQM-K148.b may also demonstrate capabilities for the content assignment of chloride, water and total structurally related impurities present at similar levels in comparable polar, hygroscopic organic materials.

Core Competency Statements and CMC support

Appendix G lists the tables containing the Core Competencies claimed by the participants in CCQM-K148.b. The information in these Tables is as provided by the participants. Details of the analytical methods used by each participant in this study are provided in Appendix E.

Eight out of twenty participants reported values for the mass fraction of oxytetracycline in the oxytetracycline HCl comparison material that did not agree with the KCRV (Figure 16). INRIM acknowledged a calculation error that affected their qNMR reported value. BVL, NIMT, VNIIM, KIMIA, KRISS, UME and NIM underestimated the water content due to insufficient sample equilibration at ambient humidity. In a few instances, the underestimation of water content did not result in disagreeing results for the main component assignment, as other impurity results compensated for the bias in water determination.

NMISA disagreement with the KCRV may be attributed to an overestimation of the related impurity content in the comparison material in relation to the consensus value (62 ± 5.6 mg/g vs. 38.3 ± 5 mg/g, k=1). The laboratory identified the impurities isochlortetracycline and chlortetracycline at 34.4 mg/g and 6.8 mg/g, respectively, neither of which was observed by any other participant.

Finally, LGC and NIST results also disagreed with the KCRV. These laboratories used qNMR to determine the oxytetracycline mass fraction value and used spectral correction techniques to account for overlapping impurities (investigated by liquid chromatography methods in the case of LGC). BAM reported value was also only based on the qNMR analysis of the comparison material.

However, their integration method based on the edited-sum approach[8] applied to the 7.2 ppm OTC signal may have better accounted for the overlapping impurities in that spectral region.

Seventeen laboratories used qNMR, either as confirmatory method, standalone method or in combination with mass balance (Table 5). The resonance signals mostly used for quantification were those in the aromatic region induced by protons H-7, H-8 and H-9 (Appendix E). Signals at 3.8 ppm (H-5) and 1.6-1.8 ppm (C-CH_3) were also used by a few participants. The signal at 4.3 ppm (H-4) was described by some participants as unsuitable for quantification due to hydrogen-deuterium exchange with the solvent. However, HSA recognized the potential lability of the H-4 proton and controlled the analysis conditions performing NMR analysis with 1-2 hours after sample dissolution. Their results obtained using H-4 were cross-checked with those quantified using the methyl protons in 0.01N DCl in D_2O and found to be comparable.

CONCLUSIONS

The reported values from the twenty CCQM-K148.b participants for the free base OTC mass fraction agreed within ca. 9 %. Value assignment approaches combining mass balance and qNMR methods presented a better overall agreement.

Water content values presented the highest variability, seemingly reflecting the challenge of measuring significantly hygroscopic materials. Hygroscopicity did not only appear to affect mass balance results, but also qNMR results as sample preparation required special attention, e.g., sufficient equilibration. The equation provided in the protocol to standardize mass determinations to the values expected at 50 % relative humidity had little impact on the results since most laboratories worked under relative humidity conditions close to the reference value of 50 %.

A consistent set of nine related structure impurities were identified by two or more participants, with one predominant impurity identified by ten participants as 2-acetyl-2-decarbamoyl-oxytetracycline. The choice of solvent to dissolve the material did not have a significant impact on the impurity profile found by participants. The instability of some impurities and a few impurity-related, unexplained NMR signals posed a significant challenge and led to a large, expanded uncertainty of the total structurally related impurity content (± 11 mg/g). A good agreement on the chloride content (± 2 mg/g expanded uncertainty) and negligeable amounts of volatiles and inorganics were found by the participants.

Participants in CCQM-K148.b demonstrated and benchmarked their ability to assign the mass fraction content of a polar and significantly hygroscopic solid organic compound having moderate molecular complexity present as the primary component in an organic material. Results from eight participants were not consistent with the KCRV within the combined 95% expanded uncertainty range of the unilateral degree of equivalence due to identified issues with their methodologies.

ACKNOWLEDGEMENTS

The study coordinators thank the participating laboratories for the extensive and comprehensive investigations undertaken to characterize the comparison material and their co-operation in checking the data and providing the information reported in this study in a timely manner.

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Appendix A: Call for participation and comparison protocol

CCQM-K148.b
Polar analyte in solid organic material:
Mass fraction of oxytetracycline in oxytetracycline hydrochloride material

Key Comparison
Track A

Study Protocol
October 2022

Gustavo Martos and Steven Westwood

Bureau International des Poids et Mesures

Pavillon de Breteuil, Sèvres, France

INTRODUCTION

Oxytetracycline (OTC) is a member of the tetracyclines group of broad-spectrum antibiotic compounds, widely used in veterinary medicine, that have a common basic structure. Because of concerns with the potential health risk to the consumer of long-term exposure to low levels of these compounds, monitoring programs for the presence of tetracycline residues in food of animal origin including meat, fish, milk, eggs and honey are in place in many countries.[1] These activities, which reduce the potential for trade barriers in this area, need to be supported by a sound reference measurement infrastructure for tetracycline analysis.

This comparison underpins core competencies of National Metrology Institutes (NMIs) for the mass fraction value assignment of high purity organic substances containing a polar analyte as the primary component (molar mass (75-500) g/mol), a core technical capability for reference material producers and providers of calibration services. Evidence of successful participation in formal, relevant international comparisons is required to establish measurement capability claims (CMCs) made by NMIs and Designated Institutes (DIs). with active programmes in organic analysis.

Food safety continues to be a priority sector of the OAWG for the 2021-2030 period. The OAWG strategy document [2] requires a planned Track A key comparison, CCQM-K148.b, to be conducted in 2022 on the value assignment of the mass fraction content of a polar analyte present as the primary component in a high-purity organic material. This comparison compliments CCQM-K148.a, completed in 2018, which examined the measurement for a non-polar organic analyte present as the primary component in a high-purity organic material.

TIMELINE

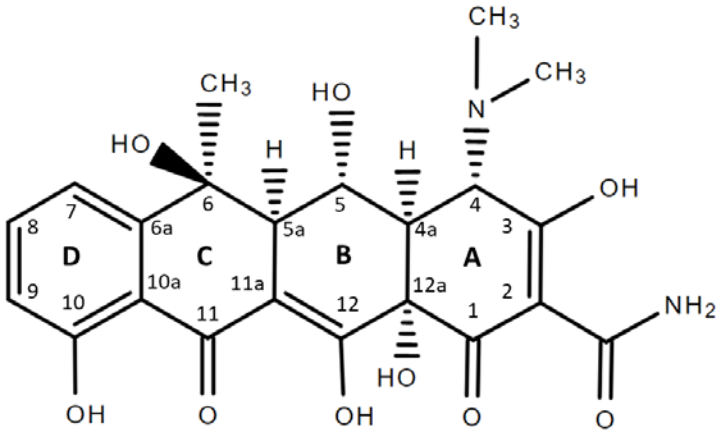
Table 1 lists the timeline for the proposed study.

Table 1:

Date	**Action**
April 2021	Sample Preparation
January 2022	Homogeneity and Stability Testing completed
October 2022	Call for participation to OAWG members
November 2022	Sample Distribution completed
March 2023	Deadline for Submission of Results
April 2023	Preliminary Discussion of Results

MEASURAND

The comparison requires the assignment of the mass fraction content, reported in mg/g, of oxytetracycline free base (OTC) in a unit of the oxytetracycline hydrochloride (OTC.HCl) comparison material under standardized conditions of relative humidity. Figure 1 below displays the molecular structure of the free base (4*S* epimer).



Oxytetracycline (OTC)

Molar mass = 460.43 g/mol; pK_{OW} ~ 0.5

Fig. 1: *Structure and conventional numbering of oxytetracycline*

STUDY MATERIAL

The comparison material was produced by TÜBITAK-UME. A bulk source material of OTC.HCl in the form of a fine yellow crystalline powder was homogenized in a 3D mixer and kept in a vacuumed container until filling to minimize moisture uptake. About 0.5 g of the material were filled into each vial of the comparison batch using an automatic filling machine.

Each participant will receive as a minimum two vials of the comparison material, each containing a minimum of 500 mg of OTC.HCl. Participants who plan to use multiple independent methods to contribute to their final property value assignment (e.g. a mass balance procedure and a separate qNMR procedure) can request an additional vial. The comparison samples will be provided in amber glass vials sealed with PTFE-lined screw-caps. They should be placed in storage at 4°C in the dark upon receipt.

Vials should be equilibrated to the laboratory's ambient temperature prior to opening. The material is significantly hygroscopic. Prior to any gravimetric operations and sampling of the bulk material the vial must be allowed to equilibrate at the laboratory ambient relative humidity (preferably maintained in the range 42-80%). Measurement results are to be reported on the material as received without additional treatment but taking into account the hygroscopicity correction described below.

Recommended Minimum Sample Amount

A minimum sample amount for analysis of 10 mg is recommended to reduce to a negligable level the potential for an influence due to between-vial inhomogeneity on the determination of the major component.

Hygroscopicity correction – IMPORTANT!

OTC.HCl has been demonstrated to be significantly hygroscopic. Figure 2 shows the reversible sorption/desorption of water from a sample of the material as a function of relative humidity (RH) and time. The figure also shows a model for the relationship between the observed mass at equilibrium at a specific RH in the range RH 40% - RH 80%. This corresponds to a relative increase of mass of a sample of the comparison material due solely to water sorption by approximately 0.4% for every 10% increase in the ambient RH (within the range RH 40% to RH 80%).

A vial used as a source of material for measurements should be equilibrated to the laboratory's ambient conditions of temperature and relative humidity (RH) prior to opening. The relative humidity in a laboratory where gravimetric or water content measurements of the material are undertaken should be maintained as far as possible in the range RH 42% - RH 80%.

Weighing protocol and correction for relative humidity

As a result of the hygroscopicity of the material, a given mass will contain a varying amount of water as a function of the ambient humidity when the sample mass was determined. It will not be feasible for each participant laboratory to operate under identical conditions of RH. As a result, in order to obtain a valid comparison of results between participants, it will be necessary to correct all mass determinations to the value expected for that sample at an agreed reference RH and to use this standardized value in all subsequent calculations.

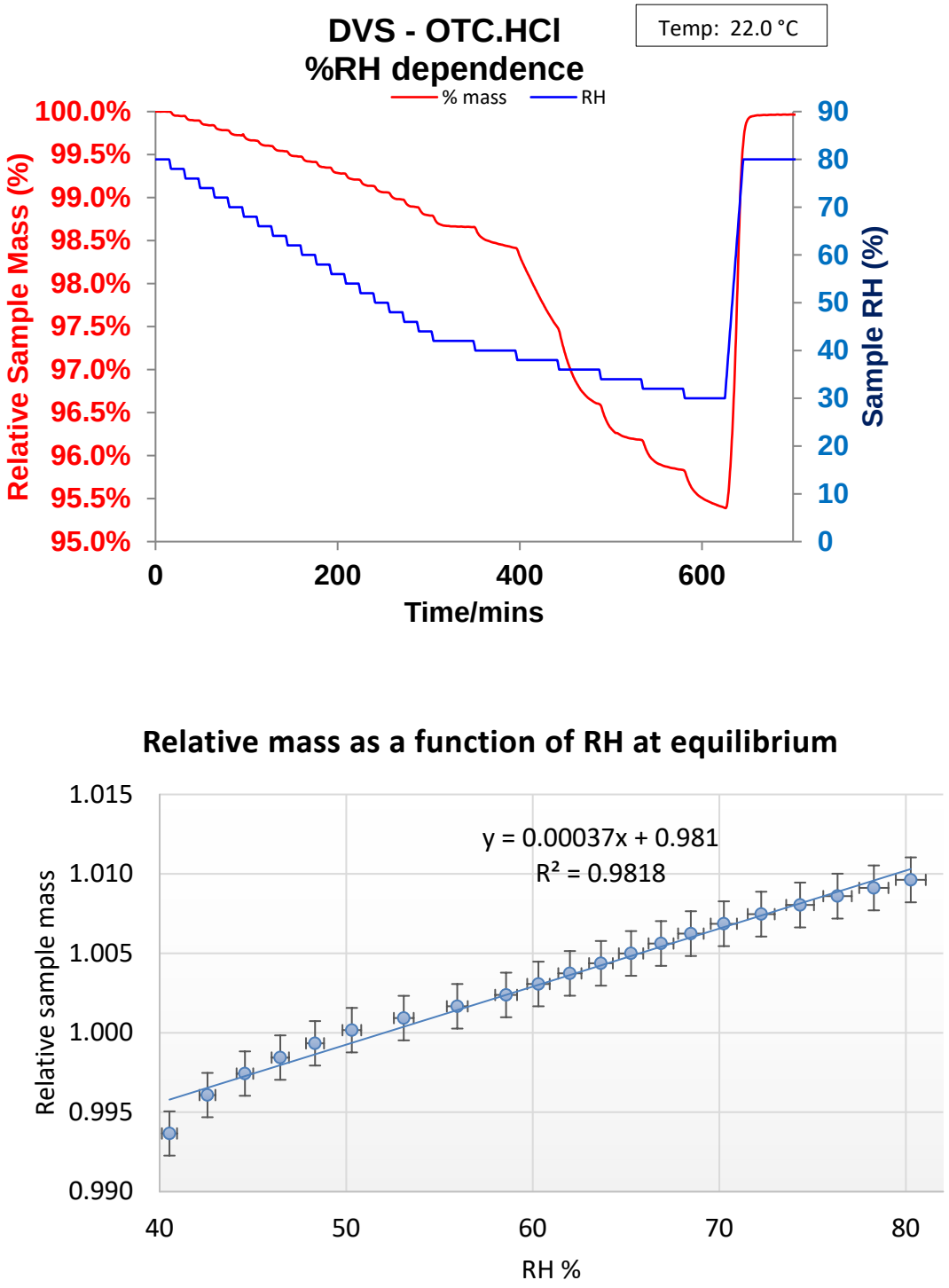


Fig. 2. *Water sorption (% mass change) as a function of time and %RH for OTC.HCl salt (Top) and the calculated linear regression function modelling the relationship between the sample mass at equilibrium and the %RH (Bottom).*

The environmental relative humidity (RH_X) at which each weighing was undertaken must be monitored and recorded. Each aliquot needs to equilibrate at the ambient RH_X before placing it in the balance pan in order to achieve a stable weighing value. In our experience the time required to reach equilibration varies depending on the size of the aliquot and it may take more than 60 min.

The observed mass of sample (m_{RH_X}) recorded at the ambient RH_X shall be normalized to the expected mass of the same sample at RH 50 % ($m_{RH_{50}}$) using the equation:

$$m_{RH_{50}} = \frac{m_{RH_X}}{1 + F(RH_X - 50)} \qquad Eq.1$$

Where F = 0.00037 and u(F) = 0.00003

For the calculation, RH_X is the numerical value of the environmental relative humidity when expressed as a percentage. The application of the equation is appropriate within the 42% RH - 80% RH range. Outside these limits assignments of m_{RH50} become less accurate. Participants are advised to verify the accuracy of their relative humidity measurements.

The standardized value, m_{RH50}, must be used for subsequent calculations (mass balance, qNMR).

Example of Mass Standardization for Hygroscopicity

A sample of the material is weighed to a constant final mass of 11.80 mg in a laboratory where RH_X is 42%. In this case RH_X = 42 and:

m_{RH_X} = 11.80 mg

$$m_{RH_{50}} = \frac{11.80}{1 + 0.00037(42 - 50)} = 11.83\ mg$$

i. For calculations of OTC free base content by qNMR, related structure impurities, chloride ion, etc (i.e. all measurements <u>other</u> than water content), the standardized value for m_{RH50} of 11.83 mg should be used as the sample mass in subsequent calculations.

ii. For assignment of water content a more careful correction is required. For example:
    a. the sample of total mass 11.80 mg of CCQM-K148.b at RH 42% has an observed mass fraction content of water of 30.0 mg/g.*
    b. the amount of water in 11.80 mg of CCQM-K148.b with mass fraction content 30.0 mg/g at RH 42% corresponds to (11.80*0.030) mg or 0.354 mg
    c. absolute water content estimated for the sample if measured at RH 50% equals 0.354 mg (content at RH 42%) adjusted for the value of the difference between m_{RH42} and m_{RH50} of (11.83 - 11.80) mg or +0.030 mg
    d. absolute water content of the sample at RH 50% is 0.384 mg (0.354 + 0.030) mg
    e. final reported value for mass fraction water content of CCQM-K148.b based on this sample, corrected to RH 50%, is 32.5 mg/g (= 0.384/11.83)

* Please note that the reported value for water content of the CCQM-K148.b material used in the example above is purely hypothetical and must not be regarded in any way as an indication of the true water content of the material.

Homogeneity Assessment of Study Material

The homogeneity of the batch was tested using an LC-UV method for the content of OTC and the main structurally related impurities. An oven-transfer, coulometric Karl Fisher titration was used for determination of water content and ion chromatography for chloride ion content. The uncertainty contribution due to inhomogeneity of the assigned values was evaluated by ANOVA. Ten vials were selected at regular intervals from the filling sequence to ensure that the results would indicate any trend in the filling process. Each vial was analyzed in a random order to ensure any trends in the bottling process were separated from possible trends resulting from the analytical sequence.

The results obtained indicated no statistically significant difference in the within- and between-vial levels of the mass fraction of each component in the material. The upper limit for the uncertainty contribution due to inhomogeneity in all cases was sufficiently small as to be unlikely to influence the effective comparison of participant results. A summary of the observed within- and between-sample variability for the major components is shown in Table 2.

Table 2. Homogeneity assessment for the main component OTC, the main related structure impurity, water and chloride in the comparison material.

ANOVA Estimate	**OTC**	**Imp A**	**H_2O**	**Cl^-**
Between-unit CV (%)	0.36%	0.77%	0.64%	0.87%
Within-unit CV (%)	0.83%	1.10%	1.03%	1.44%
Upper limit of relative uncertainty contribution due to inhomogeneity	0.27%	0.43%	0.37%	0.47%
Probability of falsely rejecting the hypothesis that all samples have the same concentration	< 5%	< 5%	< 5%	< 5%

A plot of the normalized mass fraction for each analyte obtained for the homogeneity assessment is plotted by filling sequence in Figure 3. The normalized values of repeat measurements from three aliquots taken from each individual vial are plotted.

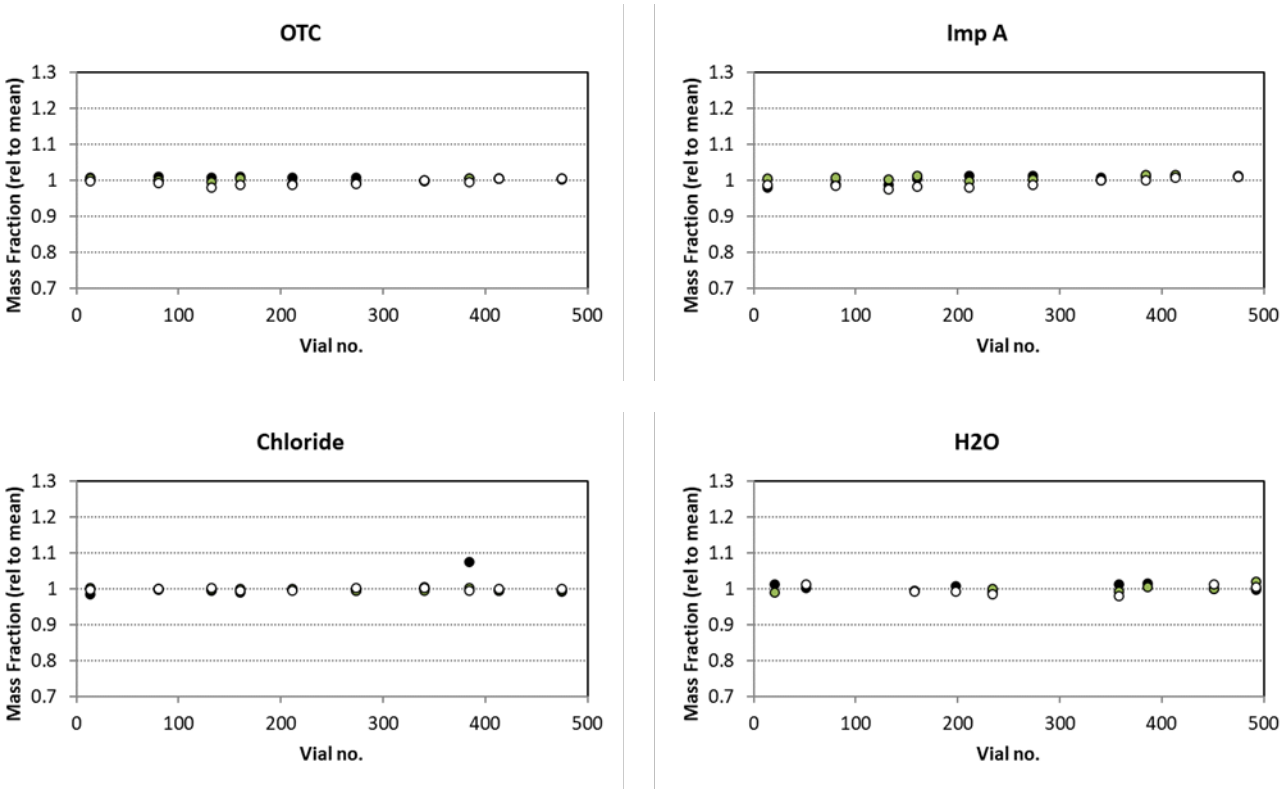


Fig. 3. *Homogeneity evaluation for OTC, the major related structure impurity A, water and chloride in the comparison material.*

Stability Assessment of Study Material

An isochronous stability study was undertaken for OTC, related structure impurities, water and chloride on storage at 4 °C, 22 °C and 40 °C in the dark. The analytical methods used were the same as in the homogeneity study. The material is sufficiently stable, within the proposed time scale of the comparison, when stored at 4 °C or 22 °C. OTC and some impurities were not stable at 40 °C. Precautions will be taken to monitor if the comparison material is exposed to temperature above 30 °C during shipment and if this occurs replacement material will be provided.

The mass fractions of OTC and chloride relative to the mean value of reference samples stored at -20 °C are shown in Figure 4 for samples stored at 4 °C and 22 °C during the stability study period. The plot displays the normalized results of duplicate analysis of samples prepared from two units of CCQM-K148.b. The upper and lower dashed lines indicate the uncertainty of the regression line, which reflects the analytical method variance in the absence of a significant instability trend.

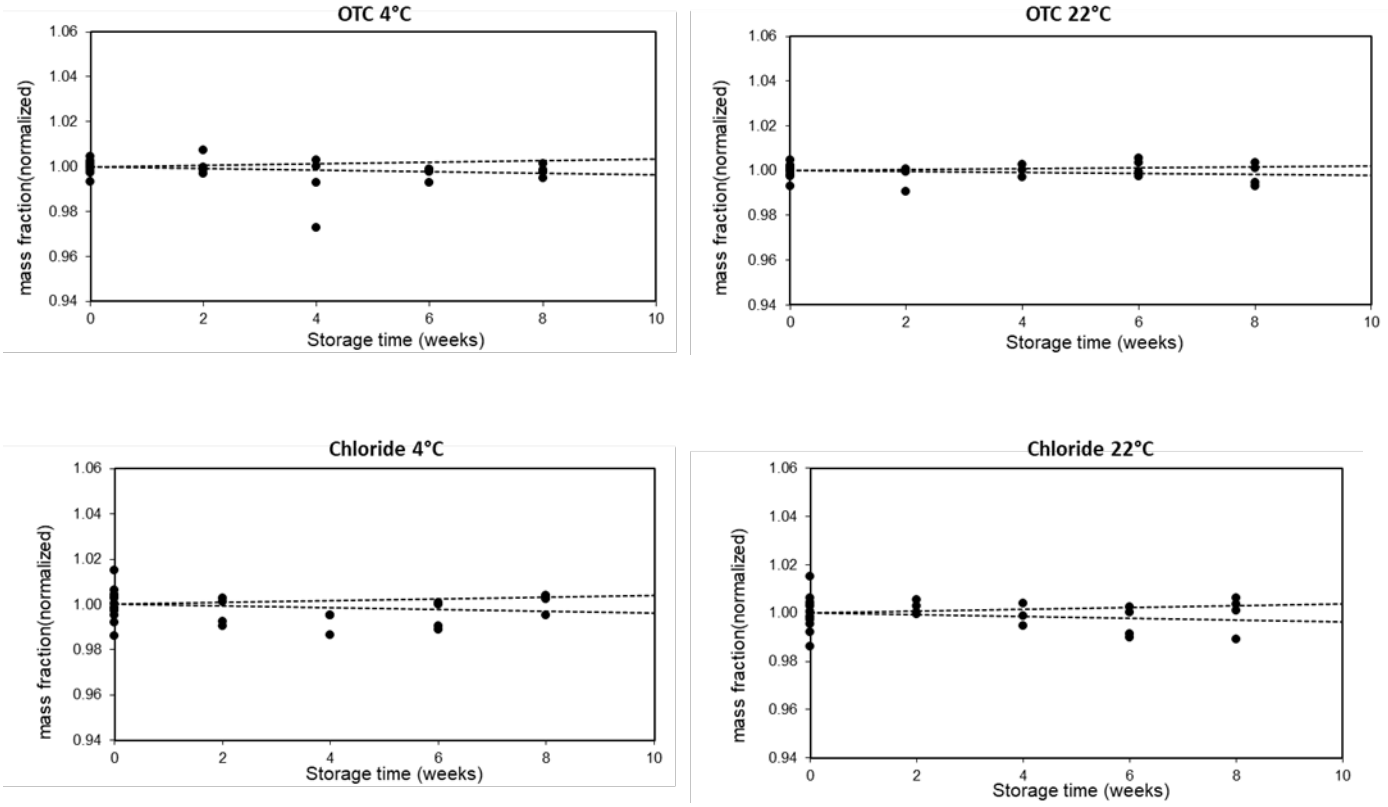


Fig. 4. *Stability evaluation of OTC and Chloride content in samples stored at 4 °C and 22 °C for 8 weeks.*

INSTRUCTIONS AND SAMPLE DISTRIBUTION

Participants are requested to notify the comparison coordinator of specific requirements for shipment documentation required to facilitate customs clearance into their country and to liaise with the coordinating laboratory during the delivery process.

Participants will be notified by the coordinating laboratory in advance of the shipment of the materials and will be given details of the carrier used for the shipment.

Participants will be asked to return a form acknowledging receipt of the samples, to advise the comparison coordinator of any damage to the vials during shipping, and to indicate based on a monitoring strip included with the shipment whether the shipping container had been exposed to a temperature in excess of 30 °C during the transport process.

RESULTS

Participants are required to report their estimate of the mass fraction of oxytetracycline as the free base present in the material in mg/g, standardized to the value expected at %50 RH. The result should be based on combined values obtained by the measurement of multiple aliquots from at least one of the vials supplied. Participants are also required to verify the accuracy of their relative humidity measurements.

There is no restriction on the use of methods to obtain data to assign the mass fraction content of OTC in the comparison material, but only one overall result can be submitted by each participant.

In addition to the quantitative results, participants will be instructed to describe their analytical methods, approach to uncertainty estimation, and the Core Competencies they felt were demonstrated in this study.

An electronic data submission form will be supplied as an EXCEL spreadsheet. The draft result reporting spreadsheet is attached to this protocol (Annex A).

The following information **shall** be included in the result reporting form:

- Laboratory information;
- Names of staff for inclusion as contributing authors in the Final Report of the comparison;
- Temperature and relative humidity in area(s) where gravimetric operations are performed and water content measurements are undertaken;
- Primary Component giving the mass fraction content of OTC free base (in mg/g) estimated if measured at RH = 50% with the combined standard uncertainty and the expanded uncertainty at a 95% confidence range;
- Measurement equation and uncertainty budget for the OTC assignment.

Participants using a mass balance approach as either the sole or a contributing method to their overall value assignment **shall** in addition report the Secondary Component (Impurity) levels in the material by providing assigned values and the associated standard uncertainty for each secondary component estimated if measured at RH = 50% contributing to the assignment of the mass fraction and standard uncertainty of OTC. This table shall include assignments for some or all of:

- total related structure impurities
- water
- residual organic solvent
- chloride ion
- total non-volatiles/inorganics

It is noted that, due to the hygroscopicity of oxytetracycline salt, reporting the value adjusted for measurement at RH = 50% is particularly important for the value of the water content.

A representative chromatogram from analysis of a sample solution shall also be provided where HPLC-based methods are used to evaluate the related structure impurity content.

Participants **may** provide further information supporting a claim for a generic water content measurement competency linked to the results obtained for this material (for those institutes wishing to make CMC claims for water content).

Participants using a qNMR approach as a contributing method to their final value assignment **shall** provide information on the:

- deuterated solvent(s) used;
- standard(s) (internal or external)
  - name and source
  - purity and associated uncertainty (in mg/g)
  - basis for the traceability of the purity of the standard(s);
- balance for gravimetric sample preparation:
  - make, model and resolution
  - repeatability (standard deviation [SD] of at least ten repeat determinations of a tared reference mass [m])
  - minimum sample weight (mass for which 2*SD/m < 0.1%)

Participants using an approach other than mass balance or qNMR as either their sole or as a contributing method to their final value assignment shall also provide a brief outline of the procedure and all critical method parameters.

When a participant combines the results of two or more independent methods to obtain the final value reported for the comparison, the individual results for each method shall be reported. A compilation of all such contributing results, including their degree of equivalence with the KCRV, will be included in an Annex to the Final Report.

USE OF RESULTS FROM CCQM-K148.b IN SUPPORT OF CALIBRATION AND MEASUREMENT CAPABILITY (CMC) CLAIMS

How Far the Light Shines

Successful participation in CCQM-K148.b will demonstrate the measurement capability for determining the mass fraction of solid organic compounds, with molar mass in the range 75 g/mol to 500 g/mol and having high polarity ($pK_{ow} > -2$), including compounds presenting significant hygroscopicity. If specifically requested, a CMC competency can also be claimed to be demonstrated for the assignment of water content present at similar levels in comparable polar, organic solids.

Core Competency Statements and CMC support

The template for the potential Core Competency claims arising from successful participation in CCQM-K148.b is provided in Annex B below.

REFERENCES

[1] Granados-Chinchilla F, Rodríguez C. Tetracyclines in Food and Feeding stuffs: From Regulation to Analytical Methods, Bacterial Resistance, and Environmental and Health Implications. J Anal Methods Chem. 2017;2017:1315497. doi: 10.1155/2017/1315497

[2] CCQM Working group on Organic Analysis: Strategy 2021-2030

Bureau International des Poids et Mesures

Appendix B: Registration Form

CCQM-K148.b/P187.b & CCQM-K179/P224
Mass fraction of oxytetracycline base (OTC) and oxytetracycline hydrochloride salt (OTC.HCl) in a solid organic material

REQUEST TO REGISTER TO PARTICIPATE IN:

- ☐ **CCQM-K148.b Track A (mass fraction of OTC)**
- ☐ **CCQM-K179 Track C (mass fraction of OTC.HCl)**
- ☐ **CCQM-P187.b (mass fraction of OTC)**
- ☐ **CCQM-P224 (mass fraction of OTC.HCl)**

(Participation in the CCQM-148.b and CCQM-179 comparisons is only permitted for National Metrology Institutes or Designated Institutes recognized under the CIPM MRA)

ORGANIZATION / DEPARTMENT / LABORATORY

[Organization Name]

CONTACT PERSON FOR THE COMPARISON

[Contact person for comparison]

E-MAIL, TELEPHONE

[Contact details]

ADDRESS FOR SHIPMENT OF SAMPLES

[Address details]

CONTACT PERSON FOR SAMPLE DELIVERY (if different)

[Contact details]

E-MAIL, TELEPHONE

[Contact details]

Date _____

Complete and return to gustavo.martos@bipm.org **before October 30, 2022**

Appendix C: Reporting form

CCQM-K148b CCQM-P187b Reporting Form 1.4
Participant identification

CCQM-K148.b / CCQM-P187.b
Mass fraction of Oxytetracycline (free base) **in high purity material**

Data Submission Form

Please complete all pages of the reporting form and submit it by email before March 1, 2023 to: gustavo.martos@bipm.org

Avoid formulas in the fill-in cells (marked in yellow). Mathematical expressions can be inserted using the "Symbols" button in the "Insert" submenu.

Registered comparison participation:	**CCQM-K148.b** **CCQM-P187.b**	(delete as appropriate)
Reporting Date		
Institute		
Submitted by (name)		
E-mail address		
Contributing authors for acknowledgement in Final Report:		

CCQM-K148b CCQM-P187b Reporting Form 1.4
Comparison Results

RESULTS

a. Mass Fraction assignment - main component

Measurand	Mass Fraction (mg/g)	Combined Standard Uncertainty (mg/g)	Coverage Factor (k)	Expanded Uncertainty (mg/g)
Oxytetracycline free base (corrected to RH 50%)				

b. Mass Fraction assignments - impurity components [required for participants using a mass balance procedure, optional otherwise]

Measurand	Mass Fraction (mg/g)	Combined Standard Uncertainty (mg/g)	Coverage Factor (k)	Expanded Uncertainty (mg/g)
Total related structure impurities				
Water content (observed at local RH)				
Water content (corrected to RH 50%)				
Chloride ion				
Total non-volatiles and inorganics				
Volatile organics content				

c. Mass Fraction assignments - individual impurity components [optional]

Measurand	Mass Fraction (mg/g)	Combined Standard Uncertainty (mg/g)	Coverage Factor (k)	Expanded Uncertainty (mg/g)
Impurity 1				
Impurity 2				
Impurity 3				
Impurity 4				
[additional entries as required]				

d. Environmental conditions

Measurement	Temperature (°C)	Relative Humidity (%)
Gravimetric operations		
Water content measurements		

CCQM-K148b CCQM-P187b Reporting Form 1.4
Analytical Method for Mass Balance procedure

Information about the procedures used

[NB - To complete your entry, please insert additional rows as necessary]

1. Related substance impurity content

Analytical instrumentation used (e.g., LC, GC, GC-MS, etc.)	
Sample amount per analysis (approximate)	mg
Number of samples analyzed	
Sample derivatization (if used)	
Sample preparation (solvent, concentration)	
Chromatographic Columns used (type and manufacturer)	
Chromatographic conditions (e.g., GC temperature program, LC mobile phase and gradient, injection size, numder of samples analyzed, number of replicates per sample)	

CCQM-K148b CCQM-P187b Reporting Form 1.4
Analytical Method for Mass Balance procedure

Assignment method (e.g., relative response, external calibration, internal standard, IDMS)	
Reference standards used (if applicable) (Please specify the compounds, source and role)	
UV wavelength(s) monitored in LC-UV (if applicable)	
SIM/MRM(s) monitored in MS (if applicable)	
Assessment of response factors (as applicable) (Please describe assumptions or investigations into the relative response factors of impurities to the main component. If no information is provided, a 1:1 response factor will be assumed)	
Any other information	

[NB - To complete your entry, please insert additional rows as necessary]

Mass balance method 4/10

CCQM-K148b CCQM-P187b Reporting Form 1.4
Analytical Method for Mass Balance procedure

2.Water content

Sample amount per analysis (approximate)	mg
Number of samples analyzed	
Instrumentation (e.g., coulometric Karl Fischer titration, TGA)	
Analytical conditions	

3. Residual solvent content

Sample amount per analysis (approximate)	mg
Number of samples analyzed	
Instrumentation (e.g., headspace GC, NMR, etc)	
Analytical conditions	

4. Combined non-volatile content

Sample amount per analysis	mg
Number of samples analyzed	
Instrumentation (e.g., TGA, EA, ICP-MS)	
Analytical conditions	

CCQM-K148b CCQM-P187b Reporting Form 1.4
Analytical Method for qNMR procedure

Information about the qNMR procedure(s) used

[NB - To complete your entry, please insert additional rows as necessary]

Solvent(s) used	
qNMR procedure (eg. internal standard, external standard, etc.)	
Name and source of standard(s)	
Purity and uncertainty of standard(s)	
Traceability source	

Gravimetry

Type of balance (make, model and resolution)	
Balance repeatability	(µg)
Minimum weight	(mg)

Sample preparation

Smallest mass of analyte	(mg)
Smallest mass of standard	(mg)
Number of independent samples prepared	

CCQM-K148b CCQM-P187b Reporting Form 1.4
Analytical Method for qNMR procedure

Number of replicate analyses per sample	

qNMR parameters

Spectrometer	
Experimental parameters	
Processing software	
Integration parameters	
Lineshape (FWHM of solvent peak)	

Signal/Noise

Standard peak	
Analyte peak	

CCQM-K148b CCQM-P187b Reporting Form 1.4
Analytical Method for qNMR procedure

Other approaches (e.g. CRAFT, QM full spin analysis, etc.)	

[NB - To complete your entry, please insert additional rows as necessary]

CCQM-K148b CCQM-P187b Reporting Form 1.4
Other Analytical Methods

Analytical instrumentation used	
Sample amount per analysis (approximate)	mg
Number of samples analyzed	
Sample preparation	

[NB - To complete your entry, please insert additional rows as necessary]

Other methods 9/10

CCQM-K148b CCQM-P187b Reporting Form 1.4
Value assignment and MU Budget

Contributing results for Oxytetracycline (free base) in CCQM-K148.b / CCQM-P187.b

Mass balance result (if used)		mg/g
qNMR result (if used)		mg/g
Other results (if used)		mg/g
Final reported result (as entered in "Results" Worksheet)		mg/g

Measurement equation
Describe both:
1. Measurement equation for individual methods

2. Measurement equation for combination of values if results of two or more methods were combined for the assignment

Uncertainty budget
(please include breakdown of the budget, describing major individual uncertainty contributions and how they were combined)

[NB - To complete your entry, please insert additional rows as necessary]

Appendix D: Core competency table template

CCQM-K148.b	*NMI*	Mass fraction of polar analyte in a solid organic material
Scope of comparison: The measurement results are representative of the laboratory's capability for the purity assignment of solid organic compounds in the molar mass range (75 – 500) g/mol with pK_{OW} > -2.		
Competency	**✓,× or N/A**	**Specific Information**
• Value assignment of Primary Reference: Main component mass fraction and uncertainty		
Identity verification		*Summary of methods used to establish the qualitative identity (e.g., comparison with independent sample, mass spec., NMR, other)*
Assignment of OTC base mass fraction content of CCQM-K148.b		*Indicate method(s) used to quantify mass fraction of OTC in the material*
Oxytetracycline content (mg/g)		*Reported comparison result (± $U_{95\%}$)*
• Value assignment of Primary Reference: Impurity class mass fraction and uncertainty (required if using a mass balance method, otherwise optional)		
Assignment of related structure impurity		*Indicate method(s) used to quantify mass fraction of related structure impurities in the material*
Total related structure impurity (mg/g)		*Reported comparison result (± $U_{95\%}$)*
Assignment of water content		*Indicate method(s) used to quantify mass fraction water content in the material*
Category of water content assignment*		*Select from list below* the applicable category of general water content assignment competency*
Water content (mg/g)		*Reported comparison result (± $U_{95\%}$)*
Assignment of residual solvent content		*Indicate method(s) used to quantify mass fraction residual solvent content in the material*
Total residual solvent (mg/g)		*Reported comparison result (± $U_{95\%}$)*
Assignment of inorganic content		*Indicate method(s) used to quantify mass fraction total non-volatile content in the material*
Total non-volatiles (mg/g)		*Reported comparison result (± $U_{95\%}$)*

General Instructions:

- Replace "*NMI*" with the acronym for your institution in the first cell of the middle column
- Place a tick, cross or N/A (not applicable) in each middle column cell as appropriate for each competency
- In each right hand column cell replace the blue text with the relevant information for your comparison result

* To be completed by NMIs intending or anticipating to make CMC claims for the assignment of water content in solid organic materials. Choose one of the following categories:

- polar organic solid, water content < 20 mg/g
- polar organic solid, water content > 20 mg/g

Appendix E: Summary of participants' analytical information

Methods in brackets used as supporting evidence, not for reporting.

Participant	SRI[1]	Water[2]	Chloride[3]	VOC	Inorganic	OTC - qNMR[4]
HSA	LC-UV 275 nm, RR (LC-UV 254, LC-MS/MS)	KFT-DA (KFT-OT)	IC (TQ-ICP-MS)	qNMR (GC-MS)	TGA, ICP-MS	MA, AceK, BA, BTFMBA (4.3 ppm)
NMISA	LC-UV 272 nm, DC	KFT-OT 125°C	IC	GC-MS	TGA	TCNB (6.9, 7.1 ppm)
NRC	LC-UV 250, 270 and 356 nm, SA (LC-hrMS)					DMTP, BA (7.6, 7.3, 6.9 ppm)
BIPM	LC-UV 275 nm, DC	KFT-OT 170°C	IC	qNMR	IC	MA (7.5, 7.0, 1.6 ppm)
NMIJ	LC-UV 270 nm, LC-CAD, DC (LC-hrMS)	KFT-OT 120°C	IC	GC-FID	TGA, IC	BTFMBA (1.8 ppm)
NMIA	LC-UV 254 nm, RRF (LC-UV 270 nm)	KFT-DA	IC	GC-MS, NMR	TGA (qNMR, EA)	MA (6.7-7.8 ppm)
NIM	LC-UV 270 nm, DC	KFT-DA	IC	GC-FID (GC-MS)	ICP-MS	Ethylparaben (1.8 ppm)
GLHK	LC-UV 270 nm, RR (LC-hrMS)	KFT-OT 160°C	ICP-MS	qNMR	TGA, ICP-MS	BTFMBA (3.8 ppm)
INMETRO	LC-UV 270 nm, RRF (LC-MS/MS)	KFT-DA	XRF	TGA, qNMR (GC-MS)	ICP-OES, ICP-MS	MA, TCNB (6.7-7.8 ppm)
EXHM	LC-UV (CAD) 254 nm, RRF (LC-MS)	KFT-OT 140°C , KFT-DA	IC	GC-MS, GC-FID	ICP-MS	MA (3.8 ppm)
BAM						MA, BTFMBA (7.2 ppm)
LGC	(LC-UV, LC-MS)	KFT-OT 160°C	ICP-MS	qNMR	ICP-MS	MA (7.5 ppm)
NIST		KFT-DA (TGA)				DMTP, TCNB (6.9, 7.1 ppm)
UME	LC-UV 275 nm, RR	KFT-OT 160°C	IC	GC-FID (NMR)		BA (7.0 ppm)
KRISS	LC-UV 270, 355 nm, RR (355 nm) (LC-MS)	KFT-OT 150°C	IC	GC-MS	TGA	BA (6.8-7 ppm)
KIMIA	LC UV 270, 288, 355 nm, RR	KFT-DA (TGA)	IC	GC-FID (GC-MS, TGA)	TGA	
BVL	LC UV 270, 288, 355 nm, RRF	KFT-OT 120°C	IC	GC-MS, TGA	GC-MS, TGA	
VNIIM	LC-UV 254 nm, DC, RRF	KFT-OT 150°C	CE-UV 374 nm	GC-FID (GC-MS)	TGA	
NIMT	LC-UV 355 nm, RR	KFT-OT 160°C		TGA		TCNB (6.8-7.7 ppm)
INRIM						BA (6.9,7.1 ppm)

Appendix E – Summary of participants' analytical information

Notes:

1) *Assignment methods: RR (relative response); RRF (relative response with estimation of response factors), DC (direct calibration), SA (Standard addition).*
2) *Karl Fischer titration (KFT) with direct sample addition (DA) or oven transfer (OT) at specified temperature.*
3) *Ion chromatography (IC), Inductively coupled plasma mass spectrometry (ICP-MS), X-ray fluorescence (XRF), Capillary electrophoresis with UV detection (CE-UV) at specified wavelength.*
4) *Internal standard(s) used (chemical shift of integrated oxytetracycline signal used for quantification).*

Appendix F: Summary of measurement equations and uncertainty budgets

Participant: HSA

Measurement equation for mass balance approach:

Mass fraction of oxytetracycline free base (mg/g) was calculated using the equation below:

$$m_{MB\,(base)} = (1000 - I_{RSI}) \times (1000 - F_{Others})/1000 \qquad (1)$$

where,

I_{RSI} is the mass fraction (mg/g) of total related structure impurities determined by HPLC-DAD;

F_{Others} is the sum of mass fraction (mg/g) of other impurities.

$$I_{RSI} = I_{LC\text{-}DAD} + I_{NR} + I_{ND} \qquad (2)$$

where,

$I_{LC\text{-}DAD}$ is the mass fraction (mg/g) of total related structure impurities detected by HPLC-DAD;

I_{NR} is the mass fraction (mg/g) of non-resolved organic impurities in HPLC-DAD (has a value of zero but has an associated uncertainty estimated from LOQ);

I_{ND} is the mass fraction (mg/g) of non-detected organic impurities in HPLC-DAD (has a value of zero but has an associated uncertainty estimated from LOD).

$$F_{Others} = F_{VO} + F_{W} + F_{IR} + F_{HCl} \qquad (3)$$

where,

F_{VO} is the mass fraction (mg/g) of residual organic solvent;

F_{W} is the mass fraction (mg/g) of water;

F_{IR} is the mass fraction (mg/g) of total non-volatiles/inorganics;

F_{HCl} is the mass fraction (mg/g) of HCl.

Measurement equation for qNMR approach:

Mass fraction of oxytetracycline free base (mg/g) was calculated using the equation below:

$$m_{qNMR} = P_{ISTD} \times (I_X / I_{ISTD}) \times (n_{ISTD} / n_X) \times (M_X / M_{ISTD}) \times (m_{ISTD} / m_X) \qquad (4)$$

where,

P_{ISTD}: mass fraction of internal standard (mg/g)

I_X: integral area of quantification peak of analyte

I_{ISTD}: integral area of quantification peak of internal standard

n_{ISTD}: number of protons of the quantification peak of internal standard

n_X: number of protons of the quantification peak of analyte

M_X: molecular weight of analyte (oxytetracycline free base) (g/mol)

M_{ISTD} molecular weight of internal standard (g/mol)

m_{ISTD} mass of internal standard (g)

m_X: mass of study sample (g)

$$m_X = m_{RH_{50}} = \frac{m_{RH_X}}{1 + 0.00037(RH_X - 50)} \qquad (5)$$

The final mass fraction of oxytetracycline free base (mg/g) using qNMR is obtained from the arithmetic mean of the four results, i.e. using acesulfame potassium as ISTD in 0.01 N DCl D2O, using maleic acid as ISTD in 0.01 N DCl D2O, using benzoic acid as ISTD in CD3OD and using 3,5 bis(trifluoromethyl)benzoic acid as ISTD in MEOD.

$$m_{qNMR(base)} = \frac{m_{qNMR(MA)} + m_{qNMR(AceK)} + m_{qNMR(BA)} + m_{qNMR(BFBA)}}{4} \qquad (6)$$

where,

$m_{qNMR(MA)}$ is the mass fraction of oxytetracycline free base determined using maleic acid as ISTD in 0.01 N DCl D2O by qNMR,

$m_{qNMR(AceK)}$ is the mass fraction of oxytetracycline free base determined using AceK as ISTD in 0.01 N DCl D2O by qNMR,

$m_{qNMR(BA)}$ is the mass fraction of oxytetracycline free base determined using benzoic acid as ISTD in CD3OD by qNMR,

$m_{qNMR(BFBA)}$ is the mass fraction of oxytetracycline free base determined using 3,5 bis(trifluoromethyl)benzoic acid as ISTD in CD3OD by qNMR.

Measurement equation for final reported result:

$$x_{report} = \frac{m_{MB(base)} + m_{qNMR(base)}}{2} \quad (7)$$

where,

x_{report} is the reported mass fraction of oxytetracycline free base,

$m_{MB(base)}$ is the mass fraction of oxytetracycline free base determined by mass balance approach,

$m_{qNMR(base)}$ is the mass fraction of oxytetracycline free base determined by qNMR approach.

Measurement uncertainty equation for mass balance approach:

The combined standard uncertainty of the mass fraction of the oxytetracycline free base using mass balance approach, $u(m_{MB\,(base)})$, is calculated from mathematical equations related to the standard uncertainty of each component (I_{RSI}, F_{VO}, F_W, F_{IR} and F_{HCl}) and the corresponding sensitivity coefficient:

$$u(m_{MB(base)}) = \sqrt{c_{I_{RSI}}^2 u_{I_{RSI}}^2 + c_{F_{VO}}^2 u_{F_{VO}}^2 + c_{F_W}^2 u_{F_W}^2 + c_{F_{IR}}^2 u_{F_{IR}}^2 + c_{F_{HCl}}^2 u_{F_{HCl}}^2} \quad (8)$$

The sensitivity coefficients of each component can be expressed as follows:

$$c_{I_{RSI}} = \frac{\delta m}{\delta I_{RSI}} = -(1000 - F_{VO} - F_W - F_{IR} - F_{HCl})/1000 \quad (9)$$

$$c_{F_{VO}} = \frac{\delta m}{\delta F_{VO}} = -(1000 - I_{RSI})/1000 \quad (10)$$

$$c_{F_W} = \frac{\delta m}{\delta F_W} = -(1000 - I_{RSI})/1000 \quad (11)$$

$$c_{F_{IR}} = \frac{\delta m}{\delta F_{IR}} = -(1000 - I_{RSI})/1000 \quad (12)$$

$$c_{F_{HCl}} = \frac{\delta m}{\delta F_{HCl}} = -(1000 - I_{RSI})/1000 \quad (13)$$

Measurement uncertainty equation for qNMR approach:

In general, the combined standard uncertainty from qNMR approach, $u(m_{qNMR(base)})$ was calculated as follows:

$$u(m_{qNMR}) = m_{qNMR} \times \sqrt{\left(\frac{u(MP)}{m_{qNMR}}\right)^2 + \left(\frac{u(P_{ISTD})}{P_{ISTD}}\right)^2 + \left(\frac{u(m_x)}{m_x}\right)^2 + \left(\frac{u(M_x)}{M_x}\right)^2 + \left(\frac{u(m_{ISTD})}{m_{ISTD}}\right)^2 + \left(\frac{u(M_{ISTD})}{M_{ISTD}}\right)^2 + \left(\frac{u(F_{Diff})}{F_{Diff}}\right)^2}$$

(14)

where,

$u(m_{qNMR})$: the uncertainty in mass fraction of oxytetracycline free base using qNMR approach

u(MP): the uncertainty in method precision

$u(P_{ISTD})$: the uncertainty in the mass fraction of the internal standard

$u(m_X)$: the uncertainty in the mass of sample weighed (including uncertainty of F and RHx in the calculation of m_{RH50})

$u(m_{ISTD})$: the mass of the internal standard weighed

$u(M_X)$: the uncertainty in the molecular weight of the analyte (oxytetracycline free base)

$u(M_{ISTD})$: the uncertainty in the molecular weight of the internal standard

$u(F_{Diff})$: the uncertainty of the factor representing bias in the results due to different parameters (e.g. neutral vs acidic solvent)

$$u(m_{qNMR(base)}) = \sqrt{\left(\frac{u(m_{qNMR(MA)})}{4}\right)^2 + \left(\frac{u(m_{qNMR(AceK)})}{4}\right)^2 + \left(\frac{u(m_{qNMR(BA)})}{4}\right)^2 + \left(\frac{u(m_{qNMR(BFBA)})}{4}\right)^2 + u_B^2}$$

(15)

where,

$u(m_{qNMR(MA)})$ is the uncertainty of mass fraction of oxytetracycline free base determined using maleic acid as ISTD in 0.01 N DCl D2O by qNMR,

$u(m_{qNMR(AceK)})$ is the uncertainty of mass fraction of oxytetracycline free base determined using acesulfame potassium as ISTD in 0.01 N DCl D2O by qNMR,

$u(m_{qNMR(BA)})$ is the uncertainty of mass fraction of oxytetracycline free base determined using benzoic acid as ISTD in CD3OD by qNMR,

$u(m_{qNMR(BFBA)})$ is the uncertainty of mass fraction of oxytetracycline free base determined using 3,5 bis(trifluoromethyl)benzoic acid as ISTD in CD3OD by qNMR,

u_B is the uncertainty from method biases expressed as the standard deviation of the results from the four methods.

Measurement uncertainty equation for final reported result:

$$u(x_{report}) = \sqrt{\left(\frac{u(m_{MB(base)})}{2}\right)^2 + \left(\frac{u(m_{qNMR(base)})}{2}\right)^2 + u_B^2} \qquad (16)$$

where,

$u(m_{MB(base)})$ is the uncertainty of mass fraction of oxytetracycline free base determined by mass balance approach,

$u(m_{qNMR(base)})$ is the uncertainty of mass fraction of oxytetracycline free base determined by qNMR approach,

u_B is the uncertainty from method bias estimated based on rectangular distribution of the difference between the two results.

APPENDIX F: Summary of measurement equations and uncertainty budgets

Table 1. Uncertainty budget for oxytetracycline free base using mass balance approach

Parameter	Source of data	Source(s) of uncertainty	Results			Type of uncertainty	Contribution to overall U, %
			I_w, mg/g	u_x, mg/g	c_x		
I_{ASI}	HPLC-DAD	The combined standard uncertainty was calculated from the standard deviation of results in HPLC-DAD measurement using ODS-AQ column, differences in results obtained using two columns (ODS-AQ vs C8) divided by two, differences in results obtained using 275 nm and 254 nm divided by two.	49.5	4.3	0.8273	Combined Type A uncertainty	36.77
		Standard uncertainty of organic impurity not detected in HPLC-DAD, $u(I_{ND})$, assuming there are 5 of them.		0.022			
		Standard uncertainty of non-resolved organic impurities, $u(I_{NR})$, assuming there are 5 of them.		0.065			
F_{Others}	F_W Karl Fischer titration	The standard deviation of the results in the measurement of moisture in the study material after correction for atmospheric moisture and drift, correction of results to RH50 according to study protocol, differences in results between direct addition and oven transfer method divided by two, the bias of the results estimated using NIST SRM 2890.	106.4	4.0	-0.9505	Type A and B uncertainty	41.59
	F_{VO} TGA	The standard uncertainty in measurement of organic solvent was estimated with the assumption of rectangular distribution. The LOD of the instrument is 2.3 mg/g. The value of the standard uncertainty is $LOD/(2\sqrt{3})$.	0.024	0.66	-0.9505	Type B uncertainty	1.17
	F_{IR} TGA	The standard uncertainty in measurement of non-volatiles/inorganics was estimated with the assumption of rectangular distribution. The LOD of the instrument is 5.0 mg/g. The value of the standard uncertainty is $LOD/(2\sqrt{3})$.	0.00	1.44	-0.9505	Type B uncertainty	5.53
	F_{HCl} IC	The combined standard uncertainty was calculated from the method precision, weighing, linear regression, method recovery, calibration standards and differences in results obtained using IC and TQ-ICP-IDMS divided by two.	66.3	2.4	-0.9505	Type A and Type B uncertainty	14.94

APPENDIX F: Summary of measurement equations and uncertainty budgets

Combined uncertainty, $u(m_{MB(base)})$, mg/g	5.8
Effective degrees of freedom (υ_{eff})	62.48
k (at 95% CI)	2.00
Expanded uncertainty (U), mg/g	11.7

Table 2. Uncertainty budget for oxytetracycline free base using qNMR approach (This table shows the MU budget using Acesulfame potassium as ISTD in 0.01 N DCl D2O)

Parameter	Value	Standard Uncertainty	Remarks
MP (mg/g)	772.8	12.3	
P_{ISTD} (mg/g)	999.2	2.5	
m_{ISTD} (g)	0.0090195	0.0000148	
m_x (g)	0.0107670	0.0000152	
M_{ISTD} (g/mol)	201.245	0.00551	
Mx (g/mol)	460.433	0.01299	
$F_{diff\_integration}$	1	0.00365	Bias in the results due to integration by different analyst
F_{diff_peak}	1	0.00973	Bias in the results due to integration on peak 4.3 ppm vs 1.8 ppm
$F_{diff\_solvent}$	1	0.01336	Bias in the results due to different solvent (0.01 N DCl D2O vs D2O)

Combined uncertainty, $u(m_{qNMR(AceK)})$, mg/g	18.1
Effective degrees of freedom (υ_{eff})	9.05
k (at 95% CI)	2.26
Expanded uncertainty (U), mg/g	41.0

Table 3. Uncertainty budget for final mass fraction of oxytetracycline free base using qNMR approach

Parameter	$m_{qNMR(MA)}$	$m_{qNMR(AceK)}$	$m_{qNMR(BFBA)}$	$m_{qNMR(BA)}$
Value, mg/g	763.8	772.8	766.9	768.2
Standard uncertainty, mg/g	9.5	18.1	1.5	6.3
Arithmetic mean, mg/g	767.9			
Combined uncertainty, mg/g	6.5			
Effective degrees of freedom (υ_{eff})	50.25			
k (at 95% CI)	2.0			
Expanded uncertainty (U), mg/g	13.1			

Table 4. Uncertainty budget for final report result of oxytetracycline free base using using both mass balance and qNMR approaches

Parameter	$m_{MB(base)}$	$m_{qNMR(base)}$
Value, mg/g	786.3	767.9
Standard uncertainty, mg/g	5.8	6.5
Arithmetic mean, mg/g	777.1	
Combined uncertainty, mg/g	6.9	
Effective degrees of freedom (υ_{eff})	112.72	
k (at 95% CI)	2.0	
Expanded uncertainty (U), mg/g	13.8	

Participant: NMISA

W_{OTC} mass balance = 1000 – (w_{imp} + w_{H20} + w_{RS} + w_{Cl} +w_H)

W_{OTC} = OTC free base mass fraction in the K148b sample (mg/g)

w_{imp} = Mass fraction of the sum of organic impurities determined by external calibration by LC (mg/g)

w_{H20} = Mass fraction of water (mg/g) determined by KF coulometry (oven transfer)

w_{RS} = Mass fraction of residual solvents (mg/g) determined by HS-GC-TOFMS

w_{Cl} = Mass fraction of chloride (mg/g) determined by IC

w_H = Mass fraction of hydrogen associated with chloride determination (mg/g) determined theoretically

Main uncertainty components: Mass balance	x	u(x)	k	U	
WRS					
Uncertainty contributors included: CRM calibrant, Bias, precission, calibration, and sample mass for all residual solvents detected (Methanol, acetonitrile)					
WNV	**x**	**u(x)**	**u(x)/x**	**vi**	**ui4/vi**
Precision (<LOQ)	1.00	0.005306	0.005	6	1.32E-10
Accuracy (CaOx)	1.02	0.000422533	4E-04	8	3.98E-15
WH2O	**x**	**u(x)**	**u(x)/x**	**vi**	**ui4/vi**
Precision	97.53	1.914974178	0.02	3.00E+00	4.48E+00
Accuracy	5.07	0.014388489	0.003	1.00E+06	4.29E-14
Bias	99.78	1.190105605	0.012	6.00E+00	3.34E-01
Wimp	**x**	**u(x)**	**k**	**U**	
Uncertainty contributors: Calibration, Purity, Precision in the quanitification of each impurity, as well as the precision of total impurities in independent replicates using different calibration curves (RF) for unknown impurities					
4-Epitetracycline (4 ETC)	1.21	0.07	2.57	0.18	
4-epioxytetracycline (4EOTC)	4.03	0.17	2.16	0.37	
Tetracycline (TC)	6.57	0.3	3.2	1.02	
Isochlortetracycline (IsoCTC)	34.4	0.5	2	0.9	
Chlortetracycline (CTC)	6.79	0.13	2	0.26	
4-epianhydrotetracycline (4EATC)	1.23	0.07	2.45	0.17	
sum of all other LC impurities (8)	8	2.2	2	4.6	
Precision of independent replicates	78.6	5.6	1.99	11	
WCl					
Purity, calibration, sample mass, Precision					
Uncertainties combined per category as relative uncertainties					

APPENDIX F: Summary of measurement equations and uncertainty budgets

Participant: NRC

Internal standard qNMR equation:

$$w_{an} = \frac{I_{an}}{I_c} \cdot \frac{N_c}{N_{an}} \cdot \frac{MW_{an}}{MW_c} \cdot \frac{m_c}{m_{an}} \cdot \frac{V_{an}}{V_c} \cdot w_c$$

External standard qNMR equation:

$$w_{an} = \frac{I_{an}}{I_c} \cdot \frac{N_c}{N_{an}} \cdot \frac{\theta_{an}^{360}}{\theta_c^{360}} \cdot \frac{NS_c}{NS_{an}} \cdot \frac{RG_c}{RG_{an}} \cdot \frac{MW_{an}}{MW_c} \cdot \frac{m_c}{m_c^{soln}} \cdot w_c \cdot \frac{m_a^{soln}}{m_a}$$

Impurity correction equation:

$$w_{imp\ corr} = \frac{MW_{an}}{N_{an}} \cdot \sum_i \frac{w_{imp\ i} \cdot N_{imp\ i}}{MW_{imp\ i}}$$

Final mass fraction:

$$w_{an\ corr} = w_{an} - w_{imp\ corr}$$

Three samples by internal standard ^{1}H-qNMR and one sample by external standard ^{1}H-qNMR. The results were averaged to generate a final value.

Where for analyte (an), calibrant (c), and impurity (imp):

w = mass fraction

I = integrated signal area

N = number of protons integrated

MW = molar mass (g/mol)

m = mass of solid (g)

V = volume by mass (g) - equivalent for analyte and calibrant for internal standard qNMR

$\boldsymbol{\theta^{360}}$ = 360 ° pulse

NS = number of scans

RG = receiver gain

m^{sol} = mass of solution

The uncertainties sources were treated as multiplicative and combined according to JCGM-100. Additional uncertainty sources were considered for external standard ^{1}H-qNMR and found to be negligible.

	Contribution	Type
Sample Preparation Overall Contribution:	**0.2%**	
Weighings of the analyte	0.1%	A
Weighing of the calibrant	0.1%	A
Molecular weight of calibrant	0.0%	A
Purity of the calibrant	0.0%	A
Molecular weight of analyte	0.0%	A
NMR Overall Contribution:	**75.8%**	
Method uncertainty due to different signals	42.1%	A
Reproducibility between samples	18.2%	A
Repeatability between replicates	9.9%	A
p360° calibration	4.5%	B
Temperature variation	0.2%	B
Peak integration (incompleteness)	0.4%	B
NMR electronics	0.4%	B
Peak integration (between analyst)	0.1%	A
LCUV Impurity Quant. Overall Contribution:	**24.0%**	
Impurity Correction	24.0%	A

APPENDIX F: Summary of measurement equations and uncertainty budgets

Participant: BIPM

The mass balance value was calculated according to equation 1.

$$w = 1000 - (\sum_i w_i + w_w + w_{VOC} + w_{NV}) \qquad \text{(Eq. 1)}$$

Where:

w : mass fraction (mg/g) of the main component in the material.

w_i: mass fraction (mg/g) of individual related structure impurity *i* in the material.

w_w: mass fraction (mg/g) of water in the material.

w_{VOC}: mass fraction (mg/g) of residual solvent in the material.

w_{NV}: mass fraction (mg/g) of non-volatile residue in the material.

qNMR assignment: Individual analyte purity uncorrected for impurities, w_a, based on a selected resonance signal was calculated according to Eq. 2.

$$w_a = \frac{I_a \cdot n_s \cdot m_s \cdot M_a}{I_s \cdot n_a \cdot m_a \cdot M_s} \cdot w_s$$

Eq. 2

Impurity-corrected, signal-specific purity values, w_c, were calculated according to Eq. 3 using information on structure related impurities

$$w_c = w_a - \frac{M_a}{n_a} \cdot \sum_i \frac{w_i \cdot n_i}{M_i} \quad or \quad w_c = w_a - F_{IC}$$

Eq. 3

Signal-specific purity values, w_c, were averaged for each replicate and sample. The mean of the impurity-corrected values assigned for each of the quantified signals at δ 1.6, 7.0 amd 7.5 ppm is the assigned value for OTC free base content.

Symbols definitions:

I_{Std}, N_{Std}, M_{Std}, m_{Std}, w_{Std} :

signal area, number of protons or fluorines, molar mass,

I_a, N_a, M_a, m_a, w_a :

weighed mass and mass fraction of the IS, respectively.

signal area, number of protons or fluorines, molecular weight,

w_i, n_i, M_i :

weighed mass and mass fraction of the analyte, respectively.

mass fractions, numbers of nuclei and molar masses of the interfering

impurities, respectively.

Both mass balance and qNMR values were combined by weighted average:

$$\bar{x} = \frac{\sum w_i k_i}{\sum w_i} \qquad k_i = \frac{1}{u_i^2}$$

Uncertainty budget - hygroscopicity correction of OTC mass fraction by qNMR

Component y	Value	Unit	Standard Uncertainty $u(y)$			Sensitivity coefficient	Contribution to $u(w_c)$ / mg/g
			Source	Type	Std. Uncert.	$c_i = \frac{\partial x}{\partial y}$	$c_i^2 \cdot u^2(y)$
w_RHX	790.81	mg/g	IC-qNMR	A, B	5.1084	0.999	26.0495
F	0.00037		Slope of the linear regression model: rel mass vs. RH%	A	0.00003	-1897.947	0.0032
RHX	47.6	%	Humidity sensor tolerance limits of 3% taken as rectangular unc distrib.	B	1.7	0.293	0.2568
w_RH50	790.11	mg/g		Combined standard uncertainty $u(w_a)$:			5.129

The uncertainty of the mass balance value was calculated by square root of the quadratic summation of the individual impurities mass fraction uncertainties.

The uncertainty of the weighted average of qNMR and mass balance values was calculated as shown below:

$$u(\bar{w}) = \frac{1}{\sum k_i}\sqrt{\sum (k_i u_i)^2} = \frac{1}{\sum (u_i)^{-2}}\sqrt{\sum \left(\frac{1}{u_i}\right)^2}$$

APPENDIX F: Summary of measurement equations and uncertainty budgets

qNMR uncertainty budget:

Uncertainty budget

Component y	Value	Unit	Standard Uncertainty $u(y)$			Sensitivity coefficient	Contribution to $u(w_c)$ / mg/g
			Source	Type	(Combined) uncertainty	$c_i = \frac{\partial x}{\partial y}$	$\lvert c_i \rvert \cdot u(y)$
I_a/I_s	1.37		considered in the overall precision, P, of w_c	A			
n_s/n_a	0.67		^{1}H isotopic abundance	B*	0.00006	1217.194	0.073
m_s	3.77	mg	weighing	A, B	0.00124	215.285	0.267
m_a	16.74	mg	weighing	A, B	0.00145	-48.480	0.070
M_a	460.44	g/mol	atomic weights uncertainties	B	0.02000	1.763	0.035
M_s	116.07	g/mol	atomic weights uncertainties	B	0.00400	-6.992	0.028
w_s	997.40	mg/g	IS certified purity	A, B	0.40000	0.814	0.325
w_a	811.59	mg/g			Combined standard uncertainty $u(w_a)$:		0.436
—	821.04	mg/g	qNMR equation factors except integrals precision	B	0.4356	1.000	0.436
—	31.81	mg/g	average correction of mass fraction by impurities (uncertainties other than $u(w_i)$ are negligeable)	B	0.5255	1.000	0.526
P	1		Precision of imp-corrected combined value, w_{cr} by ANOVA	A	0.00094	790.811	0.742
Signal bias	0		Variance between purity estimates from different signals	A	5.0079	1.000	5.008
—	790.8	mg/g			Combined standard uncertainty $u(w_c)$:		5.1
					Expanded Uncertainty (k=2), $U(w_c)$:		10.2

* Standard uncertainty from ^{1}H isotopic abundance can be estimated using the IUPAC calculator: https://ciaaw.shinyapps.io/calculator/

APPENDIX F: Summary of measurement equations and uncertainty budgets

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Participant: NMIJ

1-1. Measurement equation for Mass balance approach

$$w_{\mathrm{p}}(\mathrm{MBA}) = 1000 - w_{\mathrm{related}} - w_{\mathrm{water}} - w_{\mathrm{volatile}} - w_{\mathrm{non-volatile}} - \frac{M_{\mathrm{HCl}}}{M_{\mathrm{Cl}}} \cdot w_{\mathrm{Cl}}$$

1-2. Measurement equation for qNMR

$$w_{\mathrm{p}}(\mathrm{qNMR}) = \frac{S_{\mathrm{x}}}{S_{\mathrm{s}}} \cdot \frac{M_{\mathrm{OTC}}}{M_{\mathrm{s}}} \cdot \frac{N_s}{N_{\mathrm{x}}} \cdot \frac{m_s}{m_{\mathrm{x}}} \cdot P_{\mathrm{s}}$$

2. Measurement equation for combination of values

$$w_{\mathrm{p}} = \frac{w_{\mathrm{p}}(\mathrm{MBA}) + w_{\mathrm{p}}(\mathrm{qNMR})}{2}$$

Model equation for uncertainty evaluation of w_{p}

$$w_{\mathrm{p}} = \frac{w_{\mathrm{p}}(\mathrm{MBA}) + w_{\mathrm{p}}(\mathrm{qNMR})}{2} + f_{\mathrm{method}}$$

f_{method} = 0 mg g^{-1}

$$u(f_{\mathrm{method}}) = \frac{\left|w_{\mathrm{p}}(\mathrm{MBA}) - w_{\mathrm{p}}(\mathrm{qNMR})\right|}{\sqrt{12}}$$

Uncertainty components of w_{p} are measurement methods (mass balance approach and quantitative nuclear magnetic resonance) and difference between the methods. The standard uncertainties of the components were combined assuming they have no correlation.

Uncertainty budgets of w_{p} and w_{p}(MBA) are shown below.

APPENDIX F: Summary of measurement equations and uncertainty budgets

Uncertainty budger of w_p

Symbol	Source of uncertainty	Value x_i / mg g^{-1}	Standard uncertainty $u(x_i)$ / mg g^{-1}	c_i	$c_i\,u(x_i)$ / mg g^{-1}	Contribution
w_p(MBA)	Mass balance approach	795.86	3.78	0.5	1.89	0.292
w_p(NMR)	Quantitative nuclear magnetic resonance	786.4	2.1	0.5	1.05	0.090
f_{method}	Diferrence between measurements	0	2.74	1	2.74	0.613
$u_c(w_p)$	Combined standard uncertainty				3.50 mg g^{-1}	
$U(w_p)$	Expanded uncertainty				7.00 mg g^{-1}	
					(k = 2)	

Uncertainty budget of w_p(MBA)

Symbol	Source of uncertainty	Value x_i / mg g^{-1}	Standard uncertainty $u(x_i)$ / mg g^{-1}	c_i	$c_i\,u(x_i)$ / mg g^{-1}	Contribution
$w_{related}$	Total related structure impurities	32.08	2.60	1	2.60	0.47
w_{water}	Water content	107.04	2.71	1	2.71	0.51
w_{Cl}	Chloride ion	63.07	0.07	1.03	0.08	0.00
$w_{volatile}$	Total non-volatiles and inorganics	0.16	0.10	1	0.10	0.00
$w_{non\text{-}volatile}$	Volatile organics content	0	0.35	1	0.35	0.01
$u_c(w_p$(MBA))	Combined standard uncertainty				3.78 mg g^{-1}	
$U(w_p$(MBA))	Expanded uncertainty				7.56 mg g^{-1}	
					(k = 2)	

APPENDIX F: Summary of measurement equations and uncertainty budgets

Participant: NMIA

Purity (%) = (100 - I“Organic”)*(100 - I"Other")

I"Organic" = Mass fraction of organic impurities of similar structure.

I"Other" = Mass fraction of volatile and non-volatile impurities.

Equation for qNMR

$$P_A = \frac{I_s}{I_{Std}} \frac{n_{Std}}{n_s} \frac{M_s}{M_{std}} \frac{m_{Std}}{m} P_{std}$$

All uncertainties are combined using the square root of the sum of the squares approach, using standard uncertainties or relative standard uncertainties as appropriate.
The major components of the uncertainty budget are
Uc from Karl Fischer analysis,
Uc from HPLC organic purity analysis,
Uc from non-volatile residues,

$$u_{Purity} = P\sqrt{\left(\frac{U_{Organic}}{I_{Organic}}\right)^2 + \left(\frac{U_{Other}}{I_{Other}}\right)^2}$$

The qNMR uncertainty was calculated using the relative standard uncertainties of all componenets in the measurement equation, as shown below.

$$u_{P_{Analyte}} = P_{Analyte} x \sqrt{\left(\frac{u_{P_{Analyte}}}{P_{Analtye}}\right)^2 + \left(\frac{u_{\rho_{IS}}}{\rho_{IS}}\right)^2 + \left(\frac{u_{\rho_{Analyte}}}{\rho_{Analyte}}\right)^2 + \left(\frac{u_{P_{IS}}}{P_{IS}}\right)^2 + \left(\frac{u_{Mwt_{Analyte}}}{Mwt_{Analyte}}\right)^2 + \left(\frac{u_{Mwt_{IS}}}{Mwt_{IS}}\right)^2 + \left(\frac{u_{wt_{IS}}}{wt_{IS}}\right)^2 + \left(\frac{u_{wt_{Analyte}}}{wt_{Analtye}}\right)^2}$$

APPENDIX F: Summary of measurement equations and uncertainty budgets

APPENDIX F: Summary of measurement equations and uncertainty budgets

Participant: NIM

The measurement equation (Eqn. 1) of the Mass Balance to assign the purity of Oxytetracycine in CCQM-K148.b is:

$$P_{MB} = 1000 - X_{RS} - X_W - X_{Cl} - X_{NV} - X_V \qquad (1)$$

Where

P_{MB} : mass fraction of Oxytertracyine

X_{RS} : mass fraction of total related structure imputies

X_W : mass fraction of water content

X_{Cl} : mass fraction of Chloride ion

X_{NV} : mass fraction of total non-volatiles and inorganics

X_V : mass fraction of volatile organic content

Measurement equation for qNMR method:

$$P_{QNMR} = \frac{I_s}{I_{std}} \frac{n_{std}}{n_s} \frac{M_s}{M_{std}} \frac{m_{std}}{m_s} P_{std} \qquad (2)$$

Where

P_{QNMR} : mass fraction of sample(Oxytetracycine)

P_{std} : mass fraction of internal standard.

m_{std} : weight of internal standard.

M_{std} : molecular weight of internal standard.

n_{std} : number of hydrogen of the quantification peak of internal standard.

I_{std} : Peak area of quantification peak of internal standard.

m_s : weight of Oxytetracycine sample.

n_s : number of hydrogen of the quantification peak at the common structure part of homologues of Oxytetracycine sample.

I_s : Peak area of quantification peak of Oxytetracycine sample.

APPENDIX F: Summary of measurement equations and uncertainty budgets

The value of Oxytetracycline is :

$$P = \frac{P_{MB}+P_{QNMR}}{2} \qquad (3)$$

1. Uncertainty evaluation from Mass balance

Evaluation of measurement uncertainty of mass fractions From Eq. 1, the uncertainty of mass fration of component is:

$$u(P_{MB}) = \sqrt{[u(X_{RS})]^2 + [u(X_{Cl})]^2 + [u(X_W)]^2 + [u(X_V)]^2 + [u(X_{NV})]^2}$$

（1）$u(X_{RS})$

The relative uncertainty $u_{rel}(X_{RS1})$ of known impurities is:

$$u_{rel}(X_{RS1}) = \sqrt{u_{rel}^2(p) + u_{rel}^2(R)}$$

$u_{rel}(p)$: The relative uncertainty of impurity purity;

$u_{rel}(R)$: The relative uncertainty from the repeatability of impurity measurement;

The relative uncertainty $u_{rel}(X_{RS2})$ of unknown impurities is:

$$u_{rel}(X_{RS2}) = \sqrt{u_{rel}^2(f) + u_{rel}^2(R)}$$

$u_{rel}(f)$: The uncertainty of the average influence factor of unknown impurities;

$u_{rel}(R)$: The relative uncertainty from the repeatability of impurity measurement;

The combined uncertainty $u(X_{RS})$ is:

$$u(X_{RS}) = u_{rel}(X_{RS}) * X = 2.0 \text{ mg·g}^{-1}$$

X is the concentration of impurity, mg·g^{-1}.

Taking a 95% confidence probability with a coverage factor of *k*=2, the expanded uncertainty $U(X_{RS})$ is:

$$U(X_{RS}) = u(X_{RS}) * k = 4.0 \text{ mg·g}^{-1}$$

（2）$u(X_{Cl})$

The relative uncertainty $u_{rel}(X_{Cl})$ of chloride ion determination results is:

APPENDIX F: Summary of measurement equations and uncertainty budgets

$$u_{rel}(X_{Cl}) = \sqrt{u_{rel}^2(S) + u_{rel}^2(M) + u_{rel}^2(D) + u_{rel}^2(R)}$$

$u_{rel}(S)$:The relative uncertainty of CRM for the analysis of chloride ions in water;

$u_{rel}(M)$:The relative uncertainty from mass of smaple;

$u_{rel}(D)$:The relative uncertainty from the dilution process of standard solutions;

$u_{rel}(R)$:The relative uncertainty from measurement repeatability;

The combined uncertainty *u* (X) is:

$$u(X_{Cl}) = u_{rel}(X_{Cl}) * X = 0.0106 * 65.16 = 0.7 \text{ mg·g}^{-1}$$

X is the concentration of chloride ions, mg·g^{-1}.

Taking a 95% confidence probability with a coverage factor of *k*=2, the expanded uncertainty $U(X_{Cl})$is:

$$U(X_{Cl}) = u(X_{Cl}) * k = 1.4 \text{ mg·g}^{-1}$$

（3）$u(X_W)$

The uncertainty of water is list in the table:

Source of uncertainty	Value	Absolute uncertainty	Relative uncertainty	Comment
RHx (%)	46.2	0.1	0.22%	From hygrometer
RHx－50	-3.8	0.1	2.63%	Combined with absolute uncertainties
F	0.00037	0.00003	8.11%	From the protocol
F·(RHx－50)	-0.00141	0.00012	8.52%	Combined with relative uncertainties
1+F·(RHx－50)	0.99859	0.00012	0.01%	Combined with absolute uncertainties
m_{RHX} (mg)	10.69	0.01	0.05%	From balance
$m_{RH50}=m_{RHX}/(1+F·(RHx－50))$ (mg)	10.7051	0.0059	0.06%	Combined with relative uncertainties
Wtoal (mg)	1.1086	0.01	0.90%	From titrator
Wblank (mg)	0.2297	0.0311	3.38%	From blank detections (*n*=16)
Wsam=Wtotal-Wblank (mg)	0.8789	0.0327	3.72%	Combined with absolute uncertainties
m_{RH50}-m_{RHX} (mg)	0.0151	0.0083	54.95%	Combined with absolute uncertainties of m_{RH50} and m_{RHX}
Wsam50=Wsam+(m_{RH50}-m_{RHX}) (mg)	0.8940	0.0337	3.77%	Combined with absolute uncertainties of Wsamand (m_{RH50}-m_{RHX})
X_W=W_{sam50}/m_{RH50} (mg/mg)	0.0835	0.0031	3.77%	Combined with relative uncertainties of W_{sam50} and m_{RH50}
Repeatability			2.55%	RSD of 6 determinations
Xw of 6 detmerinations (mg/g)	89.92	4.09	4.5%	Combined with relative uncertainties of Xw and repeatibility

*For addition or subtraction, absolute uncertainties are combined by square root of sum of squares

*for multiplication or division, relative uncertainties are combined by square root of sum of squares.

（4）$u(X_V)$

The relative uncertainty of volatile organic determination results is：

$$u_{rel}(X_v) = \sqrt{u_{rel}^2(m_s) + u_{rel}^2(m_{std}) + u_{rel}^2(P_{std}) + u_{rel}^2(R)}$$

$u_{rel}(m_s)$: uncertainty from mass of sample；

$u_{rel}(m_{std})$: uncertainty from mass of standard preparation；

$u_{rel}(P_{std})$: uncertainty from purity of standard；

$u_{rel}(R)$: uncertainty from measurement repeatability.

The combined uncertainty of methanol measurement $u(X_V)$ is:

$$u(X_V) = u_{rel}(X_v) * \mathrm{X} = 0.0194 \text{ mg·g}^{-1}$$

X is the concentration, mg·g^{-1}.

Taking a 95% confidence probability with a coverage factor of k=2, the expanded uncertainty $U(X_V)$ is:

$U(X_V) = u(X_V) * k = 0.04$ mg·g^{-1}

（5）$u(X_{NV})$

The uncertainty of of total non-volatiles and inorganics is：

$$u(X_{NV}) = \sqrt{[u(P)]^2 + [u(R)]^2 + [u(L)]^2}$$

Where

$u(P)$: uncertainty from the CRM of inorganics solution;

$u(R)$: uncertainty from measurement repeatability;

$u(L)$: uncertainty from Linear of standard curve.

Taking a 95% confidence probability with a coverage factor of k=2, the expanded uncertainty $U(X_{NV})$ is:

$U(X_{NV}) = u(X_{NV}) * k = 0.02$ mg·g^{-1}

（6）the combined uncertainty of mass balance $u(P_{MB})$

$$u(P_{MB}) = \sqrt{[u(X_{RS})]^2 + [u(X_{Cl})]^2 + [u(X_W)]^2 + [u(X_V)]^2 + [u(X_{NV})]^2} = \sqrt{2.0^2 + 0.69^2 + 4.09^2 + 0.0194^2 + 0.09^2} = 4.9 \text{ mg/g}$$

2. Uncertainty evaluation from QNMR

The uncertainty evaluation for the results was carried out from weighing of sample, internal standard, molecular weight of sample and measurement of the equipment. In general, the measurement uncertainty is mainly due to measurement of the equipment .

Evaluation of measurement uncertainty of mass fractions From Eq 2, the uncertainty of mass fration of component is:

$$\frac{u(P_{QNMR})}{P_{QNMR}} = \sqrt{\left(\frac{u(I_s/I_{std})}{I_s/I_{std}}\right)^2 + \left(\frac{u(M_s)}{M_s}\right)^2 + \left(\frac{u(M_{std})}{M_{std}}\right)^2 + \left(\frac{u(m_{std})}{m_{std}}\right)^2 + \left(\frac{u(m_s)}{m_s}\right)^2 + \left(\frac{u(P_{std})}{P_{std}}\right)^2}$$

Where

$\frac{u(I_s/I_{std})}{I_s/I_{std}}$: uncertainty from NMR measurement, including baseline correction, integration of peak area and measurement repeatability.

$\frac{u(M_s)}{M_s}$: uncertainty from molecular weight of sample (Oxytetracycine).

$\frac{u(M_{std})}{M_{std}}$: uncertainty from molecular weight of internal standard.

$\frac{u(m_{std})}{m_{std}}$: uncertainty from mass of internal standard.

$\frac{u(m_s)}{m_s}$: uncertainty from mass of sample.

$\frac{u(P_{std})}{P_{std}}$: uncertainty from purity (expressed as mass fraction) of internal standard.

The combined uncertainty (u_c) can be calculated by:

$$u(P_{QNMR}) = P_{QNMR} * \frac{u(P_{QNMR})}{P_{QNMR}} = 3.04 \text{ mg·g}^{-1}$$

The expanded uncertainty U can be calculated with coverage factor *k*=2 corresponds to a confidence interval of 95%.

APPENDIX F: Summary of measurement equations and uncertainty budgets

$$U=ku_C$$

Component (units)	xi	u(xi)	u(xi)/xi (%)	
M_{std} (g mol^{-1})	166.1739	0.00421	0.00253%	
M_s (g mol^{-1})	460.4340	0.01033	0.00224%	
m_s (mg)	9.0	0.00065	0.00719%	
m_{std} (mg)	3.5	0.00029	0.00829%	
P_{std}(mg g^{-1})	999.7	0.25	0.02501%	
I_s/I_{std} (mg g^{-1})	788.6	3.0328	0.38561%	
P_{QNMR} (mg g^{-1})		788.6	3.04	0.38653%

3. The combined Uncertainty

$$u_s = \sqrt{\left(\frac{P_{MB}-P_{NNMR}}{2}\right)^2 + \left(\frac{u(P_{MB})}{2}\right)^2 + \left(\frac{u(P_{QNMR})}{2}\right)^2} = 4.9 mg/\text{g}$$

$$U = u_s \times k = 9.8\ mg/g$$

APPENDIX F: Summary of measurement equations and uncertainty budgets

Participant: GLHK

1a. Mass balance method:

$$m_{OTC} = (1000 - m_{RS,rel}) \times \left(\frac{1000 - (m_W + m_{OS} + m_{NV} + m_{Cl} + m_H)}{1000}\right) mg/g$$

1b. qNMR method:

$$P_{sample} = \frac{I_{Analyte}}{I_{IS}} \times \frac{N_{IS}}{N_{Analyte}} \times \frac{M_{Analyte}}{M_{IS}} \times \frac{m_{IS}}{m_{Sample}} \times P_{IS}$$

2. Measurement equation for combined results:

$$purity_{combined} = \sum_{i=1}^{N} w_i x_i$$

$$w_i = \frac{1}{u_i^2}$$

where w_i is the weighing factor

x_i is purity of OTC by mass balance or qNMR

1a. Mass balance method:

$U(X_{OTC}) = U(\Sigma X_{IC})$ where the major components of $U(X_{IC})$ include purities of reference standard, precision, recovery and estimation for unknown impurities

1b. qNMR method:

$U(X_{OTC}) = U(\Sigma X_{IC})$ where the major components of $U(X_{IC})$ include the following: purity of IS, integration, molecular weight of IS, molecular weight of analyte, mass of analyte, mass of IS, precision and repeatability

3. Calculation of Measurement Uncertainty of combined results:

$$u_{combined} = \frac{1}{\sqrt{w_{MB} + w_{qNMR}}}$$

$$w_i = \frac{1}{u_i^2}$$

APPENDIX F: Summary of measurement equations and uncertainty budgets

Participant: INMETRO

qNMR measurement equation

$$P_a = \frac{I_a}{I_{IS}} * \frac{M_a}{M_{IS}} * \frac{m_{IS}}{m_a} * \frac{N_{IS}}{N_a} * P_{IS}$$

Considering that we used the whole aromatic range, which is overlapped with related structure impurities, we used the LC-PDA area normalization (with the calculated response factors) value multiplied by the raw qNMR result as a correction to obtain the final qNMR result:

$$qNMR_{final} = qNMR_{RAW} * N_A$$

Mass balance measurement equation

$$w_A\,(mg/g) = (1000 - \sum w_{imp}) \times N_A$$

The combination of the results was performed by a simple average.

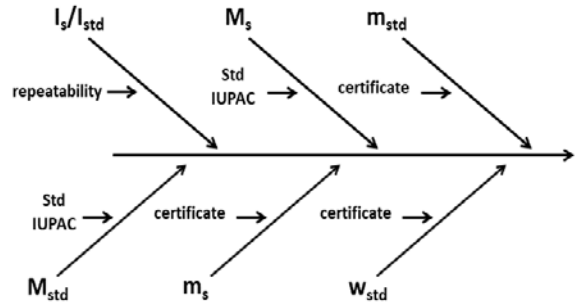
Mass balance -

The first step in the mass balance approach is to calculate (1000 - total volatile impurities - inorganic impurities). These results in a partial purity value and the uncertainties of the water content, VOCs and inorganic impurities are all combined as relative uncertainties . This value is then multiplied by the area normalization to yield the final mass balance result and the uncertainties are combined as relatives once more. For this sample, the main uncertainty source for the mass balance was the area normalization since the impurity content is relevant and each of the impurities have large uncertainties associated to their response factors.

Mass fraction:	w	u
Water	101.8108	1.2282
Volatile organic compounds	0.2286	0.0094
Total volatile impurities*	102.0394	1.2282
Inorganic impurities*	67.6859	1.0890
Nonvolatile organic impurities determined individually*		0.0000
Partial purity (disregarding area normalization)	830.2747	1.6415
Nonvolatile organic imp. determined by area normalization	30.9175	2.2660
Total nonvolatile organic impurities	30.9175	2.2660
Main compound	799.3572	2.7620

qNMR - Provided for one of the systems as an example

APPENDIX F: Summary of measurement equations and uncertainty budgets



Uncertainty sources	Value	u	u Component
IA	60545.32	-	-
IIS	40630.51	-	-
IA/IIS (repeatability)	1.490124	0.000705739	0.389708205
Analyte Molar Mass	460.4329	0.012991138	0.023216932
IS Molar Mass	260.8832	0.013175058	0.041555677
IS weighed Mass	4.9237	0.007	1.169850721
Analyte weighed Mass	5.234056	0.007	1.10048391
IS purity	997.8	1.1	0.907137616
Overall			1.885908955

The two qNMR values as well as the qNMR + Mass balance combinations are done by simple averaging while the associated measurement uncertainties are performed taking into account also the differences between the results by using the equation:

$$u_c = \sqrt{\frac{\left(\sum_{j=1}^{m}(Y_j - \bar{Y})^2/m - 1\right) + \left(\sum_{j=1}^{m} u_{(Y_j)}^2/m\right)}{m}}$$

APPENDIX F: Summary of measurement equations and uncertainty budgets

Participant: EXHM

Measurement equations

$$w_{OCT} = A_{OCT,n}(1 - \frac{w_{H_2O}+w_{vol}+w_{in}}{1000})$$

Mass balance method:

OCT fraction (mg/g) is given by the following equation:

$$w_{OCT} = A_{OCT,n}(1 - \frac{w_{H_2O}+w_{vol}+w_{in}}{1000})$$

where

w	:	mass fraction (mg/g)
$A_{OCT,n}$	:	normalized OCT peak area in the HPLC- DAD chromatogram on a mass basis
H_2O	:	water (mg/g)
vol	:	residual volatiles (mg/g)
in	:	inorganics and non-volatile material (mg/g)

The normalized OCT area on a mass basis is given by the following equation

$$A_{OCT_n} = \frac{A_{OCT}\ \frac{Rf_{OCT}}{mw_{OCT}}}{A_{OCT}\ \frac{RRf_{OCT}}{mw_{OCT}} + \sum A_{SRI,i}\ \frac{RRf_{SRI,i}}{mw_{SRI,i}}}$$

where

A_{OCT}	:	OCT peak area in the HPLC - DAD chromatogram
$A_{SRI,i}$	:	SRI_i peak area in the HPLC- DAD chromatogram
SRI_i	:	i[th] Structure Related Impurity
RRf_{OCT}	:	relative OCT response factor (= 1)
$RRf_{SRI,i}$	:	relative ith SRI response factor
mw	:	molar mass

SRI determination:

The mass fraction of each structurally-related impurity was determined as the area fraction of the respective peak in the HPLC-DAD chromatogram.

$$w_{sri} = SRI_{i,n}\ (1 - \frac{w_{H_2O}+w_{vol}+w_{in}}{1000})$$

where

$SRI_{i,n}$: normalized SRI_i peak area in the HPLC- DAD chromatogram (calculated in the same way as $A_{OCT,n}$)

Water determination:

The equation describing water determination by coulometric Karl Fisher titration is given by the following equation:

$$w_{H2O} = \frac{Q}{z\,F}\,\frac{mw}{m_{sample}} - w_{blank}$$

where, w_{H2O} = water mass fraction

Q = amount of charge

z = number of electrons exchanged

F = electrochemical equivalent

MW = molar mass

m_{sample} = sample mass

w_{blank} = water in blank

Volatile / Inorganic impurities determination:

To determine these impurities, an amount of the sample is used to form a particular solution, either by simply dissolving it in a suitable solvent system, or by using treatment such as digestion/dissolution, and determining the impurities.

The equation describing the determination of volatile and inorganic impurities by means of chromatographic and spectrometric techniques is given by the following generic equation:

$$w_{vol/in} = \frac{R_{soln}}{R_{std}}\,C_{std}\,\frac{m_{soln}}{m_{sample}}$$

where, $w_{vol/in}$ = volatile/inorganic mass fraction

$R_{soln,\,std}$ = solution/standard response

C_{std} = standard concentration

m_{soln} = solution mass

m_{sample} = sample mass

In the particular case, no volatiles nor any inorganics were determined above the LOQ (0.02 %) and therefore the value is set as zero with an uncertainty of

5b. Uncertainty budget

The uncertainty of **oxytetracycline free base and oxytetracycline hydrochloride** was calculated using the following equation:

$$u(w_{OCT,SRI}) = \sqrt{\frac{(SD_R)^2}{n} + (C_i u_{H2O})^2 + (C_i u_{vol})^2 + (C_i u_{im})^2}$$

where SD_R is the standard deviation under reproducibility conditions, n the number of determinations and C_i appropriate sensitivity coefficients.

The uncertainty of the total structure-related impurities was calculated as the sum of the uncertainties of the individual components.

The uncertainty for the **determination of residual water** is provided by the following generic equation:

$$u(w_{H2O}) = \sqrt{\frac{(SD_{R,H2O})^2}{n} + \left(C_i u_{sample\ mass}\right)^2}$$

The uncertainty for the **determination of volatile mater and inorganic/non volatile impurities** is provided by the following generic equation:

$$u(w_{vol,in}) = \sqrt{\frac{(SD_R)^2}{n} + (C_i u_{Cstd})^2 + \left(C_i m_{sample}\right)^2 + (C_i m_{soln})^2}$$

APPENDIX F: Summary of measurement equations and uncertainty budgets

qNMR

Purity was determined by qNMR and checked by the mass balance approach. The respective uncertainties were calculated via the following equations:

$$P_s = \frac{I_s}{I_{is}} \frac{N_{is}}{N_s} \frac{mw_s}{mw_{is}} \frac{m_{is}}{m_s} P_{is}$$

where

P	:	purity (mg/g)
I	:	signal intensity
N	:	number of protons
mw	:	molecular weight
m	:	mass
s	:	sample (OCT)
is	:	internal standard (maleic acid)

APPENDIX F: Summary of measurement equations and uncertainty budgets

UNCERTAINTY BUDGETS

Mass balance

OTC Normalization uncertainty budget

impurity acc. to E.P.	compound	factor	value	unc	Ci	Ci x ui	(Ci x ui)^2	NORM AREA	uncertainty	free base (mg/g)	uncertainty (mg/g)
	OTC	A	951,46	0,60	0,026	0,016	0,0002	960,25	3,12	797,50	4,67
		Rf	1,00	0	25,02	0,000	0,0000				
		MW	460,44	0,02	0,054	0,001	0,0000				
A	4epiOTC	A	3,15	0,06	0,998	0,064	0,0040	3,18	0,06	2,64	0,06
		Rf	1,00	0,01	3,143	0,031	0,0010				
		MW	460,44	0,02	0,007	0,000	0,0000				
B	TC	A	5,96	0,09	0,963	0,087	0,0076	6,23	0,09	5,18	0,08
		Rf	1,00	0,01	5,712	0,057	0,0033				
		MW	444,44	0,02	0,013	0,000	0,0000				
C	2-ADOTC	A	14,85	0,06	0,996	0,059	0,0035	15,02	0,08	12,47	0,09
		Rf	1,00	0,01	14,785	0,148	0,0219				
		MW	459,45	0,02	0,032	0,001	0,0000				
D	a-APOTC	A	6,08	0,38	0,996	0,380	0,1443	3,19	0,19	2,65	0,16
		Rf	0,50	0,01	14,785	0,148	0,0219				
		MW	442,42	0,02	0,032	0,001	0,0000				
F	AOTC	A	3,43	0,43	0,996	0,424	0,1797	3,60	0,43	2,99	0,35
		Rf	1,00	0,01	14,785	0,214	0,0457				
		MW	442,42	0,02	0,032	0,001	0,0000				
E	b-APOTC	A	5,00	0,36	0,996	0,362	0,1313	2,63	0,18	2,18	0,15
		Rf	0,50	0,01	14,785	0,148	0,0219				
		MW	442,42	0,02	0,032	0,001	0,0000				
E	b-APOTC	A	6,26	0,70	0,996	0,695	0,4825	3,29	0,35	2,73	0,29
		Rf	0,50	0,01	14,785	0,148	0,0219				
		MW	442,42	0,02	0,032	0,001	0,0000				
	ATC	A	0,55	0,02	0,996	0,019	0,0004	0,60	0,02	0,50	0,02
		Rf	1,00	0,01	14,785	0,148	0,0219				
		MW	426,40	0,02	0,032	0,001	0,0000				
	RRT 13,7	A	0,26	0,02	0,996	0,018	0,0003	0,29	0,02	0,24	0,02
		Rf	1,00	0,01	14,785	0,148	0,0219				
		MW	426,40	0,01	0,032	0,000	0,0000				
	RRT 14,4	A	0,26	0,02	0,996	0,018	0,0003	0,29	0,02	0,24	0,02
		Rf	1,00	0,02	14,785	0,296	0,0874				
		MW	426,40	0,01	0,032	0,000	0,0000				
	RRT 14,8	A	0,36	0,02	0,996	0,018	0,0003	0,40	0,02	0,33	0,02
		Rf	1,00	0,02	14,785	0,296	0,0874				
		MW	426,40	0,01	0,032	0,000	0,0000				
	RRT 15,7	A	0,31	0,01	0,996	0,012	0,0002	0,34	0,01	0,28	0,01
		Rf	1,00	0,02	14,785	0,296	0,0874				
		MW	426,40	0,01	0,032	0,000	0,0000				
	RRT 16,5	A	0,39	0,01	0,996	0,012	0,0002	0,42	0,01	0,35	0,01
		Rf	1,00	0,01	14,785	0,185	0,0342				
		MW	426,40	0,02	0,032	0,001	0,0000				
	RRT 17,7	A	0,23	0,02	0,996	0,016	0,0003	0,08	0,01	0,06	0,00
		Rf	0,31	0,01	14,785	0,185	0,0342				
		MW	426,40	0,02	0,032	0,001	0,0000				
	RRT 17,8	A	0,19	0,02	0,996	0,019	0,0004	0,20	0,02	0,17	0,02
		Rf	1,00	0,01	14,785	0,185	0,0342				
		MW	426,40	0,02	0,032	0,001	0,0000				
	unknown SRIs	A	1,41	1,04	0,959	0,994	0,9881	0,00	2,96	0,00	2,46
		Rf	1,00	0,01	1,349	0,013	0,0002				
		MW	442,42	20,00	0,003	0,061	0,0037				
								1000,00	4,51	830,51	

APPENDIX F: Summary of measurement equations and uncertainty budgets

OTC PURITY UNCERTAINTY BUDGET

component	unit	value	uncertainty
WATER	mg/g	105,33	0,66
VOLATILES	mg/g	0,00	0,01
NON-VOL	mg/g	64,16	1,40
TOTAL ORGANIC		830,51	1,55
OTC nom. fraction	mg/g	960,25	3,12
total area		1000,00	4,51
free base	mg/g	797,50	
combined uncertainty	mg/g	4,67	
k=2			
expanded uncertainty	mg/g	9,35	

KF uncertainty budget					
uncertainty component	value	units	uncertainty	uncertainty	squared RU
determination repeatability	105327,0	µg g^{-1}	643,00	0,006	0,000
blank determination	71,0	µg g^{-1}	0,10	0,001	0,000
sample mass at 43 %RH	50,000	mg	0,02	0,000	0,000
sample mass at 50 %RH	50,128	mg	0,03	0,001	0,000
water content result	105327,0	µg g^{-1}			0,00
combined standard uncertainty	660,7	µg g^{-1}			
coverage factor (k, n=6)	2,0				
expanded uncertainty (k=2)	1321,4	µg g^{-1}			

chloride uncertainty budget (standard additions)					
uncertainty component	value	units	ncertainty	ncertainty	squared RU
determination repeatabili	62,4	mg/g	1,30	0,021	1,690
sample mass	10,0	g	0,02	0,002	0,000
added Cl solution mass	100,0	mg	0,02	0,000	0,000
Cl standard solution mass	998,0	mg	0,40	0,000	0,160
standard addition model	62,6	mg	0,48	0,008	0,000
chloride mass fraction	62,4	µg g^{-1}			62,40
combined standard uncerta	1,4	µg g^{-1}			
coverage factor (k, n=8)	2,0				
expanded uncertainty (k=2	2,7	µg g^{-1}			

APPENDIX F: Summary of measurement equations and uncertainty budgets

qNMR

qNMR uncertainty budget							
uncertainty component	value	units	u_i	u_i/x_i	C_i	C_iu_i	$(C_iu_i)^2$
OXT/MA signal ratio (ppm 3.8)	0,1258		0,00102	8,108E-03	6335,14	6,4618	4,176E+01
OXT molecular mass	460,440	g mol^{-1}	0,00600	1,303E-05	1,73	0,0104	1,079E-04
MA molecular mass	116,070	g mol^{-1}	0,00600	5,169E-05	-6,87	-0,0412	1,697E-03
no of protons in signal integrated for OXT	1	nucl/mol	0,00040	1,800E-05	-796,96	-0,3188	1,016E-01
no of protons in signal integrated for MA	2	nucl/mol	0,00040	1,800E-05	398,48	0,1594	2,541E-02
OXT mass	6,6076	mg	0,00100	1,513E-04	-120,61	-0,1206	1,455E-02
MA mass	5,2772	mg	0,00100	1,895E-04	151,02	0,1510	2,281E-02
boyancy correction	1,0000		0,00000	4,065E-06	796,96	0,0032	1,050E-05
MA	999,80	mg g^{-1}	0,50000	5,001E-04	0,80	0,3986	1,588E-01
OXT purity							**796,97**
combined standard uncertainty		mg g^{-1}					**6,49**
expanded uncertainty (k=2)		mg g^{-1}					12,97

APPENDIX F: Summary of measurement equations and uncertainty budgets

Participant: BAM

$$\omega_A = \frac{N_{IC} * A_A * M_A * m_{IC}}{N_A * A_{IC} * M_{IC} * m_A} * \omega_{IC}$$

N_i = Number of nuclei m_i = Mass

A_i = Signal area ω_{IC} = Mass fraction of int. calibrant

M_i = Molar mass Indizes: A: Analyte IC: Internal calibrant

Contribution of gravimetric operations (including %RH correction for OTC)

relative uncertainty (OTC): 9.68E-04

relative uncertainty (IC): 7.15E-05

Contribution of NMR repeatability: 1.63E-03 (Example BTFMBA)

Contribution of Molar mass:

relative uncertainty (OTC): 4.34E-05

relative uncertainty (BTFMBA): 1.20E-05

relative uncertainty (MA): 3.45E-05

Contribution of IC mass fraction:

relative uncertainty (BTFMBA): 1.50E-04

relative uncertainty (Maleic Acid): 4.50E-04

Reported result by arithmetic mean and relative uncertainties combined by Root Mean Square

APPENDIX F: Summary of measurement equations and uncertainty budgets

Participant: LGC

Measurement equation for qNMR method:

$$\%Purity_{Analyte} = \frac{m_{IS}}{m_{Analyte}} \times \frac{Mwt_{Analyte}}{Mwt_{IS}} \times \frac{I_{Analyte}}{I_{IS}} \times \frac{\rho_{IS}}{\rho_{Analyte}} \times 100 P_{IS}$$

The ^{1}H NMR signal (H8) used for quantitation was corrected for the overlapping TC and ADOTC signals.

Uncertainty budget for Oxytetracycline Hydrochloride OA/22/4256+OA/22/4257 by 1H qNMR

Quantity/units	Value		u	rel u (%)
$P_{ANALYTE,mean}$/%	80.56		0.02	0.0285
$\rho_{internal\ std.}$	2		0	0.0000
$\rho_{ANALYTE}$	1		0	0.0000
$P_{internal\ std.}$/%	99.92		0.163265306	0.1634
$MW_{ANALYTE}$	460.43404		0.017885525	0.0039
$MW_{internal\ std.}$	116.07216		0.003429052	0.0030
Minternal std (mg)	10.27233333		0.01250	0.1217
Minternal std solvent (mg)	11078.42		0.05500	0.0005
Minternal std stock solution (mg)	1103.821905		0.05500	0.0050
$M_{ANALYTE}$ (mg)	10.77352		0.01250	0.1160
Mass correction	10.77828		0.00003	0.0003
P_{mean}/%	**80.56**	u_c =	**0.190**	**0.236**

Number of sub samples run =	7
k =	1.96
Purity =	**80.56**
Uncertainty at 95% C.I. = ±	**0.37**

Where,

$$u_c = P_{analyte}\sqrt{\left(\frac{u_{int\ analyte}}{I_{analyte}}\right)^2 + \left(\frac{u_{int\ std}}{I_{std}}\right)^2 + \left(\frac{u_{Ma}}{M_{analyte}}\right)^2 + \left(\frac{u_{Ms}}{M_{Std}}\right)^2 + \left(\frac{u_{ma}}{m_{analyte}}\right)^2 + \left(\frac{u_{ms}}{m_{Std}}\right)^2 + \left(\frac{u_{Std}}{P_{Std}}\right)^2}$$

Internal guidance based on biases seen within the validation campaign of qNMR for samples with purity values < 90% m/m, mandates that a minimum expanded uncertainty contribution be calculated in addition to the above standard approach for the uncertainty budget consideration. The higher of the two uncertainty values is to be reported. This minimum recommended uncertainty value was calculated to be ± 0.46% m/m and would be reported, and not the ± 0.37% m/m illustrated in the above uncertainty budget calculation. As the ^{1}H NMR signal integral used for the qNMR calculations was corrected for overlapping signals from TC and ADOTC, an additional uncertainty contribution associated with this correction was combined with the ± 0.46% m/m uncertainty value to give a final reported expanded uncertainty value of ± 0.47% m/m.

APPENDIX F: Summary of measurement equations and uncertainty budgets

Participant: NIST

Mass fraction (g/g), w_P, of oxytetracycline as the free-base form was determined via q1H-NMR using an internal standard. The measurement result was calculated through a statistical model based on the following measurement function:

$$\boldsymbol{w_{\mathrm{P}}} = \left(\frac{\boldsymbol{N_{\mathrm{I}}}}{\boldsymbol{N_{\mathrm{P}}}}\right) \times \left(\frac{\boldsymbol{M_{\mathrm{P}}}}{\boldsymbol{M_{\mathrm{I}}}}\right) \times \left(\frac{\boldsymbol{A_{\mathrm{P}}}}{\boldsymbol{A_{\mathrm{I}}}}\right) \times \left(\frac{\boldsymbol{m_{\mathrm{I}}}}{\boldsymbol{m_{\mathrm{C}}}}\right) \times \boldsymbol{P_{\mathrm{I}}}$$

N_{P} = ^{1}H multiplicity (# H/peak) of the integrated tetracycline peak
N_{I} = ^{1}H multiplicity (# H/ peak) of the integrated internal standard peak
M_{P} = relative molar mass (g/mol) of oxytetracycline free-base form
M_{I} = relative molar mass (g/mol) of internal standard
A_{P} = integral of the oxytetracycline ^{1}H peaks
A_{I} = integral of the internal ^{1}H peak
m_{C} = mass (g) of sampled BIPM oxytetracycline HCl, adjusted for relative humidity
m_{I} = mass (g) of internal standard
P_{I} = purity (g/g) of internal standard

Mass values were adjusted to 50 % relative humidity, in accordance with the K148.b protocol. Thesd adjustements were based on the measured % RH conditions at which sample materials were equilibrated prior to analyses. For each sample containing tecnazene (n=4) or dimethyl terephthalate internal(n=5) standard, an estimate of w_{P} was calculated using a hybrid statistical procedure that combined execution of a bespoke Bayesian MCM model and implementation of the NIST Consensus Builder (NICOB) Linear Pool procedure. The results are constrained to have values no greater than 1 g/g. Data from no other measurement methods were used to calculate the result, however analysis of water by Karl Fischer yielded a concordant result, providing confidence that the qNMR result is feasible.

An estimate of purity was calculated for each of the nine qNMR samples using the MCM procedure. For each variable term of the measurement equation, data for each sample was treated as having a normal distribution. Values for the μ and σ parameters were specified by the respective data inputs to the statistical model provided in Appendix A. Standard uncertainties, treated as the σ, were evaluated as follows: the $u(\frac{\boldsymbol{A_P}}{\boldsymbol{A_I}})$ was determined for each sample, based on the variation of ratios calculated using different ^{1}H NMR peaks for OTC; the $u(\frac{\boldsymbol{m_I}}{\boldsymbol{m_C}})$ was assigned a Type B relative standard uncertainty of 0.1 % to account for variability of laboratory humidity, uncertainty in sample mass adjustments based on the function relating change in relative water content to relative humidity (to 50 % RH), and the uncertainty in the weighing procedure and values indicated by the balance; the $u(\boldsymbol{P_I})$ were assigned values of 0.0009 g/g and 0.0016 g/g for tecnazene and dimethyl terephthalate, respectively; the $\boldsymbol{M_I}$, $u(\boldsymbol{M_I})$, $\boldsymbol{M_P}$, and $u(\boldsymbol{M_P})$ were calculated using the IUPAC Commission on Isotopic Abundances and Atomic Weights

(CIAAW) molecular weight calculator (https://ciaaw.shinyapps.io/calculator; no uncertainty was considered for the proton multiplicities of the primary component ($\boldsymbol{N_P}$) and internal standard (N_{I}). The nine sample results calculated from the MCM procedure were then blended using the Linear Pooling procedure option in the NICOB.

The result submitted by NIST for this key comparison is 0.806 ± 0.005 g/g, where the number after the ± symbol is the uncertainty that defines an interval of values attributable to the measurand with a level of confidence of approximately 95 percent. This estimate is based on the shortest 95% coverage interval determined from the Linear Pooling procedure.

APPENDIX F: Summary of measurement equations and uncertainty budgets

Participant: UME

Mass Balance

$wA = mA / mA + \sum mx \quad = \quad nA*M(A) / mA + \sum mx$

wA mass fraction of main component A in the material

mA mass of A in an aliquot of the material

Σmx summed mass of minor components (impurities) in the same aliquot

nA moles of A in an aliquot of the material

M(A) Molar mass of A

$w_A = 1000- (W_{RS} + W_W + W_{VOC} + W_{NV})$

w_{RS} = mass fraction of related structure impurities in the material

w_W = mass fraction of water in the material

w_{VOC} = mass fraction of residual solvent (volatile organics) in the material

w_{NV} = mass fraction of non-volatile compounds in the material

qNMR equation

$$Px = \frac{Ix}{Istd}\frac{Nstd}{Nx}\frac{Mx}{Mstd}\frac{mstd}{mx}Pstd$$

The standard uncertainty of the material of mass balance approach $u(w_{MB})$ is given by the equation below:

$$u(w_{MB}) = \sqrt{u(w_{RS})^2 + u(w_W)^2 + u(w_{VOC})^2 + u(w_{NV})^2}$$

The uncertainty of the material, qNMR approach:

$$u(P_x) = P_x\sqrt{\left(\frac{u(I_x/I_{std})}{I_x/I_{std}}\right)^2 + \left(\frac{u(M_x)}{M_x}\right)^2 + \left(\frac{u(M_{std})}{M_{std}}\right)^2 + \left(\frac{u(m_x)}{m_x}\right)^2 + \left(\frac{u(m_{std})}{m_{std}}\right)^2 + \left(\frac{u(P_{std})}{P_{std}}\right)^2}$$

APPENDIX F: Summary of measurement equations and uncertainty budgets

Participant: KRISS

1-1. LC-UV (related structure impurities)

$$P_{related\ structure\ impurity,i} = \frac{A_{impurity,i}}{A_{main} + \sum A_{impurity,i}}$$

$P_{\text{related structure impurity,I}}$: mass fraction of the related structure impurity $A_{\text{impurity,I}}$: peak area of the impurity A_{main} : peak area of the main component

1-2. KF titration (water content)

$$P_{water} = (ICEQ/10.712 - Time \times Drift - Blank)/m \times C$$

P_{water} : mass fraction of water in the sample ICEQ: total consumed electric charge Time: total KF measurement time Drift: systematic water content measured by KF titration before the analysis in time Blank: systematic water content in empty vial m: weight of the sample C: constant, 1×10^6

1-3. TGA (non-volatile impurities)

$$P_{non-volatile\ impurities} = \frac{W_{non-volatile\ impurities}}{W_{sample}}$$

$P_{\text{non-volatile imputies}}$: mass fraction of non-volatile impurities $W_{\text{non-volatile imputies}}$: weight of non-volatile impurities W_{sample} : weight of the sample

1-4. Headspace-GC/MS (volatile organics)

$$P_{volatile\ organic} = \frac{\sum W_{volatile\ organic,i}}{W_{sample}}$$

$$W_{volatile\ organic} = \frac{A_{volatile\ solvent} - y_{intercept}}{Slope}$$

$W_{\text{volatile organic,i}}$: weight of volatile organics W_{sample} : weight of the sample $y_{\text{intercept}}$: intercept of the calibration curve Slope: slope of the calibration curve

APPENDIX F: Summary of measurement equations and uncertainty budgets

1-5. IC-CD (chloride ion content)

$$P_{chloride\ \text{ion}} = \frac{C_{std} \times A_{sample}}{C_{tc} \times A_{std}}$$

Pchloride ion : mass fraction of chloride ion in sample Cstd : Concentration of chloride ions in standard solution Ctc : Concentration of tetracycline.HCl in sample solution Astd : Chloride peak area in standard solution Asample : Chloride peak area in sample solution

2. Combination of value:

$$P_{\text{OTC}} = (1 - \sum P_{impurity}) \times P_{chromatography}$$

P_{OTC} : mass fraction of oxytetracycline free base

$P_{impurity}$: mass fraction of imputities (including related structure impurities, water, non-volatile impurities, volatile organics, and chloride ion) $P_{chromatography}$: mass fraction of oxytetracyclin measured by LC-UV

3. qNMR

$$P_a = \frac{I_a N_s M_a W_s}{I_s N_a M_s W_a} P_s$$

p_a: purity of analyte

I_a: integral area of quantification peak of analyte

I_s: integral area of quantification peak of internal standard

N_s : number of protons of the quantification peak of internal standard

N_a: number of protons integrated for quantification of analyte

M_a: molecular weight of analyte

M_s: molecular weight of internal standard

W_s: weight of internal standard

W_a: weight of analyte p_s: purity of internal standard

APPENDIX F: Summary of measurement equations and uncertainty budgets

1. LC-UV (related structure impurities)

$$u_{chromatography} = \frac{SD_{main}}{\sqrt{n}}$$

SD_{main}: standard deviation of main component content measured by LC-UV n: number of sample

2. KF titration (water content)

$$u_{KF} = \frac{SD_{water}}{\sqrt{n}}$$

SD_{water}: standard deviation of water content measured by KF titration n: number of sample

3. TGA (non-volatile impurities)

$$u_{TGA} = \frac{SD_{non-volatile\ impurities}}{\sqrt{n}}$$

$SD_{non\text{-}volatile\ impurities}$: standard deviation of non-volatile impurities content measured by TGA n: number of sample

4. Headspace-GC/MS (volatile organics)

$$u_{HS-GC/MS} = \sqrt{\sum_{j=1}^{n} \left(u_{volatile\ organics,j}\right)^2}$$

case1: peak S/N < 3

$$u_{volatile\ organics} = \frac{LOD}{\sqrt{3} \times W_{sample}}$$

LOD: limit of detection W_{sample} : weight of the sample

case2: peak S/N > 3

APPENDIX F: Summary of measurement equations and uncertainty budgets

$$u_{volatile\ organics} = \frac{SD}{Slope}\sqrt{\frac{1}{p}+\frac{1}{n}+\frac{C_0 - C_m}{S_{xx}}} \div W_{sample}$$

$$S_{xx} = \sum_{j=1}^{n}\left(C_j - C_m\right)^2$$

SD : standard deviation Slope: slope of the calibration curve p : number of measurements to determine C_0 n: number of measurements for the calibration C_0 : determined volatile organic content C_m : mean value of the different calibration standards C_j : volatile organic content obtain the calibration curve W_{sample} : weight of the sample

5. IC-CD (chloride ion content)

$$u_{IC-CD} = \sqrt{(u_{std})^2 + \left(\frac{SD_{chloride}}{\sqrt{n}}\right)^2}$$

μ std : uncertainty of chloride standard solutions $SD_{chloride}$: standard deviation of chloride contents in samples measured by IC-CD n: number of sample

6. Combination of value

$$u_{free\ base} = \sqrt{\left(u_{imputies}\right)^2 + \left(u_{chromatography}\right)^2}$$

$$u_{impurities} = \sqrt{(u_{KF})^2 + (u_{TGA})^2 + \left(u_{HS-GC/MS}\right)^2 + (u_{IC-CD})^2}$$

The uncertainty was pooled with the standard uncertainty of mass balance result.
Major uncertainty contribution was from measurements of structure related impurities, water content,s and chloride ions.

7. qNMR

$$u_{NMR} = \sqrt{\left(\frac{u(I_a/I_s)}{I_a/I_s}\right)^2 + \left(\frac{u(M_a)}{M_a}\right)^2 + \left(\frac{u(M_s)}{M_s}\right)^2 + \left(\frac{u(W_a)}{W_a}\right)^2 + \left(\frac{u(W_s)}{W_s}\right)^2 + \left(\frac{u(P_s)}{P_s}\right)^2}$$

APPENDIX F: Summary of measurement equations and uncertainty budgets

Participant: KIMIA

Organic purity = 100 % – I_{SRO}

$$F_{Residual} = \frac{m_{OV}}{m_{sample}} \qquad F_{Water} = \frac{m_{w,corrected}}{m_{sample}} \qquad F_{TNV} = \frac{m_{residue}}{m_{sample}}$$

$$P_{MB} = (1000 - I_{SRO}) \times (1000 - F_{Residual} - F_{Water} - F_{Cl} - F_{TNV})/1000 \text{ mg/g}$$

$$u(P_{MB}) = \sqrt{c_{I_{SRO}}^2 u_{I_{SRO}}^2 + c_{F_{Residual}}^2 u_{F_{Residual}}^2 + c_{F_{Water}}^2 u_{F_{Water}}^2 + c_{F_{Cl}}^2 u_{F_{Cl}}^2 + c_{F_{TNV}}^2 u_{F_{TNV}}^2}$$

Components in purity analysis						
	Value	u(x)	Rel. u(x)	Sensitivity Coefficient (c)	c^2*u^2	Contribution to U
1. Structurally related organic substances (SRO)						
100% - I_{SRO} %	96.6039%	0.2028%	0.20993%			
F_{NR}	1%	0.1667%	16.66667%	0.92853	6.15637E-06	20.520%
F_{ND}	1%	0.0500%	5.00000%			
100% - I_{SRO} %	96.6039%	0.2672%	0.27661%			
2. Water	6.9418%	0.4023%	5.796%	-0.96604	1.510766E-05	50.356%
3.Chloride	7.2333%	0.2419%	3.344%	-0.96604	5.461195E-06	18.203%
4. Residual solvent	0.1800%	0.1200%	66.7%	-0.96604	1.343853E-06	4.479%
5. Total non-volatiles (TNV)	0.0251%	0.1439%	573.2%	-0.96604	1.932461E-06	6.441%
Combined	82.712%	0.548%	0.662%		3.00015E-05	

Property value of a reference material and the accosiated uncertainty can be expressed as:				
Purity	82.712%			
u(x)	0.548%			
k (at 95% level)	2			
U(x) with *k*=2	1.095%			
%U(x)	1.324%			
95% Confidence Interval			(Note that the upper limit of this purity range may exceed	Confidence Level = 95%
81.62%	82.71%	83.81%		*k* = 2

APPENDIX F: Summary of measurement equations and uncertainty budgets

Participant: BVL

Purity (mg/g) = (1000 - water (KF) - Chlorid (IC) - VOCs (TGA)) x (1000 - impurities (HPLC))/1000

Impurities (HPLC) : corrected with the related response factors of the impurities

All uncertainties were combined using the square root of the sum of the squares approach, using standard uncertainties or relative standard uncertainties as appropriate.

$$u = \sqrt{u(HPLC)^2 + u(H2O)^2 + u(Cl)^2 + u(VOCs)^2}$$

AND $U = k\,x\,u\,(k = 2)$

The main components of the uncertianty budget are:

- u (KF) from karl fischer analysis

- u (Cl) from chlorid analysis

- u (VOCs) from TGA

- u (HPLC) from organic impurities (in this case response factor correction was additionally done)

APPENDIX F: Summary of measurement equations and uncertainty budgets

Participant: VNIIM

$$w_{OTC} = 1000 - w_{RS} - w_{NV} - w_{H2O} - w_{VOC} - w_{HCl} - w_I$$

w_{RS}- mass fraction of related structure Imp.

$$w_{RS} = \sum_{i=1}^{13} w_{imp}$$

w_{NV} – mass fraction of total non-volatiles and inorganics

w_{VOC} – mass fraction of volatile organics content

$$w_{VOC} = w_{CH_3CN} + w_{CH_3OH} + w_{C_2H_5OH}$$

w_{HCl} – mass fraction of HCl

$$w_{HCl} = \frac{M_r(HCl) \cdot w_{Cl^-}}{A_r(Cl)}$$

w_{Cl^-} - mass fraction of chloride ion

w_I – mass fraction of other ions (Br^-, F^- , ets.)

Other ions (Br^-, F^- , ets.) are not detected (<0,03 mg/g)

$$U^0 = 2\sqrt{u_{RS}^2 + u_{NV}^2 + u_{H2O}^2 + u_{VOC}^2 + u_{Cl^-}^2}$$

$$u_{RS} = \sqrt{u_{imp\,1}^2 + u_{imp\,2}^2 + u_{imp\,3}^2 + u_{imp\,4}^2 + u_{imp\,5}^2 + u_{imp\,6}^2 + u_{imp\,7}^2 + u_{imp\,8}^2 + u_{imp\,9}^2 + u_{imp\,10}^2 + u_{imp\,11}^2 + u_{imp\,12}^2 + u_{imp\,13}^2}$$

$$u_{(RS)i} = \sqrt{u_A^2 + u_{cal}^2 + u_{samp}^2}$$

; i - identified *Imp. A, Imp. B, Imp. D, Imp. E*

$$u_{(RS)j} = \sqrt{u_A^2 + u_{cal}^2 + u_{samp}^2 + u_{un}^2}$$

; j - unidentified Imp. 2, Imp. (5-12)

u_A — SD of RS measurement results, mg/g

u_{cal} — uncertainty due to calibration, mg

u_{samp} - — uncertainty due to sample preparation, mg

u_{un} - — uncertainty due to unknown RRF for unidentified Imp, mg

APPENDIX F: Summary of measurement equations and uncertainty budgets

u_{NV} — combined standard uncertainty of non-volatiles mesurement,mg/g

$u_{NV} = \frac{LLOQ}{\sqrt{3}} = \frac{0{,}0004}{\sqrt{3}}$; ***LLOQ*** - low limit of quantitation of TGA method

u_{H2O} — combined standard uncertainty of water measurement, mg/g

$u_{H2O} = \sqrt{u_a^2 + u_b^2} = \sqrt{\left(\frac{SD}{\sqrt{n}}\right)^2 + \left(\frac{u_{KFtitrator}}{\sqrt{3}}\right)^2}$; $u_{KF\ titrator}$ - uncertainty due to titrator characteristics, mg

u_{Cl} — combined standard uncertainty of chloride ion measurement, mg/g

$$u_{Cl^-} = \sqrt{u_A^2 + u_{ms}^2 + u_{msolv}^2 + u_{mSRM}^2 + u_{SRM}^2 + u_{RFCl}^2}$$

u_A — SD of Cl^- measurement results, mg/g

$$u_A = \frac{SD_{Cl^-}}{\sqrt{n}}$$

u_{ms} — uncertainty due to sample weighting, mg

u_{msolv} - — uncertainty due to solvent weighting, mg

u_{mSRM} - — uncertainty due to SRM weighting, mg

u_{SRM} - — uncertainty of CRM (GSO 7436-98) reference value, mg/g

$u_{RF_{Cl^-}} = \frac{SD_{RF_{Cl}}}{\sqrt{3}}$ u_{RFCl} — uncertainty due to RF_{Cl^-} determination, mg

u_{VOC} — combined standard uncertainty of VOC mesurement, mg/g

$$u_{VOC} = \sqrt{u_{CH3CN}^2 + u_{CH3OH}^2 + u_{C2H5OH}^2}$$

$$u_{(VOC)i} = \sqrt{u_A^2 + u_{cal}^2 + u_{samp}^2}$$

i = CH3CN, CH3OH, C2H5OH

u_A — SD of VOC measurement results, mg/g

u_{cal} — uncertainty due to calibration, mg

u_{samp} - — uncertainty due to sample preparation, mg

APPENDIX F: Summary of measurement equations and uncertainty budgets

Participant: NIMT

Mass balance

$w_A = [1000 - (w_w + w_{NV} + w_{OS})] * w_{Org}$

w_w : Mass Fraction of Water in sample

w_{NV}: Mass Fraction of Nonvolatile Materials in sample

w_{OS} :Mass Fraction of Residual Organic Solvent in sample

w_{Org} :Mass fraction of related structure impurities in sample

Mass balance

$$u(w_A) = \sqrt{u(w_{Org})^2 + u(w_W)^2 + u(w_{OS})^2 + u(w_{NV})^2}$$

where;

u_{wOrg} standard uncertainty of sample–related structure impurities in sample

u_{ww} standard uncertainty of water in sample

u_{OS} standard uncertainty of organic solvent in sample

u_{NV} standard uncertainty of non-volatile in sample

Uncertainty budget

Parameter	Source of uncertainty	xi	u(xi)	u(xi)^2
M(H2O)	Mass fraction of H2O (mg/g)	59.06	16.64	276.9894
M(OTC)	Mass fraction of OTC (mg/g)	968.76	0.70	0.4900
M(V)	Mass fraction of volatiles (mg/g)	0.00	1.44	2.0822
M(NV)	Mass fraction of non-volatiles (mg/g)	5.42	0.41	0.1681
M(Cl)	Mass fraction of cholride (mg/g)	61.66	4.89	23.9121
Impurities (H_2O, NV and OS) (mg/g)		126.14		
Oxytetracycline .HCL content (mg/g)		846.56	17.42	
Expanded uncertainty (k=2) (mg/g)		34.85		

qNMR

$$P_{Analyte} = \frac{I_{analyte}}{I_{std}} x \frac{N_{std}}{N_{analyte}} x \frac{M_{analyte}}{M_{std}} x \frac{m_{std}}{m_{analyte}} x P_{std}$$

Where: $I_{analyte}$ = integrated signal area of analyte

I_{Std} = integrated signal area of standard

N_{Std} = number of H in the integrated signal area of standard

$N_{analyte}$ = number of H in the integrated signal area of analyte

APPENDIX F: Summary of measurement equations and uncertainty budgets

$M_{analyte}$ = molar masses of the analyte

M_{Std} = molar masses of the standard

m_{Std} = the mass of the standard

$m_{analyte}$ = the mass of the analyte

P_{Std} = the purity of the standard

Uncertainty budget

$$u_c(P_{Analyte}) = \sqrt{\left(\frac{u(I_{analyte}/I_{std})}{(I_{analyte}/I_{std})}\right)^2 + \left(\frac{u(M_{analyte})}{M_{analyte}}\right)^2 + \left(\frac{u(M_{std})}{M_{std}}\right)^2 + \left(\frac{u(m_{std})}{m_{std}}\right)^2 + \left(\frac{u(m_{analyte})}{m_{analyte}}\right)^2 + \left(\frac{u(P_{std})}{P_{std}}\right)^2}$$

Where: $u(I_{analyte}/I_{std})$ = the std. uncertainty of integrated signal area of analyte

$u(M_{analyte})$ = the std. uncertainty of molar masses of the analyte

$u(M_{Std})$ = the std. uncertainty of molar masses of the standard

$u(m_{Std})$ = the std. uncertainty of the mass of the standard

$u(m_{analyte})$ = the std. uncertainty of the mass of the analyte

MU budget for Oxytetracyclin (Free base) purity value, determined by ^{1}H NMR					
Source of uncertainty	Value	u(x)	rel u (%)	Veff	Rel.U4/Vi
Method Repeatability		0.0057			
BB in-homogeneity Uncertainty		0.0165			
$P_{ANALYTE}$, mean/%	84.5%	1.460%	2.062%	9	2.0079E-08
$\rho_{internal\ std.}$	1	0.000	0.000%	61	0
$\rho_{ANALYTE}$	3	0.000	0.000%	61	0
$P_{internal\ std.}$/%	99.8%	0.190%	0.190%	30	4.3807E-13
$m_{internal\ std.}$	0.055	0.001%	0.025%	4	1.0527E-15
$m_{analyte}$	0.10	0.001%	0.014%	4	9.3216E-17
$MW_{ANALYTE}$	496.8924	0.003	0.001%	4	2.9006E-22
$MW_{internal\ std.}$	260.8832	0.006	0.002%	10	2.7978E-20
	uc =	**1.46%**	K =	2.26	
	U =	**3.3%**			

mass balance combined qNMR

$$P_{final} = \frac{P_{MB} + P_{qNMR}}{2}$$

APPENDIX F: Summary of measurement equations and uncertainty budgets

Uncertainty budget

$$u_{\text{final}} = \sqrt{\left(P_{MB} - P_{qNMR}\right)^2 + u_{MB}^2 + u_{qNMR}^2}$$

Parameter	Source of uncertainty	xi	u(xi)	u(xi)^2
P_{MB}	Mass fraction of Oxytetracycline (free base) from mass balance (mg/g)	846.56	17.42	303.4564
P_{qNMR}	Mass fraction of Oxytetracycline (free base) from qNMR (mg/g)	845.00	14.60	213.16
Different value between mass balance and qNMR (mg/g)		1.56		
P_{final}	Mass fraction of Oxytetracycline (free base) from mass balance combined qNMR (mg/g)	845.8	22.78	
Expanded uncertainty (k=2) (mg/g)		45.6		

APPENDIX F: Summary of measurement equations and uncertainty budgets

Participant: INRIM

wOTC = (Is/Istd)(Nstd/Ns)*(Ms/Mstd)*(mStd/ms)*wstd*

where w_{OTC}= mass fraction (mg/g) of OTC by internal standard qNMR; I_s= Integral of the quantified signal for OTC; I_{Std}: Integral of quantified signal for internal standard; n_s= number of ^{1}H nuclei, OTC quantification signal, n_{Std}= number of ^{1}H nuclei, internal standard quantification signal, M_s = molar mass of OTC; M_{std} = molar mass of internal standard; m_s: mass of CCQM-K148.b material; m_{std} = mass of internal standard; w_{std}: mass-fraction (mg/g) content of internal standard Std.

Components:

Weighing operations

relative uncertainty (int. Standard): 0.01055 mg

relative uncertainty (Analyte): 0.00986 mg

Molar Mass Uncertainty:

relative uncertainty (int. Standard): maleic acid 0.0068 g/mol

relative uncertainty (Analyte): 0.02238 g/mol

Internal Standard Purity

relative uncertainty: Maleis Acid 0.16%;

Precision of replicate measurements:

relative uncertainty: 2.913E-3

The relative uncertainty of the reported OTC value was the quadratic combination of the component relative uncertainties; coverage factor k =2 (95%)

Appendix G: Core competency claims by participants

CCQM-K148.b	*HSA*	Mass fraction of polar analyte in a solid organic material
Scope of comparison: The measurement results are representative of the laboratory's capability for the purity assignment of solid organic compounds in the molar mass range (75 – 500) g/mol with pK_{OW} > -2.		
Competency	**✓,× or N/A**	**Specific Information**
• Value assignment of Primary Reference: Main component mass fraction and uncertainty		
Identity verification	✓	*(1) Structural elucidation by NMR spectroscopy; and (2) Comparison of retention time and UV absorption profile of the comparison material with those of the reference standard of oxytetracycline HCl from different source (Dr Ehrenstorfer).*
Assignment of OTC base mass fraction content of CCQM-K148.b	✓	*Approach 1: Deduction of four classes of impurities and HCl from 1,000 mg/g using the mass balance approach; and* *Approach 2: Direct determination of the main component (oxytetracycline free base) using quantitative nuclear magnetic resonance spectroscopy via internal standard method.*
Oxytetracycline content (mg/g)	✓	*777.1 ± 13.8*
• Value assignment of Primary Reference: Impurity class mass fraction and uncertainty (required if using a mass balance method, otherwise optional)		
Assignment of related structure impurity	✓	*(1) HPLC-DAD for identification and quantification of related structure impurities using relative peak area approach; and (2) LC-MS/MS for identification of related structure impurities.*
Total related structure impurity (mg/g)	✓	*41.0 ± 11.7*
Assignment of water content	✓	*Karl Fischer Coulometer*
Category of water content assignment*	✓	*Polar organic solid, water content > 20 mg/g*
Water content (mg/g)	✓	*106.4 ± 7.9*
Assignment of residual solvent content	✓	*(1) GC-MS for identification and estimation of residual solvent; (2) NMR for identification and quantification of residual solvent; and (3) TGA for quantification of residual solvent.*
Total residual solvent (mg/g)	✓	*0.024 ± 2.11*
Assignment of inorganic content	✓	*(1) TGA for quantification of total non-volatiles/inorganics; and (2) ICP-MS for identification and quantification of inorganics.*

APPENDIX G: Core competency claims by participants

Total non-volatiles (mg/g)	✓	*0 ± 3.27*

APPENDIX G: Core competency claims by participants

CCQM-K148.b	NMISA	Mass fraction of polar analyte in a solid organic material
Scope of comparison: The measurement results are representative of the laboratory's capability for the purity assignment of solid organic compounds in the molar mass range (75 – 500) g/mol with pK_{OW} > -2.		
Competency	**✓,× or N/A**	**Specific Information**
• Value assignment of Primary Reference: Main component mass fraction and uncertainty		
Identity verification	√	*Summary of methods used to establish the qualitative identity (e.g., comparison with independent sample, mass spec., NMR, other)* Identity verified through retention time match with authentic standards of QTC and impurities
Assignment of OTC base mass fraction content of CCQM-K148.b	√	*Indicate method(s) used to quantify mass fraction of OTC in the material* For mass balance approach: 1) structurally related impurities were determined using LC-UV 2) non-volatiles by TGA 3) residual solvent by GC-TOFMS and 4) water by KF oven transfer coulometry 5) Chloride content by IC
Oxytetracycline content (mg/g)		*Reported comparison result (± $U_{95\%}$)* *780 ± 15 mg/g*
• Value assignment of Primary Reference: Impurity class mass fraction and uncertainty (required if using a mass balance method, otherwise optional)		
Assignment of related structure impurity	√	*Indicate method(s) used to quantify mass fraction of related structure impurities in the material* LC-UV external calibration against authentic reference standards and relative response factors for unidentified impurities
Total related structure impurity (mg/g)		*Reported comparison result (± $U_{95\%}$)* *62 ± 11 mg/g*
Assignment of water content	√	*Indicate method(s) used to quantify mass fraction water content in the material* water by KF oven transfer coulometry
Category of water content assignment*		*Select from list below* the applicable category of general water content assignment competency* polar organic solid, water content > 20 mg/g
Water content (mg/g)		*Reported comparison result (± $U_{95\%}$)* *97.5 ± 5.8 mg/g*
Assignment of residual solvent content	√	*Indicate method(s) used to quantify mass fraction residual solvent content in the material* residual solvent by GC-TOFMS
Total residual solvent (mg/g)		*Reported comparison result (± $U_{95\%}$)* *0.47 ± 0.17 mg/g*

APPENDIX G: Core competency claims by participants

Assignment of inorganic content		*Indicate method(s) used to quantify mass fraction total non-volatile content in the material* Thermal Gravimetric Analysis
Total non-volatiles (mg/g)	√	*Reported comparison result (± $U_{95\%}$)* Thermal Gravimetric Analysis *<1 +0.005/-0 mg/g*

APPENDIX G: Core competency claims by participants

CCQM-K148.b	*NRC*	Mass fraction of polar analyte in a solid organic material
Scope of comparison: The measurement results are representative of the laboratory's capability for the purity assignment of solid organic compounds in the molar mass range (75 – 500) g/mol with pK_{OW} > -2.		
Competency	**✓,× or N/A**	**Specific Information**
• Value assignment of Primary Reference: Main component mass fraction and uncertainty		
Identity verification	✓	*Based on LC-MS and ^{1}H-NMR as well as a comparison with an independent sample*
Assignment of OTC base mass fraction content of CCQM-K148.b	✓	*Internal and external standard ^{1}H-qNMR (with impurity correction)*
Oxytetracycline content (mg/g)	✓	*787 ± 26 mg/g*
• Value assignment of Primary Reference: Impurity class mass fraction and uncertainty (required if using a mass balance method, otherwise optional)		
Assignment of related structure impurity	✓	*Identification by LC-HRMS and quantitation by LC-UV*
Total related structure impurity (mg/g)	✓	*47 ± 20 mg/g*
Assignment of water content	N/A	*Indicate method(s) used to quantify mass fraction water content in the material*
Category of water content assignment*	N/A	*Select from list below* the applicable category of general water content assignment competency*
Water content (mg/g)	**N/A**	*Reported comparison result (± $U_{95\%}$)*
Assignment of residual solvent content	N/A	*Indicate method(s) used to quantify mass fraction residual solvent content in the material*
Total residual solvent (mg/g)	**N/A**	*Reported comparison result (± $U_{95\%}$)*
Assignment of inorganic content	N/A	*Indicate method(s) used to quantify mass fraction total non-volatile content in the material*
Total non-volatiles (mg/g)	**N/A**	*Reported comparison result (± $U_{95\%}$)*

APPENDIX G: Core competency claims by participants

CCQM-K148.b	NMIJ	Mass fraction of polar analyte in a solid organic material
Scope of comparison: The measurement results are representative of the laboratory's capability for the purity assignment of solid organic compounds in the molar mass range (75 – 500) g/mol with pK_{OW} > -2.		
Competency	**✓,× or N/A**	**Specific Information**
• Value assignment of Primary Reference: Main component mass fraction and uncertainty		
Identity verification	✓	Comparison of mass spectrum and NMR spectrum with a commercial sample
Assignment of OTC base mass fraction content of CCQM-K148.b	✓	qNMR and Mass balance approach (LC-UV, LC-CAD, GC-FID, IC-CD, KF, TG)
Oxytetracycline content (mg/g)	✓	791.1 ± 7.0 (k = 2)
• Value assignment of Primary Reference: Impurity class mass fraction and uncertainty (required if using a mass balance method, otherwise optional)		
Assignment of related structure impurity	✓	LC (UV, CAD)
Total related structure impurity (mg/g)	✓	32.08 ± 5.20 (k = 2)
Assignment of water content	✓	Coulometric Karl Fischer titration with oven transfer
Category of water content assignment*	✓	polar organic solid, water content > 20 mg/g
Water content (mg/g)	✓	107.04 ± 5.42 (k = 2)
Assignment of residual solvent content	✓	GC (FID)
Total residual solvent (mg/g)	✓	0.00 ± 0.58 (k = 1.65)
Assignment of inorganic content	✓	TG, IC (CD)
Total non-volatiles (mg/g)	✓	65.02 ± 0.26 (k = 2)

APPENDIX G: Core competency claims by participants

CCQM-K148.b	*NIM*	Mass fraction of polar analyte in a solid organic material
Scope of comparison: The measurement results are representative of the laboratory's capability for the purity assignment of solid organic compounds in the molar mass range (75 – 500) g/mol with pK_{OW} > -2.		
Competency	**✓,× or N/A**	**Specific Information**
• Value assignment of Primary Reference: Main component mass fraction and uncertainty		
Identity verification	✓	*the qualitative identity was established by comparison with independent sample and the LC-MS/MS*
Assignment of OTC base mass fraction content of CCQM-K148.b	✓	*Indicate methods used to quantify mass fraction of OTC in the material are mass balance and QNMR*
Oxytetracycline content (mg/g)	✓	*(792.63±9.8) mg/g (± $U_{95\%}$)*
• Value assignment of Primary Reference: Impurity class mass fraction and uncertainty (required if using a mass balance method, otherwise optional)		
Assignment of related structure impurity	✓	*External calibration method was used to quantify mass fraction of related structure impurities in the material*
Total related structure impurity (mg/g)	✓	*(47.23±4.0) mg/g (± $U_{95\%}$)*
Assignment of water content	✓	*Karl Fischer titration method was used to quantify mass fraction water content in the material*
Category of water content assignment*	✓	*polar organic solid, water content > 20 mg/g*
Water content (mg/g)	✓	*(89.9±8.18) mg/g (± $U_{95\%}$)*
Assignment of residual solvent content	✓	*headspace GC method was used to quantify mass fraction residual solvent content in the material*
Total residual solvent (mg/g)	✓	*(0.89±0.04) mg/g (± $U_{95\%}$)*
Assignment of inorganic content	✓	*ICP-MS with internal standards was used to quantify mass fraction total non-volatile content in the material*
Total non-volatiles (mg/g)	✓	*(0.18±0.02) mg/g (± $U_{95\%}$)*

APPENDIX G: Core competency claims by participants

CCQM-K148.b	*GLHK*	Mass fraction of polar analyte in a solid organic material
Scope of comparison: The measurement results are representative of the laboratory's capability for the purity assignment of solid organic compounds in the molar mass range (75 – 500) g/mol with pK_{OW} > -2.		
Competency	**✓,× or N/A**	**Specific Information**
• Value assignment of Primary Reference: Main component mass fraction and uncertainty		
Identity verification	✓	NMR, LC-UV, LC-MS, comparison with independent sample
Assignment of OTC base mass fraction content of CCQM-K148.b	✓	Combination of mass balance method (indirect) and qNMR method (direct)
Oxytetracycline content (mg/g)		796.5 ± 8.6 (± $U_{95\%}$)
• Value assignment of Primary Reference: Impurity class mass fraction and uncertainty (required if using a mass balance method, otherwise optional)		
Assignment of related structure impurity	✓	LC-UV
Total related structure impurity (mg/g)		29.6 ± 6.0 (± $U_{95\%}$)
Assignment of water content	✓	Coulometric Karl Fischer titration with oven transfer, TGA as supporting
Category of water content assignment*	✓	polar organic solid, water content > 20 mg/g
Water content (mg/g)		103 ± 12 (± $U_{95\%}$)
Assignment of residual solvent content	✓	qNMR, HS GC-MS as supporting
Total residual solvent (mg/g)		0.021 ± 2 (± $U_{95\%}$)
Assignment of inorganic content	✓	TGA, ICP-MS
Total non-volatiles (mg/g)		0.017 ± 2 (± $U_{95\%}$)

APPENDIX G: Core competency claims by participants

CCQM-K148.b	*Inmetro*	Mass fraction of polar analyte in a solid organic material
Scope of comparison: The measurement results are representative of the laboratory's capability for the purity assignment of solid organic compounds in the molar mass range (75 – 500) g/mol with pK_{OW} > -2.		
Competency	**✓,× or N/A**	**Specific Information**
• Value assignment of Primary Reference: Main component mass fraction and uncertainty		
Identity verification	✓	*spectra according to OTC structure: -MS and UV (from LC-PDA-MS/MS) -NMR - X-ray fluorescence to determine the counter-ion*
Assignment of OTC base mass fraction content of CCQM-K148.b	✓	*qNMR combined with mass balance (Mass balance considered related structure substances, water, residual solvent and inorganics including chlorine)*
Oxytetracycline content (mg/g)	✓	*796.7 mg/g ± 6.6 mg/g (k=2)*
• Value assignment of Primary Reference: Impurity class mass fraction and uncertainty (required if using a mass balance method, otherwise optional)		
Assignment of related structure impurity	✓	*LC-PDA, LC-MSMS, qNMR*
Total related structure impurity (mg/g)	✓	*30.9 mg/g ± 2.3 mg/g (k=2)*
Assignment of water content	✓	*Karl Fischer direct sampling coulometric titration*
Category of water content assignment*	✓	*polar organic solid, water content > 20 mg/g*
Water content (mg/g)	✓	*101.8 mg/g ± 2.4 mg/g (k=2)*
Assignment of residual solvent content	✓	*HS-GC-MS (qualitative analysis) and qHNMR (qualitative and quantitative analysis)*
Total residual solvent (mg/g)	✓	*0.229 ± 0.019 mg/g (k=2)*
Assignment of inorganic content	✓	*Cloride= X-ray Fluorescence Elementary Analysis= ICP-MS and ICP-OES*
Total non-volatiles (mg/g)	✓	*67.6 mg/g ± 2.2 mg/g (k=2)*

APPENDIX G: Core competency claims by participants

CCQM-K148.b	*EXHM*	Mass fraction of polar analyte in a solid organic material
Scope of comparison: The measurement results are representative of the laboratory's capability for the purity assignment of solid organic compounds in the molar mass range (75 – 500) g/mol with pK_{OW} > -2.		
Competency	**✓,× or N/A**	**Specific Information**
• Value assignment of Primary Reference: Main component mass fraction and uncertainty		
Identity verification	✓	*comparison with independent EP sample, mass spectroscopy, NMR*
Assignment of OTC base mass fraction content of CCQM-K148.b	✓	*Mass balance verified by qNMR*
Oxytetracycline content (mg/g)	✓	*797.50 ± 9.35*
• Value assignment of Primary Reference: Impurity class mass fraction and uncertainty (required if using a mass balance method, otherwise optional)		
Assignment of related structure impurity	✓	*HPLC-DAD-CAD, LCqTOF-MS verified by qNMR*
Total related structure impurity (mg/g)		*33.01 ± 5.03*
Assignment of water content	✓	*Coulometric titration*
Category of water content assignment*	✓	*polar organic solid, water content > 20 mg/g*
Water content (mg/g)		*105.33 ± 1.33*
Assignment of residual solvent content	✓	*GC-FID*
Total residual solvent (mg/g)		*0.00 ± 0.02*
Assignment of inorganic content	✓	*ION CHROMATOGRAPHY, ICP-MS*
Total non-volatiles (mg/g)		*64.16 ± 2.80*

APPENDIX G: Core competency claims by participants

CCQM-K148.b	*BAM*	Mass fraction of polar analyte in a solid organic material
Scope of comparison: The measurement results are representative of the laboratory's capability for the purity assignment of solid organic compounds in the molar mass range (75 – 500) g/mol with pK_{OW} > -2.		
Competency	**✓,× or N/A**	**Specific Information**
• Value assignment of Primary Reference: Main component mass fraction and uncertainty		
Identity verification	✓	*NMR spectroscopic identification based on comparison with other sources of material and 2D NMR methods.*
Assignment of OTC base mass fraction content of CCQM-K148.b	✓	*qNMR with internal standard method*
Oxytetracycline content (mg/g)	**798.9±1.6**	*Reported comparison result (± $U_{95\%}$)*
• Value assignment of Primary Reference: Impurity class mass fraction and uncertainty (required if using a mass balance method, otherwise optional)		
Assignment of related structure impurity	×	*Indicate method(s) used to quantify mass fraction of related structure impurities in the material*
Total related structure impurity (mg/g)	×	*Reported comparison result (± $U_{95\%}$)*
Assignment of water content	×	*Indicate method(s) used to quantify mass fraction water content in the material*
Category of water content assignment*	×	*Select from list below* the applicable category of general water content assignment competency*
Water content (mg/g)	×	*Reported comparison result (± $U_{95\%}$)*
Assignment of residual solvent content	×	*Indicate method(s) used to quantify mass fraction residual solvent content in the material*
Total residual solvent (mg/g)	×	*Reported comparison result (± $U_{95\%}$)*
Assignment of inorganic content	×	*Indicate method(s) used to quantify mass fraction total non-volatile content in the material*
Total non-volatiles (mg/g)	×	*Reported comparison result (± $U_{95\%}$)*

APPENDIX G: Core competency claims by participants

CCQM-K148.b	*LGC*	Mass fraction of polar analyte in a solid organic material
Scope of comparison: The measurement results are representative of the laboratory's capability for the purity assignment of solid organic compounds in the molar mass range (75 – 500) g/mol with pK_{OW} > -2.		
Competency	**✓,× or N/A**	**Specific Information**
• Value assignment of Primary Reference: Main component mass fraction and uncertainty		
Identity verification	✓	*NMR (1D and 2D analysis) and HPLC-DAD, HPLC-MS/MS (comparison with independent sample)*
Assignment of OTC free base mass fraction content of CCQM-K148.b	✓	*qNMR approach with an internal standard*
Oxytetracycline content (mg/g)		*805.6, ± 4.7 (± $U_{95\%}$)*
• Value assignment of Primary Reference: Impurity class mass fraction and uncertainty (required if using a mass balance method, otherwise optional)		
Assignment of related structure impurity		*NMR, HPLC-DAD, HPLC-MS/MS*
Total related structure impurity (mg/g)		*Reported comparison result (± $U_{95\%}$)*
Assignment of water content	✓	*Coulometric Karl Fischer titration with oven transfer*
Category of water content assignment*		*Polar organic solid, water content > 20 mg/g*
Water content (mg/g)		*101.77, ± 8.14 (± $U_{95\%}$)*
Assignment of residual solvent content		*qNMR*
Total residual solvent (mg/g)		*Reported comparison result (± $U_{95\%}$)*
Assignment of inorganic content	✓	*ICP-MS*
Total non-volatiles (mg/g)		*0.078, ± 0.039 (± $U_{95\%}$)*

APPENDIX G: Core competency claims by participants

CCQM-K148.b	*NIST*	Mass fraction of polar analyte in a solid organic material
Scope of comparison: The measurement results are representative of the laboratory's capability for the purity assignment of solid organic compounds in the molar mass range (75 – 500) g/mol with pK_{OW} > -2.		
Competency	**✓,× or N/A**	**Specific Information**
• Value assignment of Primary Reference: Main component mass fraction and uncertainty		
Identity verification	✓	*NMR; comparison with independently sourced sample*
Assignment of OTC base mass fraction content of CCQM-K148.b	✓	*q^1H-NMR using internal standards*
Oxytetracycline content (mg/g)		*806, $U_{95\%}$ = 5 (corrected to 50 % RH)*
• Value assignment of Primary Reference: Impurity class mass fraction and uncertainty (required if using a mass balance method, otherwise optional)		
Assignment of related structure impurity	**N/A**	
Total related structure impurity (mg/g)	**N/A**	
Assignment of water content	✓	*Coulometric Karl Fischer Titration; thermogravimetric analysis as confirmatory method*
Category of water content assignment*		*polar organic solid, water content > 20 mg/g*
Water content (mg/g)		*104.4 $U_{95\%}$ = 1.5 (corrected to 50 % RH)*
Assignment of residual solvent content	**N/A**	
Total residual solvent (mg/g)	**N/A**	
Assignment of inorganic content	**N/A**	
Total non-volatiles (mg/g)	**N/A**	

APPENDIX G: Core competency claims by participants

CCQM-K148.b	*TÜBİTAK UME*	Mass fraction of polar analyte in a solid organic material
Scope of comparison: The measurement results are representative of the laboratory's capability for the purity assignment of solid organic compounds in the molar mass range (75 – 500) g/mol with pK_{OW} > -2.		
Competency	**✓,× or N/A**	**Specific Information**
• Value assignment of Primary Reference: Main component mass fraction and uncertainty		
Identity verification	✓	HPLC-UV, NMR
Assignment of OTC base mass fraction content of CCQM-K148.b	✓	Mass Balance (HPLC-UV, Karl-Fischer coulometry, HS GC-MS, Ion chromatography), qNMR
Oxytetracycline content (mg/g)	✓	816.5 ± 26.1
• Value assignment of Primary Reference: Impurity class mass fraction and uncertainty (required if using a mass balance method, otherwise optional)		
Assignment of related structure impurity	✓	Mass Balance (HPLC-UV, Karl-Fischer coulometry, HS GC-MS, Ion chromatography), qNMR
Total related structure impurity (mg/g)	✓	47.6 ± 0.8
Assignment of water content	✓	Coulometric Karl Fischer titration with oven transfer
Category of water content assignment*	✓	polar organic solid, water content > 20 mg/g
Water content (mg/g)	✓	73.4 ± 1.0
Assignment of residual solvent content	✓	HS GC-FID and qNMR
Total residual solvent (mg/g)	✓	0.17 ± 0.002
Assignment of inorganic content	✓	Ion Chromatography
Total non-volatiles (mg/g)	✓	61.3 ± 1.6 (chloride content)

APPENDIX G: Core competency claims by participants

CCQM-K148.b	KRISS	Mass fraction of polar analyte in a solid organic material
Scope of comparison: The measurement results are representative of the laboratory's capability for the purity assignment of solid organic compounds in the molar mass range (75 – 500) g/mol with pK_{OW} > -2.		
Competency	**✓,× or N/A**	**Specific Information**
• Value assignment of Primary Reference: Main component mass fraction and uncertainty		
Identity verification	✓	Comparison with independent sample, LC-UV, LC-MS, and NMR
Assignment of OTC base mass fraction content of CCQM-K148.b	✓	Mass balance method
Oxytetracycline content (mg/g)	✓	(819.4 ± 5.0) mg/g (with 95% of confidence level, *k*=2.0)
• Value assignment of Primary Reference: Impurity class mass fraction and uncertainty (required if using a mass balance method, otherwise optional)		
Assignment of related structure impurity	✓	LC-UV
Total related structure impurity (mg/g)	✓	(35.9 ± 1.6) mg/g (with 95% of confidence level, *k*=2.1)
Assignment of water content	✓	Coulometric KF titration with oven method
Category of water content assignment*	✓	polar organic solid, water content > 20 mg/g
Water content (mg/g)	✓	(78.1 ± 3.7) mg/g (with 95% of confidence level, *k*=2.1)
Assignment of residual solvent content	✓	Headspace GC-MS
Total residual solvent (mg/g)	✓	(0.1 ± 3.3) mg/g (with 95% of confidence level, *k*=2.0)
Assignment of inorganic content	✓	TGA
Total non-volatiles (mg/g)	✓	(0.1 ± 1.3) mg/g (with 95% of confidence level, *k*=2.0)

APPENDIX G: Core competency claims by participants

CCQM-K148.b	*KIMIA*	Mass fraction of polar analyte in a solid organic material
Scope of comparison: The measurement results are representative of the laboratory's capability for the purity assignment of solid organic compounds in the molar mass range (75 – 500) g/mol with pK_{OW} > -2.		
Competency	**✓,× or N/A**	**Specific Information**
• Value assignment of Primary Reference: Main component mass fraction and uncertainty		
Identity verification	✓	*1) Comparison with reference standard* *2) FT-IR*
Assignment of OTC base mass fraction content of CCQM-K148.b	✓	*1) HPLC-UV-PDA: Structurally related organic compound* *2) Coulometric Karl Fischer Titration: Water* *3) Headspace GC-FID: Residual solvent* *4) TGA: Total non-volatiles*
Oxytetracycline content (mg/g)	✓	*827.12 mg/g ± 10.96 mg/g*
• Value assignment of Primary Reference: Impurity class mass fraction and uncertainty (required if using a mass balance method, otherwise optional)		
Assignment of related structure impurity	✓	*HPLC-UV-PDA*
Total related structure impurity (mg/g)	✓	*33.96 mg/g ± 5.34 mg/g*
Assignment of water content	✓	*Coulometric Karl Fischer Titration* *TGA (as a consistency check)*
Category of water content assignment*	✓	*Polar organic solid, water content > 20 mg/g*
Water content (mg/g)	✓	*69.42 mg/g ± 8.04 mg/g*
Assignment of residual solvent content	✓	*Headspace GC-FID* *GC-MS (direct injection) &* *TGA (as consistency check)*
Total residual solvent (mg/g)	✓	*1.80 mg/g ± 2.40 mg/g*
Assignment of inorganic content	✓	*TGA (under high-temperature oxidative conditions)*
Total non-volatiles (mg/g)	✓	*0.25 mg/g ± 2.88 mg/g*

APPENDIX G: Core competency claims by participants

CCQM-K148.b	*BVL*	Mass fraction of polar analyte in a solid organic material
Scope of comparison: The measurement results are representative of the laboratory's capability for the purity assignment of solid organic compounds in the molar mass range (75 – 500) g/mol with pK_{OW} > -2.		
Competency	**✓,× or N/A**	**Specific Information**
• Value assignment of Primary Reference: Main component mass fraction and uncertainty		
Identity verification	✓	*Summary of methods used to establish the qualitative identity (e.g., comparison with independent sample, mass spec., NMR, other).* *QToF and Orbi-trap , HS-GC/MS*
Assignment of OTC base mass fraction content of CCQM-K148.b	✓	*Indicate method(s) used to quantify mass fraction of OTC in the material: HPLC-UV, KF, IC, TGA-GC/MS, HS-GC/MS*
Oxytetracycline content (mg/g)	✓	*835,46 (± 10,28)*
• Value assignment of Primary Reference: Impurity class mass fraction and uncertainty (required if using a mass balance method, otherwise optional)		
Assignment of related structure impurity	✓	*HPLC-UV (at 270 nm)*
Total related structure impurity (mg/g)	✓	*43,96 (± 7,29)*
Assignment of water content	✓	*Karl Fischer and TGA*
Category of water content assignment*	✓	*polar organic solid, water content > 20 mg/g*
Water content (mg/g)	✓	*51,73 (± 4,46)*
Assignment of residual solvent content	✓	*TGA and HS-GC/MS*
Total residual solvent (mg/g)	✓	*5,96 (± 6,11) (Acetonitrile)*
Assignment of inorganic content	✓ ×	*Ion chromatography for Chloride* *Other than chloride with TGA*
Total non-volatiles (mg/g)	✓	*Cl = 66,55 (± 3,01)*

APPENDIX G: Core competency claims by participants

CCQM-K148.b	*VNIIM*	Mass fraction of polar analyte in a solid organic material
Scope of comparison: The measurement results are representative of the laboratory's capability for the purity assignment of solid organic compounds in the molar mass range (75 – 500) g/mol with pK_{OW} > -2.		
Competency	**✓,× or N/A**	**Specific Information**
• Value assignment of Primary Reference: Main component mass fraction and uncertainty		
Identity verification	✓	*LC/MS mass-spectra*
Assignment of OTC base mass fraction content of CCQM-K148.b	✓	*Mass balance approach:* *Related structure imp.- LC/DAD;* *Residual solvent – GC/MS, GC/FID;* *Water - KF titration with oven;* *Non-volatiles – TGA* *Chloride ion - CE*
Oxytetracycline content (mg/g)	✓	*844,5 ± 5,4*
• Value assignment of Primary Reference: Impurity class mass fraction and uncertainty (required if using a mass balance method, otherwise optional)		
Assignment of related structure impurity	✓	*LC/DAD*
Total related structure impurity (mg/g)	✓	*17,75 ± 1,86*
Assignment of water content	✓	*KF titration with oven*
Category of water content assignment*	**N/A**	
Water content (mg/g)	✓	*62,34 ± 2,42*
Assignment of residual solvent content	✓	*GC/FID*
Total residual solvent (mg/g)	✓	*0,560 ± 0,014*
Assignment of inorganic content	✓	*TGA*
Total non-volatiles (mg/g)	✓	*< 0,004*

APPENDIX G: Core competency claims by participants

CCQM-K148.b	*NIMT*	Mass fraction of polar analyte in a solid organic material
Scope of comparison: The measurement results are representative of the laboratory's capability for the purity assignment of solid organic compounds in the molar mass range (75 – 500) g/mol with pK_{OW} > -2.		
Competency	**✓,× or N/A**	**Specific Information**
• Value assignment of Primary Reference: Main component mass fraction and uncertainty		
Identity verification	✓	comparison with commercial OTC.HCl salt standard (Supelco) using HPLC-PDA and ^{1}H- NMR
Assignment of OTC base mass fraction content of CCQM-K148.b	✓	Mass balance and qNMR
Oxytetracycline content (mg/g)		845.8 ± 45.6 (mg/g)
• Value assignment of Primary Reference: Impurity class mass fraction and uncertainty (required if using a mass balance method, otherwise optional)		
Assignment of related structure impurity	✓	HPLC-PDA
Total related structure impurity (mg/g)	✓	31.23 ± 1.42 (mg/g)
Assignment of water content	✓	Karl Fischer Titration (KFT)
Category of water content assignment*	✓	polar organic solid, water content > 20 mg/g
Water content (mg/g)	✓	59.07 ± 33.28 (mg/g)
Assignment of residual solvent content	✓	Ion chromatography
Total residual solvent (mg/g)	✓	0 ± 2.88 (mg/g)
Assignment of inorganic content	✓	Thermogravimetric Analysis (TGA)
Total non-volatiles (mg/g)	✓	5.42 ±0.82 (mg/g)

APPENDIX G: Core competency claims by participants

CCQM-K148.b	*INRIM*	Mass fraction of polar analyte in a solid organic material
Scope of comparison: The measurement results are representative of the laboratory's capability for the purity assignment of solid organic compounds in the molar mass range (75 – 500) g/mol with pK_{OW} > -2.		
Competency	**✓, × or N/A**	**Specific Information**
• Value assignment of Primary Reference: Main component mass fraction and uncertainty		
Identity verification	✓	*NMR*
Assignment of OTC base mass fraction content of CCQM-K148.b	✓	*qNMR*
Oxytetracycline content (mg/g)	✓	*861.7(± 6.14)*
• Value assignment of Primary Reference: Impurity class mass fraction and uncertainty (required if using a mass balance method, otherwise optional)		
Assignment of related structure impurity	**N/A**	*Indicate method(s) used to quantify mass fraction of related structure impurities in the material*
Total related structure impurity (mg/g)	**N/A**	*Reported comparison result (± $U_{95\%}$)*
Assignment of water content	**N/A**	*Indicate method(s) used to quantify mass fraction water content in the material*
Category of water content assignment*	**N/A**	*Select from list below* the applicable category of general water content assignment competency*
Water content (mg/g)	**N/A**	*Reported comparison result (± $U_{95\%}$)*
Assignment of residual solvent content	**N/A**	*Indicate method(s) used to quantify mass fraction residual solvent content in the material*
Total residual solvent (mg/g)	**N/A**	*Reported comparison result (± $U_{95\%}$)*
Assignment of inorganic content	**N/A**	*Indicate method(s) used to quantify mass fraction total non-volatile content in the material*
Total non-volatiles (mg/g)	**N/A**	*Reported comparison result (± $U_{95\%}$)*

Appendix H: HB-REM parameters for KCRV calculations

The NIST Consensus Builder developed by Antonio Possolo -NIST, version of 2024-May, (https://consensus.nist.gov/) was used to implement the Hierarchical Bayes procedure as described in Koepke et al.[5]

The Hierarchical Bayes Random Effects Model (HB REM, Gaussian) estimator was calculated for the measurands chloride, water, volatile and inorganic contents using as input the participant results listed in Table 11. The model was also used to calculate a qNMR value (Figure 15) based on data from Table 5 after excluding values from UME, NIM, KRISS, NIMT and INTI.

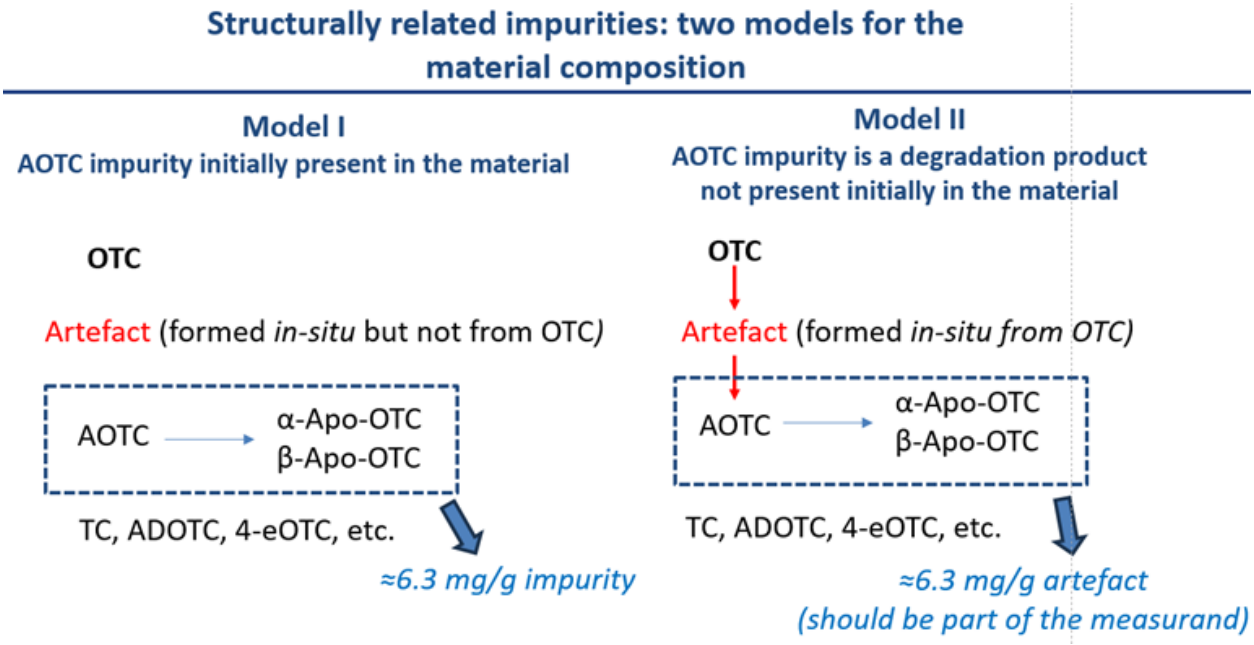
The following (default) settings of the NIST Consensus Builder application were used:

- Scale for half-Cauchy prior on between laboratory variance: median of the absolute values of the differences between the measured values and their median.
- Scale for half-Cauchy prior on within laboratory variances: median of participant standard uncertainties.
- total number of iterations = 250000
- length of burn in = 50000
- thinning rate = 25

Appendix I: Investigation into potential degradation and stability of sample solutions (March 2024 report)

Objective

This work was initiated by BIPM as a result of findings shared by LGC on the detection of potential degradants forming in solutions used for NMR analysis. A dedicated group (LGC, NMIA, NRC and INMETRO) was formed to share their findings and shed some light on the proposed models for the oxytetracycline (OTC) material composition.



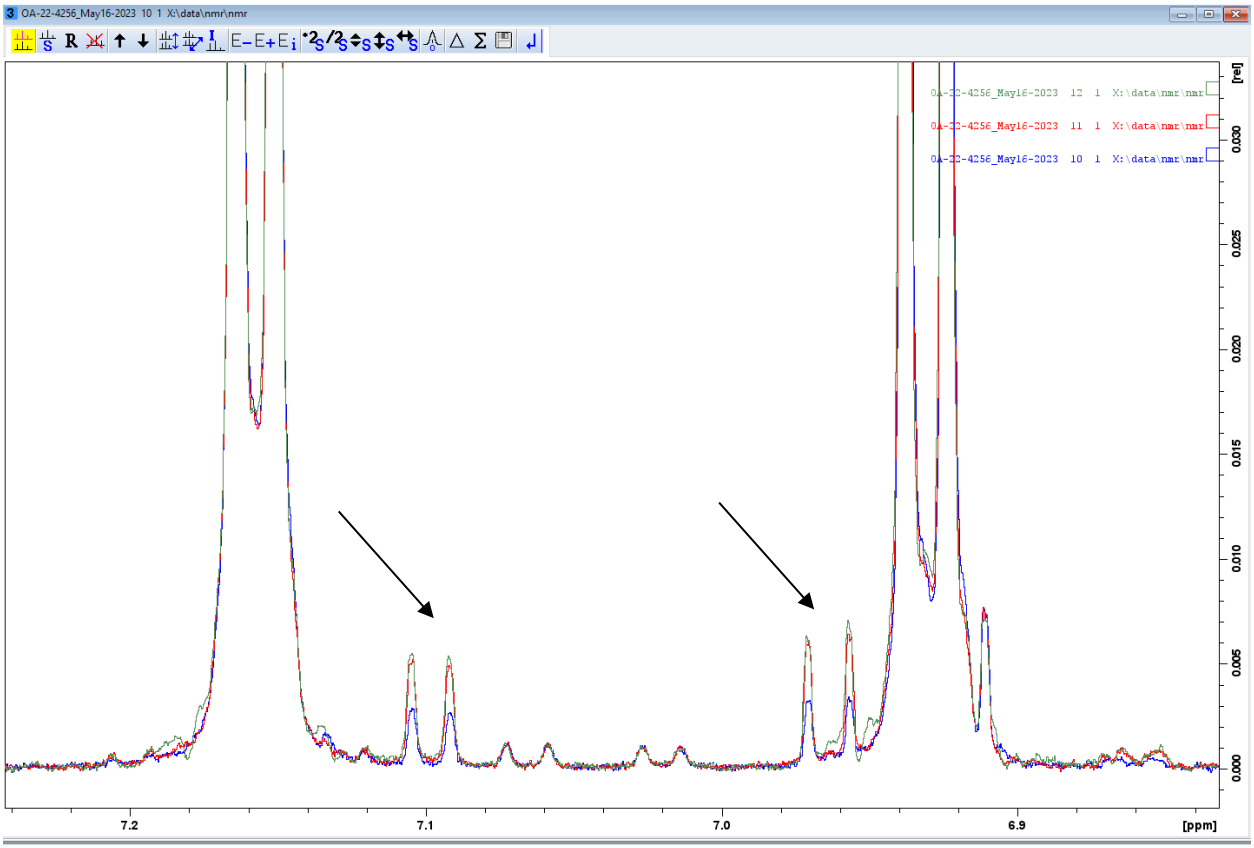
As the investigation relates to observations for NMR data, which was acquired soon after sample preparation (within 10 mins), this investigation has an NMR focus, and in some instances is supported by orthogonal methods.

APPENDIX I: Investigation into potential degradation and stability of sample solutions

Overview of discussion and results

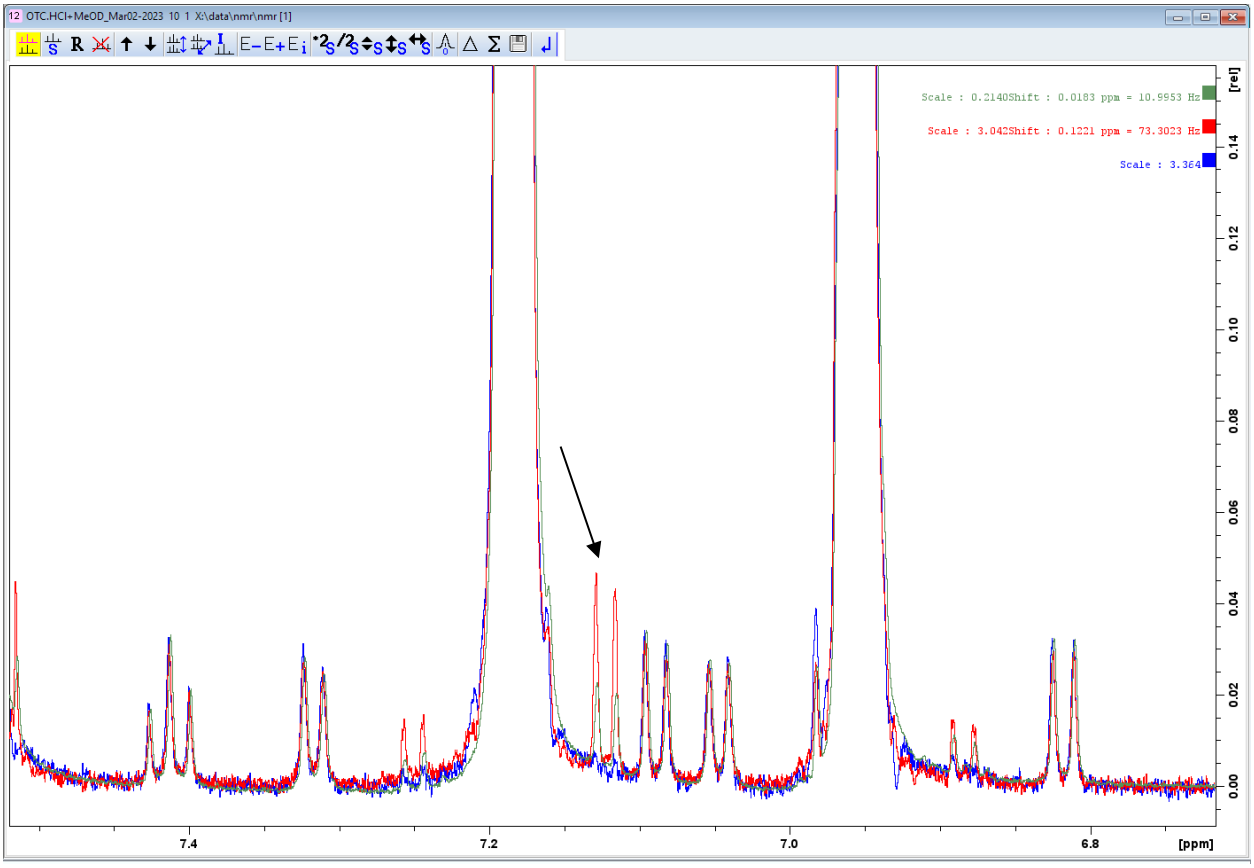
LGC used a mass balance approach as well as a qNMR approach for the OTC mass fraction determination. After concerns about the formation of degradation products *in* situ, the mass balance methods (LC-UV, LC-MS, KF, ICP-MS) were used as supporting data for the mass fraction determination and identification of impurities, and the qNMR data was used to report the mass fraction of OTC. The qNMR method used maleic acid as an internal standard in D_2O and this was recertified against the NIST BA standard. The OTC H8 signal was used for quantitation and overlap of this signal with the equivalent TC and ADOTC signals was corrected for. During the qNMR method development it was noted that a degradant appeared to be forming *in situ*, and the rate of formation was dependent on the deuterated solvent used for sample preparation and varied with time. The degradant appeared to plateau after 10 minutes (example signals shown at δ 7.08 ppm and 6.96 ppm), and a question was raised as to whether this was being considered as a degradant and was included as part of the OTC measurand or was it being reported as an impurity. The formation of the degradant appeared to be dependent on the solvent with the rate of formation in D_2O > in CD_3OD.

LGC: OTC in D_2O solution, NMR analysis (within 10 mins, after ~ 30 mins, after ~ 1 hour)



APPENDIX I: Investigation into potential degradation and stability of sample solutions

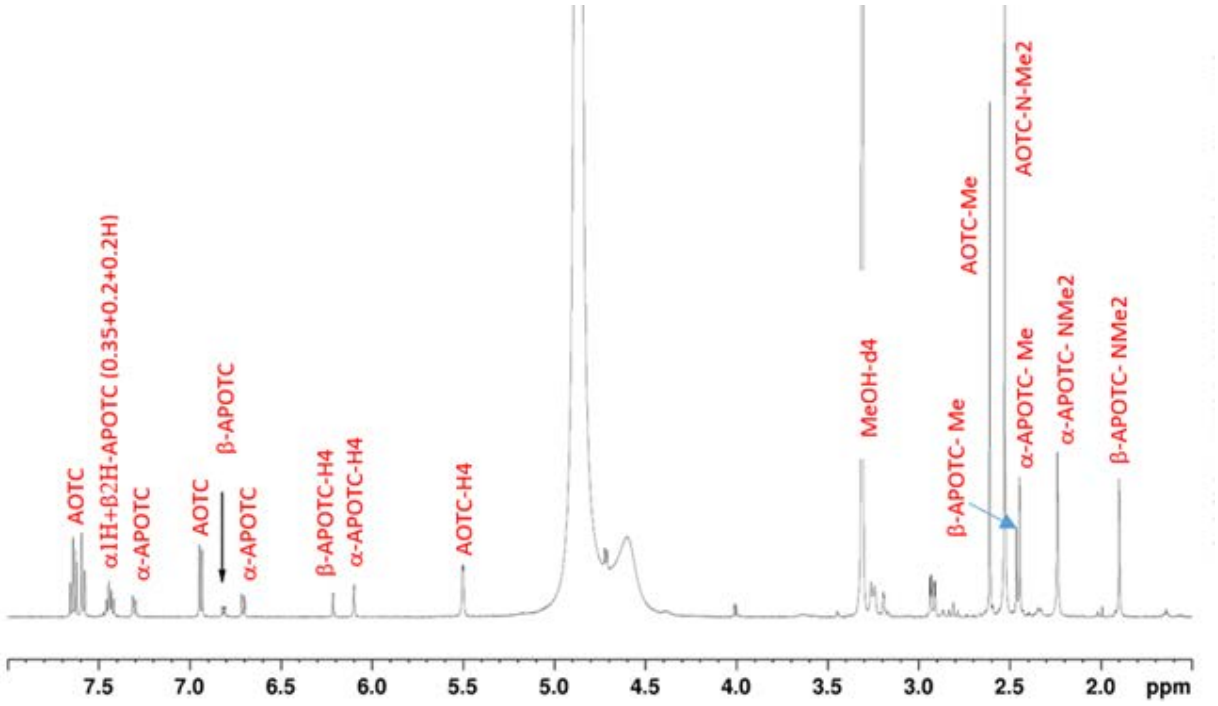
LGC: OTC in CD_3OD solution, NMR analysis (within 10 mins, after ~ 30 mins, after ~ 1 hour)



Examination of the NMR data acquired in different deuterated solvents did not show any obvious signals from AOTC and the isomers α- and β-APOTC, and it was questioned whether the degradant forming then went on to form AOTC and the more stable APOTC isomers. However, closer examination of LGC's NMR data run in D_2O and in CD_3OD, within a few minutes of sample preparation, shows evidence for the presence of low levels of AOTC, but not for the APOTC isomers. It was noted that the signals attributed to AOTC (H4 and aromatic signals) were only apparent in D_2O when NMR experiments were acquired within a few minutes, and by 10 minutes these signals have disappeared, suggesting instability in D_2O. The expected appearance of the characteristic signals (H4) for the APOTC isomers are not detected with the simultaneous disappearance of the AOTC signals, but this is thought to be due to the very low concentration of these impurities and the LOD of NMR.

APPENDIX I: Investigation into potential degradation and stability of sample solutions

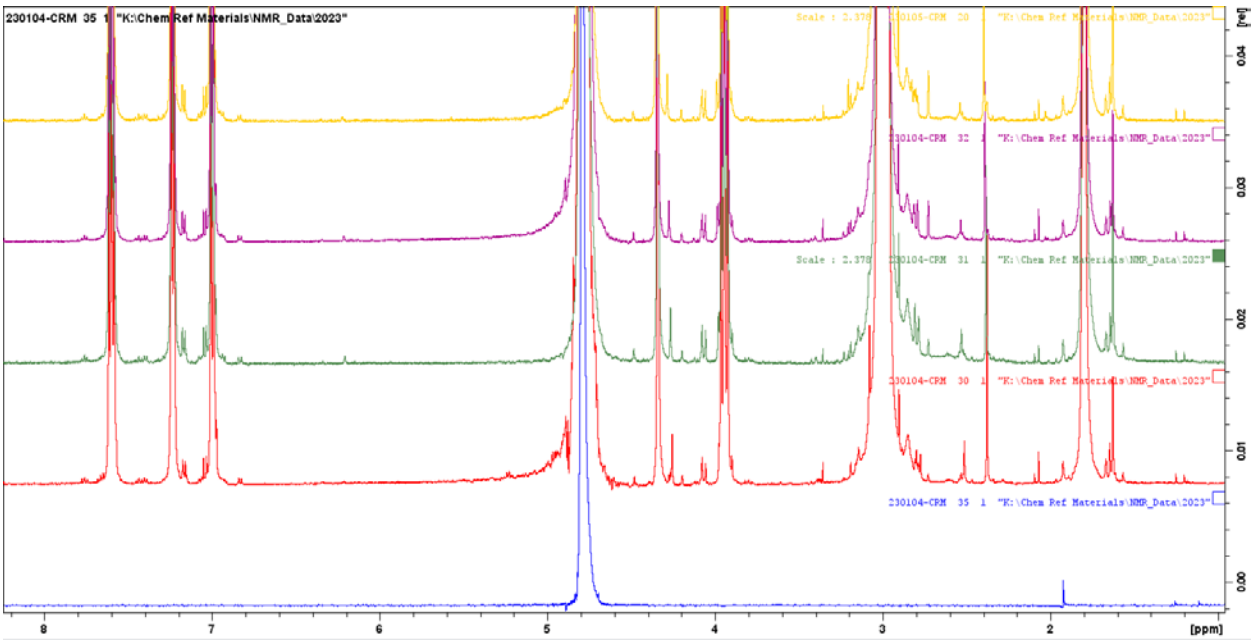
A reference ^{1}H NMR spectrum of AOTC, α-APOTC and β-APOTC was provided by NMIA and was particularly useful in the absence of AOTC being commercially available.



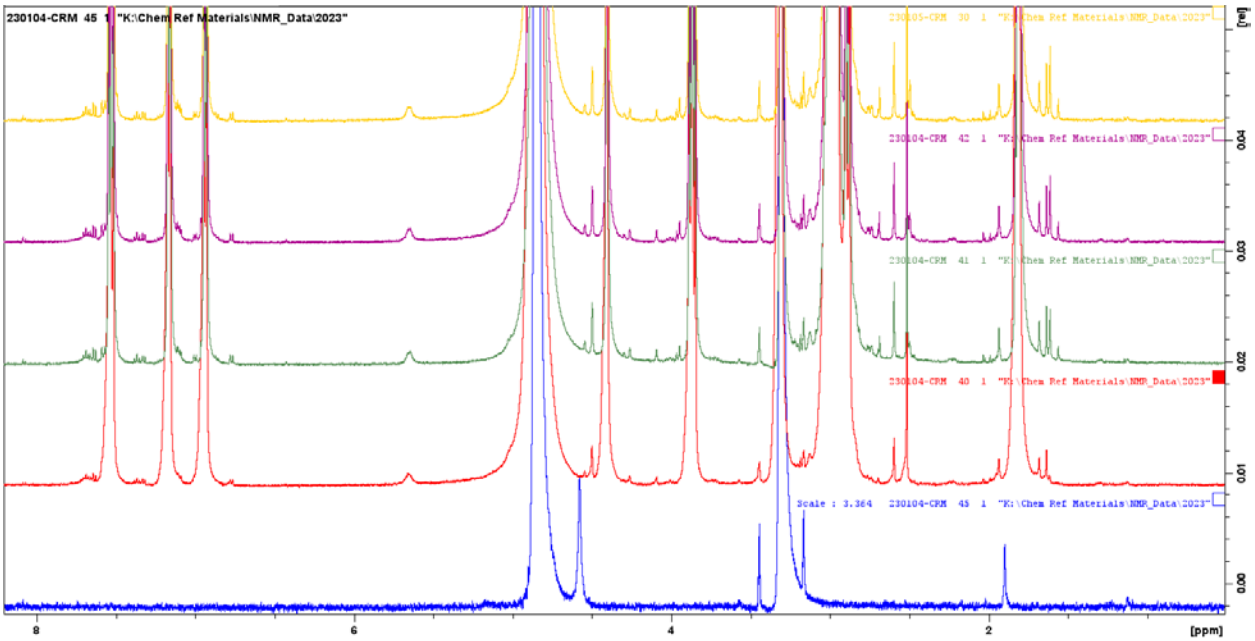
Supporting evidence for the presence of AOTC and the α-and β-APOTC isomers was provided by NMIA, where NMR stability data was performed in various solvents. These impurities appear to be dependent on the NMR solvents (MeOH-d_4, D_2O, DMSO-d_6) and time.

APPENDIX I: Investigation into potential degradation and stability of sample solutions

NMIA: ^{1}H NMR spectra of OTC in D_2O



NMIA: ^{1}H NMR spectra of OTC in CD_3OD

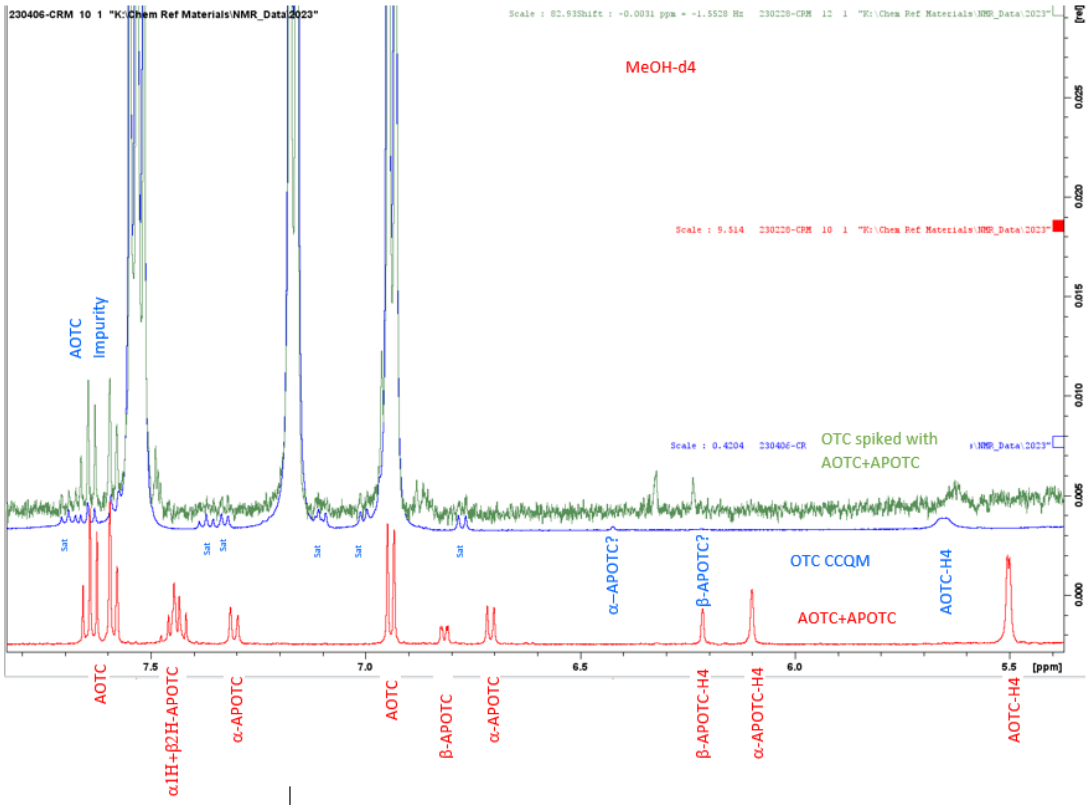


APPENDIX I: Investigation into potential degradation and stability of sample solutions

NMIA commented on the assignment of the ^{1}H NMR signals:

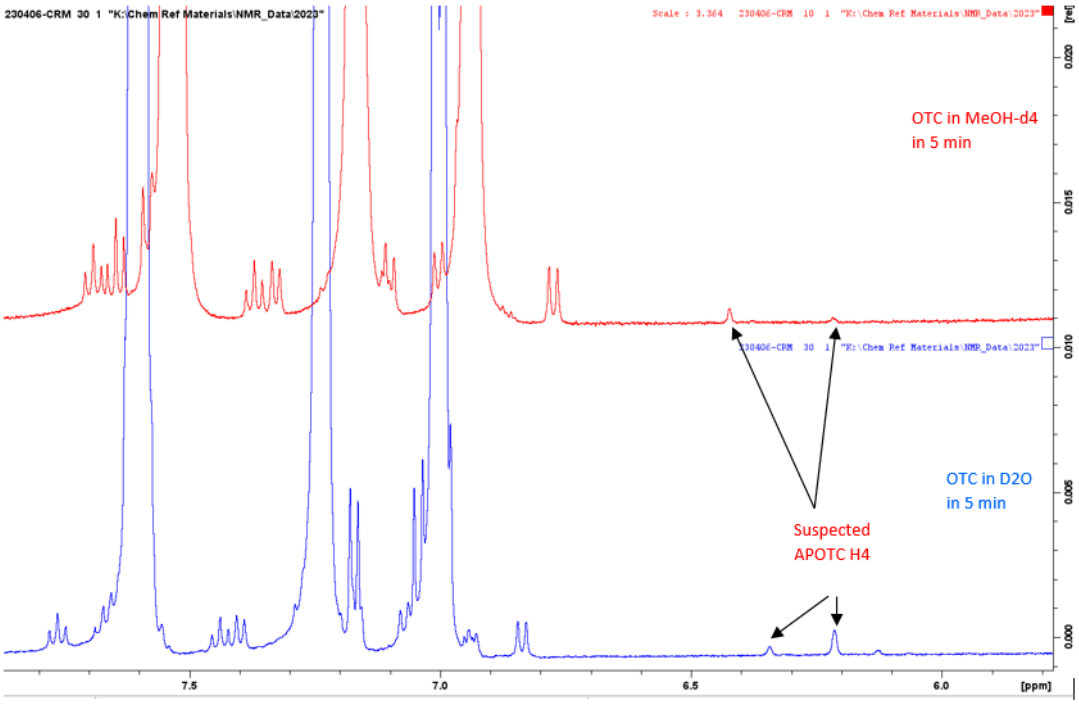
We have very clearly seen two AOTC aromatic protons at 7.60 ppm and 7.64 ppm and C6-Me peak at 2.61 ppm in the MeOH-d_4 (5 minutes sample) and confirmed its presence by spike. In MeOH-d_4 the peak at 6.4 ppm suspected as α-APOTC H4, peak at 6.2 ppm suspected as β-APOTC H4 and peak at 5.6 ppm suspected as AOTC-H4. Similar spiking experiments in D_2O were inconclusive due to AOTC, APOTC standard solubility issues in D_2O. However, suspected APOTC-H4s can be seen both in MeOH-d_4 and D_2O samples. The impurity observation and quantification match the HPLC mass balance impurity giving us further confidence of the analysis.

NMIA: ^{1}H NMR spectra



APPENDIX I: Investigation into potential degradation and stability of sample solutions

NMIA: ^{1}H NMR spectra

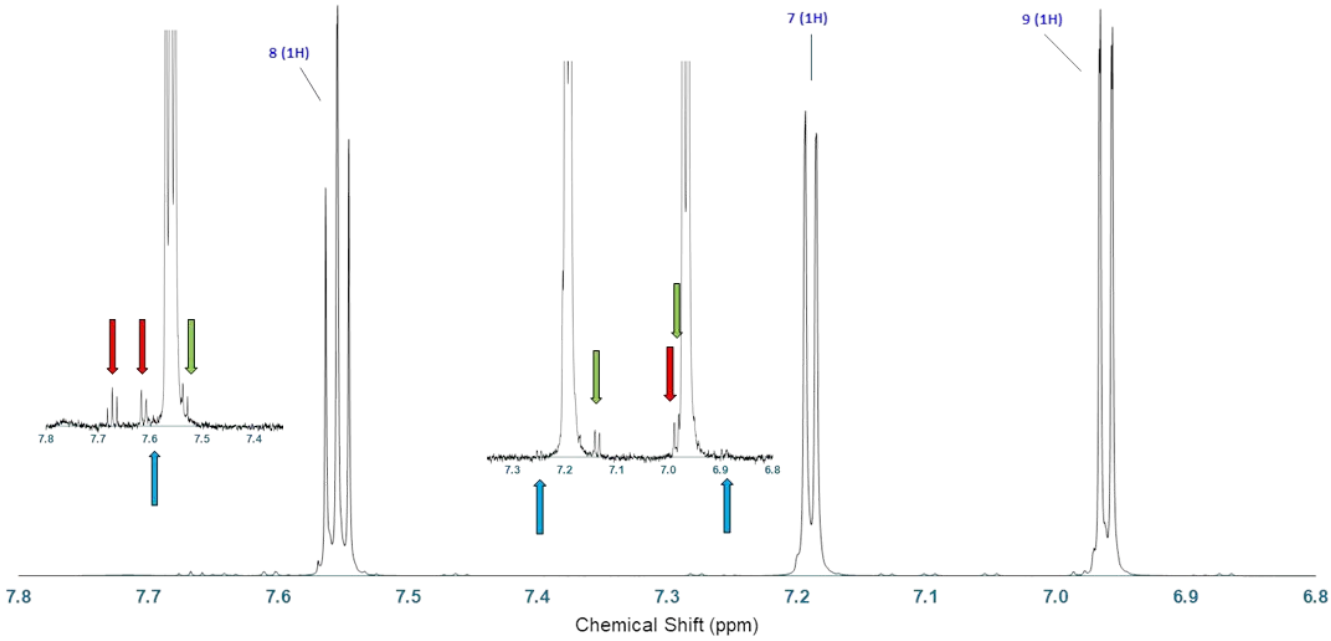


Evidence for the presence of AOTC and the α-and β-APOTC isomers was also provided by NRC, that ran 1D selective TOCSY experiments to observe the correlations between spin systems. NRC commented:

We are confident that the green is not AOTC, as this was identified as the red integrals below. We came to this conclusion because the peak pattern for the 3 aromatic protons is very different from the other OTC derivatives. We only saw this pattern (2 protons close to each other at 7.7 ppm and one far away on the lower end) in alpha and beta. Now, alpha and beta differ from OTC and other derivatives since they don't have an OH group at position C6. AOTC also doesn't have an OH at that position. Furthermore, it was observed that these peaks disappear in water over time. This correlates with the degradation from AOTC to alpha and beta. We believe these signals also match those in NMIA's ^{1}H spectrum for AOTC at around 7.6 ppm.

APPENDIX I: Investigation into potential degradation and stability of sample solutions

NRC: ^{1}H NMR spectrum



These observations were further supported by INMETRO's NMR data:

In our NMR spectra for OTC in methanol we found impurity peaks in 7.64 ppm (triplet), 7.59 ppm (doublet), and 5.64 ppm (broad peak). We believe these peaks belong to AOTC because they are consistent with AOTC spectrum obtained by NMIA and they match the pattern for aromatic peaks mentioned by NRC for α-APOTC and β-APOTC, whose aromatic moiety is similar to AOTC. The broadness of the 5.64 ppm peak could be explained by the labile character of H4.

Conclusion on AOTC and α-APOTC and β-APOTC impurities

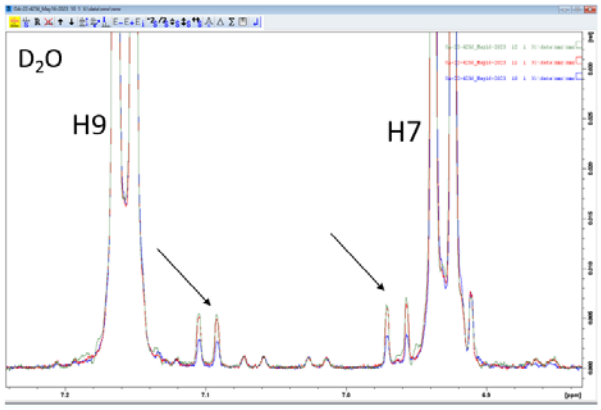
Considering the above evidence provided by NMIA, NRC and INMETRO, and combined with LGC's observations that AOTC can be observed in D_2O sample preparations if data is acquired within a few minutes of sample preparation (< 5 min), it can be concluded that AOTC and the more stable isomers appear to be there in the sample and are not degradation products as proposed by Model II on page 1. The evidence provided would support Model I.

APPENDIX I: Investigation into potential degradation and stability of sample solutions

This does not explain what the potential degradation product is and why the signals in question are changing in intensity over time. This was observed by LGC and BIPM (see spectra below). NMIA did not see a change in signal intensity for theses signals in D_2O over time. NRC commented that they did not monitor the stability of the samples under 30 minutes.

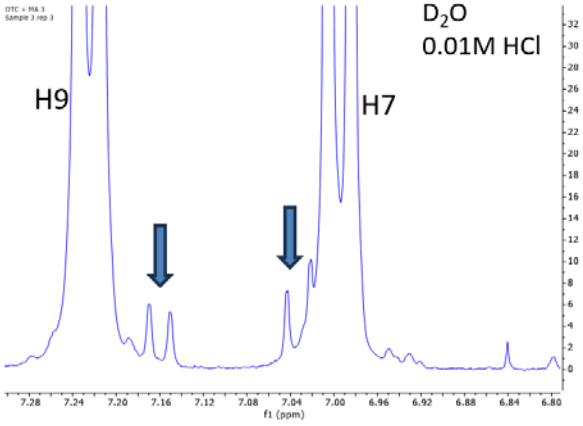
LGC and BIPM: ^{1}H NMR spectra in D_2O over time

LGC



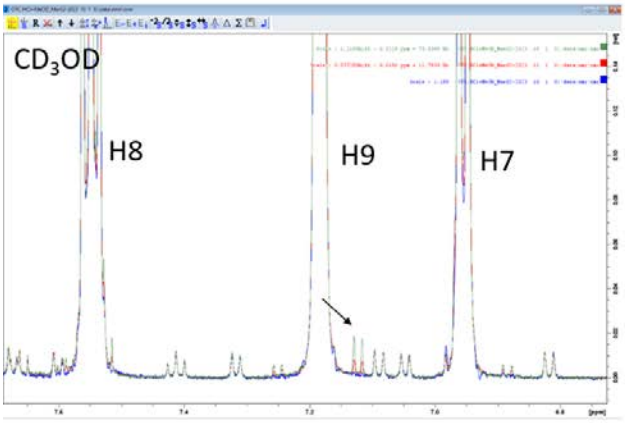
- within 10 min (blue)
- after ~ 30 min (red)
- after ~ 1 hour (green)

BIPM



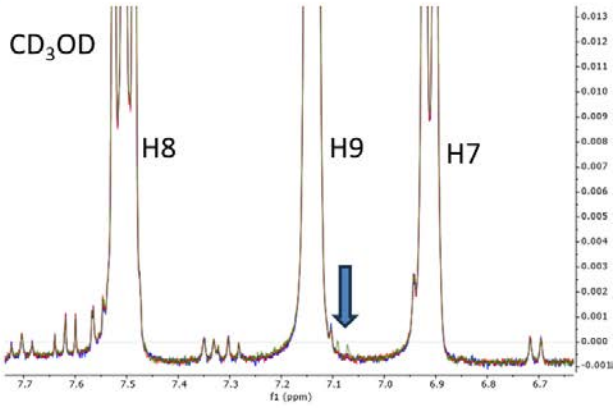
LGC and BIPM: ^{1}H spectra in CD_3OD over time

LGC



- within 10 min (blue)
- after ~ 30 min (red)
- after ~ 1 hour (green)

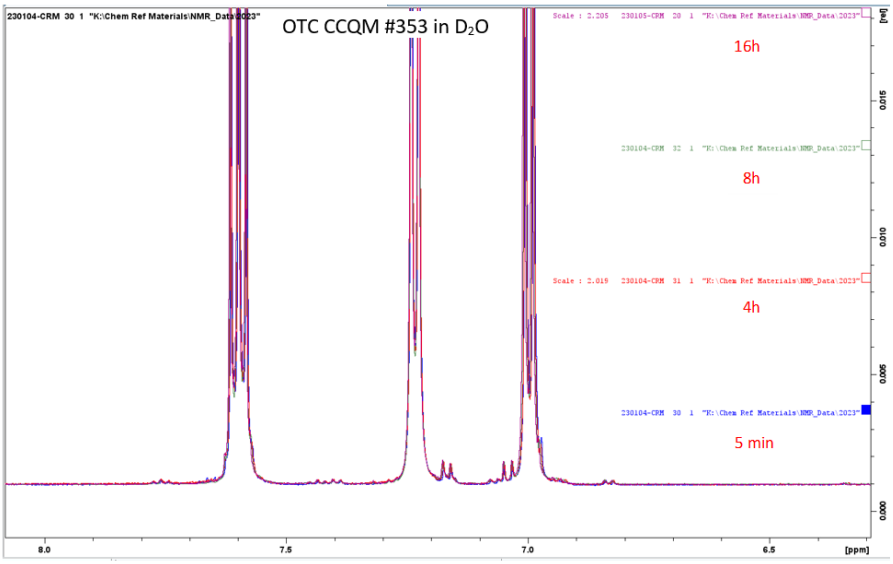
BIPM



- within 8 min (blue)
- after ~ 17 min (red)
- after ~ 2 hour (green)

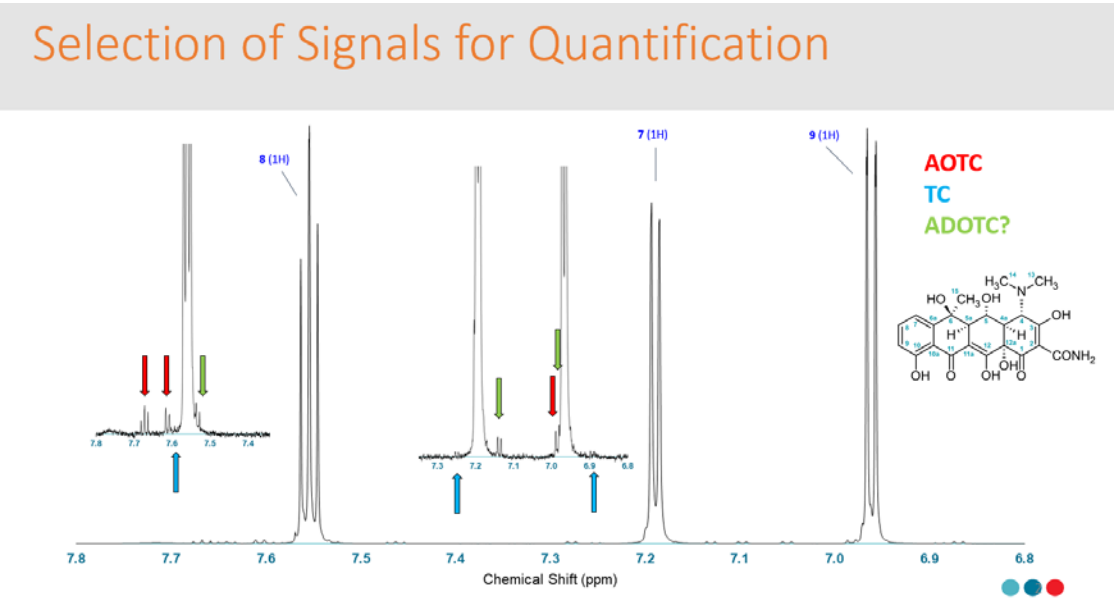
APPENDIX I: Investigation into potential degradation and stability of sample solutions

NMIA: ^{1}H NMR spectra in D_2O over time



NRC commented:

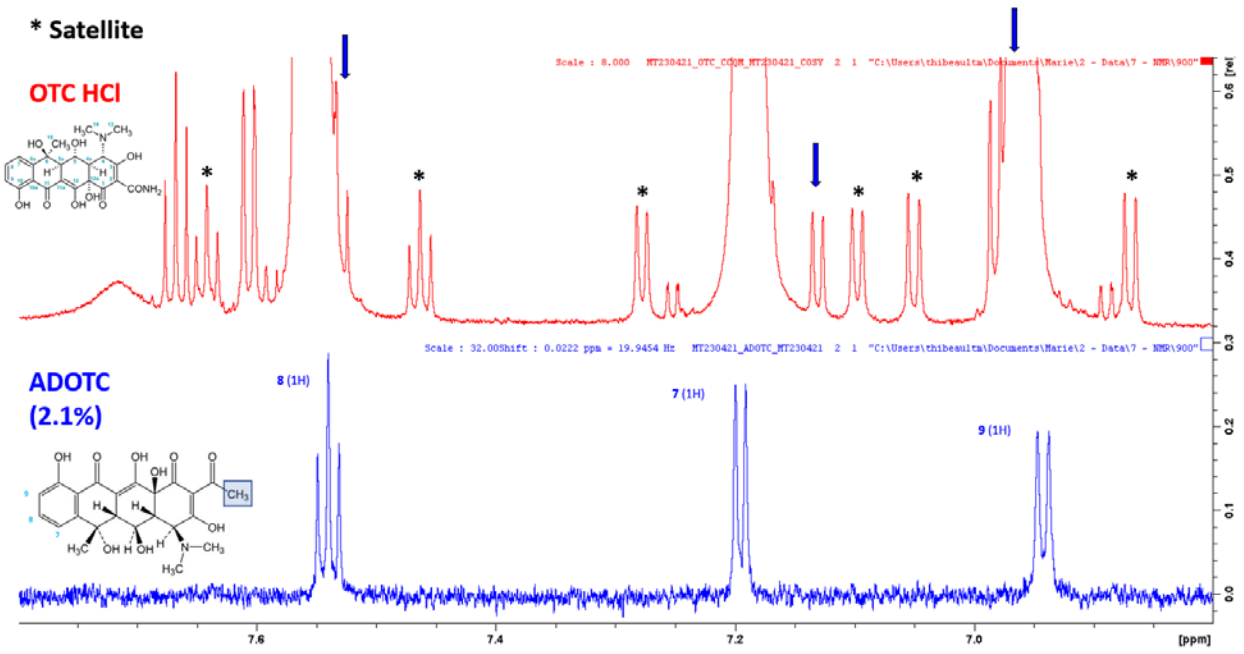
We identified the red integrals as AOTC, the blue as TC, and the green as ADOTC. However, we cannot confidently say that the green is ADOTC but we do know that ADOTC is present in the sample considering our LC-MS and LC-UV data.



APPENDIX I: Investigation into potential degradation and stability of sample solutions

While NRC purchased ADOTC, they did not have enough to do an experiment where they spiked it into the OTC sample to obtain a reference NMR spectrum.

NRC: ^{1}H NMR spectra of OTC and ADOTC



From the relative intensity of the signals (δ 7.08 ppm and 6.96 ppm), expected chemical shift values and splitting patterns, it would appear that these signals may be assigned to ADOTC. However, the COCH3 signal (about 2.3 ppm in D_2O) assigned to ADOTC (supported by HMBC and HSQC experiments) does not show a similar pattern with regards to the signal intensity increasing within 30 minutes. Furthermore, it was observed by LGC that ADOTC is not stable over an extended time period (60 h) and the intensity of the ADOTC COCH3 signal can be seen to decrease in intensity over this extended time. The aromatic signals in question (δ 7.08 ppm and 6.96 ppm) do not decrease over this time and remain at a constant level.

Remaining questions

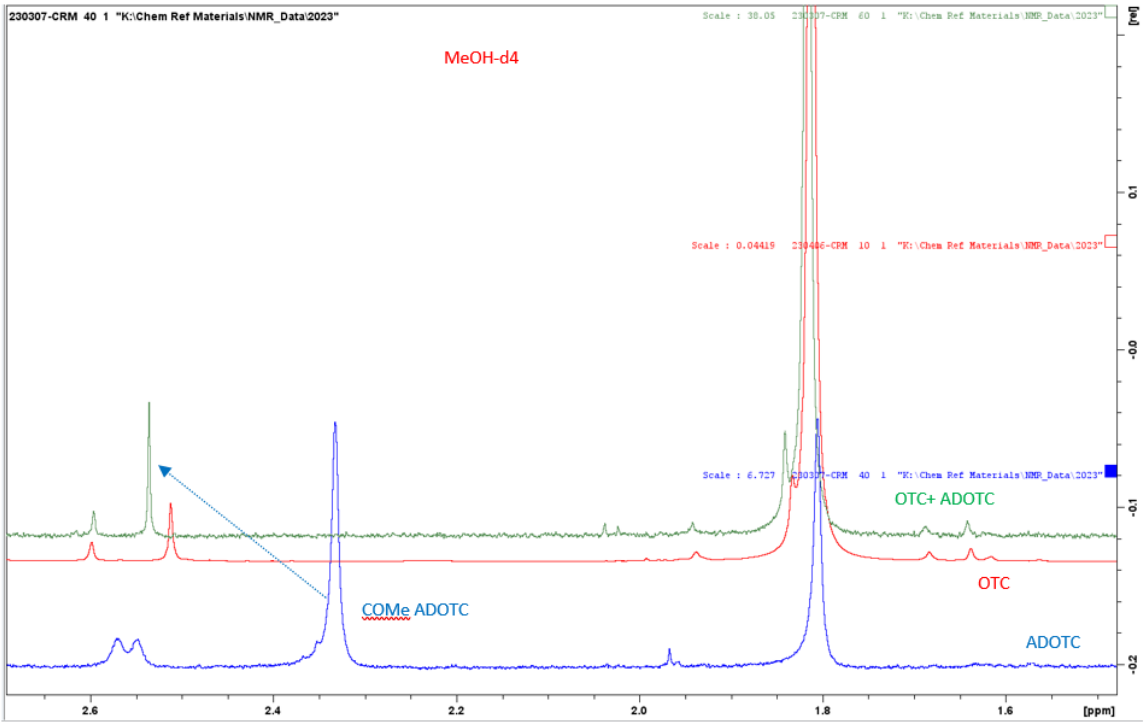
If these signals are not from ADOTC, what are they from and why is this component increasing within the first 10 minutes in D_2O preparations and at a slower rate in CD_3OD (as observed by

LGC and BIPM)? An estimate of the relative amount of this component, purely based on an approximate integral region relative to an OTC signal integral, is approximately 2%. From the collective results from participants, the only impurity close to this level is ADOTC. Is it ADOTC that is forming in solution initially?

NMIA purchased ADOTC to confirm its presence in K148.b OTC.HCl using HPLC-UV (co-elution study) and ^{1}H NMR (spiking experiment). They noted that the commercial ADOTC was free base and the -C=OMe resonated at 2.337 ppm in MeOH-d$_4$, in line with literature precedents. Upon spiking into the OTC.HCl solution the -C=OMe resonance shifted downfield to ~2.5 ppm due to the residual acidity resulting from dissociation and equilibration of the OTC.HCl salt. NMIA commented:

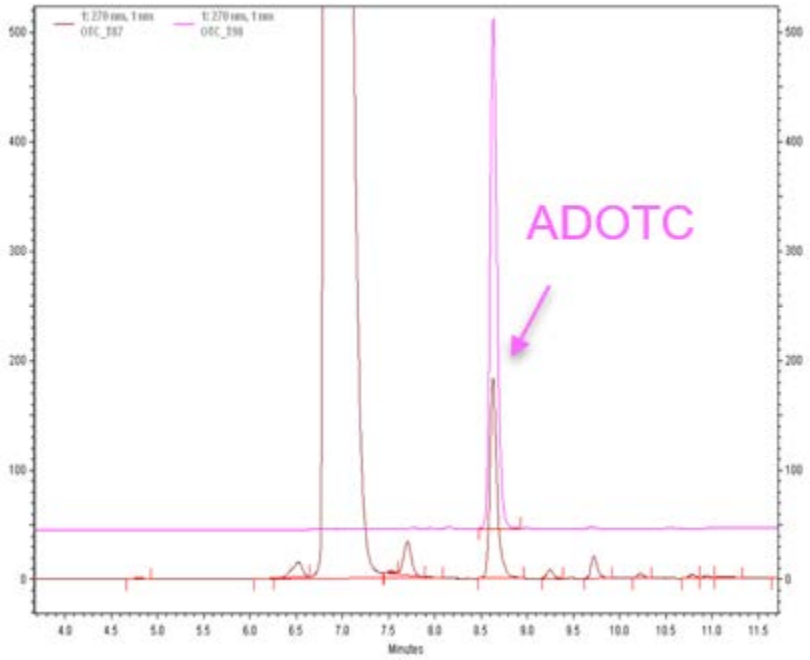
I don't think there is any doubt that ADOTC was present in the K148.b sample. It is simply not possible for OTC (with amide functionality) to decompose to ADOTC (with -C=OMe functionality). What I can't rule out is that the ADOTC, at 2.3%, is isomerising to the epi-ADOTC, although this isn't evident from our HPLC-UV analysis. It is worth noting that in D$_2$O we evidenced two doublets at 7.04 and 7.17 ppm at ~ 2% in line with our mass fraction assignment for ADOTC by HPLC-UV. These doublets were not resolved from the corresponding OTC resonances in MeOH-d$_4$.

NMIA: ^{1}H NMR spectra of OTC and ADOTC



APPENDIX I: Investigation into potential degradation and stability of sample solutions

NMIA: HPLC chromatogram of OTC and ADOTC



In relation to the changing ADOTC signal intensity, INMETRO commented:

In NMR, we also observed a decrease in the 2.49 ppm peak in methanol-d_4 (assigned to ADOTC) over time, as already mentioned by some of you. Impurity peaks in the aromatic region did not decrease in the same rate, neither in methanol-d_4 nor in D_2O. For example, we could see the doublet in 7.17 ppm in the D_2O spectrum with consistent ADOTC intensity, but it did not change over time. ADOTC epimerization, as raised by Steve, could explain a shift in the 2.49 ppm peak (closer to C4) not followed by a similar shift in the more distant aromatic region. However, our HPLC results did not confirm this – ADOTC peak was stable in all the conditions that we tested (diluents water, methanol and DMSO, mobile phase FA 0.1 % and acetonitrile). But maybe the time in solution for HPLC analyses was not enough to allow ADOTC reaction, or epi-ADOTC co-elutes with ADOTC.

These observations were explained by NMIA:

Upon reading Wagner's reply, I had a eureka moment and believe I now understand why the ADOTC -COMe signal at 2.49 ppm is decreasing while the aromatic protons assigned to the same molecule do not. The three protons on the -COMe are exchanging with the D_2O and MeOH-d_4. We use this chemistry all the time to introduce deuterium into steroids to prepare isotopically labelled internal standards.

Further comments from NMIA on the stability of AOTC and its detection were communicated:

APPENDIX I: Investigation into potential degradation and stability of sample solutions

I think we all agree that AOTC degrades to the α- and β-apoOTC isomers. The nice work at INMETRO suggests that this degradation occurs even in neutral conditions (pH 7) and is, no doubt, accelerated at lower pH created by simple dissolution of the OTC hydrochloride salt. This behaviour was evident at NMIA and, no doubt, elsewhere.

Food for thought: The degradation of AOTC to the α- and β-apoOTC isomers will continue as the chromatographic run is underway. This will result in on-going loss of AOTC and, more importantly, a continual stream of the α- and β-apoOTC isomers which, post injection, will be lost in the baseline and never measured. This "loss" of the α- and β-apoOTC isomers in the baseline means that we are never able to accurately measure the total mass fraction of AOTC, the α-apoOTC and β-apoOTC by HPLC. In principle this can only be achieved by ^{1}H NMR, assuming we can see all relevant peaks. On a positive note, I don't think the rate of decomposition of AOTC to the α- and β-apoOTC isomers is significant enough to create a significant bias in this case – as evidenced by the relative mass fraction of all related structure impurities being reasonably consistent throughout the HPLC-UV analysis at NMIA (10 sub samples in duplicate).

INMETRO responded to the observations shared by NMIA:

Steve, your eureka moment really shed light on the decreasing $COCH_3$ peak for ADOTC. Bruno recalled that ^{1}H signals close to deuterium are usually shifted to a smaller chemical shift compared to a hydrogen in the same position due to isotope effect. And we did see an increase of impurity peaks to the right of $COCH_3$ signal, while the $COCH_3$ peak itself was decreasing. Those signals might indicate that $COCH_2D$ and $COCHD_2$ are being produced as intermediates for $COCD_3$ conversion.

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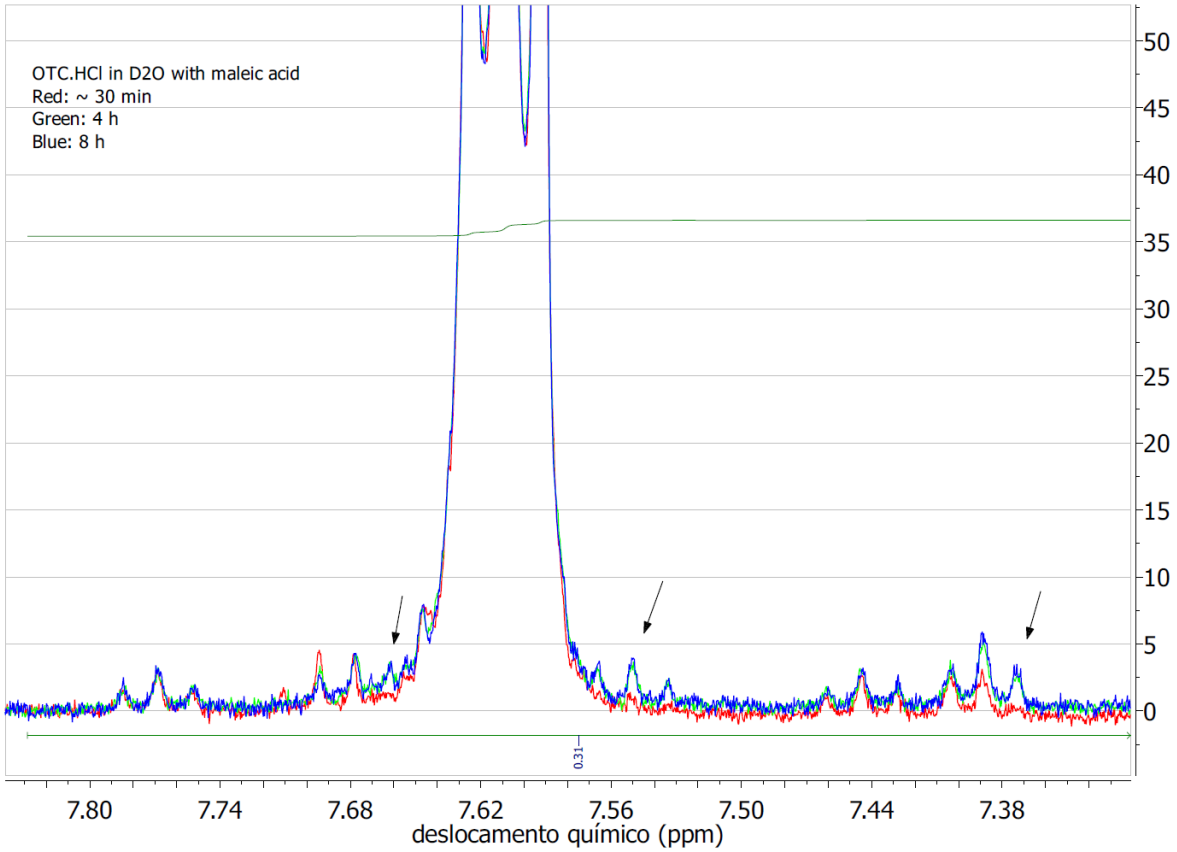
INMETRO provided some ^{1}H NMR spectra acquired over time for:

1. A sample of OTC in D_2O with maleic acid. They have a 4-hour difference each because we analysed other tubes in-between. We have obtained those in May 2023 and the arrows show the impurity peaks I had mentioned before.



2. After reading your last message we decided to prepare a fresh OTC.HCl solution in D_2O (this time without maleic acid) and analyze it quickly. And we had a result similar to yours: the most intense impurity peaks in the aromatic region (assigned to ADOTC) are smaller in the "almost-time-zero" acquisition and plateau in the next acquisitions. As you mentioned, this observation does not correlate with $COCH_3$ signal and unfortunately, we also don't have an explanation for this. The positions of impurity peaks and ^{13}C satellites are a bit different in our spectra compared to yours because we used 500 MHz while you probably used 600 MHz, right? (Correct, LGC have a 600 MHz instrument) .

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