CCQM-K180
Polar analyte in high protein food matrix - metronidazole in porcine muscle

Key Comparison
Track A

Final Report
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U. Mülow-Stollin, J. Polzer
Federal Office of Consumer Protection and Food Safety (BVL)
EU Reference Laboratory for Residues (EURL)
Diedersdorfer Weg 1 ● 12277 Berlin ● Germany
P. O. Box 11 02 60 ● 10832 Berlin ● Germany

With contributions from:

Lesley Johnston
National Measurement Institute Australia (NMIA)
North Ryde, Australia

Eliane C. P. do Rego, Fernando G. M. Violante, Rodrigo V. P. Leal, Tânia M. Monteiro, Waldemar S. Souza, Jane L. N. Fernandes
National Institute of Metrology, Quality and Technology (INMETRO)
Xerém, Brazil

Garnet McRae
National Research Council of Canada (NRC)
Ottawa, ON, Canada

Xiquin Li, Shi Ling, Zhang Qinghe
National Institute of Metrology China (NIM)
Beijing, China

Andrés Sebastián Salinas Trujillo, Julian Herney Pulido, Fabian Yebrail Niño, Andrés Madrid Montoya
Instituto Nacional de Metrología de Colombia (INM)
Bogotá, Colombia

F. Tadjine, K. Heider
Federal Office of Consumer Protection and Food Safety (BVL)
EU Reference Laboratory for Residues
Berlin, Germany

Elias Kakoulides, E. Skotidaki, E. Stathoudaki, V. Schoina
Chemical Metrology Laboratory (EXHM)
Athens, Greece

Ng Chi-Shing, Shiu Hoi Yan Fiona
Government Laboratory of Hong Kong (GLHK)
Pok Fu Lam, Hong Kong

Seok-Won Hyung, Kihwan Choi
Korea Research Institute of Standard and Science (KRISS)
Daejeon, Republic of Korea

Laura Regalado Contreras, Mauricio Maldonado Torres, Evelina Berenice Mercado Pedraza,Mariana Arce Osuna
Centro Nacional de Metrología (CENAM)
Querétaro, Mexico

Alena Mikheeva
D.I. Mendeleyev Institute for Metrology (VNIIM)
Saint-Petersburg, Russia

Abdulrahman R. Al-Askar
National Measurement and Calibration Center of Saudi-Arabia (SASO-NMCC)
Riyadh, Saudi-Arabia

Cheow Pui Sze, Wang Juan, Lu Ting, Teo Tang Lin
Health Sciences Authority (HSA)
Singapore

Caitlin Swiegelaar
National Metrology Institute of South Africa (NMISA)
Pretoria, South Africa

Kittiya Shearman
The National Institute of Metrology Thailand (NIMT)
Klong Luang, Thailand

Hanen Klich, Wided Bellazreg, Soumaya Chaieb, Ons Kesraoui, Ines Ben Amor, Emna Jridette, Imen Ben Mbarek, Kamel Alimi
Institut National de Recherche et d'Analyse Physico-Chimique (INRAP)
Ariana, Tunisia

Mine Bilsel, Simay Gunduz, Hasibe Yilmaz, Ilker Un, Gokhan Bilsel
TUBITAK UME
Gebze Yerleşkesi, Turkey

Christopher Hopley, Vanshni Vekerya
LGC
Teddington, United Kingdom

SUMMARY

Nitroimidazoles are a group of synthetic chemicals derived from azomycin (2-nitroimidazole) and different pharmaceutical activity (e.g. anthelmintic, antibiotic and coccidiostat properties). Among the most important nitroimidazoles from a pharmaceutical perspective is metronidazole. However, due to its proven genotoxic and suspected carcinogenic properties no acceptable daily intake (ADI) could be derived. Therefore, metronidazole (and other nitroimidazoles) are banned worldwide in many countries (e.g., Canada, the European Union, the United States) for use in animals from which products for human consumption are derived, and effective food control is necessary to ensure consumer protection.

Evidence of successful participation in formal, relevant international comparisons is needed to document measurement capability claims (CMCs) made by national metrology institutes (NMIs) and designated institutes (DIs). At the CCQM Organic Analysis Working Group (OAWG) meetings in 2021 it was decided to organize a Track A comparison for polar analytes in a protein rich matrix in order to provide support of existing CMC claims (e.g. based on CCQM-K85 and CCQM-K141).

18 National Metrology Institutions registered in the Track A Key Comparison CCQM-K180 “Polar analyte in high protein food matrix - metronidazole in porcine muscle”, 17 thereof were able to import the test samples. Participants were requested to evaluate the mass fractions, expressed in µg/kg, of metronidazole in lyophilized porcine muscle material on a dry mass basis and should include standard and expanded uncertainties (95 % level of confidence). Finally, all 17 National Metrology Institutions provided results, 15 of which were considered for the calculation of the key comparison reference value (KCRV). Applying the decision tree for KCRV selection to the final data set, it was decided to use the DerSimonian-Laird estimator (NIST consensus builder) for the KCRV calculation. Accordingly, the KCRV for metronidazole was calculated as 8.41 +/- 0.08 µg/kg (with an associated dark uncertainty τ of 0.18 µg/kg).

All participants provided results based on LC-MS measurements, generally using LC-MS/MS instruments. Various extraction methods (water, acetonitrile, ethyl acetate, sometimes with acidification) were used, in some cases with repeated extractions or enzymatic hydrolysis. Various clean-up methods (Extrelut, SPE, sometimes with a defattening step) as well as no purification were used to obtain the final extracts for injection into the LC system. Judging from the method details submitted to the study organizer, no evidence for a correlation between the analytical method employed by the participants and their results could be detected.

Successful participation in CCQM-K180 demonstrates the following measurement capabilities in determining mass fraction of organic compounds, with molecular mass of 100 g/mol to 500 g/mol, having high polarity pKow > -2 , in mass fraction range from 0.10 [µg/kg] to 200 [µg/kg] in a protein rich food sample (food triangle categories 4, 8, 9, e.g. animal tissue, AOAC International food triangle [6]).

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ACRONYMS

CCQM	Consultative Committee for Amount of Substance: Metrology in Chemistry and Biology
CMC	Calibration and Measurement Capability
CRM	certified reference material
CV	coefficient of variation, expressed in %: CV = $100 \cdot s/\bar{x}$
DI	designated institute
DoE	degrees of equivalence
HPLC-DAD	high pressure liquid chromatography with diode array detection
LC-HRMS	liquid chromatography with high-resolution mass spectrometry detection
LC-MS	liquid chromatography with mass spectrometry detection
LC-MS/MS	liquid chromatography with tandem mass spectrometry detection
ID	isotope dilution
IDMS	isotope dilution mass spectrometry
KC	Key Comparison
KCRV	Key Comparison Reference Value
LC	liquid chromatography
MADe	median absolute deviation from the median (MAD)-based estimate of s: MADe = $1.4826 \cdot$MAD, where MAD = median($\lvert x_i$-median(x_i)$\rvert$)
MNZ	metronidazole
MRM	multiple reaction monitoring
NMI	national metrology institute
NMR	nuclear magnetic resonance spectroscopy
OAWG	Organic Analysis Working Group
pKow, logP	logarithm of the octanol-water partition coefficient
pka	acid dissociation constant
PSE	pressurized solvent extraction
qNMR	quantitative nuclear magnetic resonance spectroscopy
QuEChERS	“Quick, Easy, Cheap, Effective, Rugged, Safe” liquid/solid extraction
RMP	Reference Measurement Procedure
SIM	selected ion monitoring
SPE	solid phase extraction
SRM	Selected reaction monitoring

SYMBOLS

d_i	degree of equivalence: x_i - KCRV
$\%d_i$	percent relative degree of equivalence: $100 \cdot d_i$/KCRV
k	coverage factor: $U(x) = k \cdot u(x)$
n	number of quantity values in a series of quantity values
s	standard deviation of a series of quantity values: $s = \sqrt{\sum_{i=1}^{n}(x_i - \bar{x})^2/(n-1)}$
t_s	Student's *t*-distribution expansion factor
$u(x_i)$	standard uncertainty of quantity value x_i
$\bar{u}(x)$	pooled uncertainty: $\bar{u}(x) = \sqrt{\sum_{i=1}^{n} u^2(x_i)/n}$
$U(x)$	expanded uncertainty
$U_{95}(x)$	expanded uncertainty defined such that $x \pm U_{95}(x)$ is asserted to include the true value of the quantity with an approximate 95 % level of confidence
$U_{k=2}(x)$	expanded uncertainty defined as $U_{k=2}(x) = 2 \cdot u(x)$
x	a quantity value
x_i	the i^{th} member of a series of quantity values
$\bar{x}$	mean of a series of quantity values: $\bar{x} = \sum_{i=1}^{n} x_i/n$

INTRODUCTION

Nitroimidazoles are a group of synthetic chemicals derived from azomycin (2-nitroimidazole), a compound which was first isolated from a Streptomyces species in the 1950s [1]. Many nitroimidazoles exhibit anthelmintic properties and are also potent antibiotics and coccidiostats. The most important nitroimidazoles from a pharmaceutical perspective are metronidazole and dimetridazole. However, due to their proven genotoxic and suspected carcinogenic properties no acceptable daily intake (ADI) [2] can be derived, which means that the setting of a maximum residue level (MRL) in food in accordance with Reg. (EC) No. 470/2009 [3] is not possible. Dimetridazole and metronidazole are therefore included in Table 2 of Commission Regulation (EU) No. 37/2010 [4] as prohibited compounds and hence any confirmed presence in food samples is considered a non-compliance with EU food legislation. Also in many countries worldwide nitroimidazoles are banned from use in animals from which products for human consumption are derived. For muscle, the European Reference Laboratory (EURL) Berlin recommends 1 µg/kg as the minimum method performance requirement (MMPR) [5] for the detection of nitroimidazole residues.

At the CCQM Organic Analysis Working Group (OAWG) Meetings in 2021 suggestions for Track A comparisons for polar analytes in a protein rich matrix were discussed according to the OAWG multi-annual strategy plan for the support of existing CMC claims (e.g. based on CCQM-K85 and CCQM-K141). Finally, it was decided to start a study with metronidazole in pig muscle as representative for this purpose. This study helps to support core competencies in the area of analytes with a molecular mass range of 100 g/mol to 500 g/mol, of analytes having high polarity pKow > -2 in a mass fraction range from 0.10 [µg/kg] to 200 [µg/kg] in a protein rich food sample (food triangle categories 4, 8, 9, e.g. animal tissue, AOAC International food triangle [6]).

As a Track A comparison, it was expected that all NMIs or DIs who have, or expect to have services related to the capabilities related to the “How far does the light shine” statement for this key comparison would participate.

TIMELINE

Table 1: Adjusted timeline for CCQM-K180.

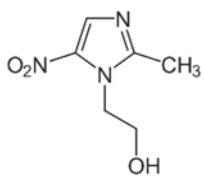
November 2021 – January 2022	Preparation of sample
January/February 2022	Homogeneity testing
February/March/August 2022 and ongoing	Stability testing
August 2022	Call for participation to OAWG members
September 2022	Deadline for registration
February 2023	Dispatch of samples
1 September 2023	Initial deadline for submission of results
15 September 2023	Extended deadline for submission of results
September/October 2023	Preliminary Discussion of results
January 2024	Draft A report distributed to OAWG
October 2024	Follow-up discussion on technical aspects with participants – KCRV calculation options
December 2024	Final feedback from participants, withdrawal of single results (VNIIM and SASO-NMCC)
April 2025	Discussion of KCRV calculation options based on the final data set
October 2025	Draft B report distributed to OAWG
November 2025	Final report approved by OAWG

MEASURAND

The measurand of this study is the mass fraction of free metronidazole in porcine muscle on a dry mass basis in µg/kg. Physico-chemical information for metronidazole is provided in Table 2. The sample material also contains dimetridazole, as well as metabolites of metronidazole (metronidazole hydroxide) and dimetridazole (2-hydroxymethyl-1-methyl-5-nitro-1H-imidazole (HMMNI)).

Table 2: Selected physico-chemical parameters of metronidazole [7].

Name	metronidazole
Abbreviation	MNZ
IUPAC name	2-(2-methyl-5-nitro-1H-imidazol-1-yl)ethanol
CAS	443-48-1
Sum formula	C6H9N3O3
Molecular weight	171.15 g/mol
logP	-0.1
pKa	2.5



STUDY MATERIALS

The matrix porcine muscle is a matrix rich in protein and partly also rich in fat and therefore falls into category 4, 8, or 9 of the AOAC International food triangle [6], depending on the individual muscle composition. For this study, materials produced in an animal study initiated by the BVL veterinarian in 2021, as well as blank porcine muscle samples purchased from the retail market, were used. The animal study was conducted on two 5-month old male, castrated rearing pigs (body weight 90 kg) which were treated with the nitroimidazole-containing pharmaceutical preparations given below.

Metronidazole: Eradia suspension 125 mg/mL (Virbac Switzerland AG, Glattbrugg, Switzerland), 2x/day per oral over the course of 3 days, 18 hours waiting time before slaughter

Dimetridazole: chevi-col powder 400 mg/g (Chevita GmbH, Pfaffenhofen, Germany), 2x/day per oral over the course of 3 days, 18 hours waiting time before slaughter

The study material with the respective desired concentration in the range 0.1-200 µg/kg was produced from the incurred material and the blank material as a total batch from a number of sub-samples. All sub-samples were combined and homogenised under addition of dry ice. Subsequently, the sample material was lyophilised, passed through a sieve and homogenised again ("Grindomix"). Finally, individual portions of around 4.3 g lyophilised material were weighed into 50 mL plastic centrifuge tubes. The material was stored at -20 °C.

The aliquots contain 4.3 – 4.4 g of lyophilized muscle corresponding to approximately 16 g fresh muscle (1 g fresh muscle = 0.27 g lyophilisate).

Reconstitution of a lyophilised sample is achieved by adding the amount of water lost during lyophilisation. The amount of lyophilised sample to be reconstituted shall not be smaller than the amount used in the homogeneity study (0.540 g). The vessels should be at room temperature before reconstitution, as the samples are hygroscopic.
The recommended method for the reconstitution of lyophilised samples is:

- Shake the sample by hand or agitate using a vortex-mixer or an overhead shaker
- Weigh out an adequate amount of lyophilised material
- Add respective amount of water as ultra-pure water and vortex-mix
- If vortex-mixing does not yield a visually uniform sample, treat the sample in an ultrasonic bath or homogenise using an overhead shaker for at least 15 min at ambient temperature.

Ideally, the reconstituted sample is stored at +4 °C for 1 hour before the analysis procedure is begun.

Recommended Minimum Sample Amount

Participants received 4 samples of P220048. The samples were to be stored at -20 °C or below under the absence of light. Before opening, the samples should be allowed to equilibrate to room temperature. As the minimum sample intake 0.540 g lyophilised muscle (corresponding to 2 g of reconstituted sample) is recommended.

Dry Mass Determination

Participants were also requested to carry out dry mass correction. The determination of dry mass correction should be conducted with a recommended size of at least 0.3 g. The test sample portion taken for dry mass correction should be placed over anhydrous calcium sulphate (DRIERITE®) in a desiccator at room temperature for a minimum of 20 days until a constant mass is reached. Dry mass correction should be carried out at the same time as the test sample portion is to be analyzed in the same package of sample.

Homogeneity Assessment of the Study Material

Homogeneity of the incurred samples was assessed in accordance with ISO 13528:2020 Annex B.2.2 [8]. For this method a suitable number of test items is chosen at random and analysed in duplicate. Subsequently, the standard deviation between the samples s_s and the analytical standard deviation s_w are calculated from the determined analyte contents. The standard deviation between the samples s_s is negligible if the sum of mean squares between the samples is smaller than the sum of the mean squares of the repeated analysis.

In order to assess the sample homogeneity for this study, a number of test items of the sample P220048 were chosen at random (Table 3). The samples were analysed in accordance with the validated EURL method NIIM_009 (available upon request).

In order to control for any possibly undetected heterogeneity, an F-test was also carried out. The result of the F-test was that no significant heterogeneity was detected (Table 4). Graphical representations of the results from homogeneity analysis are given in Figure 1.

Table 3: Samples randomly selected for the homogeneity assessment.

Sample	Test item production numbers
P220048	003, 036, 078, 092, 122, 146, 163, 199, 210, 229, 239

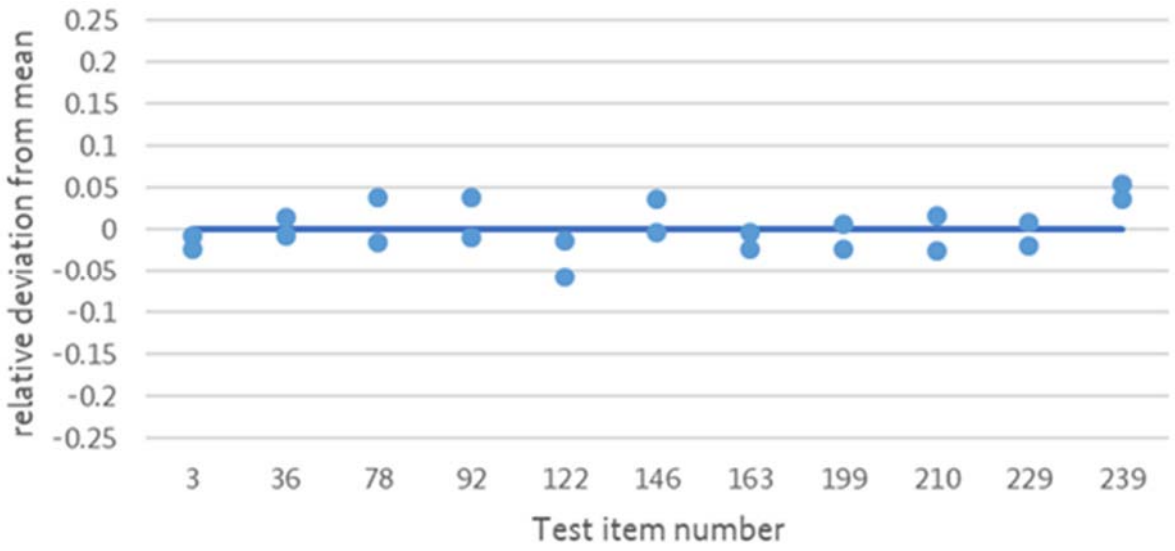


Figure 1: Results of the homogeneity assessment for P220048 (Metronidazole in pig muscle; sample size 0.540 g lyophilized pig muscle).

Table 4: Results of ANOVA homogeneity assessment for metronidazole in muscle.

ANOVA Estimate	**metronidazole**
Within-sample, s_w	1.2 %
Between-sample, s_s	2.5 %
Total analytical variability, CV	2.8 %
$F_{crit.}$ ($F_{0.05,10,22}$)/F	4.35 / 0.10
p-value (Probability of falsely rejecting the hypothesis that all samples have the same concentration)	0.75

Stability Assessment of Study Material

The short-term stability of the material was assessed in a stability study over the course of four weeks. This time period was chosen in order to assure sample stability during shipment, preparation, and analysis. Individually packaged aliquots of P220048 were stored at -80 °C (reference), -25 °C, +4 °C, and room temperature (dark). For every selected temperature-time combination two samples were analysed in duplicate in accordance with EURL method NIIM_009 (available upon request). The results of the stability study are displayed in Figure 2. The stability study is ongoing and more data will be provided once available (Table 5).

Considering the data obtained on the samples P220048, as well as results from previous stability studies on nitroimidazoles in muscle it was concluded that the samples were sufficiently stable for the purpose of this key comparison. No degradation is to be expected over the course of the study.

Table 5: Stability study outline: temperature-time combinations to be considered for the stability study (data are currently available until 6 months of storage).

Duration	**-20 °C**	**+4 °C**	**Room temperature (dark)**
28 days	X	X	X
6 months	X	X	X
2 years	X	X	X
3 years	X	X	
5 years	X	X	

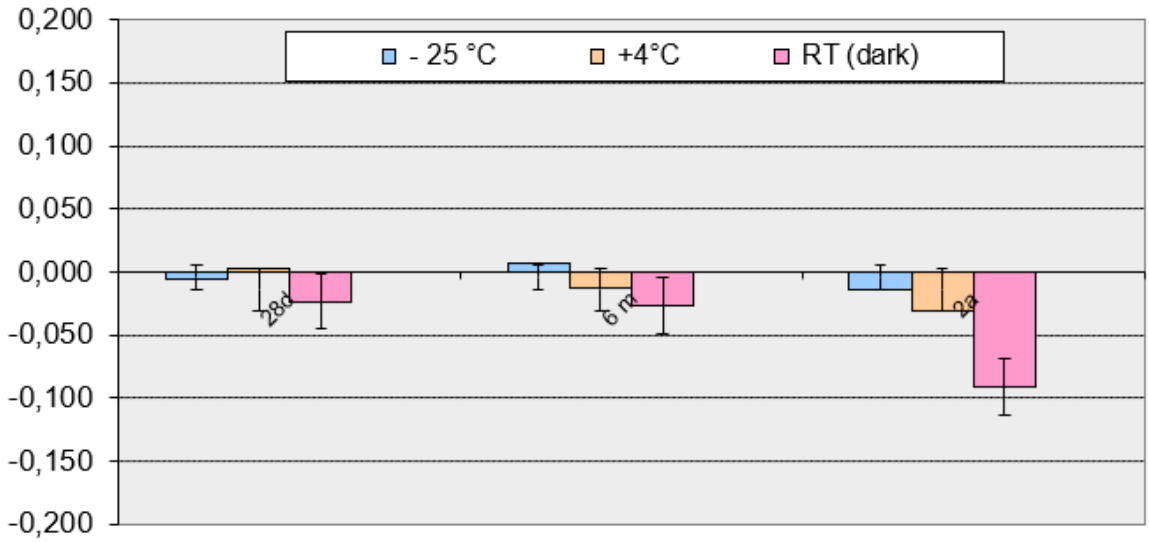


Figure 2: Relative deviation of MNZ concentration from the reference concentration (-80 °C) in sample P220048 after 28 days, 6 months and 2 years. Error bars represent the standard deviation of the multiple analyses.

PARTICIPANTS, INSTRUCTIONS AND SAMPLE DISTRIBUTION

The call for participation was made in June 2022 during the OAWG meeting with the intent to distribute samples in September 2022 and receive results in February 2023. The shipment date had to be adjusted in order to be able to provide the participants with an MNZ CRM supplied by INMETRO. The deadline for result submission was adjusted in response to requests by participants. See Table 1 for study timeline. Appendix A reproduces the Call for Participation; Appendix B reproduces the study Protocol. Table 6 lists the institutions that registered for CCQM-K180.

Table 6: Institutions registered for CCQM-K180.

NMI or DI	Code	Country	Contact
Bundesamt für Verbraucherschutz und Lebensmittelsicherheit	BVL	Germany	Ferial Tadjine, Katrin Heider
Centro Nacional de Metrología	CENAM	Mexico	Laura Regalado Contreras, Claudia Marcela Salazar Arzate, Mariana Arce Osuna, Jannet Hernandez
Chemical Metrology Laboratory	EXHM	Greece	Elias Kakoulides, E. Skotidaki, E. Stathoudaki, V. Schoina
Government Laboratory of Hong Kong	GLHK	Hong Kong	Ng Chi-Shing, Shiu Hoi Yan Fiona
Health Sciences Authority	HSA	Singapore	Cheow Pui Sze
Instituto Nacional de Metrología de Colombia	INM Colombia	Colombia	Andrés Sebastián Salinas Trujillo
National Institute of Metrology, Quality and Technology	INMETRO	Brazil	Eliane C. Rego
Institut National de Recherche et d'Analyse Physico-Chimique	INRAP	Tunisia	Hanen Klich
Korea Research Institute of Standard and Science	KRISS	Republic of Korea	Seok-Won Hyung
LGC Ltd.	LGC	United Kingdom	Christopher Hopley
National Institute of Metrology China	NIM China	China	Xiquin Li
The National Institute of Metrology Thailand	NIMT	Thailand	Kittiya Shearman
National Metrology Institute of South Africa	NMI South Africa	South Africa	Caitlin Swiegelaar
National Measurement Institute Australia	NMIA	Australia	Lesley Johnston
National Research Council of Canada	NRC	Canada	Garnet McRae
National Measurement and Calibration Center of Saudi-Arabia	SASO-NMCC	Saudi-Arabia	Abdulrahman R. Al-Askar

TÜBİTAK Gebze Yerleşkesi	UME	Turkey	Mine Bilsel
D.I. Mendeleyev Institute for Metrology	VNIIM	Russia	Alena Mikheeva

Participants received 4 samples of P220048 shipped on cooling packs. Upon request participants were also provided with one unit of INMETRO 8365 metronidazole CRM.

NMI South Africa had to withdraw from participation because their import permit for the samples expired and could not be re-obtained.

RESULTS

The participants were asked to report a single result for the mass fraction of free metronidazole. The results were to be provided based on a single test item in µg free metronidazole per kg of dry sample mass. The respective standard uncertainties and expanded uncertainties (95 % level of confidence) were also to be reported.

In addition to the quantitative results, participants were asked to provide information on the measurement procedure (extraction, clean-up, column and chromatographic conditions, quantification approach), calibration standards, internal standards, any quality control materials, number of replicates, the means of calculation of the results. Furthermore, any details regarding the estimation of measurement uncertainty were to be included, generally any information relevant to the core competencies that the participants in this study were seeking to demonstrate.

Results for the study CCQM-K180 were received from all 17 institutions who registered for the study and received study material.

Employed Calibration Materials

Participants established the metrological traceability of their results by using certified reference materials (CRMs) with stated traceability and/or commercially available high purity materials for which they determined the purity. Table 7 lists the CRMs of metronidazole neat substance which were reported by the participants as the traceable reference. Some participants also reported the application of matrix CRM, information on which is given in Table 8. Any internal standards which were employed by the participants are given in Table 9.

Table 7: Metronidazole calibration standards used as source of traceability for MNZ in CCQM-K180.

Item No.	Certified value	Provider	Reference	Used by	In-house purity assignment of CRM
8365	998.9 ± 1.1 mg/g	INMETRO	[9]	BVL, EXHM, GLHK, INM Colombia, INMETRO, INRAP, KRISS, LGC, NMIA[1], NRC, SASO-NMCC, NIMT[2]	EXHM (mass balance and qNMR 998.5 mg/g ± 1.0 mg/g)
GBW(E)060908	99.9 % ± 0.2 %	NIM China	[10]	HSA, NIM China, NIMT	
	Not certified	Hubei Hongyuan Pharmaceutical		VNIIM	VNIIM (mass balance 99.3 % ± 0.3 %)
M3761	Not certified	Supelco		UME	UME (qNMR 99.5 % ± 0.2 %)
PHR1052 Lot LRAC6503	99.9 % ± 0.2 %	Supelco		CENAM	CENAM (99.92 % ± 0.01 %)
1442009 Lot JOC316	Not certified	United States Pharmacopeia		INMETRO[3]	INMETRO (qNMR 1000.33 mg/g ± 1.86 mg/g)

Table 8: Metronidazole matrix CRM employed by the participants.

Item	Item No.	Certified value	Provider	Reference	Used by
Nitroimidazoles in porcine muscle	ERM-BB124	(1.93±0.15) µg/kg	JRC	[11]	BVL, CENAM, EXHM, HSA, KRISS, NIM China, NMIA, NRC, UME, VNIIM

Table 9: Internal standards for metronidazole employed by the participants.

Item	Used by
Methomyl	INM Colombia
Metronidazole-2-^{13}C-1,3-$^{15}N_2$	BVL,CENAM, GLHK, HSA, INMETRO, KRISS, LGC, NIM China, NIMT, NMIA, VNIIM
Metronidazole-d4 (ethylene)	INRAP, UME
Metronidazole-d3 (methyl)	EXHM, NRC

[1] CRM was used for quantification, the calibrant provided by NIM China only for comparison
[2] CRM was only used for comparison to the calibrant provided by NIM China
[3] used exclusively for preparation of control samples

Analytical Methods

The participants were free in their choice of analytical method. The homogeneity and stability assessment had been carried out using the EURL method NIIM_009 which was also provided to the participants for information purposes. This method comprises the following basic steps:

- sample reconstitution
- addition of internal standard
- addition of buffer and protease
- Extrelut clean-up
- evaporation to dryness and reconstitution
- LC-MS/MS measurement

Only a single participant (BVL) provided results which were obtained from a procedure including an enzymatic hydrolysis. EXHM reported that an alternative method including an enzymatic hydrolysis had been tested.

For the extraction of the sample the participants often added water, followed by acetonitrile (5 participants, 29 %) or ethyl acetate (10 participants, 59 %). In some cases the organic solvents were acidified. The sample mixture was then subjected to vortexing, shaking or ultrasonication for one minute to two hours. Several participants extracted the sample repeatedly (9 participants, 53 %).

Eight participants (47 %) included a defatting step using hexane. About half of the reported analytical methods also included an Extrelut or (d)SPE clean-up (9 participants, 53 %) of the crude extract. Four participants (24 %) did not follow up the extraction with any kind of clean-up except filtration.

All of the participants measured the final extracts by LC-MS. In one case, a 2D-LC was applied (NMIA). In another the measurement was conducted using an LC coupled to an HRMS device (UME). The remainder of the participants employed either ion trap or triple quadrupole instruments. Details on the analytical methods employed by the participants are given in APPENDIX F: Summary of Participants' Analytical Information.

Participant Results

The results for CCQM-K180 for the determination of metronidazole, including the moisture of the sample, are given in Table 10 and presented graphically in Figure 3 and Figure 4.

Table 10: Results reported for the mass fraction of metronidazole and the moisture of the sample P220048.

	%	Metronidazole, µg/kg			
NMI	Moisture	x	$u(x)$	k	$U(x)$
BVL	1.58	8.19	0.59	2	1.18
CENAM	0.115	8.79	0.40	2	0.80
EXHM	1.158	8.128	0.281	2.13	0.598
GLHK	2.35	8.78	0.31	2	0.63
HSA	1.85	8.16	0.42	2	0.85
INM Colombia	0.80	8.02	0.22	2	0.44
INMETRO	2.07	8.61	0.19	2	0.39
INRAP	1.03	7.68	0.29	2	0.57
KRISS	1.2	8.71	0.10	2.31	0.24
LGC	0.996	8.55	0.15	2	0.30
NIM China	1.63	8.13	0.20	2	0.40
NIMT	1.738	8.33	0.175	2	0.35
NMIA	1.3	8.42	0.064	2.02	0.13
NRC	1.13	8.45	0.30	2	0.60
SASO-NMCC	0.57	4.76	0.12	2	0.25
UME	1.3	9.16	0.52	2	1.03
VNIIM	1.2	10.48	0.47	2	0.94
n	17	17			
$\bar{x}$	1.29	8.31			
s	0.548	1.10			
$\bar{u}$		0.319			
CV	42.3	13.2			

n = number of results included in summary statistics; $\bar{x}$ = mean; s = standard deviation; CV = $100 \cdot s/\bar{x}$; $\bar{u} = \sqrt{\sum_i^n u^2(x_i)/n}$, the "average" reported uncertainty

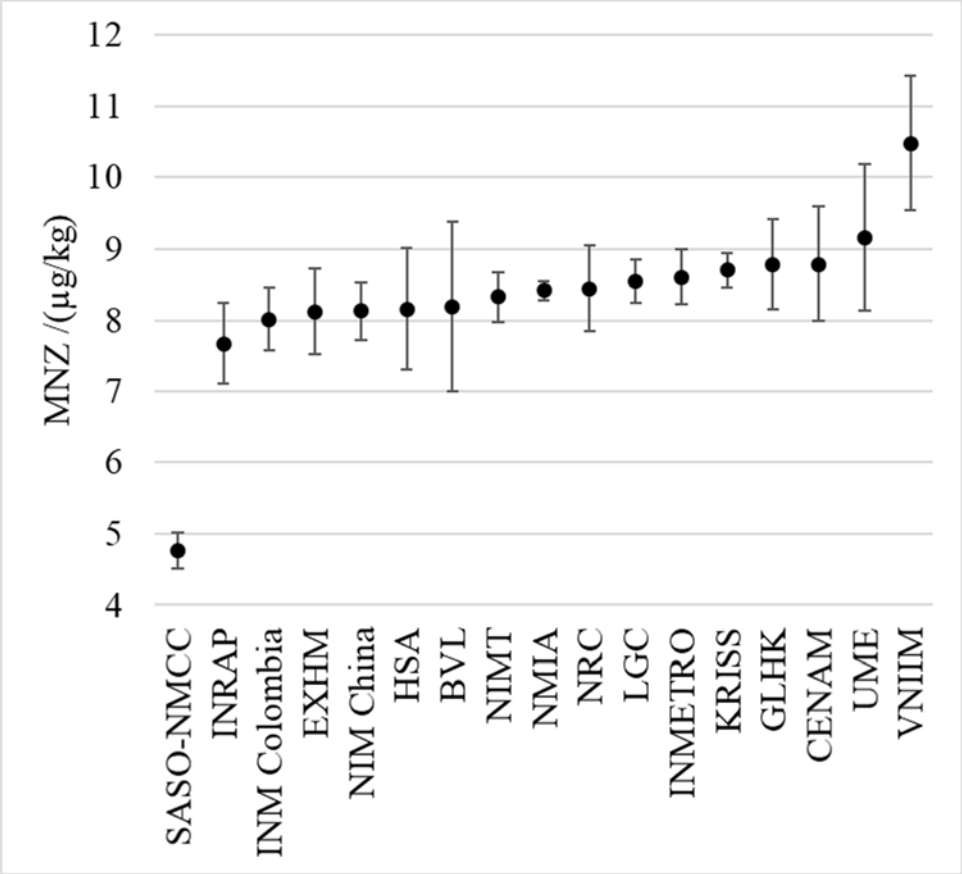


Figure 3: Dot-and-Bar plot of the results reported for the mass fraction of metronidazole in the sample P220048 in µg/kg dry mass. The dots represent the reported mean values, x, the bars their 95 % expanded uncertainties, U(x).

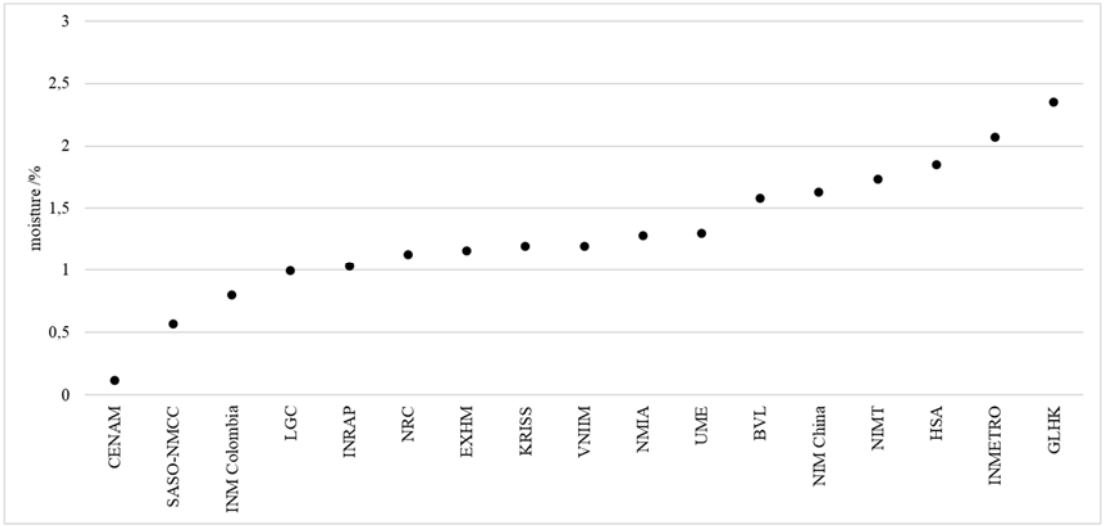


Figure 4: Plot of the results reported for the moisture in the sample P220048 in %. The dots represent the reported mean values, x. Uncertainties were not provided for this measurand.

An initial visual inspection of the results indicated that the result submitted by SASO-NMCC differed significantly from the remainder of the data set. Taking into account the expanded uncertainties SASO-NMCC's result is significantly different from the remaining results. Also, the result submitted by VNIIM just overlaps with CENAM's result. A Grubbs' test on the measurement results for MNZ at the 0.99 significance level marked the results submitted by SASO-NMCC and VNIIM as outliers. This is also represented in the kernel-density estimations for the complete data set which exhibits three modes (Figure 6), the data set with the results submitted by SASO-NMCC removed (Figure 7), which is bi-modal, and the Grubbs' outlier removed data set which is unimodal (Figure 8). Judging from the method details submitted to the study organizer, no evidence for a correlation between the analytical method employed by these two participants and their results could be detected.

The standard uncertainties reported by the participants for MNZ vary in a wide range between 0.76 % (NMIA) and 7.2 % (BVL) (percentages given in relation to the participants' measurement result). Taking into account all submitted results, a χ^2 test indicates that the dataset is inconsistent ($\chi^2_{observed} >> \chi^2_{critical}$ and $\chi^2_{observed} > (n-1)$). Upon exclusion of SASO-NMCC's and VNIIM's results from the χ^2 test, the test results still suggest inconsistent data. In this edited data set INRAP's and KRISS' contribution to $\chi^2_{observed}$ are the largest, indicating that their measurement results differ noticeably from the uncertainty-weighted mean or their reported measurement uncertainty is smaller than expected. All of the above is also indicated by a plot relating the individual results to the median and the individual reported standard uncertainties (Figure 5).

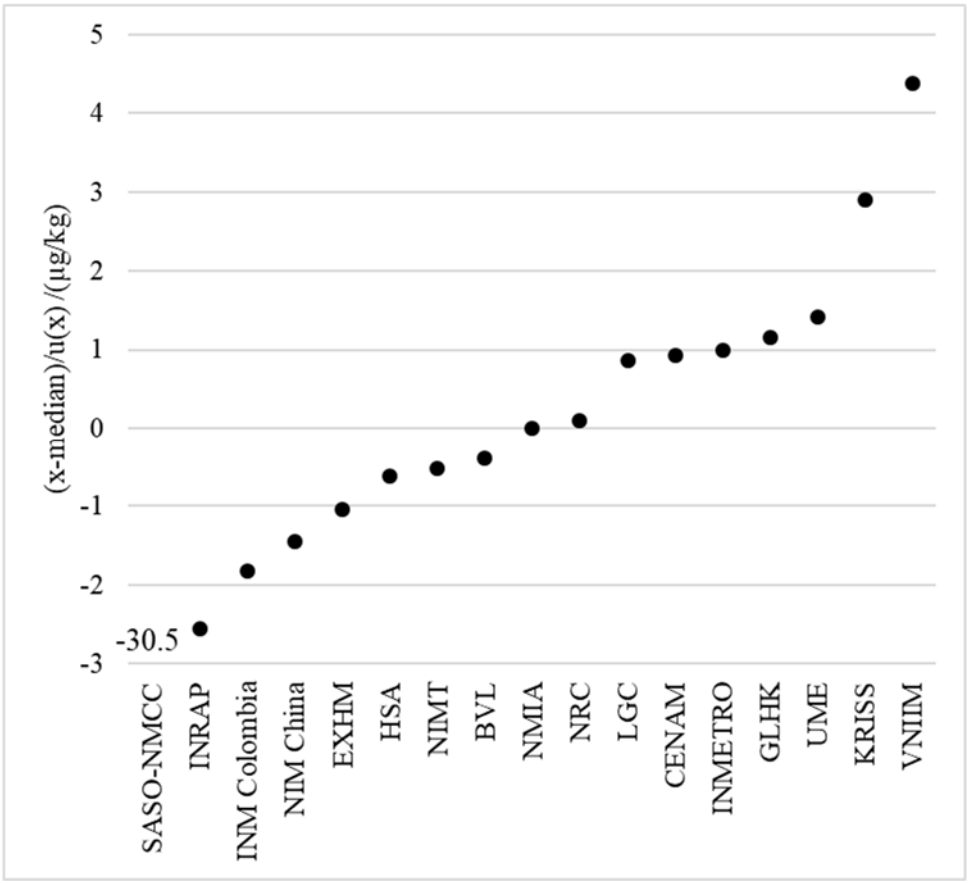


Figure 5: Plot of the results reported for the mass fraction of metronidazole in the sample P220048 in µg/kg dry mass corrected for the median and related to the respective standard uncertainties.

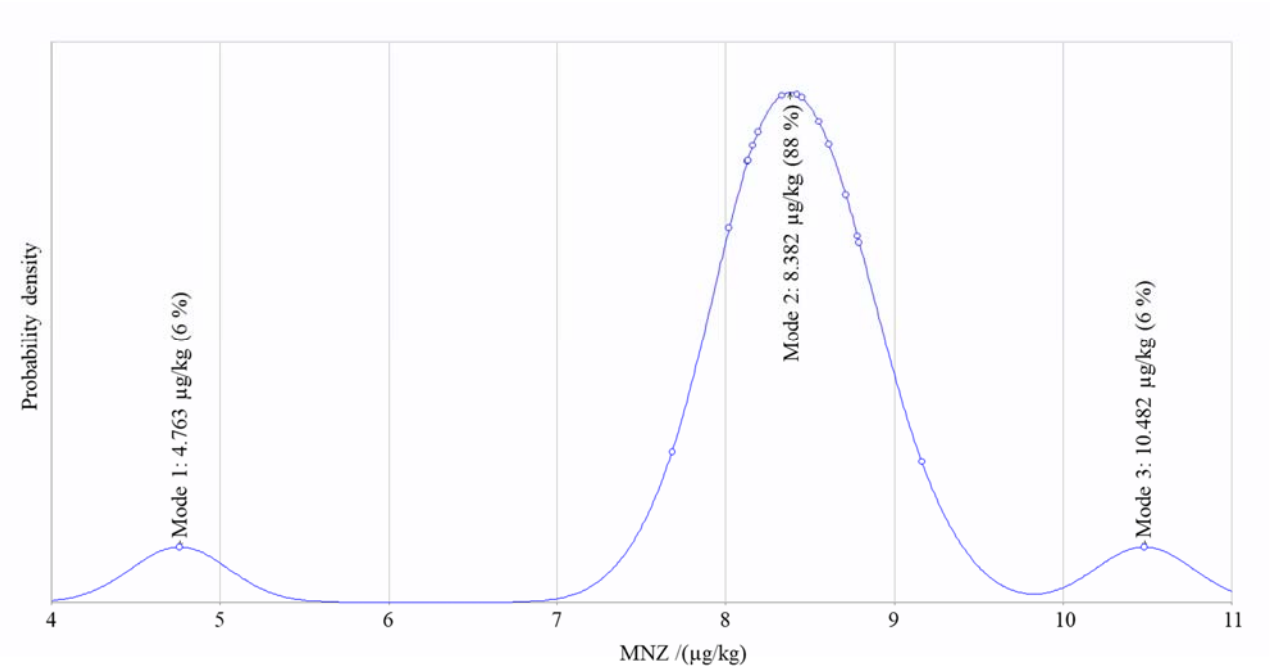


Figure 6: Kernel-density estimation for the complete data set (bandwidth 0.9s*/n^0.2).

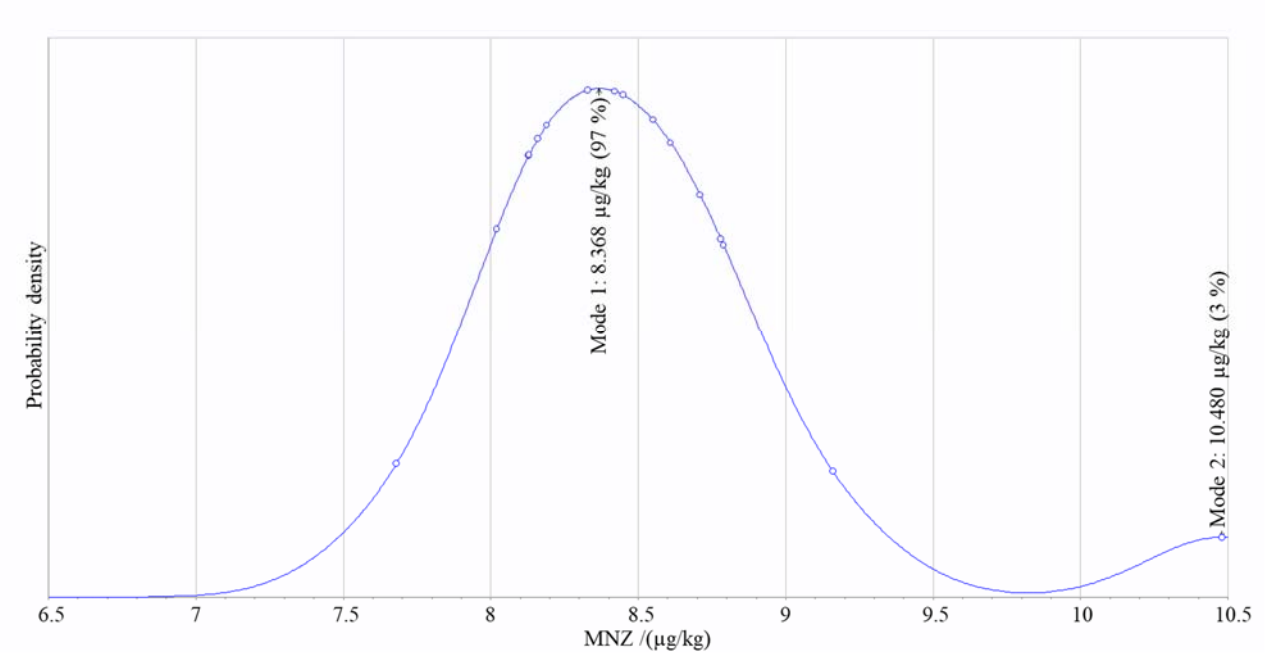


Figure 7: Kernel-density estimation for the edited data set A (results submitted by SASO-NMCC not considered removed) (bandwidth 0.9s*/n^0.2).

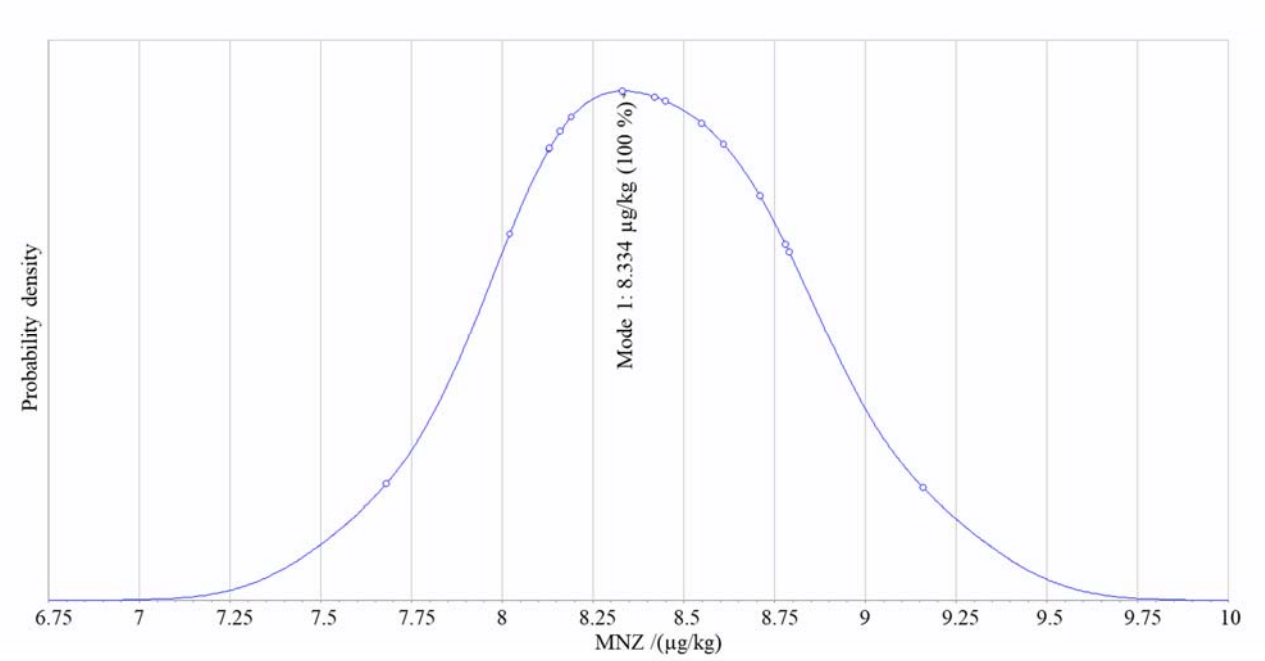


Figure 8: Kernel-density estimation for the edited data set B (results submitted by SASO-NMCC and VNIIM not considered) (bandwidth 0.9s*/n^0.2).

Withdrawal of Results

The results for CCQM-K180 were discussed at the OAWG meeting in April 2024 and during a subsequent discussion of technical aspects with participants in October 2024. Following these discussions and internal follow-up discussions, SASO-NMCC and VNIIM are withdrawing their values for the KCRV calculation.

The reason for SASO-NMCC's withdrawal of the values was the discovery of extraction difficulties that led to lower values. VNIIM conducted extensive follow-up investigations with additional sample material (additional sample aliquots were sent to VNIIM). The results of their follow-up investigation showed that, based on their extraction conditions, a triple extraction process led to significantly higher values than a double extraction process, even when using an isotopically labeled internal standard (after 2 extractions – 8.84 ± 0.36 ug/kg, after 3 extractions – 9.50 ± 0.40 ug/kg). However, these results were below the originally reported value (10.48 ± 0.47 ug/kg), and VNIIM could not identify a reason for this. It therefore withdrew its results from the KCRV calculation.

KEY COMPARISON REFERENCE VALUE (KCRV)

After withdrawal of two results 15 results were taken into account for the KCRV calculation. For the assignment of a suitable KCRV the authors referred to the decision tree given in Figure 9. First the presence of extreme values for x and u(x) should be inspected. The results of the χ^2 test and the Grubbs' outlier test described in the previous section indicate that there were still minor anomalies (decision tree node 1a). These findings were discussed during the OAWG meeting in 2024 and in April 2025. No indication of invalidity of these seemingly anomalous results was found (decision tree node 1b). It was therefore decided to derive the KCRV based on all 15 remaining submitted results. With n=15 the study is considered to be under control (decision tree node 2) and the uncertainties were considered generally believable (decision tree node 3). The data set now contains no outliers (decision tree node 4) and with n>4 it is recommended to apply the DerSimonian-Laird procedure for the value assignment. The study participants agreed on this KCRV calculation during the April 2025 OAWG meeting. The calculations were conducted with the NIST Consensus Builder [14] using the method "DerSimonian-Laird"[4]. This method also estimates the dark uncertainty τ which was 0.18 µg/kg.

Table 11: Key Comparison Reference Value for the valid data set.

		Metronidazole, µg/kg				
Estimator	u?[a]	X	$u(X)$	n	k	$U_{95}(X)$[b]
DerSimonian-Laird	YES	8.41	0.08	15	2.15	0.16

a) Does the estimator utilize the information in the reported uncertainties?
b) $U_{95}(X) = t_s \cdot u(X)$, where t_s is the appropriate two-tailed Student's t critical value for 95 % coverage.

Other estimators ("Laplace Rem", "HB Rem") give nearly identical KCVR with a slightly higher dark uncertainty τ of 0.02 µg/kg.

[4] NIST Consensus Builder (version 10.01.2024), - DerSimonian-Laird without mod. Knapp-Hartung adjustment, without parametric bootstrap for uncertainty evaluation, without entering Degrees of Freedom; DoEs conforming to MRA; Bootstrap replicates: 10000; 95 % coverage interval ranges from 8.26-8.56.

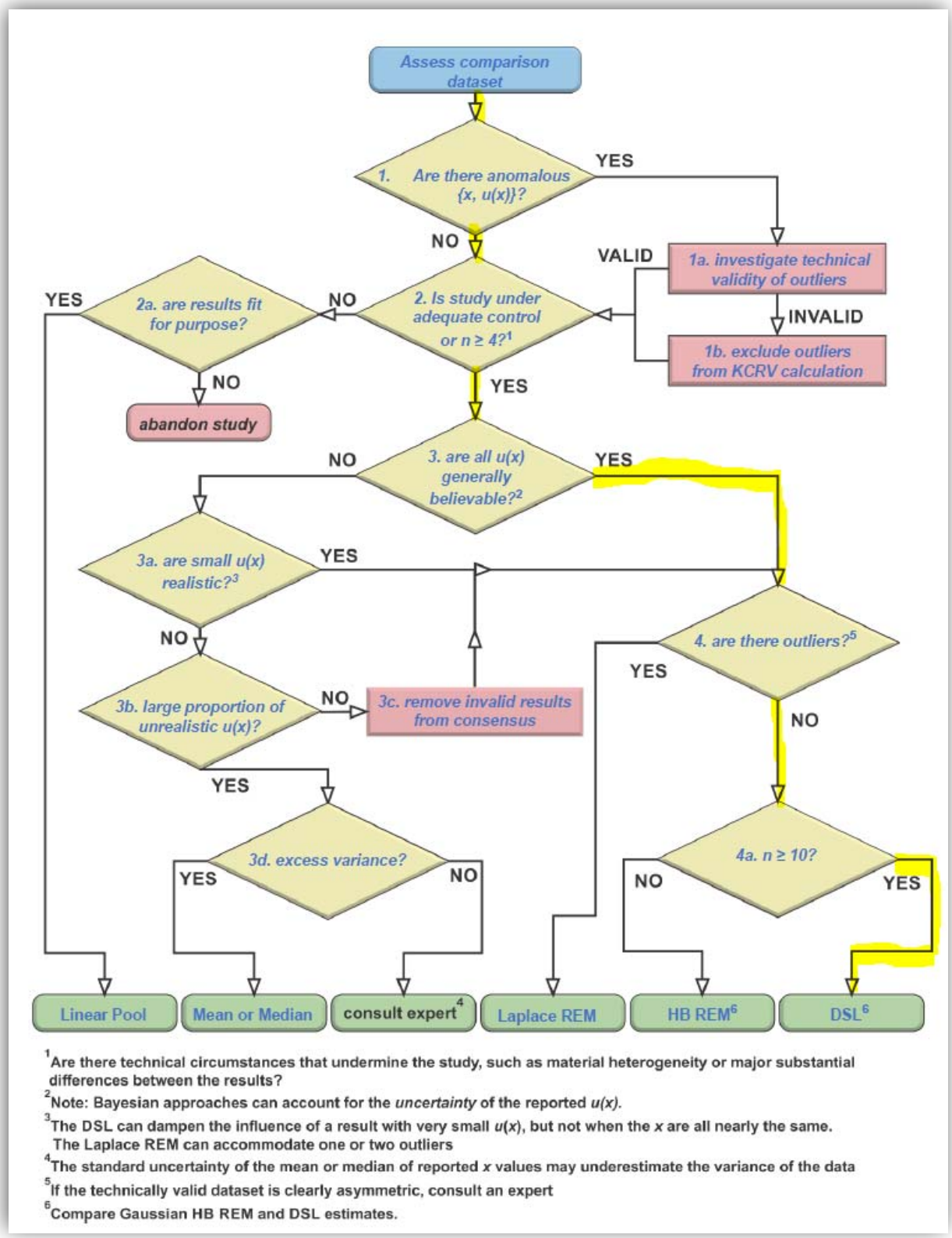


Figure 9: Decision tree for KCRV selection, reproduced from [13] with the path for the data set of CCQM-OAWG-K180 highlighted in yellow.

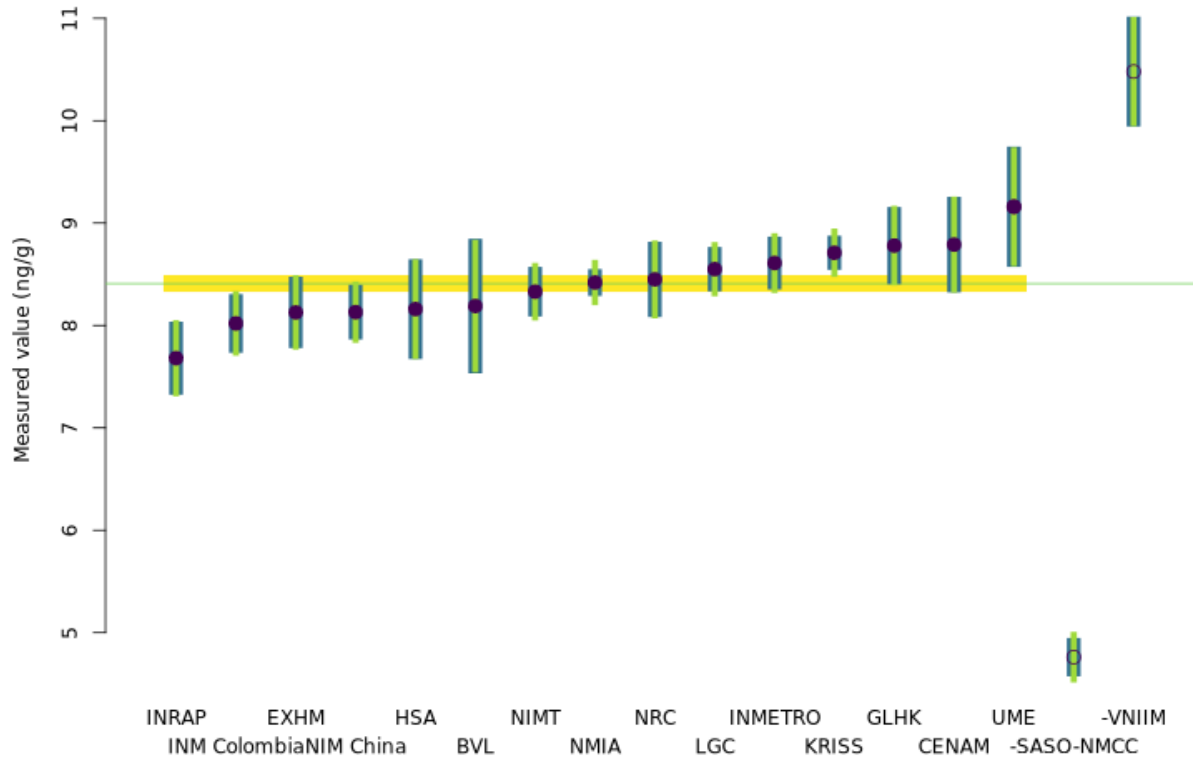


Figure 10: Dot-and-Bar plot of the results reported for the mass fraction of metronidazole in the sample P220048 in µg/kg dry mass. The dots represent the reported mean values, x, the blue bars their standard uncertainties, u(x), the green bars their standard uncertainties combined with the dark uncertainty τ. The DerSimonian Laird estimator is given as a green line, the yellow area reflects the associated uncertainty u. The calculated upper and lower limit of $U(x)_{95}$ are 8.56 and 8.26 respectively.

DEGREES OF EQUIVALENCE (DoE)

The unilateral degrees of equivalence (DoE) are a measure of agreement between the KCRV and the individual measurement results. They are expressed as the deviation of the measurement result from the KCRV and the uncertainty of this deviation at 95 % confidence level (DoE U_{95}). Participant results are considered to be in agreement with the KCRV if the DoE U_{95} exceeds the DoE's absolute value. For study K180 this is the case for all participants except SASO-NMCC and VNIIM (Table 12, Figure 11) which were also found to be Grubb's outliers and for INRAP.

Table 12: Degrees of equivalence (DoE), uncertainty of the DoE at 95 % confidence level (DoE U95), upper and lower value of credible DoE interval.

Laboratory	DoE	DoE U_{95}	DoE lower	DoE upper
INRAP	-0.73	0.656	-1.39	-0.0762
INM Colombia	-0.39	0.547	-0.937	0.156
EXHM	-0.282	0.651	-0.922	0.377
NIM China	-0.28	0.515	-0.783	0.241
HSA	-0.25	0.883	-1.14	0.63
BVL	-0.22	1.19	-1.41	0.967
NIMT	-0.0797	0.486	-0.562	0.41
NMIA	0.0103	0.376	-0.363	0.388
NRC	0.0403	0.674	-0.642	0.711
LGC	0.14	0.444	-0.309	0.583
INMETRO	0.2	0.507	-0.301	0.712
KRISS	0.3	0.408	-0.107	0.71
GLHK	0.37	0.678	-0.31	1.05
CENAM	0.38	0.838	-0.46	1.22
UME	0.75	1.08	-0.331	1.83
SASO-NMCC	-3.65	0.449	-4.1	-3.2
VNIIM	2.07	0.997	1.07	3.07

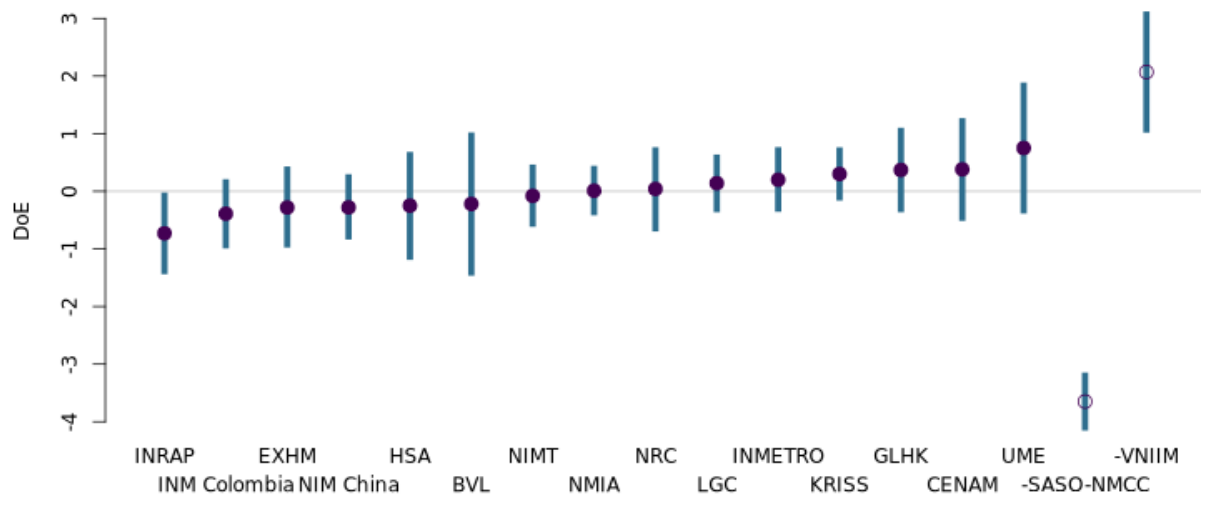


Figure 11: Estimated degrees of equivalence (DoE) and their 95 % coverage interval.

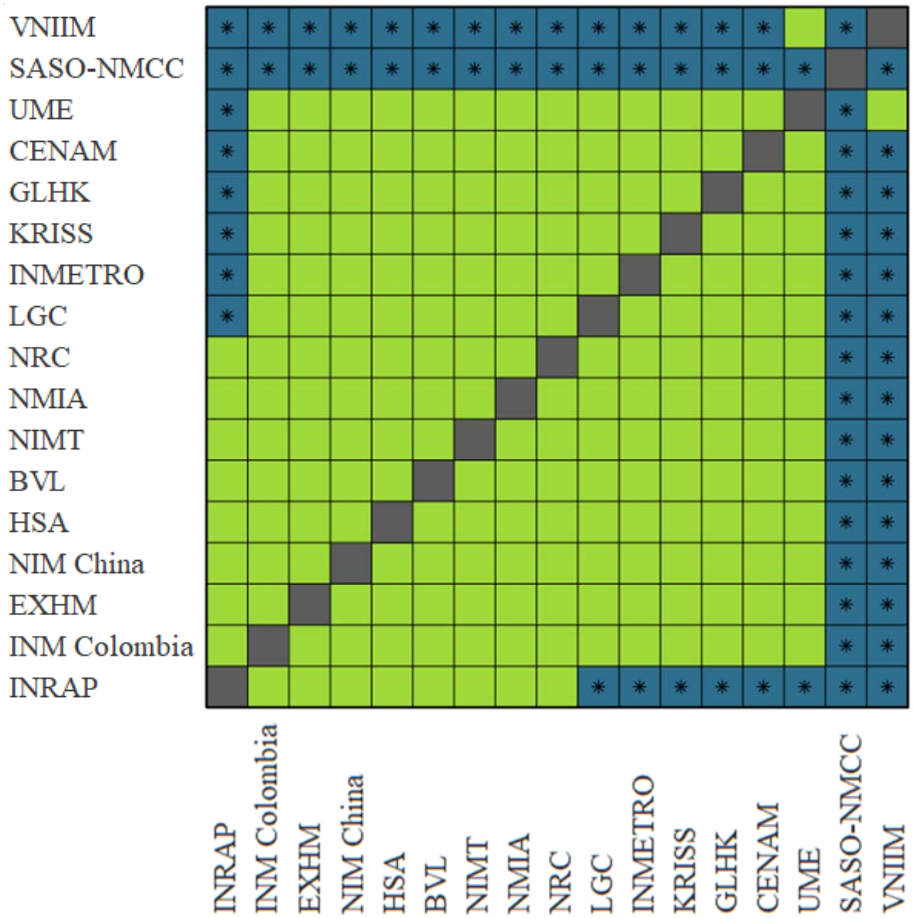


Figure 12: Bilateral degrees of equivalence; blue squares (with black asterisks in the center) indicate results that differ significantly from 0 at 95% coverage. Green squares indicate results that do not differ significantly at 95% coverage. Dark grey squares are space-fillers for results when compared to themselves.

USE OF CCQM-K180 (metronidazole in porcine muscle) IN SUPPORT OF CALIBRATION AND MEASUREMENT CAPABILITY (CMC) CLAIMS

How Far the Light Shines

Successful participation in CCQM-K180 will demonstrate the following measurement capabilities: determination of mass fraction of organic compounds, with molecular mass of 100 g/mol to 500 g/mol, having high polarity $pK_{ow} > -2$ in mass fraction range from 0.1 µg/kg to 200 µg/kg in a protein rich food sample (food triangle categories 4, 8, 9, e.g. animal tissue, AOAC International food triangle [6]).

Furthermore additional measurement capabilities might be demonstrated (if applicable):

- value assignment of primary reference standards (if in-house purity assessment carried out)
- extraction of analytes of interest from the matrix,
- cleanup and separation of analytes of interest from interfering matrix or extract components,
- separation and quantification using gas or liquid chromatographic analytical systems.

Core Competency Statements and CMC support

The core competencies claimed by the participants in CCQM-K180 are given below. The information in these tables is as provided by the participants.

Core competencies demonstrated in CCQM-K180 by BVL

CCQM-K180	***Federal office of consumer protection and food safety***	**Polare analyte in a protein rich matrix – metronidazole in muscle**
Scope of Measurement: : Successful participation in this study may demonstrate participants' capabilities in determining high-polarity analytes (pKow > -2) with molecular mass range from 100 to 500 g/mol at mass fraction levels of 0.1 to 200 µg/kg in a high protein food matrix. This may include demonstration of measurement capabilities such as: (1) value assignment of primary reference standards; (2) extraction of analyte of interest from the matrix; (3) cleanup and separation of analyte of interest from other interfering matrix or extract components; (4) separation and quantification using techniques such as LC-MS/MS, LC-MS, LC-HRMS, GC/MS		
Competency	**✓,×, or N/A**	**Specific Information as Provided by *NMI/DI***
Competencies for Value-Assignment of Calibrant		
Calibrant: Did you use a "highly-pure substance" or calibration solution?	✓	*A highly-pure substance consisting of Metronidazole CRM (MRC 8365.0001a), Mass Fraction: (998.9 ± 1.1) mg/g from Inmetro was used*
Identity verification of analyte(s) in calibration material.	✓	*Retention time, mass spectrometry ion ratios by LC-ESI-MS/MS (Sciex 6500+)*
For calibrants which are a highly-pure substance: Value-Assignment / Purity Assessment method(s).	✓	*Mass balance was the p*urity assessment method for the *MRC (Inmetro)*
For calibrants which are a calibration solution: Value-assignment method(s).	x	-----
Sample Analysis Competencies		
Identification of analyte(s) in sample	✓	*LC-MS/MS precursor ion, daughter ion and ion ratios* *Metronidazole Q1: 172,0 and Q3: 128,1; 82,0; 111,8* *Internal standard: 13C2,15N2-MNZ: Q1 : 176,6 and Q3 : 132,1; 85,9, 114,9*

Extraction of analyte(s) of interest from matrix	✓	*The sample was first reconstituted. For this purpose, 2,70 ± 0,02 g of lyophilised sample were weighted accurately and an appropriate amount of water 7,30 ± 0,02 g was added. 3 x sub-samples of the reconstituted sample were weighted accurately (1,0 ± 0,02 g) in IKA tubes and 15,47 ± 0,75 mg (20 µl) of an isotope standard solution (MNZ-13C2,15N2, 0,1 µg/ml in EtOH) are added. The samples are equilibrated for 15 min. 5 ml buffer solution (5.84 g NaCl and 13.61 g KH2PO4 are dissolved in approximately 950 ml of H2O. The pH value is adjusted to 3 with 20 or 25 % HCl using a pH-electrode. The solution is then filled up to 1000 ml with H2O.) and 0.5 ml protease solution (800 mg of protease are filled up with 10 ml of 0.002 M HCl. The solution has to be prepared daily) are added. The samples are homogenised for 1 min in an IKA Ultraturrax at 6000 rpm. The samples should have a pH value of 3 (+0/-0.5, check with pH-indicator). The samples are then hydrolysed at 37°C overnight. The supernatant has to be separate in a 50-mL Falcon tube and approximately 3 ml buffer solution is added again to the remainder, vortexed and centrifuged. The aqueous phase are combined and the pH value has to be controlled and if necessary readjusted to pH 3 using 20-25 % HCl. 5 ml hexane is added to the combined aqueous phase and the extraction is to be carried out by carefully shaking the sample with an overhead thumbler for 2 min. After the defatting step the sample is centrifuged at 4000 rpm and 4°C for 10 minutes and the hexane layer is discarded. The remaining aqueous phase is adjusted to pH 6 (+1/-0, check with pH-indicator) by using 5 M NaOH solution and filled up with water to approximately 9 ml if necessary.*

Cleanup - separation of analyte(s) of interest from other interfering matrix components (if used)	✓	*SPE Clean up: The buffer solution can be applied directly onto the XTR cartridge without further preparation of the cartridge. Not more and not considerably less than 9 ml of aqueous solution should be added to the XTR cartridge. After 20 min equilibration time, 9 ml ethyl acetate/tBME (1:1 v/v) are added. After another 15 min equilibration time, twice 9 ml ethyl acetate/tBME solution are added to elute the analytes. The combined eluates are concentrated to dryness in a TurboVap evaporator. The dry residues is re-dissolved in 200 µl of a mixture of Eluent A using a vortexer. The solutions are transferred into an UltraCentrifugal Devices (NanoSep 100 kDa Omega) and centrifuged for 10 min at 12000 rpm. The clear solution is carefully transferred into dark glass vials with inserts and analysed by LC-MSMS.*
Transformation - conversion of analyte(s) of interest to detectable/measurable form (if used)	x	-----
Analytical system	✓	*LC-MS/MS (Sciex 6500+ coupled to an Agilent InfinityII)*
Calibration approach for value-assignment of analyte(s) in matrix	✓	*Internal standard*
Verification method(s) for value-assignment of analyte(s) in sample (if used)	✓	*One in-house quality control sample and a CRM from JRG Geel were used as quality control sample ERM-BB124.*
Other	x	-----

Core competencies demonstrated in CCQM-K180 by CENAM

CCQM-K180	***NMI/DI***	**Polare analyte in a protein rich matrix – metronidazole in muscle**
Scope of Measurement: : Successful participation in this study may demonstrate participants' capabilities in determining high-polarity analytes (pKow > -2) with molecular mass range from 100 to 500 g/mol at mass fraction levels of 0.1 to 200 µg/kg in a high protein food matrix. This may include demonstration of measurement capabilities such as: (1) value assignment of primary reference standards; (2) extraction of analyte of interest from the matrix; (3) cleanup and separation of analyte of interest from other interfering matrix or extract components; (4) separation and quantification using techniques such as LC-MS/MS, LC-MS, LC-HRMS, GC/MS		
Competency	**✓,×, or N/A**	**Specific Information as Provided by *NMI/DI***
Competencies for Value-Assignment of Calibrant		
Calibrant: Did you use a "highly-pure substance" or calibration solution?	✓	*Highly pure substance was used.* *In house purity assignment was done to a reference material from Supelco PHR1052.*
Identity verification of analyte(s) in calibration material.	✓	*LC-MS was used.* *Metronidazole identity confirmed using retention time and mass spectra.*
For calibrants which are a highly-pure substance: Value-Assignment / Purity Assessment method(s).	✓	*Mass balance was used, where LC-UV was applied to assign analyte mass fraction for related organic impurities, ashes for inorganic impurities and Karl-Fischer for water content*
For calibrants which are a calibration solution: Value-assignment method(s).	NA	
Sample Analysis Competencies		
Identification of analyte(s) in sample	✓	*The method used to identify analytes in the sample were retention time and mass spec ion ratios.* *Metronidazole identity confirmed in samples using retention and MS*
Extraction of analyte(s) of interest from matrix	✓	*Liquid/liquid extraction was used*
Cleanup - separation of analyte(s) of interest from other interfering matrix components (if used)	✓	*Solid phase extraction (SPE) was used to clean up the sample extract*
Transformation - conversion of analyte(s) of interest to detectable/measurable form (if used)	**N/A**	
Analytical system	✓	*LC-MS/MS*
Calibration approach for value-assignment of analyte(s) in matrix	✓	*a) IDMS* *b) calibration mode used: single-point calibration*
Verification method(s) for value-assignment of analyte(s) in sample (if used)	✓	*Used in house spiked solutions and ERM-BB124 Pork muscle (metronidazole) as control.*
Other	**N/A**	

Core competencies demonstrated in CCQM-K180 by EXHM

CCQM-K180	***EXHM***	**Polar analyte in a protein rich matrix – metronidazole in muscle**
Scope of Measurement: : Successful participation in this study may demonstrate participants' capabilities in determining high-polarity analytes (pKow > -2) with molecular mass range from 100 to 500 g/mol at mass fraction levels of 0.1 to 200 µg/kg in a high protein food matrix. This may include demonstration of measurement capabilities such as: (1) value assignment of primary reference standards; (2) extraction of analyte of interest from the matrix; (3) cleanup and separation of analyte of interest from other interfering matrix or extract components; (4) separation and quantification using techniques such as LC-MS/MS, LC-MS, LC-HRMS, GC/MS		
Competency	**✓,×, or N/A**	**Specific Information as Provided by *NMI/DI***
Competencies for Value-Assignment of Calibrant		
Calibrant: Did you use a "highly-pure substance" or calibration solution?	✓	*INMETRO MRC 8365.0001a (metronidazole CRM)*
Identity verification of analyte(s) in calibration material.	✓	*NMR, MS*
For calibrants which are a highly-pure substance: Value-Assignment / Purity Assessment method(s).	✓	*Certified value used, confirmed by mass balance (LC-DAD, KF, HS-GCMS, ICP-MS) and qNMR (value 998.5 ± 1.0 mg/g)*
For calibrants which are a calibration solution: Value-assignment method(s).	*x*	
Sample Analysis Competencies		
Identification of analyte(s) in sample	✓	*Retention time, mass spec ion ratios*
Extraction of analyte(s) of interest from matrix	✓	*solid/liquid extraction with acetonitrile*
Cleanup - separation of analyte(s) of interest from other interfering matrix components (if used)	✓	*desalting with sodium chloride after the extraction procedure. The organic layer was collected, evaporated in a vacuum oven and reconstituted in 1 mL of water:acetonitrile/formic acid (80:20:0.1%)*
Transformation - conversion of analyte(s) of interest to detectable/measurable form (if used)	x	*none*
Analytical system	✓	*LC-MS/MS, LC-HRMS*
Calibration approach for value-assignment of analyte(s) in matrix	✓	*IDMS, single-point calibration at exact matching*
Verification method(s) for value-assignment of analyte(s) in sample (if used)	✓	*Alternative method with enzymatic hydrolysis Analysis of IRMM ERM BB124*
Other	*x*	*Indicate any other competencies demonstrated.*

Core competencies demonstrated in CCQM-K180 by GLHK

CCQM-K180	***NMI/DI***	**Polare analyte in a protein rich matrix – metronidazole in muscle**
Scope of Measurement: : Successful participation in this study may demonstrate participants' capabilities in determining high-polarity analytes (pKow > -2) with molecular mass range from 100 to 500 g/mol at mass fraction levels of 0.1 to 200 µg/kg in a high protein food matrix. This may include demonstration of measurement capabilities such as: (1) value assignment of primary reference standards; (2) extraction of analyte of interest from the matrix; (3) cleanup and separation of analyte of interest from other interfering matrix or extract components; (4) separation and quantification using techniques such as LC-MS/MS, LC-MS, LC-HRMS, GC/MS		
Competency	**✓,×, or N/A**	**Specific Information as Provided by *NMI/DI***
Competencies for Value-Assignment of Calibrant		
Calibrant: Did you use a "highly-pure substance" or calibration solution?	✓	*Neat standard from Inmetro (MRC 8365.0001a)*
Identity verification of analyte(s) in calibration material.	N/A	
For calibrants which are a highly-pure substance: Value-Assignment / Purity Assessment method(s).	N/A	
For calibrants which are a calibration solution: Value-assignment method(s).	N/A	
Sample Analysis Competencies		
Identification of analyte(s) in sample	✓	*Retention time and MRM transition ratio*
Extraction of analyte(s) of interest from matrix	✓	*Extract sample with chilled 1% acetic acid in acetonitrile by 30 min ultrasonic agitation and 30 min vertical shaking*
Cleanup - separation of analyte(s) of interest from other interfering matrix components (if used)	✓	*Clean up by mixed-mode cationic SPE cartridge and liquid chromatographic separation*
Transformation - conversion of analyte(s) of interest to detectable/measurable form (if used)	N/A	
Analytical system	✓	*LC-MS/MS Sciex Triple Quad™ 7500 LC-MS/MS System - QTRAP® Ready coupled with Agilent 1290 Infinity II LC System*
Calibration approach for value-assignment of analyte(s) in matrix	✓	*a) IDMS* *b) single-point calibration*
Verification method(s) for value-assignment of analyte(s) in sample (if used)	N/A	
Other	N/A	

Core competencies demonstrated in CCQM-K180 by HSA

CCQM-K180	*HSA*	Polar analyte in a protein rich matrix – metronidazole in muscle
Scope of Measurement: Successful participation in this study may demonstrate participants' capabilities in determining high-polarity analytes (pKow > -2) with molecular mass range from 100 to 500 g/mol at mass fraction levels of 0.1 to 200 µg/kg in a high protein food matrix. This may include demonstration of measurement capabilities such as: (1) value assignment of primary reference standards; (2) extraction of analyte of interest from the matrix; (3) cleanup and separation of analyte of interest from other interfering matrix or extract components; (4) separation and quantification using techniques such as LC-MS/MS, LC-MS, LC-HRMS, GC/MS		
Competency	**✓,×, or N/A**	**Specific Information as Provided by *NMI/DI***
Competencies for Value-Assignment of Calibrant		
Calibrant: Did you use a "highly-pure substance" or calibration solution?	✓	High purity certified reference material (CRM) of metronidazole (GBW(E)060908) from the National Institute of Metrology (NIM), China with a purity value of 99.9% ± 0.2% was used as the calibrant.
Identity verification of analyte(s) in calibration material.	✓	The identity of metronidazole was confirmed by comparing its mass spectrum with the metronidazole certified reference material 8365 (998.9 ± 1.1 mg/g) from the National Institute of Metrology, Quality and Technology (INMETRO), based on retention time and m/z ratio of the parent and daughter ions on the LC-MS/MS.
For calibrants which are a highly-pure substance: Value-Assignment / Purity Assessment method(s).	N/A	
For calibrants which are a calibration solution: Value-assignment method(s).	N/A	
Sample Analysis Competencies		
Identification of analyte(s) in sample	✓	Retention time and m/z ratio of the parent and daughter ions on the LC-MS/MS.
Extraction of analyte(s) of interest from matrix	✓	The study material was weighed into a 50 mL centrifuge tube and about 1.46 g of water was added. The mixture [sample blend (SB)] was vortexed and stored at about 4 °C for at least 1 hour after gravimetrically spiking with an appropriate amount of isotope-labelled metronidazole internal standard solution. Following the equilibration, the SB was vortexed and shaken on a multitube shaker for 15 to 30 min. The SB was added with 15 mL of 1 % formic acid in ethyl acetate, vortexed for 2 min, sonicated for 5 min and shaken on a multitube shaker for 10 min. After the sample was centrifuged at 4,200 rpm for 5 min, the supernatant solution was collected. The extraction with 1 % formic acid in ethyl acetate was repeated one more time. The combined supernatant was then dried under nitrogen at 40 °C. The dried residue was redissolved in 1.5 mL of 0.1 mol/L hydrochloric acid. Then, 2.5 mL of n-hexane was added and mixed well. After centrifugation at 4,200 rpm for 5 min, the water layer was retained and the n-hexane layer was removed.

Cleanup - separation of analyte(s) of interest from other interfering matrix components (if used)	✓	The water layer was transferred into an amicon Ultra-0.5 centrifugal filter (consisting of regenerated cellulose membrane), and was centrifuged at 13,400 rpm for 10 min before injecting the solution for LC-MS/MS analysis.
Transformation - conversion of analyte(s) of interest to detectable/measurable form (if used)	N/A	
Analytical system	✓	Thermo Scientific TSQ Altis™ triple quadrupole LC-MS/MS system
Calibration approach for value-assignment of analyte(s) in matrix	✓	A single-point calibration, using exact-matching IDMS method.
Verification method(s) for value-assignment of analyte(s) in sample (if used)	✓	Method recovery was investigated using ERM®-BB124 (certified mass fraction of metronidazole in pig muscle (reconstituted material) = 1.93 ± 0.15 µg/kg). The recovery results fall within the expanded uncertainty range of the certified mass fraction of metronidazole in pig muscle (ranged between 97.3 % to 101.5 %) and were used to estimate the uncertainty of method recovery (Frec). Method recovery was also investigated using the comparison study material spiked gravimetrically with metronidazole solution prepared from GBW(E)060908 (NIM). The spiked material was analysed in parallel with the SBs for quality control (QC). Each QC was subjected to the same extraction and clean-up as the study sample. The recovery results ranged between 97.5% to 99.2%.
Other		

Core competencies demonstrated in CCQM-K180 by INM Colombia

CCQM-K180	***Instituto Nacional de Metrología de Colombia (INM-Colombia)***	**Polare analyte in a protein rich matrix – metronidazole in muscle**
Scope of Measurement: : Successful participation in this study may demonstrate participants' capabilities in determining high-polarity analytes (pKow > -2) with molecular mass range from 100 to 500 g/mol at mass fraction levels of 0.1 to 200 µg/kg in a high protein food matrix. This may include demonstration of measurement capabilities such as: (1) value assignment of primary reference standards; (2) extraction of analyte of interest from the matrix; (3) cleanup and separation of analyte of interest from other interfering matrix or extract components; (4) separation and quantification using techniques such as LC-MS/MS, LC-MS, LC-HRMS, GC/MS		
Competency	**✓,×, or N/A**	**Specific Information as Provided by *NMI/DI***
Competencies for Value-Assignment of Calibrant		
Calibrant: Did you use a "highly-pure substance" or calibration solution?	✓	*A highly-pure substance consisting of Metronidazole MRC 8365.0001a, Mass Fraction: (998.9 ± 1.1) mg/g from Inmetro was used*
Identity verification of analyte(s) in calibration material.	✓	*Retention time, mass spectrometry ion ratios by LC-ESI-MS/MS (QqQ)*
For calibrants which are a highly-pure substance: Value-Assignment / Purity Assessment method(s).	✓	*Mass balance was the p*urity assessment method for the *MRC 8365.0001a*
For calibrants which are a calibration solution: Value-assignment method(s).	x	-----
Sample Analysis Competencies		
Identification of analyte(s) in sample	✓	*Retention time, mass spectrometry ion ratios* *Metronidazole Q1: 171.580/ Q3: 128.000* *Internal standard Q1: 163.024/ Q3: 87.900*
Extraction of analyte(s) of interest from matrix	✓	*A 0.600 g sub-sample (with a precision 0.1 mg) of the KC item was reconstituted according to the protocol and an aliquot of 0,200 g of internal standard solution (methomyl in acetonitrile) was added. Then, a 10 mL aliquot of water was added. Subsequently, 10 mL of acetonitrile was added (its weight was recorded with a precision of 0.1 mg). This mixture was vigorously shaken by hand for two minutes and an additional 30 seconds in a vortex at 3000 rpm. After this, the buffered saline mixture of 4.0 g of magnesium sulfate, 1.0 g of sodium chloride, 0.5 g of sodium citrate sesquihydrate, and 1.0 g of sodium citrate trihydrate was added, shaking immediately by hand for at least three minutes. Finally, the tubes were centrifuged at 6000 xg for 5 minutes at 0 °C.*

Cleanup - separation of analyte(s) of interest from other interfering matrix components (if used)	✓	*6 mL of the crude extract was subjected to cleaning by dispersive solid-phase extraction (d-SPE) using a mixture consisting of 900 mg of anhydrous magnesium sulfate, 150 mg of PSA, and 150 mg of C18, followed by shaking for 30 s. Finally, the mixture was centrifuged at 6000 xg for 5 minutes at 0 ° C.* *1 mL of the clean extract were dried under a nitrogen stream at 35 °C and reconstituted with 1 mL of water/methanol/Formic acid 80:20:0.1*
Transformation - conversion of analyte(s) of interest to detectable/measurable form (if used)	x	-----
Analytical system	✓	*Measurements were carried out using a SCIEX chromatographic system, Exion LC/AD model, made up of a mobile phase degassing unit, two single pumps, an autosampler with cooling, and a column heating compartment, coupled through a controller to a mass spectrometer SCIEX model QTrap 4500 triple quadrupole (QqQ) with ESI(+) interface.*
Calibration approach for value-assignment of analyte(s) in matrix	✓	*Internal standard* *Bracketing.*
Verification method(s) for value-assignment of analyte(s) in sample (if used)	✓	*A recovery trial of spiked blank-matrix was carried out.* *An In-house quality control was used for the method and extraction procedures development.*
Other	x	-----

Core competencies demonstrated in CCQM-K180 by INMETRO

CCQM-K180	***INMETRO***	**Polare analyte in a protein rich matrix – metronidazole in muscle**
Scope of Measurement: : Successful participation in this study may demonstrate participants' capabilities in determining high-polarity analytes (pKow > -2) with molecular mass range from 100 to 500 g/mol at mass fraction levels of 0.1 to 200 µg/kg in a high protein food matrix. This may include demonstration of measurement capabilities such as: (1) value assignment of primary reference standards; (2) extraction of analyte of interest from the matrix; (3) cleanup and separation of analyte of interest from other interfering matrix or extract components; (4) separation and quantification using techniques such as LC-MS/MS, LC-MS, LC-HRMS, GC/MS		
Competency	**✓,×, or N/A**	**Specific Information as Provided by *NMI/DI***
Competencies for Value-Assignment of Calibrant		
Calibrant: Did you use a "highly-pure substance" or calibration solution?	✓	*Highly-pure substance: Metronidazole (CRM 8365.0001a, INMETRO)*
Identity verification of analyte(s) in calibration material.	✓	*Analyte in the calibration material was identified by its MS/MS ion transitions, by HPLC-MS/MS. Transition 1: 172.1 > 128.3; transition 2(confirmation):172.1 > 82.3.*
For calibrants which are a highly-pure substance: Value-Assignment / Purity Assessment method(s).	✓	*CRM 8365.0001a certificate: (998.9 ± 1.1) mg/g (k=2, 95%).*
For calibrants which are a calibration solution: Value-assignment method(s).	✓	*The following calibrant was used only to prepare control samples: metronidazole from United States Pharmacopeia Reference Standard (USP), lot: JOC316. Purity was determined by ^{1}H-qNMR at INMETRO (1000.33 mg/g ± 1.86 mg/g, K = 2) using Maleic Acid, CRM 8792.0001 – INMETRO, as internal standard.*
Sample Analysis Competencies		
Identification of analyte(s) in sample	✓	*Analyte in the sample was identified by its MS/MS ion transitions, by HPLC-MS/MS. Transition 1: 172.1 > 128.3; transition 2(confirmation):172.1 > 82.3.*
Extraction of analyte(s) of interest from matrix	✓	*Analyte was extracted from matrix by LLE (by adding NaCl (0.2 g/ml) solution followed by two extractions with 5 mL of ethyl acetate).The two extracts were combined, solvent evaporated with nitrogen gas flow at 40 °C, and reconstituted with 1.5 mL of HCl (0.1 mol/L).*
Cleanup - separation of analyte(s) of interest from other interfering matrix components (if used)	✓	*One step of clean up was performed by adding 5 mL of hexane to the reconstituted extract in order to remove lipid components.*
Transformation - conversion of analyte(s) of interest to detectable/measurable form (if used)	N/A	*No transformation was performed*
Analytical system	✓	*LC-MS/MS*
Calibration approach for value-assignment of analyte(s) in matrix	✓	*a) Quantification mode used: IDMS.* *b) Calibration mode used: bracketing calibration.*
Verification method(s) for value-assignment of analyte(s) in sample (if used)	✓	*Two calibration solutions were prepared completely independently and used for cross-confirmation of the results. Control samples were prepared by spiking blank matrix samples with metronidazole solution prepared from other standard than the used for calibration solutions. The mean recovery obtained for the control samples was very close to 100 %. The*

		metronidazole standard used to prepare the control samples was: United States Pharmacopeia Reference Standard (USP), lot: JOC316. Purity was determined by ^{1}H-qNMR at Inmetro (1000.33 mg/g ± 1.86 mg/g, k=2). Maleic Acid, CRM 8792.0001 - INMETRO, used as internal standard in qNMRq purity determination.
Other	N/A	*Indicate any other competencies demonstrated.*

Core competencies demonstrated in CCQM-K180 by INRAP

CCQM-K180	***NMI/DI***	**Polare analyte in a protein rich matrix – metronidazole in muscle**
Scope of Measurement: : Successful participation in this study may demonstrate participants' capabilities in determining high-polarity analytes (pKow > -2) with molecular mass range from 100 to 500 g/mol at mass fraction levels of 0.1 to 200 µg/kg in a high protein food matrix. This may include demonstration of measurement capabilities such as: (1) value assignment of primary reference standards; (2) extraction of analyte of interest from the matrix; (3) cleanup and separation of analyte of interest from other interfering matrix or extract components; (4) separation and quantification using techniques such as LC-MS/MS, LC-MS, LC-HRMS, GC/MS		
Competency	**✓,×, or N/A**	**Specific Information as Provided by *NMI/DI***
Competencies for Value-Assignment of Calibrant		
Calibrant: Did you use a "highly-pure substance" or calibration solution?		*Indicate if you used a "pure material" or a calibration solution. Indicate its source and ID, eg CRM identifier*
Identity verification of analyte(s) in calibration material.	N/A	*Indicate method(s) you used to identify analyte(s)*
For calibrants which are a highly-pure substance: Value-Assignment / Purity Assessment method(s).	N/A	*Indicate how you established analyte mass fraction/purity (i.e., mass balance (list techniques used), qNMR, other)*
For calibrants which are a calibration solution: Value-assignment method(s).	N/A	*Indicate how you established analyte mass fraction in calibration solution*
Sample Analysis Competencies		
Identification of analyte(s) in sample	✓	*Indicate method(s) you used to identify analyte(s) in the sample (i.e., Retention time, mass spec ion ratios, other) Retention time, mass spec ion ratios*
Extraction of analyte(s) of interest from matrix	✓	*Indicate extraction technique(s) used, if any, (i.e. Liquid/liquid, Soxhlet, ASE, other) Liquid/liquid*
Cleanup - separation of analyte(s) of interest from other interfering matrix components (if used)	N/A	*Indicate cleanup technique(s) used, if any (i.e., SPE, LC fractionation, other)*
Transformation - conversion of analyte(s) of interest to detectable/measurable form (if used)	N/A	*Indicate chemical transformation method(s), if any, (i.e., hydrolysis, derivatization, other)*
Analytical system	✓	*Indicate analytical system (i.e., LC-MS/MS, GC-HRMS, GC-ECD, other) LCMS/MS*
Calibration approach for value-assignment of analyte(s) in matrix	✓	*a) Indicate quantification mode used (i.e., IDMS, internal standard, external standard, other) internal standard* *b) Indicate calibration mode used (i.e., single-point calibration, bracketing, x-point calibration curve, other) x-point calibration curve*
Verification method(s) for value-assignment of analyte(s) in sample (if used)	N/A	*Indicate any confirmative method(s) used, if any.*
Other		*Indicate any other competencies demonstrated.*

Core competencies demonstrated in CCQM-K180 by KRISS

CCQM-K180	***NMI/DI***	**Polare analyte in a protein rich matrix – metronidazole in muscle**
Scope of Measurement: : Successful participation in this study may demonstrate participants' capabilities in determining high-polarity analytes (pKow > -2) with molecular mass range from 100 to 500 g/mol at mass fraction levels of 0.1 to 200 µg/kg in a high protein food matrix. This may include demonstration of measurement capabilities such as: (1) value assignment of primary reference standards; (2) extraction of analyte of interest from the matrix; (3) cleanup and separation of analyte of interest from other interfering matrix or extract components; (4) separation and quantification using techniques such as LC-MS/MS, LC-MS, LC-HRMS, GC/MS		
Competency	**✓,×, or N/A**	**Specific Information as Provided by *NMI/DI***
Competencies for Value-Assignment of Calibrant		
Calibrant: Did you use a "highly-pure substance" or calibration solution?	✓	Metronidazole CRM (MRC 8365.0001a) was provided by INMETRO.
Identity verification of analyte(s) in calibration material.	✓	ID-LC/MS
For calibrants which are a highly-pure substance: Value-Assignment / Purity Assessment method(s).	✓	Purity value measured by INMETRO was used (99.89 ± 0.11%).
For calibrants which are a calibration solution: Value-assignment method(s).	✓	Calibration solutions were gravimetrically prepared in KRISS using the neat CRM from INMETRO and verified by cross-checking of multiple calibration solutions.
Sample Analysis Competencies		
Identification of analyte(s) in sample	✓	LC retention time: 12.15 min Mass spec ion ratios: 0.89
Extraction of analyte(s) of interest from matrix	✓	Liquid/liquid extraction using DI water and ethyl acetate.
Cleanup - separation of analyte(s) of interest from other interfering matrix components (if used)	✓	Liquid/liquid extraction
Transformation - conversion of analyte(s) of interest to detectable/measurable form (if used)	×	-
Analytical system	✓	LC-MS/MS
Calibration approach for value-assignment of analyte(s) in matrix	✓	a) IDMS b) Single-point calibration
Verification method(s) for value-assignment of analyte(s) in sample (if used)	✓	ERM-BB124 (Nitroimidazoles in porcine muscle) and gravimetrically fortified pork samples were used for the verification of analytical method.
Other	*N/A*	-

Core competencies demonstrated in CCQM-K180 by LGC

CCQM-K180	*LGC*	Polar analyte in a protein rich matrix – metronidazole in muscle
Scope of Measurement: : Successful participation in this study may demonstrate participants' capabilities in determining high-polarity analytes (pKow > -2) with molecular mass range from 100 to 500 g/mol at mass fraction levels of 0.1 to 200 µg/kg in a high protein food matrix. This may include demonstration of measurement capabilities such as: (1) value assignment of primary reference standards; (2) extraction of analyte of interest from the matrix; (3) cleanup and separation of analyte of interest from other interfering matrix or extract components; (4) separation and quantification using techniques such as LC-MS/MS, LC-MS, LCHRMS, GC/MS		
Competency	**✓, ×, or N/A**	**Specific Information as Provided by *NMI/DI***
Competencies for Value-Assignment of Calibrant		
Calibrant: Did you use a "highly-pure substance" or calibration solution?	✓	*CRM obtained from Inmetro (Batch: MRC8365.0001a)*
Identity verification of analyte(s) in calibration material.	N/A	
For calibrants which are a highly-pure substance: Value-Assignment / Purity Assessment method(s).	N/A	
For calibrants which are a calibration solution: Value-assignment method(s).	N/A	
Sample Analysis Competencies		
Identification of analyte(s) in sample	✓	*Retention time and ion ratio of 2 qualifier ions compared to CRM standard*
Extraction of analyte(s) of interest from matrix	✓	Liquid extraction
Cleanup - separation of analyte(s) of interest from other interfering matrix components (if used)	✓	*Centrifugation and Solid Phase Extraction (SPE)*
Transformation - conversion of analyte(s) of interest to detectable/measurable form (if used)	N/A	
Analytical system	✓	*LC-MS/MS*
Calibration approach for value-assignment of analyte(s) in matrix	✓	*a) DEM-IDMS* *b) Bracketed double exact matching*
Verification method(s) for valueassignment of analyte(s) in sample (if used)	X	
Other	N/A	

Core competencies demonstrated in CCQM-K180 by NIM China

CCQM-K180	***NIM***	**Polar analyte in a protein rich matrix – metronidazole in muscle**
Scope of Measurement: : Successful participation in this study may demonstrate participants' capabilities in determining high-polarity analytes (pKow > -2) with molecular mass range from 100 to 500 g/mol at mass fraction levels of 0.1 to 200 µg/kg in a high protein food matrix. This may include demonstration of measurement capabilities such as: (1) value assignment of primary reference standards; (2) extraction of analyte of interest from the matrix; (3) cleanup and separation of analyte of interest from other interfering matrix or extract components; (4) separation and quantification using techniques such as LC-MS/MS, LC-MS, LC-HRMS, GC/MS		
Competency	**✓, ×, or N/A**	**Specific Information as Provided by *NMI/DI***
Competencies for Value-Assignment of Calibrant		
Calibrant: Did you use a "highly-pure substance" or calibration solution?	✓	Certified reference material Pure Metronidazole GBW(E)060908 99.9±0.2% traceable to SI
Identity verification of analyte(s) in calibration material.	N/A	/
For calibrants which are a highly-pure substance: Value-Assignment / Purity Assessment method(s).	N/A	/
For calibrants which are a calibration solution: Value-assignment method(s).	N/A	/
Sample Analysis Competencies		
Identification of analyte(s) in sample	✓	Retention time on two columns with different stationary phases compared against calibrant. Ratios of two MRM responses compared against calibrant
Extraction of analyte(s) of interest from matrix	✓	Sample was extracted using solid-liquid extraction
Cleanup - separation of analyte(s) of interest from other interfering matrix components (if used)	✓	The extract was filtered through a PP membrane filter (0.22µm) prior to the analysis.
Transformation - conversion of analyte(s) of interest to detectable/measurable form (if used)	×	/
Analytical system	✓	LC-MS/MS
Calibration approach for value-assignment of analyte(s) in matrix	✓	Exact matching IDMS with single point calibration
Verification method(s) for value-assignment of analyte(s) in sample (if used)	✓	The comparison study material was spiked gravimetrically with metronidazole solution prepared from GBW(E)060908 (NIM). The spiked material was analyzed in parallel with the sample for quality control (QC). Each QC was subjected to the same extraction as the study sample. The recovery results ranged between 97.2 % to 99.0 % were used to estimate the uncertainty of spiked recovery. ERM-BB124（Nitroimidazoles in the reconstituted material） was also investigated using for verification of the method.
Other	N/A	/

Core competencies demonstrated in CCQM-K180 by NIMT

CCQM-K180	*NIMT*	Polare analyte in a protein rich matrix – metronidazole in muscle
Scope of Measurement: : Successful participation in this study may demonstrate participants' capabilities in determining high-polarity analytes (pKow > -2) with molecular mass range from 100 to 500 g/mol at mass fraction levels of 0.1 to 200 µg/kg in a high protein food matrix. This may include demonstration of measurement capabilities such as: (1) value assignment of primary reference standards; (2) extraction of analyte of interest from the matrix; (3) cleanup and separation of analyte of interest from other interfering matrix or extract components; (4) separation and quantification using techniques such as LC-MS/MS, LC-MS, LC-HRMS, GC/MS		
Competency	**✓,×, or N/A**	**Specific Information as Provided by *NMI/DI***
Competencies for Value-Assignment of Calibrant		
Calibrant: Did you use a "highly-pure substance" or calibration solution?	✓	Yes, high pure standard metronidazole (CRM) GBW(E)060908 from NIM China
Identity verification of analyte(s) in calibration material.	✓	based on retention time and m/z ratio on the LC-MS/MS
For calibrants which are a highly-pure substance: Value-Assignment / Purity Assessment method(s).	-	
For calibrants which are a calibration solution: Value-assignment method(s).	-	
Sample Analysis Competencies		
Identification of analyte(s) in sample	✓	Chromatographic retention time (LC-MS/MS), MRM mode with two ion pairs for identification
Extraction of analyte(s) of interest from matrix	✓	Liquid-solid extraction, Liquid-liquid extraction
Cleanup - separation of analyte(s) of interest from other interfering matrix components (if used)	✓	SPE cleanup
Transformation - conversion of analyte(s) of interest to detectable/measurable form (if used)	-	
Analytical system	✓	LC-MS/MS
Calibration approach for value-assignment of analyte(s) in matrix	✓	a) Exact-matching double IDMS (matrix-matched calibration blends) b) single-point, bracketing calibration
Verification method(s) for value-assignment of analyte(s) in sample (if used)	✓	-Internal standard six-point calibration curve -two sources of pure CRM (NIM-GBW(E)060908 and INMETRO- 8365) were compared.
Other	-	

Core competencies demonstrated in CCQM-K180 by NMIA

CCQM-K180	*NMI/DI*	Polare analyte in a protein rich matrix – metronidazole in muscle
Scope of Measurement: : Successful participation in this study may demonstrate participants' capabilities in determining high-polarity analytes (pKow > -2) with molecular mass range from 100 to 500 g/mol at mass fraction levels of 0.1 to 200 µg/kg in a high protein food matrix. This may include demonstration of measurement capabilities such as: (1) value assignment of primary reference standards; (2) extraction of analyte of interest from the matrix; (3) cleanup and separation of analyte of interest from other interfering matrix or extract components; (4) separation and quantification using techniques such as LC-MS/MS, LC-MS, LC-HRMS, GC/MS		
Competency	**✓,×, or N/A**	**Specific Information as Provided by *NMI/DI***
Competencies for Value-Assignment of Calibrant		
Calibrant: Did you use a "highly-pure substance" or calibration solution?		*Pure material: INMETRO 8365*
Identity verification of analyte(s) in calibration material.	✓	*Certified by INMETRO, who have a CMC for the material*
For calibrants which are a highly-pure substance: Value-Assignment / Purity Assessment method(s).	✓	*Certified by INMETRO, who have a CMC for the material*
For calibrants which are a calibration solution: Value-assignment method(s).	**N/A**	
Sample Analysis Competencies		
Identification of analyte(s) in sample	✓	*Retention time, mass spec ion ratios*
Extraction of analyte(s) of interest from matrix	✓	*Liquid/solid extraction with a polar organic solvent*
Cleanup - separation of analyte(s) of interest from other interfering matrix components (if used)	✓	*Solid phase extraction (HLB – reversed phase – and MCX – ion-exchange)*
Transformation - conversion of analyte(s) of interest to detectable/measurable form (if used)	×	*none*
Analytical system	✓	*2D - LC-MS/MS*
Calibration approach for value-assignment of analyte(s) in matrix	✓	*a) Exact-matching double IDMS* *b) Single point bracketed*
Verification method(s) for value-assignment of analyte(s) in sample (if used)	**N/A**	*Verification was by multiple SPE and chromatographic conditions*
Other	**N/A**	.

Core competencies demonstrated in CCQM-K180 by NRC Canada

CCQM-K180	*NRC*	Polar analyte in a protein rich matrix – metronidazole in muscle
Scope of Measurement: Successful participation in this study may demonstrate participants' capabilities in determining high-polarity analytes (pKow > -2) with molecular mass range from 100 to 500 g/mol at mass fraction levels of 0.1 to 200 µg/kg in a high protein food matrix. This may include demonstration of measurement capabilities such as: (1) value assignment of primary reference standards; (2) extraction of analyte of interest from the matrix; (3) cleanup and separation of analyte of interest from other interfering matrix or extract components; (4) separation and quantification using techniques such as LC-MS/MS, LC-MS, LC-HRMS, GC/MS		
Competency	**✓,×, or N/A**	**Specific Information as Provided by *NMI/DI***
Competencies for Value-Assignment of Calibrant		
Calibrant: Did you use a "highly-pure substance" or calibration solution?	✓	Highly pure substance: INMETRO CRM: MRC 8365.0001a
Identity verification of analyte(s) in calibration material.	N/A	N/A
For calibrants which are a highly-pure substance: Value-Assignment / Purity Assessment method(s).	N/A	N/A
For calibrants which are a calibration solution: Value-assignment method(s).	N/A	N/A
Sample Analysis Competencies		
Identification of analyte(s) in sample	✓	Identification of metronidazole and its d3 IS were performed via HPLC retention time, and MS/MS monitoring of two ion transitions.
Extraction of analyte(s) of interest from matrix	✓	The analyte was extracted via a double liquid-solid extraction of the matrix (QUECHERS) using acetonitrile and water with NaCl and Mg2SO4 salts.
Cleanup - separation of analyte(s) of interest from other interfering matrix components (if used)	✓	A hexane cleanup was used to reduce fat content in the acetonitrile extract, followed by dry-down, reconstitution in 0.1% formic acid water and centrifugation to remove solid particulates.
Transformation - conversion of analyte(s) of interest to detectable/measurable form (if used)	N/A	N/A
Analytical system	✓	LC-MS/MS: Agilent 1290 Infinity I UPLC and Thermo Quantiva Mass Spectrometer
Calibration approach for value-assignment of analyte(s) in matrix	✓	a) ID2MS - Single-point b) SA-ID2MS - two-point
Verification method(s) for value-assignment of analyte(s) in sample (if used)	N/A	N/A
Other	N/A	*N/A*

Core competencies demonstrated in CCQM-K180 by SASO-NMCC

Remark: results were withdrawn – competencies cannot be claimed.

CCQM-K180	***NMI/DI***	**Polare analyte in a protein rich matrix – metronidazole in muscle**
Scope of Measurement: : Successful participation in this study may demonstrate participants' capabilities in determining high-polarity analytes (pKow > -2) with molecular mass range from 100 to 500 g/mol at mass fraction levels of 0.1 to 200 µg/kg in a high protein food matrix. This may include demonstration of measurement capabilities such as: (1) value assignment of primary reference standards; (2) extraction of analyte of interest from the matrix; (3) cleanup and separation of analyte of interest from other interfering matrix or extract components; (4) separation and quantification using techniques such as LC-MS/MS, LC-MS, LC-HRMS, GC/MS		
Competency	**✓,×, or N/A**	**Specific Information as Provided by *NMI/DI***
Competencies for Value-Assignment of Calibrant		
Calibrant: Did you use a "highly-pure substance" or calibration solution?	✓	*CRM* *DIMCI 0744 / 2012h / INMETRO* *MRC 8365.0001a*
Identity verification of analyte(s) in calibration material.	N/A	
For calibrants which are a highly-pure substance: Value-Assignment / Purity Assessment method(s).	N/A	
For calibrants which are a calibration solution: Value-assignment method(s).	N/A	
Sample Analysis Competencies		
Identification of analyte(s) in sample	✓	*SRM ms2 172 [127.5-128.5]*
Extraction of analyte(s) of interest from matrix	✓	*Liquid/liquid*
Cleanup - separation of analyte(s) of interest from other interfering matrix components (if used)	✓	*SPE*
Transformation - conversion of analyte(s) of interest to detectable/measurable form (if used)	N/A	
Analytical system	✓	*LC MS/MS*
Calibration approach for value-assignment of analyte(s) in matrix	✓	*a) External standard* *b) calibration curve*
Verification method(s) for value-assignment of analyte(s) in sample (if used)	×	
Other		*Indicate any other competencies demonstrated.*

Core competencies demonstrated in CCQM-K180 by UME

CCQM-K180	***TUBITAK UME***	**Polar analyte in a protein rich matrix – metronidazole in muscle**
Scope of Measurement: : Successful participation in this study may demonstrate participants' capabilities in determining high-polarity analytes (pKow > -2) with molecular mass range from 100 to 500 g/mol at mass fraction levels of 0.1 to 200 µg/kg in a high protein food matrix. This may include demonstration of measurement capabilities such as: (1) value assignment of primary reference standards; (2) extraction of analyte of interest from the matrix; (3) cleanup and separation of analyte of interest from other interfering matrix or extract components; (4) separation and quantification using techniques such as LC-MS/MS, LC-MS, LC-HRMS, GC/MS		
Competency	**✓,×, or N/A**	**Specific Information as Provided by *TUBITAK UME***
Competencies for Value-Assignment of Calibrant		
Calibrant: Did you use a "highly-pure substance" or calibration solution?	✓	Pure material Metronidazole, Sigma Aldrich (M3761)
Identity verification of analyte(s) in calibration material.	✓	In house value assignment by qNMR
For calibrants which are a highly-pure substance: Value-Assignment / Purity Assessment method(s).	✓	Purity assessment by qNMR
For calibrants which are a calibration solution: Value-assignment method(s).	N/A	-
Sample Analysis Competencies		
Identification of analyte(s) in sample	✓	Retention time
Extraction of analyte(s) of interest from matrix	✓	Liquid/Solid extraction
Cleanup - separation of analyte(s) of interest from other interfering matrix components (if used)	N/A	-
Transformation - conversion of analyte(s) of interest to detectable/measurable form (if used)	N/A	-
Analytical system	✓	LC-HRMS
Calibration approach for value-assignment of analyte(s) in matrix	✓	IDMS, 3-point calibration curve
Verification method(s) for value-assignment of analyte(s) in sample (if used)	N/A	-
Other		*Indicate any other competencies demonstrated.*

Core competencies demonstrated in CCQM-K180 by VNIIM

Remark: results were withdrawn – competencies cannot be claimed.

CCQM-K180	***NMI/DI***	**Polare analyte in a protein rich matrix – metronidazole in muscle**
Scope of Measurement: : Successful participation in this study may demonstrate participants' capabilities in determining high-polarity analytes (pKow > -2) with molecular mass range from 100 to 500 g/mol at mass fraction levels of 0.1 to 200 µg/kg in a high protein food matrix. This may include demonstration of measurement capabilities such as: (1) value assignment of primary reference standards; (2) extraction of analyte of interest from the matrix; (3) cleanup and separation of analyte of interest from other interfering matrix or extract components; (4) separation and quantification using techniques such as LC-MS/MS, LC-MS, LC-HRMS, GC/MS		
Competency	**✓,×, or N/A**	**Specific Information as Provided by *NMI/DI***
Competencies for Value-Assignment of Calibrant		
Calibrant: Did you use a "highly-pure substance" or calibration solution?	✓	*Pure material Metronidazole (produced by Hubei Hongyuan Pharmaceutical Technology Co.,Ltd)*
Identity verification of analyte(s) in calibration material.	✓	*Agilent 6530 Q-TOF LC/MS*
For calibrants which are a highly-pure substance: Value-Assignment / Purity Assessment method(s).	✓	*Metronidazole purity was evaluated in-house by mass balance approach (99,9±0,3) %*
For calibrants which are a calibration solution: Value-assignment method(s).	**N/A**	*Indicate how you established analyte mass fraction in calibration solution*
Sample Analysis Competencies		
Identification of analyte(s) in sample	✓	*Retention time, mass spec ion ratios*
Extraction of analyte(s) of interest from matrix	✓	*Extraction to organic solvent by vortex*
Cleanup - separation of analyte(s) of interest from other interfering matrix components (if used)	✓	*Bond Elut SCX cartriges (Agilent, 200 mg/3 ml)*
Transformation - conversion of analyte(s) of interest to detectable/measurable form (if used)	**N/A**	*Indicate chemical transformation method(s), if any, (i.e., hydrolysis, derivatization, other)*
Analytical system	✓	*LC-MS/MS Agilent TripleQuard 6460)*
Calibration approach for value-assignment of analyte(s) in matrix	✓	*a) IDMS* *b) single-point calibration*
Verification method(s) for value-assignment of analyte(s) in sample (if used)	✓	*Extraction procedure was validated by ERM-BB124 - PORK MUSCLE*
Other	**N/A**	*Indicate any other competencies demonstrated.*

CONCLUSIONS

Eighteen National Metrology Institutions registered for the study, 17 laboratories submitted results, 15 results were taken into account for the calculation of the KCRV. Due to the very homogenous data set it was quite easy to agree upon an approach for the KCRV calculation. It was decided to use the DerSimonian-Laird approach which yielded a KCRV for metronidazole of 8.41 +/- 0.08 µg/kg (and a estimated dark uncertainty τ of 0.18 µg/kg)

Other estimators ("Laplace Rem", "HB Rem") provide nearly identical KCRV.

The DoE calculation showed that 14 participant results are in agreement with the calculated KCRV proving the excellent performance of the participants.

The same material was also analysed in the framework of a proficiency test for the European network of National Reference Laboratories for food control. The assigned value calculated for this study is also in good agreement with the KCRV – but showing a significantly higher uncertainty (8.38 +/- 0.32 µg/kg; n=32) [15]. This once again demonstrates the excellent results achieved by the National Metrology Institutions.

The provision of pure calibration reference materials by INMETRO and NIM China enabled traceability to SI on the one hand and helped the laboratories to focus on the challenge of analysing the requested measurand in incurred matrix material on the other.

The idea of analysing additional measurands (also present in the sample) was not followed for this study. The idea of analysing an additional sample for the same measurand, but in a different matrix (e.g a different muscle or milk) was also not considered. Both approaches could have provided data for a better understanding of the dark uncertainty and additional evidence for stating broad claim CMCs. This approach should be considered for future key comparisons – if possible.

The shipment of animal matrix samples (study material porcine muscle) was expected to be very difficult. However, it was possible in close exchange with the participants (individual veterinary certificates, partly individual delivery services, difficulties with delivery addresses and limited availability by email) to deliver the material in good condition to the 17 of the 18 registered participants. In general, detailed discussion of the study protocol and the results template with the participants after registration for the study might be helpful in order to avoid misunderstandings/misinterpretations in the course of the study.

ACKNOWLEDGEMENTS

The study coordinators would like to thank the participating laboratories for their cooperation and for providing the information required for this study. The efforts of Dr. Ferial Tadjine and Ms. Katrin Heider who prepared the study material and conducted the homogeneity and stability studies are gratefully acknowledged.

Provision of pure calibration reference materials substances by NIM China and INMETRO is gratefully acknowledged. Special thanks to INMETRO who provided a higher number of aliquots of pure calibration reference materials substances to us via PTB which offered the possibility to send calibrants together with the study material.

Financial support of the European Commission for the European Reference Laboratory (EURL) at the BVL is gratefully acknowledged.

REFERENCES

[1] K. Maeda, T. Osato, H. Umezawa, The Journal of antibiotics 1953, 6, 182.

[2] Committee for veterinary medicinal products, Metronidazole summary report, 1997.

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[14] NIST Consensus Builder https://consensus.nist.gov/ (Accessed 10.01.2024)

[15] Nitroimidazoles in porcine muscle: Proficiency test NIIM1122 - Final report on results; U. Mülow-Stollin, F. Tadjine, J. Polzer; (assigned values are reported here as mass fraction in reconstituted muscle material).

APPENDIX A: Call for Participation

Fr 12.08.2022 13:14

Steven WESTWOOD <swestwoo@bipm.org>

Information for the OAWG

An: A. Alaskar; Abdullah Alosaimi; Adriana Rocco; aksytov; Alejandro Perez Castorena; Alharthi, Majed - SASO-NMCC; Alicia Saez; Allen Tongson-Juncay; Almirón, Florencia - LATU; alper isleyen; Amandine Boeuf; André Henriot; Andrea Giovannozzi; andreas.brinkmann@nrc-cnrc.gc.ca; Andres Salinas; abotha@nmisa.org; Annarita Baldan; Annie Wong; Augustin Camparella; Bahadoor, Adilah; Baldan, Annarita; Bales, Jennifer; Beach, Daniel; Beatrice Lalere; Bei Xu; Benny Tong; bernd.guettler@ptb.de; Boris Wong; Bruno Garrido; Bryan Calderon-Jiménez; byungjoo; C. S. Ng; Cailean Clarkson; carlos.gonzalez@nist.gov; Carmen Sanchez; Chaieb; 'Charun Yafa'; cheerapa@nimt.or.th; Chi-Kin Koo; Chris Hopley; christine.brauckmann@ptb.de; Chun Wai Tse; Claudia Swart; cristian-gabriel.arsene@ptb.de; Davies, Stephen.; Desiree Prevoo; dyah.sfpa@bsn.go.id; eferri@ilatu.org.uy; Eliane Pires; Elias Kakoulides (IAPR, GR); Estela Kneeteman; Evaldas Naujalis; Evelyn Mendoza; f.durbiano@inrim.it; fanny Ganfoni; Fong Liu; Fuhai Su; Garnet McRae; Gavin O Connor; Gmena Colombo; gisela.umbricht@metas.ch; gmolina@lacomet.go.cr; Gustavo MARTOS; hapeong.kwon@kriss.re.kr; hanan-n@aist.go.jp; Hashim; haslina@snsm.my; Henry Rotich; hgasca@cenam.mx; Hiroko Ueno; Hongmei Hua Tang; huangfmg@nim.ac.cn; i.toloza@iaea.org; iagonzalez@inm.gov.co; ibrahim.tahoun@nrc.sci.eg; Isaac Mugenya;

Sie haben am 27.10.2022 10:31 auf diese Nachricht geantwortet.
Diese Nachricht wurde in das Nur-Text-Format konvertiert.

CCQM-OAWG-K180_protocol.pdf
302 KB

Dear OAWG colleagues,

On behalf of the OAWG Chair I am providing a short update on progress with two actions arising from the June 2022 video conferences and in particular participation in the Track A comparisons CCQM-K180 and CCQM-K148.b

Please find attached the Comparison Protocol for the CCQM-K180 Track A key comparison: Polar analyte in high protein food matrix -metronidazole in porcine muscle. This e-mail constitutes the call for participation in the comparison. NMIs wishing to participate can register by e-mail to Joachim Polzer (joachim.polzer@bvl.bund.de <mailto:joachim.polzer@bvl.bund.de>) with a cc: to eurlvetdrug@bvl.bund.de <mailto:eurlvetdrug@bvl.bund.de> . The deadline for registration is 30th September 2022.

The circulation of the final protocols for the CCQM-K148.b Track A key comparison: Mass fraction of oxytetracycline in oxytetracycline hydrochloride material and the parallel Track C comparison CCQM-K179: Mass fraction of oxytetracycline HCl salt have been delayed in order to include the full protocol for control of the hygroscopicity of the material and for the reporting of water content results. The protocol with the call for participation in the comparisons will now be distributed in September and sample distribution will commence shortly thereafter.

Finally I would also like to bring your attention to the CCQM Workshop on Particle Metrology which will be hosted by the BIPM from 25th to 27th October as an online-only meeting. An outline of the workshop programme is available in the "Working Documents" section. The full workshop agenda with invited speakers will be available shortly.

Free registration for the Workshop is now open through the online link:
https://www.bipm.org/en/committees/cc/ccqm/wg/ccqm-ws/2022-10-25

Best Regards,

Steven Westwood
Principal Research Chemist
Bureau International des Poids et Mesures
Pavillon de Breteuil
Sevres, France 92312

APPENDIX B: Protocol

CCQM-K180
Polar analyte in high protein food matrix - metronidazole in porcine muscle

Key Comparison
Track A

Study Protocol
August 2022

U. Mülow-Stollin, J. Polzer
Federal Office of Consumer Protection and Food Safety (BVL)
EU Reference Laboratory for Residues (EURL)
Diedersdorfer Weg 1 ● 12277 Berlin ● Germany
P. O. Box 11 02 60 ● 10832 Berlin ● Germany

INTRODUCTION

Nitroimidazoles are a group of synthetic chemicals derived from azomycin (2-nitroimidazole), a compound which was first isolated from a Streptomyces species in the 1950s [1]. Many nitroimidazoles exhibit antihelmintic properties and are also potent antibiotics and coccidiostats. The most important nitroimidazoles from a pharmaceutical perspective are metronidazole and dimetridazole. However, due to their proven genotoxic and suspected carcinogenic properties no acceptable daily intake (ADI) [2] can be derived, which means that the setting of a maximum residue level (MRL) in food in accordance with Reg. (EC) No. 470/2009 [3] is not possible. Dimetridazole and metronidazole are therefore included in Table 2 of Commission Regulation (EU) No 37/2010 [4] as prohibited compounds and hence any confirmed presence in food samples is considered a non-compliance with EU food legislation. Also worldwide in many countries nitroimidazoles are banned from use in animals from which products for human consumption are derived. For muscle, the European Reference Laboratory (EURL) Berlin recommends 1 µg/kg as the minimum method performance requirement (MMPR) [5] for the detection of nitroimidazole residues.

At the CCQM Organic Analysis Working Group (OAWG) Meetings in 2021 suggestions for track A comparisons for polar analytes in a protein rich matrix were discussed according to the OAWG multi-annual strategy plan for the support of existing CMC claims (e.g. based on CCQM-K85 and CCQM-K141). Finally, it was decided to start a study with metronidazole in pig muscle as representative for this purpose. This study helps to support core competencies in the area of analytes with a molecular mass range of 100 g/mol to 500 g/mol, of analytes having high polarity pKow > -2 in a mass fraction range from 0.10 [ng/g] to 200 [µg/kg] in a protein rich food sample (food triangle categories 4, 8, 9, e.g. animal tissue, AOAC International food triangle [6]).

As a Track A comparison, it is expected that all NMIs or DIs who have, or expect to have services related to the capabilities related to the "How far does the light shine" statement for this key comparison will participate.

TIMELINE

Table 1: Proposed timeline for CCQM-K180

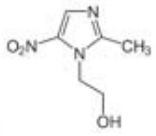
November 2021 – January 2022	Preparation of sample
January/February 2022	Homogeneity testing
February/March/August 2022 and ongoing	Stability testing
August 2022	Call for participation to OAWG members
September 2022	Deadline for registration
October / November 2022	Dispatch of samples
1 June 2023	Deadline for submission of results
September/October 2023	Preliminary Discussion of results

MEASURANDS

The measurand of this study is the mass fraction of metronidazole in porcine muscle on a dry mass basis in µg/kg. Physico-chemical information for metronidazole is provided below. The sample material also contains dimetridazole, as well as metabolites of metronidazole (metronidazole hydroxide) and dimetridazole (2-hydroxymethyl-1-methyl-5-nitro-1H-imidazole (HMMNI)).

Table 2: Selected physico-chemical parameters of metronidazole [7].

Name	metronidazole
Abbreviation	MNZ
IUPAC name	2-(2-methyl-5-nitro-1H-imidazol-1-yl)ethanol
CAS	443-48-1
Sum formula	C6H9N3O3
Molecular weight	171.15 g/mol
logP	-0.1
pKa	2.5



STUDY MATERIALS

The matrix porcine muscle is a matrix rich in protein and partly also rich in fat and therefore falls into category 4, 8, or 9 of the AOAC International food triangle [6], depending on the individual muscle composition. For this study, materials produced in an animal study initiated by the BVL veterinarian in 2021, as well as blank porcine muscle samples purchased from the retail market, were used. The animal study was conducted on two 5-month old male, castrated rearing pigs (body weight 90 kg) which were treated with nitroimidazole-containing pharmaceutical preparations given below.

Metronidazole: Eradia suspension 125 mg/mL (Virbac Switzerland AG, Glattbrugg, Switzerland), 2x/day per oral over the course of 3 days, 18 hours waiting time before slaughter

Dimetridazole: chevi-col powder 400 mg/g (Chevita GmbH, Pfaffenhofen, Germany), 2x/day per oral over the course of 3 days, 18 hours waiting time before slaughter

The study material with the respective desired concentration in the range 0.01-50 µg/kg was produced from the incurred material and the blank material as a total batch from a number of sub-samples. All sub-samples were combined and homogenised under addition of dry ice. Subsequently, the sample material was lyophilised, passed through a sieve and homogenised again ("Grindomix"). Finally, individual portions of around 4.3 g lyophilised material were weighed into 50 mL plastic centrifuge tubes. The material was stored at -20 °C.

The aliquots contain 4.3 – 4.4 g of lyophilized muscle referring to approximately 16 g fresh muscle (1 g fresh muscle = 0.27 g lyophilisate). Current methods need approximately 1-2 g fresh muscle for a single analysis.

Reconstitution of a lyophilised sample is realised by adding the amount of water lost during lyophilisation. In any case, the amount of lyophilised sample to be reconstituted shall not be smaller than the amount used in the homogeneity study (0.540 g). The vessels should be at room temperature before reconstitution, as the samples are hygroscopic.
The recommended method for the reconstitution of lyophilised samples is:

- Shake the sample by hand or agitate using a vortex-mixer or an overhead shaker
- Weigh out an adequate amount of lyophilised material
- Add respective amount of water as ultra-pure water and vortex-mix
- If vortex-mixing does not yield a visually uniform sample, treat the sample in an ultrasonic bath or homogenise using an overhead shaker for at least 15 min at ambient temperature.

Ideally, the reconstituted sample is stored at +4 °C for 1 hour before the analysis procedure begins.

Recommended Minimum Sample Amount

Participants will receive 4 samples of P220048. The samples are to be stored at -20°C or below under the absence of light. Before opening, the samples should be allowed to equilibrate to room temperature. As minimum sample intake 0.540 g lyophilized muscle (referring to 2 g of reconstituted sample) is recommended.

Dry Mass Determination

Participants are also requested to carry out dry mass correction. The determination of dry mass correction should be conducted with a recommended size of at least 0.3 g. The test sample portion taken for dry mass correction should be placed over anhydrous calcium sulphate (DRIERITE®) in a desiccator at room temperature for a minimum of 20 days until a constant mass is reached. Dry mass correction should be carried out at the same time as the test sample portion is to be analyzed in the same package of sample.

Homogeneity Assessment of Study Material

Homogeneity of the incurred samples was assessed in accordance with ISO 13528:2020 Annex B.2.2 [8]. For this method a suitable number of test items is chosen at random and analysed in duplicate. Subsequently, the standard deviation between the samples s_s and the analytical standard deviation s_w are calculated from the determined analyte contents. The standard deviation between the samples s_s is negligible if the sum of mean squares between the samples is smaller than the sum of the mean squares of the repeated analysis.

In order to assess the sample homogeneity for this study, a number of test items of the sample P220048 were chosen at random (Table 3). The samples were analysed in accordance with the validated EURL method NIIM_009 (available upon request).

In order to control for any possibly undetected heterogeneity, an F-test was also carried out. The result of the F-test was that no significant heterogeneity was detected (Table 4). Graphical representations of the results from homogeneity analysis are given in Figure 1.

Table 3: Samples randomly selected for the homogeneity assessment.

Sample	Test item production numbers
P220048	003, 036, 078, 092, 122, 146, 163, 199, 210, 229, 239

Figure 1 : Results of the homogeneity assessment for P220048 (Metronidazole in pig muscle; sample size 0.540 g lyophilized pig muscle)

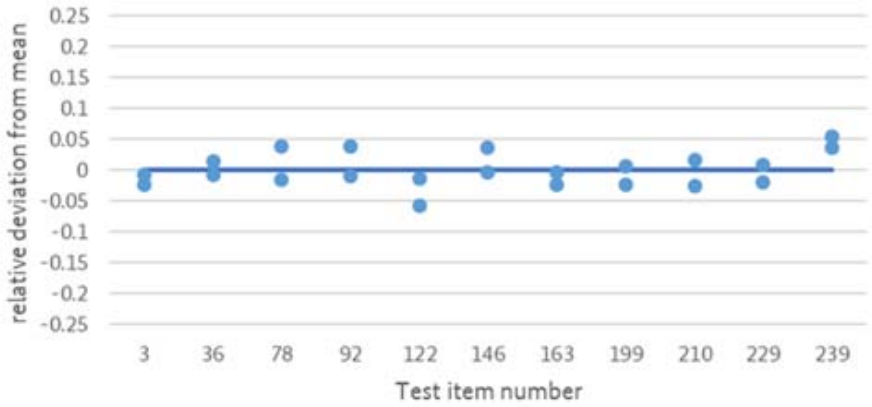


Table 4 : Results of ANOVA homogeneity assessment for metronidazole in muscle.

ANOVA Estimate	metronidazole
Within-packet, CV_{wth}:	1.2 %
Between-packet, CV_{btw}:	2.5 %
Total analytical variability, CV:	2.8 %
F / F_{crit} ($F_{0.05,10,22}$)	4.35 / 0.10
p-value (Probability of falsely rejecting the hypothesis that all samples have the same concentration):	0.75

Stability Assessment of Study Material

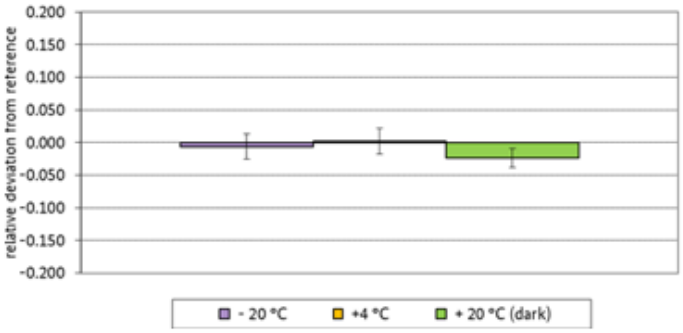
The short-term stability of the material was assessed in a stability study over the course of four weeks. This time period was chosen in order to assure sample stability during shipment, preparation, and analysis. Individually packaged aliquots of P220048 were stored at -80 °C (reference), 25 °C, +4 °C, and room temperature (dark). For every selected temperature-time combination two samples were analysed in duplicate in accordance with EURL method NIIM_009 (available upon request). The results of the stability study are displayed in Figure 3. The stability study is ongoing and more data will be provided once available (Table 5).

Considering the data obtained on the samples P220028, as well as results from previous stability studies on nitroimidazoles in muscle it was concluded that the samples were sufficiently stable for the purpose of this key comparison. No degradation is to be expected over the course of the study.

Table 5: Stability study outline: temperature-time combinations to be considered for the stability study (presently finalized time 28 days) .

Duration	-20 °C	+4 °C	Room temperature (dark)
28 days	X	X	X
6 months (August 2022)	X	X	X
2 years	X	X	X
3 years	X	X	
5 years	X	X	

Figure 2: Relative deviation of MNZ concentration from the reference concentration (-80 °C) in sample P220048 after 28 d. Error bars represent the standard deviation of the multiple measurements.



INSTRUCTIONS AND SAMPLE DISTRIBUTION

Participants should investigate the particular local customs and quarantine requirements for the samples to be sent to their countries. If special permits are required, please inform the organizer. The organizer will not be responsible for any charges such as import taxes or related charges for the importation of the samples.

Samples will be sent with cool packs, shipping on dry ice is not necessary due to the short term stability of the material at room temperature.

At the time of sample dispatch, participants will be informed individually per email including a tracking number. A sample receipt form will be provided electronically to all participants and must be filled in and returned to the study coordinator on receipt of the shipments.

RESULTS

The results should be reported in the unit of µg/kg for metronidazole on a dry mass basis and should include standard and expanded uncertainties (95 % level of confidence).

In addition to the quantitative results, participants should provide information on the measurement procedure (extraction, clean-up, column and conditions, quantification approach), calibration standards, internal standards, any quality control materials, number of replicates, the means of calculation of the results. Furthermore, any details regarding the estimation of measurement uncertainty should be included, generally any information relevant to the core competencies that the participants in this study are seeking to demonstrate.

Participants are required to carry out a dry mass correction. Dry mass correction should be carried out at the same time as the test sample portion is to be analyzed in the same package of sample.

The results reporting form will be provided electronically to each participant and must be completed and returned to the study coordinator before the submission deadline.

All the results of the key comparison will be evaluated against the key comparison reference value (KCRV). The KCRV will be determined from the results of all NMIs/DIs participating in the key comparison that have used appropriately validated methods with demonstrated metrological traceability. The draft A report will provide candidate estimates of the KCRV and its uncertainty for review and discussion by the OAWG.

Available Calibration Materials

Participants may establish 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 6 and 7 lists the CRMs that are available for use for this study.

Table 6: Metronidazole calibration CRMs available from NMIs/DIs.

Item	Item No.	Certified value	Producer	Reference
Metronidazole	8365	998.9 ±1.1 mg/g	INMETRO	[9]
Metronidazole	GBW(E)060908	99.9 % +/- 0.2 %	NIM China	[10]

Table 7: Available metronidazole matrix CRM.

Item	Item No.	Certified value	Producer	Reference
Nitroimidazoles in porcine muscle	ERM-BB124	(1.93±0.15) µg/kg	JRC	[11]

Table 8: Selection of available isotopically labelled internal standards for metronidazole.

Item	Item No.	Producer
Metronidazole-2-^{13}C-1,3-$^{15}N_2$	NM014	WITEGA Laboratorien Berlin-Adlershof GmbH, Berlin, Germany
Metronidazole-2-^{13}C-1,3-$^{15}N_2$	32744	Supelco, Merck KgaA, Darmstadt, Germany
Metronidazole-d4 (ethylene)	DRE-C15201001	Dr. Ehrenstorfer, Augsburg, Germany

Metronidazole-d4 (ethylene) 100 µg/mL in acetonitrile	DRE-A15201001AL-100	Dr. Ehrenstorfer, Augsburg, Germany
Metronidazole-d4 (ethylene)	TRC-M338882	Toronto Research Chemicals, Toronto, Canada
Metronidazole-d3 (methyl)	TRC-M978800	Toronto Research Chemicals, Toronto, Canada

USE OF CCQM-K180 (metronidazole in porcine muscle) IN SUPPORT OF CALIBRATION AND MEASUREMENT CAPABILITY (CMC) CLAIMS

How Far the Light Shines

Successful participation in CCQM-K180 will demonstrate the following measurement capabilities: determination of mass fraction of organic compounds, with molecular mass of 100 g/mol to 500 g/mol, having high polarity $pK_{ow} > -2$ in mass fraction range from 0.1 [ng/g] to 200 [ng/g] in an unprocessed food matrix of animal origin with a high protein content (e.g. animal tissue).

Furthermore additional measurement capabilities might be demonstrated (if applicable):

- value assignment of primary reference standards (if in-house purity assessment carried out)
- extraction of analytes of interest from the matrix,
- cleanup and separation of analytes of interest from interfering matrix or extract components,
- separation and quantification using gas or liquid chromatographic analytical systems.

Core Competency Statements and CMC support

An example of a Core Competency Table that will be used to claim competencies by the participants in this study is in the Annex and a respective template will be provided.

REFERENCES

[1] K. MAEDA, T. OSATO, H. UMEZAWA, The Journal of antibiotics 1953, 6, 182.

[2] Committee for veterinary medicinal products, Metronidazole summary report, 1997.

[3] Regulation (EC) No 470/2009 of the European Parliament and the Council of 6 May 2009 laying down Community procedures for the establishment of residue limits of pharmacologically active substances in foodstuffs of animal origin, repealing Council Regulation (EEC) No 2377/90 and amending Directive 2001/82/EC of the European Parliament and of the Council and Regulation (EC) No 726/2004 of the European Parliament and of the Council. Reg. (EC) 470/2009, 2009.

[4] Commission Regulation (EU) No 37/2010 of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin. Commission Regulation (EU) No 37/2010, 2009.

[5] EURL, EURL Guidance on minimum method performance requirements (MMPRs) for specific pharmacologically active substances in specific animal matrices, 2022 (https://eurl-residues.eu/ ; https://eurl-residues.eu/eurl-portal/portal-guidance-documents/).

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[8] International Organization for Standardization, Statistical methods for use in proficiency testing by interlaboratory comparison, ISO, Geneva, Switzerland.

[9] R. Nogueira, W. F. C. Rocha, T. E. Silva, E. C. P. Rego, G. F. Moreira, W. Wollinger, J. M. Rodrigues, J. Braz. Chem. Soc. 2012.

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[11] R. Zeleny, H. Schimmel, F. Ulberth, H. Emons, Certification of mass fractions of nitroimidazoles in pork meat, ERM-BB124, 2008.

APPENDIX A: Reporting Form

Template for reporting of results (will be provided in Excel or Word).

Bundesamt für Verbraucherschutz und Lebensmittelsicherheit

CCQM Key Comparison/Pilot Study
CCQM-K180 / P ?
Metronidazole in porcine muscle
Results Report Form

European Union Reference Laboratory supported by the European Commission

Please use this excel sheet for reporting.
Please submitted this report electronically to **joachim.polzer@bvl.bund.de** and **502@bvl.bund.de**
Please fill in all required information and use the requested units.
Please provide any extra information in the comments section or on a separate sheet if necessary.

Part I: Participant's Information

Laboratory Name:	
Contact person/ submitted by:	
Reporting Date: (dd/mm/yyyy)	
Programme Participated: (CCQM-K180 / P ?)	

Part II: Results:

Sample No. used for reporting	K180 -
Moisture content (w/w %):	

Analyte/ Mass Fraction	Mass fraction (µg/kg) reconstituted muscle	Combined Standard uncertainty u (µg/kg)	Coverage factor (k)	Expanded uncertainty U (µg/kg)	Number of replicates (n)
metronidazole					

Note: Please refer to the OAWG guidance document on significant figures when reporting values

Part III: Technical Information of Methodology Used	
Sample size used for analysis (g)	
Moisture content method (Please briefly describe the moisture determination procedure)	
Extraction method (Please briefly describe the extraction procedures, e.g. Liquid/Solid extraction, Soxhlet, etc., solvents, volumes, time, temperature etc.)	
Post extraction clean-up method and the transformation procedures, if any (e.g., SPE, etc) (Please briefly describe the clean-up and transformation procedures including any dilutions or concentration steps prior to analysis)	
Derivatization, if any	
Analytical instrument(s) used (e.g., LC-MS/MS,HPLC-FLD. etc) (Please specify the model)	
Chromatographic column	
The chromatographic condition(s) (e.g. LC Mobile phase gradient, etc)	
Method of quantification (e.g., external calibration, Internal standard calibration, IDMS, etc)	
Type of calibration (e.g., single-point, bracketing, three-point calibration curve, etc.)	
Native calibration standards: source, confirmation of identity, value assignment, uncertainty and traceability	
Reference material used for calibration is in compliance with the requirements for Traceability in CIPM MRA (Document No.: CIPM 2009-24; Latest update: Revised 13 Oct. 2009):	
Internal standards used (Please specify the compounds, and at which stage of analysis were the internal standards added.)	
Indicate ions /MRMs measured in the mass spectrometer instrument.	

Part IV: Uncertainty budget

The measurement equations used to calculate the mass fraction of each analyte. Please provide details of all the factors listed in the equations and indicate how these values were determined.

Estimation of uncertainties for each factor. Give a complete description of how the estimates were obtained and combined to calculate the overall uncertainty. Please provide a table detailing the full uncertainty budget.

Part V: Comments / additional information

Other information, observations or evidences, if any, that can further support your results.

APPENDIX E: Core Competency Table

CCQM OAWG: Competency Template for metronidazole in porcine muscle

CCQM-K180	*NMI/DI*	Polare analyte in a protein rich matrix – metronidazole in muscle
Scope of Measurement: : Successful participation in this study may demonstrate participants' capabilities in determining high-polarity analytes (pKow > -2) with molecular mass range from 100 to 500 g/mol at mass fraction levels of 0.1 to 200 µg/kg in a high protein food matrix. This may include demonstration of measurement capabilities such as: (1) value assignment of primary reference standards; (2) extraction of analyte of interest from the matrix; (3) cleanup and separation of analyte of interest from other interfering matrix or extract components; (4) separation and quantification using techniques such as LC-MS/MS, LC-MS, LC-HRMS, GC/MS		
Competency	**✓, ×, or N/A**	**Specific Information as Provided by *NMI/DI***
Competencies for Value-Assignment of Calibrant		
Calibrant: Did you use a "highly-pure substance" or calibration solution?		*Indicate if you used a "pure material" or a calibration solution. Indicate its source and ID, eg CRM identifier*
Identity verification of analyte(s) in calibration material.		*Indicate method(s) you used to identify analyte(s)*
For calibrants which are a highly-pure substance: Value-Assignment / Purity Assessment method(s).		*Indicate how you established analyte mass fraction/purity (i.e., mass balance (list techniques used), qNMR, other)*
For calibrants which are a calibration solution: Value-assignment method(s).		*Indicate how you established analyte mass fraction in calibration solution*
Sample Analysis Competencies		
Identification of analyte(s) in sample		*Indicate method(s) you used to identify analyte(s) in the sample (i.e., Retention time, mass spec ion ratios, other)*
Extraction of analyte(s) of interest from matrix		*Indicate extraction technique(s) used, if any, (i.e. Liquid/liquid, Soxhlet, ASE, other)*
Cleanup - separation of analyte(s) of interest from other interfering matrix components (if used)		*Indicate cleanup technique(s) used, if any (i.e., SPE, LC fractionation, other)*
Transformation - conversion of analyte(s) of interest to detectable/measurable form (if used)		*Indicate chemical transformation method(s), if any, (i.e., hydrolysis, derivatization, other)*
Analytical system		*Indicate analytical system (i.e., LC-MS/MS, GC-HRMS, GC-ECD, other)*
Calibration approach for value-assignment of analyte(s) in matrix		*a) Indicate quantification mode used (i.e., IDMS, internal standard, external standard, other)* *b) Indicate calibration mode used (i.e., single-point calibration, bracketing, x-point calibration curve, other)*
Verification method(s) for value-assignment of analyte(s) in sample (if used)		*Indicate any confirmative method(s) used, if any.*
Other		*Indicate any other competencies demonstrated.*

Instructions:

- In the middle column place a tick, cross or say the entry is not applicable for each of the competencies listed (the first row does not require a response)
- Fill in the right hand column with the information requested in blue in each row
- Enter the details of the calibrant in the top row, then for materials which would not meet the CIPM traceability requirements the three rows with a * require entries.

Updated protocol of 14 October 2022

The desired concentration range of metronidazole in the study material was adapted to 0.1-200 g/kg.

Information was added that the results should only be submitted for a single unit of the provided sample material.

Updated protocol of 31 January 2023

The proposed timeline was adjusted.

Updated protocol of 28 June 2023

It was clarified that the results should be reported for mass fraction of free metronidazole.

APPENDIX C: Registration Form

No formal registration form was provided. The participants registered via e-mail.

APPENDIX D: Reporting Form

The template for reporting of the results was provided as an Excel file.

Bundesamt für Verbraucherschutz und Lebensmittelsicherheit

CCQM Key Comparison/Pilot Study
CCQM-K180 / P ?
Metronidazole in porcine muscle
Results Report Form

European Union Reference Laboratory supported by the European Commission

Please use this excel sheet for reporting.
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Sample No. used for reporting	K180 -
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metronidazole					

Note: Please refer to the OAWG guidance document on significant figures when reporting values

Part III: Technical Information of Methodology Used	
Sample size used for analysis (g)	
Moisture content method (Please briefly describe the moisture determination procedure)	
Extraction method (Please briefly describe the extraction procedures, e.g. Liquid/Solid extraction, Soxhlet, etc., solvents, volumes, time, temperature etc.)	
Post extraction clean-up method and the transformation procedures, if any (e.g., SPE, etc) (Please briefly describe the clean-up and transformation procedures including any dilutions or concentration steps prior to analysis)	
Derivatization, if any	-
Analytical instrument(s) used (e.g., LC-MS/MS,HPLC-FLD. etc) (Please specify the model)	
Chromatographic column	
The chromatographic condition(s) (e.g. LC Mobile phase gradient, etc)	
Method of quantification (e.g., external calibration, Internal standard calibration, IDMS, etc)	
Type of calibration (e.g., single-point, bracketing, three-point calibration curve, etc.)	
Native calibration standards: source, confirmation of identity, value assignment, uncertainty and traceability	
Reference material used for calibration is in compliance with the requirements for Traceability in CIPM MRA (Document No.: CIPM 2009-24; Latest update: Revised 13 Oct. 2009):	
Internal standards used (Please specify the compounds, and at which stage of analysis were the internal standards added.)	
Indicate ions /MRMs measured in the mass spectrometer instrument.	

Part IV: Uncertainty budget
The measurement equations used to calculate the mass fraction of each analyte. Please provide details of all the factors listed in the equations and indicate how these values were determined.
Estimation of uncertainties for each factor. Give a complete description of how the estimates were obtained and combined to calculate the overall uncertainty. Please provide a table detailing the full uncertainty budget.
Part V: Comments / additional information
Other information, observations or evidences, if any, that can further support your results.

APPENDIX E: Core Competency Table Form

CCQM OAWG: Competency Template for metronidazole in porcine muscle

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Scope of Measurement: : Successful participation in this study may demonstrate participants' capabilities in determining high-polarity analytes (pKow > -2) with molecular mass range from 100 to 500 g/mol at mass fraction levels of 0.1 to 200 µg/kg in a high protein food matrix. This may include demonstration of measurement capabilities such as: (1) value assignment of primary reference standards; (2) extraction of analyte of interest from the matrix; (3) cleanup and separation of analyte of interest from other interfering matrix or extract components; (4) separation and quantification using techniques such as LC-MS/MS, LC-MS, LC-HRMS, GC/MS		
Competency	**✓,×, or N/A**	**Specific Information as Provided by *NMI/DI***
Competencies for Value-Assignment of Calibrant		
Calibrant: Did you use a "highly-pure substance" or calibration solution?		*Indicate if you used a "pure material" or a calibration solution. Indicate its source and ID, eg CRM identifier*
Identity verification of analyte(s) in calibration material.		*Indicate method(s) you used to identify analyte(s)*
For calibrants which are a highly-pure substance: Value-Assignment / Purity Assessment method(s).		*Indicate how you established analyte mass fraction/purity (i.e., mass balance (list techniques used), qNMR, other)*
For calibrants which are a calibration solution: Value-assignment method(s).		*Indicate how you established analyte mass fraction in calibration solution*
Sample Analysis Competencies		
Identification of analyte(s) in sample		*Indicate method(s) you used to identify analyte(s) in the sample (i.e., Retention time, mass spec ion ratios, other)*
Extraction of analyte(s) of interest from matrix		*Indicate extraction technique(s) used, if any, (i.e. Liquid/liquid, Soxhlet, ASE, other)*
Cleanup - separation of analyte(s) of interest from other interfering matrix components (if used)		*Indicate cleanup technique(s) used, if any (i.e., SPE, LC fractionation, other)*
Transformation - conversion of analyte(s) of interest to detectable/measurable form (if used)		*Indicate chemical transformation method(s), if any, (i.e., hydrolysis, derivatization, other)*
Analytical system		*Indicate analytical system (i.e., LC-MS/MS, GC-HRMS, GC-ECD, other)*
Calibration approach for value-assignment of analyte(s) in matrix		*a) Indicate quantification mode used (i.e., IDMS, internal standard, external standard, other)* *b) Indicate calibration mode used (i.e., single-point calibration, bracketing, x-point calibration curve, other)*
Verification method(s) for value-assignment of analyte(s) in sample (if used)		*Indicate any confirmative method(s) used, if any.*
Other		*Indicate any other competencies demonstrated.*

Instructions:

- In the middle column place a tick, cross or say the entry is not applicable for each of the competencies listed (the first row does not require a response)

- Fill in the right hand column with the information requested in blue in each row
- Enter the details of the calibrant in the top row, then for materials which would not meet the CIPM traceability requirements the three rows with a # require entries.

APPENDIX F: Summary of Participants' Analytical Information

Table 13 (sample preparation) and Table 14 (chromatography) list the methodological details provided by the participants. They have been edited for brevity and uniformity.

Table 13: Details of the analytical methods employed by the participants of CCQM-K180 for the determination of metronidazole.

Lab	Sample size	Hydrolysis	Extraction	Defatting	Clean-up	Calibration	Quantification
BVL	1 g reconstituted sample	protease	5 mL buffer (5.84 g NaCl + 13.61 g KH2PO4 in 1 L water @pH=3) and 10 mL protease solution; 1 min in IKA Ultraturrax @ 6000 rpm	hexane	Extrelut	six points	internal standard, gravimetric
CENAM	1.08 g	no	2x20 mL ethyl acetate	no	SPE MCX 6cc	single point	IDMS
EXHM	1 g reconstituted sample	no[5]	10 mL acetonitrile+0.1 % formic acid; 3 min vortex	no	none except water removal	single point	exact matching IDMS
GLHK	0.54 g	no	5 mL acetonitrile+1 % acetic acid; 30 min ultrasonic and vertical shaking	hexane	SPE MCX	single point	IDMS
HSA[6]	0.54-0.57 g lyophilised sample, reconstituted with water to 2 g prior to analysis	no	15 mL ethyl acetate+1 % formic acid; 2 min vortex, 5 min ultrasonication, 10 min multitube shaker	hexane	none except ultracentrifugation filtration	single point	exact matching IDMS
INM Colombia	0.6 g lyophilised sample reconstituted with water prior to analysis	no	10 mL water, 10 mL acetonitrile; 2 min shaking by hand, 30 s vortex @ 3000 rpm; saline buffer 4 g MgSO4+ 1 g NaCl + 0.5 g sodium citratesesquihydrate+ 1 g sodium citratetrihydrate; 3 min shaking by hand	no	dSPE with 900 mg MgSO4, 150 mg PSA, 150 mg C18	bracketing, matrix-matched	internal standard
INMETRO	0.54 g lyophilised sample reconstituted with water prior to analysis	no	5 mL NaCl solution (0.2 g/mL), 2x5 mL ethyl acetate; 2 min vortex, 20 min ultrasonication	hexane	none	bracketing	IDMS
INRAP	1.08 g lyophilised sample reconstituted with water prior to analysis	no	8 mL ethyl acetate	no	none	matrix calibration	internal standard
KRISS	0.54 g lyophilised sample	no	9.46 mL water; 1 h equilibration at 4 °C, 5 min ultrasonication, 10 min shaking; supernatant extracted with 2x20 mL ethyl acetate; 5 min shaking	no	none except PVDF filtration	single point	IDMS

[5] The laboratory reported that enzymatic digestion and clean-up using Extrelut provided equivalent results.

[6] The laboratory reported that they studied extraction by ethyl acetate, methanol and 0.1% formic acid in ethyl acetate and estimated the uncertainty of different sample extraction solvents from the results. They had also studied the influence of multiple extraction cycles but had found no significant improvement of the extraction efficiency with three cycles as opposed to two cycles.

Lab	Sample size	Hydrolysis	Extraction	Defatting	Clean-up	Calibration	Quantification
LGC	0.54 g lyophilised sample	no	8 mL water; equilibration overnight; 20 mL MeOH+200 µL acetic acid; 2 h extraction in rotary mixer	no	SPE Oasis HLB	bracketed single point exact matching	double exact matching IDMS
NIM China	0.54 g lyophilised sample reconstituted with water prior to analysis	no	2x15 mL ethyl acetate; 45 min shaking	no	none except PP membrane filtration	single point	exact matching IDMS
NIMT[7]	0.7 g	no	5 mL water; equilibration 1 h at 4 °C; 10 mL ethyl acetate, 5 mL ethyl acetate; 30 min vortex and mechanical shaker	hexane	SPE Oasis MCX 6 mL/150 mg	single point, bracketing	exact matching double IDMS
NMIA	0.55 g	no	acetonitrile (6+4+4 mL) + 1.5 g MgSO4:NaCl (4:1); 20 min tumbling, 2x15 min ultrasonication	no	SPE MCX	single point, bracketing	exact matching double IDMS
NRC	0.5 g lyophilised sample reconstituted with water prior to analysis	no	5 mL acetonitrile; 30 s vortex; 0.5 g NaCl+2 g MgSO4; 2 min vortex; 5 mL acetonitrile; 2 min vortex	hexane	none	matrix calibration, standard addition	exact matching double IDMS
SASO-NMCC	0.7 g, 1.25 g	no	10+5+5 mL ethyl acetate; vortex, 30 min shaking	hexane	SPE C18/OH 1g/6 mL	multiple points	external calibration
UME	1 g lyophilised sample reconstituted with water prior to analysis	no	10 mL ethyl acetate; 5 min vortex	hexane	none	three points	IDMS
VNIIM	0.6 g lyophilised sample reconstituted with water prior to analysis	no	3x7 mL ethyl acetate; vortex	no	SPE SCX 200 mg/3 mL	single point	IDMS

[7] The laboratory reported that they had compared equilibrating the reconstituted test material for one hour and overnight and had found no significant differences in the overall results.

Table 14: Details of the chromatography and the ion transitions employed by the participants of CCQM-K180 for the determination of metronidazole.

<table>
<tr><th>Lab</th><th>Separation, Detection</th><th>Ion transitions</th></tr>
<tr><td>BVL</td><td>Sciex Triple Quad 6500+ LC-MS/MS System coupled to Agilent 1290 Infinity II LC System
column: Phenomenex Luna Omega C18, (100 x 2.1) mm, 1.6µm, C18 pre-column
mobile phase A: 2 mM aqueous ammoniumformate + 0.1 % formic acid
mobile phase B: acetonitrile + 0.1% formic acid
flow: 300 µL/min
injection volume: 5 µL
Gradient:<table><tr><th>t /min</th><th>%A</th></tr><tr><td>0.0</td><td>95</td></tr><tr><td>2.0</td><td>95</td></tr><tr><td>5.0</td><td>80</td></tr><tr><td>7.0</td><td>50</td></tr><tr><td>9.0</td><td>10</td></tr><tr><td>10.5</td><td>10</td></tr><tr><td>11.0</td><td>95</td></tr><tr><td>12.0</td><td>95</td></tr></table></td><td>MNZ : m/z=172.0 >128.1; 82.0; 111.8
Internal standard: m/z=176.6 >132.1; 85.9; 114.9</td></tr>
<tr><td>CENAM</td><td>Waters Xevo-TQS LC-MS/MS
column: Waters Xbridge C18 (2.1 x 150) mm, 5 µm
mobile phase: 1mM aqueous ammonium acetate:ACN (10:90, v:v), pH 3.57</td><td>m/z=172; 176</td></tr>
<tr><td>EXHM</td><td>Thermo Finnigan Surveyor MS pump+ and autosampler coupled to Thermo Finnigan TSQ Quantum Ultra AM
column: Agilent Zorbax Eclipse XDB-C18, (2.1 x 150) mm, 3.5 µm
mobile phase A: water + 0.1 % formic acid
mobile phase B: acetonitrile + 0.1 % formic acid
flow: 300 µL/min
gradient:<table><tr><th>t /min</th><th>%A</th></tr><tr><td>0</td><td>90</td></tr><tr><td>5</td><td>10</td></tr><tr><td>6</td><td>10</td></tr><tr><td>7</td><td>90</td></tr><tr><td>10</td><td>90</td></tr></table></td><td>m/z=172.000>82.000; 110.800; 128.100
m/z=175.000>85.000; 113.800; 131.100</td></tr>
<tr><td>GLHK</td><td>Sciex Triple QuadTM 7500 LC-MS/MS System - QTRAP® Ready coupled to Agilent 1290 Infinity II LC System
column: Waters Acquity UPLC HSS T3 column 100Å, 1.8 µm, (2.1 x 150) mm
mobile phase A : 0.1 % (v/v) formic acid in water
mobile phase B: 0.1 % (v/v) formic acid in methanol
gradient: At 0 min - 10 min, 10% B (0.3 mL/min)
At 10.5 min - 12 min, 95% B (0.3 mL/min)
At 12.5 min - 20 min, 10% B (0.3 mL/min)</td><td>MNZ: m/z=172>128 (Quantification); 82; 111
Internal standard: m/z=176>132 (Quantification); 86; 115</td></tr>
</table>

<table>
<tr><th>Lab</th><th>Separation, Detection</th><th>Ion transitions</th></tr>
<tr><td>HSA[8]</td><td>Thermo Scientific TSQ AltisTM triple quadrupole LC-MS/MS system
Set-up 1:
column: Agilent Zorbax SB-Aq (2.1 x 100) mm, 3.5 µm
mobile phase A: 0.1 % formic acid in water
mobile phase B: 0.1 % formic acid in acetonitrile
flow: 0.3 mL/min
gradient:
<table>
<tr><td>t /min</td><td>%B</td></tr>
<tr><td>0</td><td>5</td></tr>
<tr><td>5</td><td>90</td></tr>
<tr><td>6</td><td>5</td></tr>
<tr><td>10</td><td>Stop</td></tr>
</table>
Set-up 2:
column: Agilent Poroshell 120 EC-C18 (4.6 x 50) mm, 2.7 µm
mobile phase A: 0.1 % formic acid in water
mobile phase B: 0.1 % formic acid in acetonitrile
flow: 0.3 mL/min
gradient:
<table>
<tr><td>t /min</td><td>%B</td></tr>
<tr><td>0.0</td><td>5</td></tr>
<tr><td>1.0</td><td>5</td></tr>
<tr><td>8.0</td><td>90</td></tr>
<tr><td>8.5</td><td>5</td></tr>
<tr><td>11.0</td><td>Stop</td></tr>
</table>
Set-up 3:
column: Waters Xterra MS C18 (2.1 x 100) mm, 3.5 µm
mobile phase A: 0.1 % formic acid in water
mobile phase B: 0.1 % formic acid in acetonitrile
flow: 0.3 mL/min
gradient:
<table>
<tr><td>t /min</td><td>%B</td></tr>
<tr><td>0</td><td>5</td></tr>
<tr><td>5</td><td>5</td></tr>
<tr><td>6</td><td>90</td></tr>
<tr><td>8</td><td>5</td></tr>
<tr><td>13</td><td>Stop</td></tr>
</table></td><td>MNZ: m/z=172 >127.97 (Quantification) 82.13
Internal standard: m/z=176 >132.2 (Quantification); 86</td></tr>
</table>

[8] The participants reported that they had also used a Kinetex Polar C18 (100 x 2.1 mm, 2.6 µm) column in order to estimate the uncertainty arising from the application of different chromatographic columns.

<table>
<tr><th>Lab</th><th>Separation, Detection</th><th>Ion transitions</th></tr>
<tr><td>INM
Colombia</td><td>Sciex QTRAP 4500 coupled to Sciex ExionLC
column: Themro Scientific Acclaim RSLC 120 A C18 (2.1 x 100) mm, 2.2 µm
column temperature: 50 °C
mobile phase A: water/methanol/formic acid 97/3/0.1
mobile B: methanol/formic acid 99.9/0.1
gradient:
<table><tr><th>t /min</th><th>%A</th></tr><tr><td>0.0</td><td>98</td></tr><tr><td>2.0</td><td>98</td></tr><tr><td>2.5</td><td>80</td></tr><tr><td>3.5</td><td>60</td></tr><tr><td>4.0</td><td>30</td></tr><tr><td>5.0</td><td>0</td></tr><tr><td>6.0</td><td>0</td></tr><tr><td>7.0</td><td>60</td></tr><tr><td>8.0</td><td>90</td></tr><tr><td>9.0</td><td>98</td></tr></table></td><td>MNZ: m/z=171.850>128.000
Internal standard: m/z=163.024>87.900</td></tr>
<tr><td>INMETRO</td><td>Waters Xevo TQ
column: Waters Acquity BEH Phenyl 1.7 µm, (2.1 x 100) mm
mobile Phase A: water + 0.1 % acetic acid
mobile phase B: acetonitrile
flow: 0.4 mL/min
gradient:
<table><tr><th>t /min</th><th>%A</th></tr><tr><td>0.0</td><td>90</td></tr><tr><td>1.0</td><td>50</td></tr><tr><td>1.5</td><td>90</td></tr><tr><td>3.0</td><td>90</td></tr></table></td><td>MRM with ESI(+)
MNZ: m/z=172.1 > 82.3; 128.3
Internal standard: m/z=176.1 > 132.3</td></tr>
<tr><td>INRAP</td><td>LC-MS/MS
column: C18 (150 x 3.5) mm, 2.1 µm
mobile phase A : water + 0.2 % formic acid (v/v)
mobile phase B : acetonitrile</td><td>m/z=172 > 82.00; 128.00</td></tr>
<tr><td>KRISS</td><td>LC-MS/MS
column: Waters HSS T3 (2.1 × 100) mm, 1.8 µm
mobile phase A: 10 mM aqueous ammonium formate
mobile phase B: methanol</td><td>MNZ: m/z=172>128
Internal standard: m/z=176>132</td></tr>
</table>

<table>
<tr><th>Lab</th><th>Separation, Detection</th><th>Ion transitions</th></tr>
<tr><td>LGC</td><td>Waters premier LC system coupled to Waters TQ Absolute Triple Quad
column: Avantor ACE Excel 2 C18 PFP (150 x 3) mm, 2 µm
mobile phase A: water + 0.1 % FA
mobile Phase B: methanol + 0.1 % FA
flow: 0.3 mL/min
injection volume: 4 µL
temperature: 40 °C
gradient:
<table>
<tr><th>t /min</th><th>%A</th></tr>
<tr><td>0</td><td>95</td></tr>
<tr><td>2</td><td>95</td></tr>
<tr><td>5</td><td>70</td></tr>
<tr><td>10</td><td>70</td></tr>
<tr><td>11</td><td>20</td></tr>
<tr><td>16</td><td>20</td></tr>
<tr><td>17</td><td>95</td></tr>
<tr><td>27</td><td>95</td></tr>
</table>
</td><td>MNZ: m/z=172.2>82.1 (Quantification); 128.0; 111.2
Internal standard: m/z=176.1>86.0 (Quantification); 115.1; 132.1</td></tr>
</table>

<table>
<tr><th>Lab</th><th>Separation, Detection</th><th>Ion transitions</th></tr>
<tr><td>NIM
China</td><td>Thermo TSQ Altis MS/MS instrument coupled to Dionex UltiMate 3000 UHPLC system
Set-up 1:
column: Waters Acquity UPLC BEH C18 (2.1 x 100) mm, 1.7 µm
mobile phase A: water + 0.1 % formic acid
mobile phase B: methanol
flow: 0.3 mL/min
gradient:
<table>
<tr><th>t /min</th><th>%B</th></tr>
<tr><td>0.0</td><td>5</td></tr>
<tr><td>0.5</td><td>5</td></tr>
<tr><td>1.5</td><td>10</td></tr>
<tr><td>8.5</td><td>12</td></tr>
<tr><td>12.5</td><td>90</td></tr>
<tr><td>13.5</td><td>90</td></tr>
<tr><td>13.6</td><td>5</td></tr>
<tr><td>17.5</td><td>5</td></tr>
</table>
Set-up 2:
column: Waters Acquity UPLC HSS T3 (2.1 x 100) mm, 1.8 µm
mobile phase A: water + 0.1 % formic acid
mobile phase B: methanol
flow: 0.3 mL/min
gradient:
<table>
<tr><th>t /min</th><th>%B</th></tr>
<tr><td>0.0</td><td>5</td></tr>
<tr><td>0.5</td><td>5</td></tr>
<tr><td>1.5</td><td>10</td></tr>
<tr><td>5.5</td><td>12</td></tr>
<tr><td>7.5</td><td>90</td></tr>
<tr><td>9.5</td><td>90</td></tr>
<tr><td>9.6</td><td>5</td></tr>
<tr><td>14.0</td><td>5</td></tr>
</table></td><td>MNZ: m/z=172.1>127.9 (Quantification); 82.07
Internal standard: m/z=176.1> 132.0 (Quantification); 86.07</td></tr>
<tr><td>NIMT</td><td>Shimadzu LC system coupled to Sciex API 4000
column: Agilent Zorbax SB-C18, 4.6 µm, (150 x 4.6) mm
mobile phase A: water + 0.1 % formic acid
mobile phase B: acetonitrile + 0.1 % formic acid
flow: 0.6 mL/min
isocratic elution: 80:20 A:B, 0-15 min</td><td>MNZ: m/z=172>128 (Quantification); 82
Internal standard: m/z=176>132 (Quantification); 86</td></tr>
</table>

<table>
<tr><th>Lab</th><th>Separation, Detection</th><th>Ion transitions</th></tr>
<tr><td>NMIA</td><td>Two-dimensional LC-MSMS using a Sciex 7500 coupled to ExionLC binary LC pumps
D1 column 1: Waters Acquity UPLC HSS T3 (50 x 2.1) mm, 1.8 µm
D1 column 2: Avantor ACE UltraCore SuperC18 (50 x 2.1) mm, 2.5 µm
D2 column 3: Restek Force Biphenyl (50 x 2.1) mm, 1.8 µm
D2 column 4: Avantor ACE UltraCore SuperPhenylHexyl (50 x 2.1) mm, 2.5 µm
D1 organic modifier: methanol
D2 organic modifer: acetonitrile
D1 aqueous phase: water or 5 mM ammonium formate
D2 aqueous phase: 0.01 % formic acid or 5 mM ammonium formate
Column ovens set to 40 °C
see table for details
<table>
<tr><td></td><td>T3-PhHex</td><td></td><td>C18-PhHex</td><td></td><td>C18-Bip</td><td></td></tr>
<tr><td>D1 column</td><td>column 1</td><td></td><td>column 2</td><td></td><td>column 2</td><td></td></tr>
<tr><td>D1 organic modifier</td><td>methanol</td><td></td><td>methanol</td><td></td><td>methanol</td><td></td></tr>
<tr><td>D1 aqueous modifier</td><td>5 mM ammonium formate</td><td></td><td>water only</td><td></td><td>water only</td><td></td></tr>
<tr><td>D1 gradient time</td><td>0 min</td><td>4.7 min</td><td>0 min</td><td>3.4 min</td><td>1 min</td><td>3.4 min</td></tr>
<tr><td>D1 % organic</td><td>2 %</td><td>5 %</td><td>1 %</td><td>5 %</td><td>1 %</td><td>5 %</td></tr>
<tr><td>MNZ transfer</td><td>4.00 - 4.55 min</td><td></td><td>2.35 - 2.85 min</td><td></td><td>2.35 - 2.90 min</td><td></td></tr>
<tr><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>D2 column</td><td>column 4</td><td></td><td>column 4</td><td></td><td>column 3</td><td></td></tr>
<tr><td>D2 organic modifier</td><td>acetonitrile</td><td></td><td>acetonitrile</td><td></td><td>acetonitrile</td><td></td></tr>
<tr><td>D2 aqueous modifier</td><td>5 mM ammonium formate</td><td></td><td>0.01% formic acid</td><td></td><td>5 mM ammonium formate</td><td></td></tr>
<tr><td>D2 gradient time</td><td>4.6 min</td><td>7 min</td><td>2.86 min</td><td>4.4 min</td><td>2.91 min</td><td>6.9 min</td></tr>
<tr><td>D2 % organic</td><td>3 %</td><td>7 %</td><td>3 %</td><td>10 %</td><td>3 %</td><td>5.5 %</td></tr>
<tr><td>MNZ elution</td><td>6.1 min</td><td></td><td>4.0 min</td><td></td><td>6.1 min</td><td></td></tr>
</table></td><td>MNZ: m/z=172.0>128.0; 82.0; 111.0
Internal standard: m/z=176.0>132.0; 86.0; 115.0</td></tr>
<tr><td>NRC</td><td>Thermo Quantiva triple quadrupole MS coupled to Agilent 1290 Infinity I UPLC
column: Phenomenex Kinetix C18, (100 x 3) mm, 1.7 µm + guard column Phenomenex SecurityGuard C18, 2.1mm
mobile phase A: water:formic acid 100:0.1
mobile Phase B: acetonitrile:formic acid 100:0.1
flow: 0.5 mL/min
gradient:
<table>
<tr><td>t /min</td><td>%B</td></tr>
<tr><td>0.0</td><td>5</td></tr>
<tr><td>0.5</td><td>5</td></tr>
<tr><td>3.0</td><td>40</td></tr>
<tr><td>3.2</td><td>90</td></tr>
<tr><td>4.0</td><td>90</td></tr>
<tr><td>4.2</td><td>5</td></tr>
<tr><td>6.5</td><td>5</td></tr>
</table></td><td>MNZ: m/z=172.1>128.1; 82.1
Internal standard: m/z=175.1>131.1; 85.1</td></tr>
<tr><td>SASO-NMCC</td><td>LC-MS/MS
column: Thermo Scientific Hypersil GOLD 3 µm (150 x 4.6) mm
mobile phase: water:methanol (70:30) + 0.2 % formic acid</td><td>MNZ: m/z=172>127.5; 128.5</td></tr>
<tr><td>UME</td><td>Thermo Scientific QExactive ORBITRAP Ultimate 3000
column: Phenomenex Luna C18 3µm 100A, (150 x 2) mm
mobile Phase: A: methanol:water 10:90 + 0.1 % formic acid
mobile phase B: methanol
Flow rate: 0.3 mL/min
isocratic elution: 10:90 A:B</td><td>MNZ: m/z=172.07
Internal standard: m/z=176.09</td></tr>
</table>

<table>
<tr><th>Lab</th><th>Separation, Detection</th><th>Ion transitions</th></tr>
<tr><td>VNIIM</td><td>LC-MS/MS Agilent TripleQuad 6460
column: Agilent Zorbax Bonus-RP (4.6 x 150mm), 5 µm
mobile phase A: water + 0.1 % formic acid
mobile phase B: acetonitrile + 0.1 % formic acid
gradient:
<table>
<tr><th>t /min</th><th>%B</th></tr>
<tr><td>0</td><td>10</td></tr>
<tr><td>5</td><td>10</td></tr>
<tr><td>6</td><td>40</td></tr>
<tr><td>7</td><td>80</td></tr>
<tr><td>12</td><td>80</td></tr>
<tr><td>13</td><td>10</td></tr>
<tr><td>18</td><td>10</td></tr>
</table></td><td>MNZ: m/z=172>128; 86
Internal standard: m/z=176>132</td></tr>
</table>

APPENDIX G: Summary of Participants' Uncertainty Estimation Approaches

The following are pictures of the uncertainty-related information provided by the participants in the "Analytical Information" worksheet of the "Reporting Form" Excel workbook. Information is grouped by participant and presented in alphabetized acronym order.

Uncertainty Information of BVL

Part IV: Uncertainty budget

The measurement equations used to calculate the mass fraction of each analyte. Please provide details of all the factors listed in the equations and indicate how these values were determined.

$$\text{Mass fraction (MNZ) } (\mu g/kg) = \frac{a.A_{(mnz)}.C_{(IS)}+b.C_{(IS)}.A_{(IS)}}{A_{(IS)}.m_{sample}(1-DW)} = \frac{a\,.\,A_{(mnz)}.m_{(IS)}+b\,.\,m_{(IS)}.A_{(IS)}}{A_{(IS)}.V_{IS}.m_{sample}(1-DW)}$$

Estimation of uncertainties for each factor. Give a complete description of how the estimates were obtained and combined to calculate the overall uncertainty. Please provide a table detailing the full uncertainty budget.

$$u_{(mnz)} = x\,(mnz)\,.\sqrt{(u_x/x)_{rep.}{}^2 + (u_x/x)_{calib}{}^2 + (u_x/x)_{sample\ weight.}{}^2 + (u_x/x)_{moisture}{}^2 + (u_x/x)_{sample\ spike(IS)}{}^2}$$

Part V: Comments / additional information

Other information, observations or evidences, if any, that can further support your results.

contributions to measurement uncertainty:

	u	(unit)	target	(unit)	u(x)/X [%]	(u(x)/X)^2
u calibration solution:	0,01923	ng/g	12,766	ng/g	0,1506	0,02269
u sample weight:	0,02444	mg	1000,000	mg	0,0024	0,00001
u sample spike:	0,02105	mg	15,470	mg	0,1361	0,01852
u dry mass:	0,00074	g	0,493	g	0,1510	0,02280
reproducibility method:	0,15264	ng/g	2,120	ng/g	7,2000	51,84000

Uncertainty Information of CENAM

METRONIDAZOLE		CCQM K180										
#	X Source of uncertainty	Value	Units	Source of information	Original uncertainty or s	Type of Uncertainty	Distribution Type	Standard uncertainty	Combined uncertainty u c	U relative u i(y) Value	Combined uncertainty u c (preparation)	U relative u i(y) Value
1	sample mass	1.08	g						0.000024	0.000022		0.000022
	balance repeatability		g	Certificate of calibration CNM-CC-730-185/2022	0.00004	B	Rectangular	0.000023				
	indication error		g	Certificate of calibration CNM-CC-730-185/2023	0.00005	B	Rectangular	0.000050				
	balance resolution		g	Certificate of calibration CNM-CC-730-185/2024	0.00001	B	Rectangular	0.000006				
2	Calibrant preparation uncertainty	0.99	µg/kg								0.001334	0.001346
	Calibrant purity MTNDZ	99.92	g/100 g	CENAM value for LRAC8503	0.0050	B	Rectangular	0.0050	0.0050	0.000050		
	Calibrant mass (stock solution)	1.185	mg						0.001291	0.001089		
	balance repeatability		mg	Certificate of calibration CNM-CC-730-133/2022	0.002	B	Rectangular	0.001155				
	indication error		mg	Certificate of calibration CNM-CC-730-133/2022	0.00315	B	Rectangular	0.003150				
	balance resolution		mg	Certificate of calibration CNM-CC-730-133/2022	0.001	B	Rectangular	0.000577				

	Total mass of the solution	58.59506	g						0.046188	0.000788		
	balance repeatability		g	Certificate of calibration CNM-CC-730-185/2022	0.08	B	Rectangular	0.046188				
	indication error		g	Certificate of calibration CNM-CC-730-185/2023	0.000055	B	Rectangular	0.000055				
	balance resolution		g	Certificate of calibration CNM-CC-730-185/2024	0.00001	B	Rectangular	0.000006				
	Calibrant mass (work solution)	1.47266	g						0.000024	0.000016		
	balance repeatability		g	Certificate of calibration CNM-CC-730-185/2022	0.00004	B	Rectangular	0.000023				
	indication error		g	Certificate of calibration CNM-CC-730-185/2023	0.00005	B	Rectangular	0.000050				
	balance resolution		g	Certificate of calibration CNM-CC-730-185/2024	0.00001	B	Rectangular	0.000006				
	Total mass of the solution	30.01663	g						0.000024	0.000001		
	balance repeatability		g	Certificate of de calibration CNM-CC-730-185/2022	0.00004	B	Rectangular	0.000023				
	indication error		g	Certificate of calibration CNM-CC-730-185/2023	0.000055	B	Rectangular	0.000055				
	balance resolution		g	Certificate of calibration CNM-CC-730-185/2024	0.00001	B	Rectangular	0.000006				
3	**Repetibility of MTNDZ Measurement**	9.2348	µg/kg	Measurements made	0.218	A	Normal	0.10920	0.1092	0.011825		0.109202
4	**Reproducibility of MTNDZ Measurement**	8.7889	µg/kg	Measurements made	0.668	A	Normal	0.38578	0.3858	0.043894		0.385781

Relative combined uncertainty	uc/average value	=	0.0118
	Combined uncertainty u_c	=	0.401
		=	0.8022

Metronidazole =	**8.789**	**±**	**0.802**	**µg/kg**

Relative Uncertainty 9.1 %

Uncertainty Information of EXHM

The measurement equation is:

$$\left[w_{M,S} = \frac{w_{M,C}}{(1-H) \times R} \times \frac{m_{is,S}}{m_{M,S}} \times \frac{m_{M,C}}{m_{is,C}} \times \frac{R_S}{R_C} \right]$$

where
- $w_{M,S}$ = dry mass fraction of measurand in the sample, (µg/kg)
- $w_{M,C}$ = mass fraction of measurand in the calibration blend, (µg/kg)
- H = sample moisture content (g/g)
- R = recovery (as a fraction)
- $m_{is,S}$ = mass of internal standard added to the sample blend, (µg/kg)
- $m_{M,S}$ = mass of test material in the sample blend, (µg/kg)
- $m_{M,C}$ = mass of measurand added to the calibration blend, (µg/kg)
- $m_{is,C}$ = mass of internal standard added to calibration blend, (µg/kg)
- R_S = measured peak area ratio of the selected ions in the sample blend
- R_C = measured peak area ratio of the selected ions in the calibration blend

The equation used to estimate standard uncertainty is :

$$u(w_{BS}) = \sqrt{\left(SDr/\sqrt{n}\right)^2 + \sum\left(C_j u(m_i)\right)^2 + \sum\left(C_j u(R_i)\right)^2 + \left(C_{MC} u(w_{MC})\right)^2 + \left(C_H u(H)\right)^2 + \left(C_R u(R)\right)^2}$$

where SD_r is the standard deviation under reproducibility conditions, n the number of determinations (12) and C_j the sensitivity coefficients associated with each uncertainty component. The uncertainty of the peak area ratios was considered to have been included in the estimation of method precision.

Uncertainty estimation was carried out according to JCGM 100: 2008. The standard uncertainties were combined as the sum of the squares of the product of the sensitivity coefficient (obtained by partial differentiation of the measurement equation) and standard uncertainty to give the square of the combined uncertainty. The square root of this value was multiplied by a coverage factor (95% confidence interval) from the t-distribution at the total effective degrees of freedom obtained from the Welch-Satterthwaite equation to give the expanded uncertainty.

uncertainty component	typical values	sensitivity coefficient	standrard uncertainty	relative uncertainty	$C_i \times u_i$	$(C_i \times u_i)^2$
metronidazole mass fraction (ng/g, method precision)	**8,128**	1	0,264	0,032	0,2640	0,070
mass fraction of metronidazole in the calibration solution, (ng/g)	26,57	0,306	0,0106	0,000	0,0032	0,000
sample moisture content, (g/g,in the reconstituted sample)	0,734	30,535	0,0006	0,001	0,0179	0,000
recovery (expressed as a fraction)	1,000	8,128	0,0115	0,012	0,0939	0,009
mass of metronidazole-d_3 solution added to the sample blend, (mg)	220,75	0,037	0,0149	0,000	0,0005	0,000
mass of test material in the sample blend, (mg)	1060	0,008	0,0185	0,000	0,0001	0,000
mass calibration solution added to the calibration blend, (mg)	85,97	0,095	0,0144	0,000	0,0014	0,000
mass of metronidazole-d_3 solution added to calibration blend, (mg)	222,65	0,037	0,0149	0,000	0,0005	0,000
measured peak area ratio of the selected ions in the sample blend	0,409		considered to be included in the			
measured peak area ratio of the selected ions in the calibration blend	0,404		estimation of method precision			
combined standard uncertainty (µg/kg)	**0,281**					
effective degrees of freedom	15					
coverage factor	2,13					
expanded uncertainty (µg/kg)	**0,598**					

Uncertainty Information of GLHK

Part IV: Uncertainty budget

The measurement equations used to calculate the mass fraction of each analyte. Please provide details of all the factors listed in the equations and indicate how these values were determined.

$$C_X = C_Z \times \frac{M_y}{M_X} \times \frac{M_{ZC}}{M_{YC}} \times \frac{R_b}{R_{bc}} / (1 - W)$$

Where			
	M_y	=	Mass of the isotope-labeled standard added to the sample blend (g)
	M_x	=	Mass of the sample (g)
	M_{zc}	=	Mass of the calibration standard solution added to the calibration blend (g)
	M_{yc}	=	Mass of isotope-labelled standard added to the calibration blend (g)
	R_b	=	Measured ratio (peak area of Met/ peak area of Met-$^{13}C_2$,$^{15}N_2$) in the sample blend
	R_{bc}	=	Measured ratio (peak area of Met/ peak area of Met-$^{13}C_2$,$^{15}N_2$) in the calibration blend
	C_z	=	Mass fraction of Met standard (μg/kg)
	W	=	Moisture content

Estimation of uncertainties for each factor. Give a complete description of how the estimates were obtained and combined to calculate the overall uncertainty. Please provide a table detailing the full uncertainty budget.

Symbol	Description	Value x	Standard uncertainty $u(x_i)$	Relative standard uncertainty $u(x_i)/x_i$	Contribution to total u_c (%)	$u(y,x_i) = (dy/dx_i)*u(x_i)$	Sensitivity coefficient $(dy/dx_i) = u(y,x_i)/u(x_i)$	Uncertainty evaluation
c_{z0} (mg/g)	Mass fraction of neat standard	998.90	1.10	0.0011	0.0956	0.0097	0.01	B
c_{z1}(g)	Mass of neat standard	0.03128	0.0000	0.0011	0.0877	0.0093	281.92	B
c_{z2} (g)	Mass of solvent used to dissolve the neat standard	15.14666	0.0000	0.0000	0.0000	0.0000	-0.58	B
c_{z3} (g)	Mass of stock solution taken	0.10084	0.0000	0.0003	0.0084	0.0029	87.45	B
c_{z4} (g)	Mass of solvent used	14.34752	0.0000	0.0000	0.0000	0.0000	-0.61	B
c_{z5}(g)	Mass of 1st Inter solution taken	0.10097	0.0000	0.0003	0.0084	0.0029	87.33	B
c_{z6} (g)	Mass of solvent used	8.59567	0.0000	0.0000	0.0000	0.0000	-1.03	B
c_{z7} (g)	Mass of 2nd Inter solution taken	0.81662	0.0000	0.0000	0.0001	0.0004	10.80	B
c_{z8} (g)	Mass of solvent used	11.26165	0.0000	0.0000	0.0000	0.0000	-0.78	B
m_y (g)	Weight of internal standard in sample blend	0.11487	0.0000	0.0003	0.0065	0.0025	76.77	B
m_x (g)	Weight of sample	0.54154	0.0000	0.0001	0.0003	-0.0005	-16.28	B
m_{yc} (g)	Weight of internal standard in calibration blend	0.42262	0.0000	0.0001	0.0005	-0.0007	-20.86	B
m_{zc} (g)	Weight of standard added to calibration blend	1.36338	0.0000	0.0000	0.0000	0.0002	6.47	B
R_b	Isotope ratio of sample	1.1368	0.0128	0.0113	10.0551	0.0996	7.76	A
R_{bc}	Isotope ratio of calibration blend	1.1156	0.0078	0.0070	3.8442	-0.0616	-7.85	A
R	Run to run variability	1.0000	0.0080	0.0080	5.0811	0.0708	8.82	A
Rm	Matrix CRM accuracy (Rm)	1.0000	0.0204	0.0204	32.7015	0.1796	8.82	A
Rm'	Spike recovery (Rm)	1.0000	0.0238	0.0238	44.6304	0.2099	8.82	A
W	Moisture content	0.9765	0.0065	0.0067	3.4802	-0.0586	-8.97	A
$c_{x,i}$ (μg/kg)		8.781			S $A_{contrib.}$ =	99.8		
$u_c(c_{x,i})$		0.313	μg/kg		S $B_{contrib.}$ =	0.2		
U (k=2)		0.626	μg/kg		Total	100.0		
RSU		7.13	%					

Uncertainty Information of HSA

Part IV: Uncertainty budget

The measurement equations used to calculate the mass fraction of each analyte. Please provide details of all the factors listed in the equations and indicate how these values were determined.

$$C_X = C_Z \cdot \frac{m_Y \cdot m_{Zc}}{m_X \cdot m_{Yc}} \cdot \frac{R_Y - R_B}{R_B - R_X} \cdot \frac{R_{Bc} - R_Z}{R_Y - R_{Bc}} \cdot \frac{1}{(1 - F_{mois})}$$

where

Factors	Details of the factors	How the values were determined
C_Z =	Mass fraction of metronidazole in the calibration standard solution (μg/kg) used to prepare the calibration blend	Gravimetric values of serial dilution of the calibration solution and the certified purity value of metronidazole calibrant
F_{mois} =	Dry mass correction factor based on moisture determination of the study sample Cx = Cx' - 1/(1-F_{mois}), where F_{mois} represents the moisture content and Cx' represents the mass fraction of metronidazole in pig muscle powder based on wet mass of study sample	Gravimetric values of study sample before and after drying.
m_Y =	Mass of internal standard solution (g) added to the sample blend	Weighing
m_{Yc} =	Mass of internal standard solution (g) added to the calibration blend	Weighing
m_{Zc} =	Mass of standard solution (g) added to the calibration blend	Weighing
m_X =	Mass of study material (g) in the sample blend	Weighing
R_X =	Observed isotope abundance ratio in the study material	Peak area ratio of (172>127.97)/(176>132.2) in the study material
R_Y =	Observed isotope abundance ratio in the internal standard	Peak area ratio of (172>127.97)/(176>132.2) in the internal standard solution
R_Z =	Observed isotope abundance ratio in the calibration standard	Peak area ratio of (172>127.97)/(176>132.2) in the calibration standard solution
R_B =	Observed isotope abundance ratio in the sample blend	Peak area ratio of (172>127.97)/(176>132.2) in the sample blend
R_{Bc} =	Observed isotope abundance ratio in the calibration blend	Peak area ratio of (172>127.97)/(176>132.2) in the calibration blend

Estimation of uncertainties for each factor. Give a complete description of how the estimates were obtained and combined to calculate the overall uncertainty. Please provide a table detailing the full uncertainty budget.

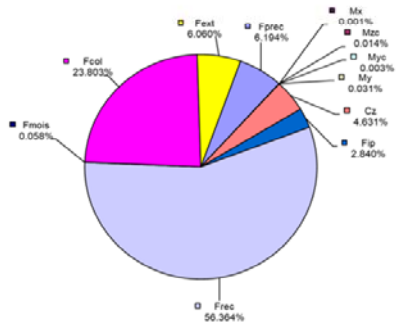
$$C_X = F_{prec} \cdot F_{rec} \cdot F_{ip} \cdot F_{col} \cdot F_{ext} \cdot C_Z \cdot \frac{m_Y \cdot m_{Zc}}{m_X \cdot m_{Yc}} \cdot \frac{R_Y - R_B}{R_B - R_X} \cdot \frac{R_{Bc} - R_Z}{R_Y - R_{Bc}} \cdot \frac{1}{(1 - F_{mois})}$$

where

additional factors (F) contributing to biases in the result value of metronidazole were included, with an uncertainty associated to each factor.

F_{prec} =	Factor representing method precision
F_{rec} =	Factor representing method recovery
F_{ip} =	Factor representing any bias in the result due to choice of different ion pair
F_{col} =	Factor representing any bias in the result due to the choice of different LC column
F_{ext} =	Factor representing any bias in the result due to the choice of different sample extraction solvent

The full uncertainty budget for the determination of metronidazole is given in the Table below:



Parameter	x_i	u_i	u_i/x_i	Contribution	Sources of uncertainty
F_{prec}	1	0.01291	1.2911%	6.194%	Standard deviation of four independent determinations on the study material using LC-MS/MS.
F_{rec}	1	0.03895	3.8948%	56.364%	Method recovery using European Reference Material ERM-BB124 (metronidazole in pork muscle).
F_{ip}	1	0.00874	0.8743%	2.840%	Comparison of results obtained using different ion pairs on the same subsamples.
F_{mois}	0.01851	0.00123	6.6280%	0.058%	• Uncertainty in weighing before and after drying based on balance calibration certificate. • Standard deviation of the mean of moisture content determined using three subsamples. • Uncertainty due to possible re-absorption of moisture content in the study sample.
F_{col}	1	0.02531	2.5311%	23.803%	Comparison of results obtained using different LC columns on the same subsamples.
F_{ext}	1	0.01277	1.2771%	6.060%	Comparison of results obtained using different extraction solvents, i.e. ethyl acetate vs methanol.
C_Z	102.2198	1.14114	1.1164%	4.631%	• Uncertainty in the purity value of metronidazole certified reference material (GBW(E)060908). • Uncertainty in weighing based on balance calibration certificate. • Bias in the preparation of calibration solutions.
m_Y	0.0924	0.0000849	0.0918%	0.031%	Uncertainty in weighing based on balance calibration certificate.
m_{Yc}	0.2804	0.0000849	0.0303%	0.003%	
m_{Zc}	0.1358	0.0000849	0.0625%	0.014%	
m_X	0.5427	0.0000849	0.0156%	0.001%	
R_X, R_Y, R_Z	Negligible				
R_B, R_{Bc}	Uncertainty included in method precision				

Uncertainty Information of INM Colombia

Part IV: Uncertainty budget

The measurement equations used to calculate the mass fraction of each analyte. Please provide details of all the factors listed in the

$$C_{BH} = A * C_{MNZ} * Z * R_3 * \frac{R_2(Y_3 - Y_1) - R_1(Y_3 - Y_2)}{(Y_2 - Y_1)}$$

$$C_{BS} = C_{BH} * \frac{1}{(1 - H)}$$

$$\%H = \left(\left(1 - \left(\frac{M_3 - M_1}{M2}\right)\right) * 100\right) + F$$

C_{BH} =Sample concentration, wet basis
A= Method repeatability factor
Z= IS dilution factor
R_3= Mass ratio (IS/Sample)
R_2= Calibration blend 2 mass ratio (MNZ/IS)
R_1= Calibration blend 1 mass ratio (MNZ/IS)
C_{MNZ}= MNZ concentration for calibration blends
Y_3= Sample relative response
Y_2= Calibration blend 2 Relative response
Y_1= Calibration blend 1 Relative response
C_{BS}=Sample concentration, dry basis
H= Moisture
M_3= weight of containter+ dried residue
M_2=Sample weight
M_1= Container weight
F= Repeatability factor

Estimation of uncertainties for each factor. Give a complete description of how the estimates were obtained and combined to calculate the overall uncertainty. Please provide a table detailing the full uncertainty budget.

Code	Value	u(x)	units	Ci	(ci*u(x))^2	Relative uncertainty	combined standard u (ug/kg)
A	1.00	2.18E-02	-	7.96	3.02E-02	66.3%	0.21
C_MNZ	9.99	4.80E-02	ug/kg	0.80	1.46E-03	3.2%	
Z	6.10E+00	3.47E-03	-	1.31	2.05E-05	0.05%	
R1	3.64E-01	1.53E-04	g /g	14.96	5.25E-06	0.01%	
R2	0.47	1.59E-04	g /g	5.35	7.24E-07	0.002%	
R3	3.33E-01	1.96E-04	g /g	23.89	2.18E-05	0.05%	
Y1	1.46E-02	2.51E-04	-	-427.11	1.15E-02	25.3%	
Y2	1.83E-02	3.16E-04	-	-152.66	2.32E-03	5.1%	
Y3	1.56E-02	-	-	579.77			

Code	Value	u(x)	units	Ci	(ci*u(x))^2	Relative uncertainty	combined standard u (ug/kg)
C_BH	7.96	0.213	µg/kg	1.01	4.63E-02	99.88%	0.21
H	7.98E-03	9.30E-04	g/g	8.09	5.66E-05	0.12%	

Code	Value	units	u(x)	Ci	Ci u	Relative uncertainty	combined standard u (g/100 g)
M2	0.403	g	1.08E-04	246.259	0.027	8.18%	0.09
M1	25.434	g	1.08E-04	247.905	0.027	8.29%	
M3	25.833	g	1.08E-04	-247.905	-0.027	8.29%	
F	0	g/100 g	0.0806	1	0.081	75.23%	

Uncertainty Information of INMETRO

Part IV: Uncertainty budget

The measurement equations used to calculate the mass fraction of each analyte. Please provide details of all the factors listed in the equations and indicate how these values were determined.

$$w = \left[\frac{(y - y_0)(x_1 - x_0)}{y_1 - y_0} + x_0\right] \times \frac{m_{IS}}{m_s}$$

w: Mass fraction of metronidazole in the sample (obtained by the measurement equation;
y: Area ratio (analyte/IS) of the sample;
y_0: Area ratio (analyte/IS) of the lower level calibration blend;
y_1: Area ratio (analyte/IS) of the higher level calibration blend;
x_0: Mass ratio (analyte/IS solution) of the lower level calibration blend;
x_1: Mass ratio (analyte/IS solution) of the higher level calibration blend;
m_{IS}: Mass of internal standard solution added to the sample;
m_s: Mass of sample.

Estimation of uncertainties for each factor. Give a complete description of how the estimates were obtained and combined to calculate the overall uncertainty. Please provide a table detailing the full uncertainty budget.

Source of uncertainty		Source	Uncertainty component (µg/kg)	Contribution (%)
Measurement equation soucers	x_0 (Mas of IS solution)	balance certificate	2.308E-03	0.014
	x_0 (Mass of analyte)	gravimetric preparation of the lower solution - balance certificate	8.838E-03	0.208
	x_1 (Mas of IS solution)	balance certificate	5.071E-03	0.069
	x_1 (Mass of analyte)	gravimetric preparation of the higher solution - balance certificate	1.203E-02	0.386
	y	Standard devition of the between subsamples mean	1.703E-01	77.342
	y_0	Standard devition of the between subsamples mean	2.748E-02	2.015
	y_1	Standard devition of the between subsamples mean	4.260E-02	4.840
	m_{IS}	balance certificate	4.893E-04	0.001
	m_s	balance certificate	4.001E-04	0.000
w (repeatability)		Standard devition of the between subsamples mean	7.477E-02	14.911
Conversion to dry mass basis		Determination of total solids by desiccation (balance certificate)	8.935E-03	0.213
Combined standard uncertainty (u_c)			**0.19**	**100**

Uncertainty Information of INRAP

Part IV: Uncertainty budget

The measurement equations used to calculate the mass fraction of each analyte. Please provide details of all the factors listed in the equations and indicate how these values were determined.

x=(y-b)/a with a: slope, b:intercept, y: ratio STD/ISTD ; x mass fraction of analyte in fresh sample (µg/kg)

X=x*f1*f2 with f1: correction of weighted sample, f2: correction of lyphlisation; X: mass fraction of analyte in dry sample (µg/kg)

Estimation of uncertainties for each factor. Give a complete description of how the estimates were obtained and combined to calculate the overall uncertainty. Please provide a table detailing the full uncertainty budget.

$U=V(u_L^2 + u_{MRC}^2 + u_{Rep}^2)$ with u_L: uncertainty of matrix calibration curve inclusing precision of weighted sample, u_{MRC}: uncertainty of RMC, u_{Rep}: uncertainty of precision

Uncertainty Information of KRISS

Part IV: Uncertainty budget

The measurement equations used to calculate the mass fraction of each analyte. Please provide details of all the factors listed in the equations and indicate how these values were determined.

$$C_{sample} = f \times \frac{M_{is-sol.,spiked} \times AR_{sample} \times M_{s-sol.,std.,mix.} \times C_{s-sol.}}{M_{sample} \times AR_{std.,mix.} \times M_{is-sol.,std.,mix.}}$$

where,

C_{sample} is the concentration of the analyte in the sample;

f is the dry mass correction factor, given by f=1/(1-x), x is the moisture content of the sample;

$M_{is\text{-}sol.,spiked}$ is the mass of the isotope standard solution spiked to the sample aliquot;

$M_{s\text{-}sol.,std.,mix.}$ is the masse of the standard solution blended into the isotope ratio standard solution;

$M_{is\text{-}sol.,std.,mix.}$ is the masse of the isotope standard solution blended into the isotope ratio standard solution;

$C_{s\text{-}sol.}$ is the concentration of the standard solution of the target analyte;

M_{sample} is the mass of the sample used for the analysis;

AR_{sample} is the area ratio of analyte and its isotope standard for sample extract obtained by LC/MS;

$AR_{std.,mix.}$ is the area ratio of analyte and its isotope standard for the isotope ratio standard solution obtained by LC/MS.

Estimation of uncertainties for each factor. Give a complete description of how the estimates were obtained and combined to calculate the overall uncertainty. Please provide a table detailing the full uncertainty budget.

$$u(C_{mean}) = \sqrt{u_{systematic}^2 + s^2}$$

Systematic uncertainty	u,sys (re/%)	DOF
purity of primary standard	0.11	∞
Gravimetric preparation for standard solution	0.55	3
Gravimetric mixing for calibration isotope standard mixtures	0.14	4
Area ratio of MNZ/MNZ-$^{13}C_2$,$^{15}N_2$ for the calibration standard mixture, observed by LC/MS	0.91	4
Dry mass correction	0.06	2
Sum	1.08	6

s: standard deviation of multiple measurement results from 5 subsamples (s=0.51%)

Uncertainty Information of LGC

Part IV: Uncertainty budget

The measurement equations used to calculate the mass fraction of each analyte. Please provide details of all the factors listed in the equations and indicate how these values were determined.

Each of the sample injections was quantified using the calibration blend injected immediately before and after the sample. The amount of analyte was calculated for each of the last 5 injections using the reduced form of the IDMS equation:

$$w'_X = w_Z \cdot \frac{m_Y}{m_X} \cdot \frac{m_{Zc}}{m_{Yc}} \cdot \frac{R'_b}{R'_{bc}} \cdot$$

w_X	mass fraction in sample
w_Z	mass fraction in calibration standard
m_Y	mass of spike solution in sample blend
m_X	mass of sample in sample blend
m_{Zc}	mass of standard solution in calibration blend
m_{Yc}	mass of spike solution in calibration blend
R_b	observed isotope amount ratio in sample blend
R_{bc}	observed isotope amount ratio in calibration blend
R_X	natural isotope amount ratio of sample
R_Y	natural isotope amount ratio of spike
R_Z	natural isotope amount ratio of calibration standard
ΣR_X	sum of all isotope amount ratios of sample
ΣR_Z	sum of all isotope amount ratios of standard
R_{bc}	gravimetric value of the isotope amount ratio of calibration blend

Estimation of uncertainties for each factor. Give a complete description of how the estimates were obtained and combined to calculate the overall uncertainty. Please provide a table detailing the full uncertainty budget.

Variable	Value	u (%)	Budget
R_{SB}	0.958	1.59%	
R_{CB}	0.972	1.00%	
R_{CB} (bracketing)	0.971	0.72%	
R_{SB}/R_{CB} (bracketed)	0.987	2.10%	99.32%
w_Z	25.076	0.06%	0.08%
m_X	1.743	0.01%	0.00%
$m_{Y,SB}$	0.199	0.09%	0.19%
m_Z	0.188	0.10%	0.21%
$m_{Y,CB}$	0.199	0.09%	0.18%
Dilution factor X	1.000	-	-
Dilution factor Z	1.000	-	-
Dilution factor Y	1.000	-	

RSB/RCB = the ratio measurements of the calibration and sample blends
wZ = the mass fraction of the calibration solution
mX = the mass of sample used
mY, SB = the mass of label added to the sample
mZ = the mass of natural added to the calibration blend
mY, CB = the mass of label added to the calibration blend

Individual uncertainties were combined using the following equation:

$$u(w_{totalMNZ}) = \frac{\sqrt{u(w_{MNZ})^2 + u(sw_{MNZ})^2}}{n}$$

uw = represents the average of the moisture corrected standard uncertainty for each measurement
usw = the variability of the measurements

Uncertainty Information of NIM China

Part IV: Uncertainty budget

The measurement equations used to calculate the mass fraction of each analyte. Please provide details of all the factors listed in the equations and indicate how these values were determined.

$$C_X = C_Z \times \frac{M_y}{M_x} \times \frac{M_{zc}}{M_{yc}} \times \frac{R_b}{R_{bc}} \times F_{mois} \times P \times F_{bias}$$

Factors	Details of the factors	How the values were determined
C_x (μg/kg)	Concentration of analytes in the sample(μg/kg)	Calculated from measurement equation
C_z (ng/g)	Mass fraction of metronidazle (MDZ) in the calibration standard solution (μg/kg) used to prepare the calibration blend	Gravimetric values of serial dilution of the calibration solution and the certified mass fraction of MDZ calibrant
M_{zc} (g)	Mass of MDZ standard solution added to the calibration blend	Weighing
M_{yc} (g)	Mass of internal standard(IS-MDZ) added to the calibration blend	Weighing
M_y (g)	Mass of IS-MDZ added to the sample blend	Weighing
M_x (g)	Mass of sample	Weighing
R_b	Peak area ratio between MDZ and IS-MDZ in sample blend	Measured by peak area responses determined by LC-MSMS
R_{bc}	Peak area ratio between MDZ and IS-MDZ in calibration blend	Measured by peak area responses determined by LC-MSMS
F_{mois}	Factor related to mass fraction correction to dry weight basis	Gravimetric values of study sample before and after drying.
P	Precision of the analytical method	Numerically set to 1, used to incorporate measurement precision into the uncertainty budget
F_{bias}	Factor related to bias from extraction	Numerically set to 1, used to incorporate an estimate of potential bias from extraction conditions

Estimation of uncertainties for each factor. Give a complete description of how the estimates were obtained and combined to calculate the overall uncertainty. Please provide a table detailing the full uncertainty budget.

Metronidazole	Value x	Standard uncertainty $u(x_i)$	Relative standard uncertainty $u(x_i)/x_i$	Description
C_z (ng/g)	35	0.05075	0.145%	Uncertainty in the certified mass fraction value of certified reference material (GBW(E)060908), combined with uncertainty in weighing for the preparation of the working standard, based on balance calibration certificate.
M_{zc} (g)	0.1133	0.000112	0.0989%	Uncertainty in weighing of the working standard added to calibration blend, based on balance calibration certificate
M_{yc} (g)	0.1210	0.000112	0.0926%	Uncertainty in weighing of the internal standard added to calibration blend, based on balance calibration certificate
M_y (g)	0.1300	0.000112	0.0862%	Uncertainty in weighing of the internal standard added to sample blend, based on balance calibration certificate
M_x (g)	0.54	0.000112	0.0207%	Uncertainty in weighing of sample, based on balance calibration certificate
R_b	1.035	Considered as part of precision	/	Considered to be incorporated into P
R_{bc}	1.015	Considered as part of precision	/	Considered to be incorporated into P
F_{mois}	1.016570849	0.0009603	0.0945%	Standard deviation of the mean of 6 determinations of the moisture correction factor, combined with uncertainty in weighing before and after drying based on balance calibration certificate
P	1	0.02397	2.397%	Standard deviation of the mean of six analytical batch analyses
F_{bias}	1	0.0062	0.620%	Estimation of additional uncertainty due to extraction processes, based on expert opinion according to spike recovery.
C_x (μg/kg)	8.13	0.20	2.49%	Combined standard uncertainty
K		2		Coverage factor (k)
U(μg/kg)		0.40		Expanded uncertainty U

Uncertainty Information of NIMT

Part IV: Uncertainty budget

The measurement equations used to calculate the mass fraction of each analyte. Please provide details of all the factors listed in the equations and indicate how these values were determined.

Where:
wx = mass fraction of MNZ in porcine muscle
wzc = mass fraction of MNZ in the calibration solution used to prepare the calibration blend
my = mass of spike solution (internal standard) added to sample blend
myc = mass of spike solution (internal standard) added to calibration blend
mzc= mass of standard solution added to calibration blend
mx = mass of sample added to sample blend
F_E = extraction efficiency factor, given a value of 1
F_I = interference effect, given a value of 1
F_P = method precision factor, given a value of 1
F drymass = dry mass correction factor obtained from moisture content analysis
R'b and R'bc = observed isotope amount ratios in the sample blend and the calibration blend, respectively

$$w_x = F_P.F_E.F_I.w_{zc} \cdot \frac{m_y \cdot m_{zc}}{F_{drymass}.m_x \cdot m_{yc}} \cdot \frac{R'_b}{R'_{bc}}$$

Estimation of uncertainties for each factor. Give a complete description of how the estimates were obtained and combined to calculate the overall uncertainty. Please provide a table detailing the full uncertainty budget.

$$\frac{u(w_x)}{w_x} = \sqrt{\left(\frac{u(w_{ZC})}{w_{ZC}}\right)^2 + \left(\frac{u(m_y)}{m_y}\right)^2 + \left(\frac{u(m_{yc})}{m_{yc}}\right)^2 + \left(\frac{u(m_{zc})}{m_{zc}}\right)^2 + \left(\frac{u(m_x)}{m_x}\right)^2 + \left(\frac{u(F_{drymass})}{F_{drymass}}\right)^2 + \left(\frac{u(R'_b)}{R'_b}\right)^2 + \left(\frac{u(R'_{bc})}{R'_{bc}}\right)^2 + \left(\frac{u(F_I)}{F_I}\right)^2 + \left(\frac{u(F_E)}{F_E}\right)^2 + \left(\frac{u(F_P)}{F_P}\right)^2}$$

Where;
u(wz,c) is the standard uncertainty of the mass fraction of analyte in the calibration solution used to prepare the calibration blend. The value was evaluated from the purity of MNZ standard, masses weighed for preparation of stock solutions and uncertainty using different standards (standard comparison).

u(my), u(my,c), u(mx) and u(mz,c) are standard uncertainties of the masses. These values were evaluated from the bias and precision effect of the balance.

u (F_P) is the standard uncertainty of the precision factor. This value was evaluated from standard deviation of the multiple IDMS results.

u(F_I) is the standard uncertainty of the interference effect. This value was evaluated from potential bias between primary ion pair and secondary ion pair of the MRM program.

u(F_E) is the standard uncertainty of the extraction efficiency factor which was estimated from the liquid-solid extraction and solid-phase –extraction.

u(Fdrymass) is the standard uncertainty of the dry mass correction factor which was evaluated from the moisture content analysis.

u(R'b) andvu(R'b,c) are standard uncertainties of the measured isotope amount ratios of the analyte and the internal standard in the sample and calibration blend. These value were evaluated from the precison on these ratios.

Combination of Uncertainties

Factor	Values	Uncertainties	
	x	u(x)	u(x)/(x)
Measurement equation factors			
Method Precision	1.0000	0.00422	0.422%
mzc	0.30032	0.000017	0.0057%
my	0.30954	0.000017	0.0055%
myc	0.31900	0.000017	0.0054%
Fdrymass	0.98262	0.000794	0.0808%
mx	0.70544	0.000017	0.0024%
wz	18.8436	0.126680	0.6723%
R'b	1.0565	0.012013	1.1370%
R'bc	1.0561	0.012893	1.2208%
Additional Factors		Enter u(x) = 0 and veff = 1 for unused factors.	
Extraction effects	1.000	0.0100	1.000%
Interference from two different ion pairs	1.000	0.0002	0.019%

Uncertainty Analysis Results

wx=	**8.33**	ng/g
u(x) =	0.175	ng/g
u(x)/x =	2.10%	
Veff(total) =	151.407	
k=	2.0	(@ 95% level)
U(x) =	**0.35**	
%U(x) =	**4.20%**	

Uncertainty Information of NMIA

$$W_x = W_z \cdot \frac{m_y}{m_z} \cdot \frac{m_{zc}}{m_{yc}} \cdot \frac{R'_b}{R'_{bc}} \cdot P \cdot F_{MRM} \cdot F_{Bias} \cdot F_{MC}$$

Term	Description	Determination	Estimation of Uncertainty	value	u_{rel}	v_{eff}
W_x	**Mass fraction of sample, dry weight basis**	**Calculated from measurement equation components**	**Combination of uncertainty components**	**8.42**	**0.76%**	**40.2**
m_z	Mass of sample in sample blend	Gravimetry	Uncertainty due to bias of measurement of mass, derived from balance calibration. Uncertainty due to precision of the balance used is considered to be incorporated into P.	0.56	0.0073%	400
m_y	Mass of internal standard added to sample blend	Gravimetry	Uncertainty due to bias of measurement of mass, derived from blanace calibration. Uncertainty due to precision of the balance used is considered to be incorporated into P.	0.55	0.0074%	400
m_{zc}	Mass of calibration standard added to calibration blend	Gravimetry	Uncertainty due to bias of measurement of mass, derived from blanace calibration. Uncertainty due to precision of the balance used is considered to be incorporated into P.	0.28	0.015%	400
m_{yc}	Mass of internal standard added to calibration blend	Gravimetry	Uncertainty due to bias of measurement of mass, derived from blanace calibration. Uncertainty due to precision of the balance used is considered to be incorporated into P.	0.50	0.0082%	400
W_z	Mass fraction of calibration standard	Calculated from gravimetric preparation of the working standard	Uncertainty due to bias and precision of the balance used to prepare the working standard, combined with uncertainty determined by ANOVA from comparisons between multiple independent preparations of working standards.	0.015	0.41%	11
R'_b	Ratio of analyte and internal standard responses in the sample blend	Measured by peak area responses determined by 2D-LC-MSMS	Considered to be incorporated into P			
R'_{bc}	Ratio of analyte and internal standard responses in the calibration blend	Measured by peak area responses determined by 2D-LC-MSMS	Considered to be incorporated into P			
P	Precision of the analytical method	Numerically set to 1, used to incorporate measurement precision into the uncertainty budget	Standard deviation of the mean of three analytical batch analyses. The result of each batch was determined by the mean of two or three replicate aliquots analysed using two complimentary clean-up and three 2D-LC-MSMS methods.	1	0.063%	2.0
F_{MRM}	Factor related to the extent of agreement between MRMs used for analysis	Numerically set to 1, used to incorporate differences between MRM results into the uncertainty budget	Maximum uncertainty calculated from pairwise t-tests between MRM pairs within each analytical method	1	0.58%	20
F_{Bias}	Factor related to bias from extraction, evidence from different SPE extracts and different LCMS conditions	Numerically set to 1, used to incorporate an estimate of potential bias from extraction conditions	Maximum uncertainty calculated from pairwise t-tests between result from each SPE extract/LCMS method combination	1	0.28%	20
F_{MC}	Factor related to mass fraction correction to dry weight basis	Gravimetry of undried and dried sample aliquots	Standard deviation of the mean of the moisture correction factor in each of four bottles, determined from three to five dry weighings and corrected for a blank vial weighing, combined with uncertainty due to bias of measurement of mass.	1.013	0.032%	4.1

Uncertainty Information of NRC Canada

Part IV: Uncertainty budget

The measurement equations used to calculate the mass fraction of each analyte. Please provide details of all the factors listed in the equations and indicate how these values were determined.

ID2-MS:

$$w_A = -w_{A^*}\frac{m_{A^*\text{-}1}m_{B\text{-}2}(R_1 - R_{A^*})(R_B - R_2)}{m_{A\text{-}2}m_{B\text{-}1}(R_B - R_1)(R_A - R_2)}$$

Symbol description: A is MNZ in the sample (natural isotopic composition), A* is MNZ in the primary standard (natural isotopic composition), B is MNZ-d3 in the isotopic standard (isotopically enriched composition), AB is the blend of sample (A) and isotopic standard (B), A*B is the blend of the primary standard (A*) and isotopic standard (B), W_A is the mass fraction of A (natural) in the sample, W_{A^*} is the mass fraction of A* (natural) in the primary standard, $m_{A^*\text{-}1}$ is the mass of A (natural) in blend-1 (A*B), $m_{B\text{-}1}$ is the mass of B (isotopic IS) in blend-1 (A*B), $m_{A\text{-}2}$ is the mass of matrix sample in blend-2 (AB), $m_{B\text{-}2}$ is the mass of isotopic standard in blend-2 (AB), R1 is the measured isotope ratio in blend-1 (A*B), R_2 is the measured isotopic ratio in blend-2 (AB), RA is the measured isotope ratio in blend-3 (A), RA* is the measured isotope ratio in blend-4 (A*), and R_B is the measured isotope ratio in blend-5 (B).

SA-ID2-MS:

$$w_A = -w_{A^*}\frac{m_{A^*\text{-}1}m_{B\text{-}2}(R_1 - R_{A^*})(R_B - R_2) + m_{A^*\text{-}2}m_{B\text{-}1}(R_B - R_1)(R_{A^*} - R_2)}{m_{A\text{-}1}m_{B\text{-}2}(R_1 - R_A)(R_B - R_2) + m_{A\text{-}2}m_{B\text{-}1}(R_B - R_1)(R_A - R_2)}$$

Symbol description: A is MNZ in the sample (natural isotopic composition), A* is MNZ in the primary standard (natural isotopic composition), B is MNZ-d3 in the isotopic standard (isotopically enriched composition), AB is the blend of sample (A) and isotopic standard (B), AA*B is the blend of the sample (A), the primary standard (A*), and isotopic standard (B), WA is the mass fraction of A (natural) in the sample, WA* is the mass fraction of A* (natural) in the primary standard, mA-1 is the mass of matrix sample in blend-1 (AA*B-1), mA*-1 is the mass of A (natural) in blend-1 (AA*B-1), mB-1 is the mass of B (isotopic IS) in blend-1 (AA*B-1), (Note: A*=0 in blend-1), mA-2 is the mass of matrix sample in blend-2 (AA*B-2), mA*-2 is the mass of A (natural) in blend-2 (AA*B-2), mB-2 is the mass of isotopic standard in blend-2 (AA*B-2), R1 is the measured isotope ratio in blend-1 (AA*B-1), R2 is the measured isotopic ratio in blend-2 (AA*B-2), RA is the measured isotope ratio in blend-3 (A), RA* is the measured isotope ratio in blend-4 (A*), and RB is the measured isotope ratio in blend-5 (B).

Mass fractions were measured for each sample using both ion transitions and both isotope dilution methods. The average mass fractions (WA) were obtained for each sample using each of the isotope methods, and the results were then converted to a dry mass basis (WA(dry)) using the calculated moisture content of the sample and the following equation:

$$w_{A(dry)} = \frac{w_A}{(1 - \frac{M_L}{100})}$$

The average mass fractions of the samples were determined for each of the isotope dilution methods and then a final mass fraction was determined using the DerSimonian-Laird random effetcs model (DSL).

Estimation of uncertainties for each factor. Give a complete description of how the estimates were obtained and combined to calculate the overall uncertainty. Please provide a table detailing the full uncertainty budget.

A linear mixed effects statistical model was used to model the uncertainties arising from the following effects: choice of the measurement model (ID2MS vs SA-ID2MS), method bias (estimated from a certified reference material ERM BB124), choice of ion transition (172 to 128 or 172 to 82), the effect of sample homogeneity (from two sample units provided) and the effect of dry weight correction.
The repeatability uncertainty associated with each measurement result was evaluated using Gauss formula of the error propagation.
The mixed effects measurement model was fit to data in Stan using Bayesian methods resulting in the following overall relative contributions of these sources of uncertainties to the reported result:

34%	Repeatability
28%	Method Bias
13%	Measurement model
23%	Ion transition
2%	Sample homogeneity
0%	Dry weight

The participant reported that the uncertainty arising from weighing of the sample was considered negligible and that ERM-BB-124 (nitroimidazoles in porcine muscle) was used to verify the LC-MS/MS method. The bias between the certified mass fraction and determined mass fraction was included as an uncertainty component.

Uncertainty Information of SASO-NMCC

Part IV: Uncertainty budget

The measurement equations used to calculate the mass fraction of each analyte. Please provide details of all the factors listed in the equations and indicate how these values were determined.

Cun: concentration of the unknown sample (mg/kg),
intercept, a: slope of the calibration line
Aun: area of unknown sample

$$C_{un} = \frac{(A_{un} - b)}{a}$$

b:

Estimation of uncertainties for each factor. Give a complete description of how the estimates were obtained and combined to calculate the overall uncertainty. Please provide a table detailing the full uncertainty budget.

slope, $u(a) = \sqrt{\frac{S^2}{\sum_{i=1}^{n}(x_i - \bar{x})^2}}$

recovery $u_R = \sqrt{\left(\frac{\partial f}{\partial C_{obs}} . u_{obs}\right)^2 + \left(\frac{\partial f}{\partial C_{spike}} . u_{spike}\right)^2 + \left(\frac{\partial f}{\partial C_{spike}} . u_{spike}\right)^2}$

intercepte $u(b) = \sqrt{\frac{S^2 \sum_{i=1}^{n} x_i^2}{n \sum_{i=1}^{n}(x_i - \bar{x})^2}}$

area of Unknown $u_{Aun} = \frac{SD}{\sqrt{n}}$

combined uncertanity $u_c = \sqrt{\left(\frac{\partial f}{df_{Aun}} . u_{Aun}\right)^2 + \left(\frac{\partial f}{df_a} . u_a\right)^2 + \left(\frac{\partial f}{df_b} . u_b\right)^2 + \left(\frac{\partial f}{df_{Rec}} . u_{Rec}\right)^2}$

Uncertainty Information of UME

Part IV: Uncertainty budget

The measurement equations used to calculate the mass fraction of each analyte. Please provide details of all the factors listed in the equations and indicate how these values were determined.

$$C_{sample} = \frac{Area_{sample}}{Area_{IS}} \frac{C_{Std}}{C_{IS}} \frac{Area_{STD-IS}}{Area_{Sample}} C_{Sample\ -IS}$$

$$\frac{U_c(A)}{C_A} = \sqrt{(\frac{u(W_{SM})}{W_{SM}})^2 + (\frac{u(W_{IS})}{W_{IS}})^2 + (\frac{u(C_{NS})}{C_{NS}})^2 + (\frac{u(C_{LS})}{C_{LS}})^2 + (\frac{u(Cal)}{c_0})^2 + (\frac{u(\mathrm{Rec})^2}{\mathrm{Rec}}) + u(r)^2}$$

SI: Sample mass
IS: Internal standard
NS: Native stock solution
LS: Labeled stock solution
r: repeatibility
Rec: Recovery
Cal: Calibration curve

Estimation of uncertainties for each factor. Give a complete description of how the estimates were obtained and combined to calculate the overall uncertainty. Please provide a table detailing the full uncertainty budget.

Uncertainty budget of Metronidazole (LC-HRMS)				
	Value	u(x)	u(x)/x	
Weighing of sample (mg)	1000	1,31E-02	1,31E-05	
Weighing of IS (mg)	100	2,32E-06	2,32E-08	
Standard stock solution (µg/kg)	1000	1,21E+00	1,21E-03	
Internal stock solution (µg/kg)	1000	3,78E+00	3,78E-03	
Intermediate precision	100	6,50E-01	6,50E-03	
Recovery	1	3,84E-02	3,84E-02	
Repeatability	100	4,05E-02	4,05E-04	
Calibration graph	0,4	1,62E-02	4,06E-02	
			5,64E-02	
Result (µg/kg)	9,16			
Combined uncertainty	0,52			
Expanded uncertainty	1,03			
% Relative uncertainty	11,28			

Uncertainty Information of VNIIM

Part IV: Uncertainty budget

The measurement equations used to calculate the mass fraction of each analyte. Please provide details of all the factors listed in the equations and indicate how these values were determined.

w- mass fraction of MNZ in the sample, mkg/kg;
mis - mass of IS added to sample before sample preparation, ng;
m - mass of the sample (dry mass), g;
SMNZ - peak area of MNZ;
Sis - peak area of IS.
RRF - relative response factor.
mis - mass of IS in calibration solution; mMNZ - mass of MNZ in calibration solution;
SMNZcal - peak area of MNZ in calibration solution; Siscal - peak area of IS in calibration solution

$$w = \frac{S_{MNZ} \cdot m_{IS}}{S_{IS} \cdot RRF \cdot m}$$

$$RRF = \frac{S_{MNZ} \cdot m_{IS}}{S_{IS} \cdot m_{MNZ}}$$

Estimation of uncertainties for each factor. Give a complete description of how the estimates were obtained and combined to calculate the overall uncertainty. Please provide a table detailing the full uncertainty budget.

$$u_c = \sqrt{u_A^2 + u_B^2} = \sqrt{u_{MZN}^2 + u_{RRF}^2 + u_{cal}^2 + u_{pur}^2 + u_{sol}^2 + u_m^2 + u_{IS}^2 + +u_{dry}^2 + u_{blank}^2}$$

u_{MNZ} - the standard uncertainty of MNZ measuring
u_{RRF} - the standard uncertainty of RFF determination
u_{cal} - the standard uncertainty of calibration solution preparation
u_{pur} - the standard uncertainty of calibrant
u_{sol} - the standard uncertainty of MNZ solutions preparation
u_m - the standard uncertainty of sample weighing
u_{IS} - the standard uncertainty of IS adding into the sample
u_{dry} - the standard uncertainty of dry mass determination
u_{blanc} - the standard uncertainty of method blank

Source of uncertainty		u, (µg/kg)
u_m	B	0.0004
u_{pur}	B	0.015
u_{sol}	B	0.061
u_{cal}	B	0.0068
u_{RRF}	A	0.074
u_{IS}	B	0.033
u_{MNZ}	A	0.35
u_{dry}	B	0.065
u_{blank}	B	0.29
Combined standard uncertainty, u_c		0.47
Expanded standard uncertainty, U		0.94

APPENDIX H: Participants' Quantitative Results as Reported

The following are pictures of the quantitative results as provided by the participants in the "Results" worksheet of the "Reporting Form" Excel workbook. Information is grouped by participant and presented in alphabetized acronym order.

Quantitative Results of BVL

Part II: Results:					
Sample No. used for reporting	K180 - 203				
Moisture content (w/w %):	**1,58 %**				
Analyte/ Mass Fraction	Mass fraction (µg/kg) dry mass basis	Combined Standard uncertainty u (µg/kg)	Coverage factor (k)	Expanded uncertainty U (µg/kg)	Number of replicates (n)
metronidazole	8.19	0.59	2.0	1.18	3

Quantitative Results of CENAM

Part II: Results:					
Sample No. used for reporting	K180 - 208, 26 and 251				
Moisture content (w/w %):	**0.115**				
Analyte/ Mass Fraction	Mass fraction (µg/kg) dry mass basis	Combined Standard uncertainty u (µg/kg)	Coverage factor (k)	Expanded uncertainty U (µg/kg)	Number of replicates (n)
Metronidazole	8.79	0.40	2	0.80	6

Quantitative Results of EXHM

Part II: Results:					
Sample No. used for reporting	K180 - 134				
Moisture content (w/w %):	**1.158**				
Analyte/ Mass Fraction	Mass fraction (µg/kg) dry mass basis	Combined Standard uncertainty u (µg/kg)	Coverage factor (k)	Expanded uncertainty U (µg/kg)	Number of replicates (n)
metronidazole	8.128	0.281	2.13	0.598	9

Quantitative Results of GLHK

Part II: Results:					
Sample No. used for reporting	K180 - 142				
Moisture content (w/w %):	**2.35**				
Analyte/ Mass Fraction	Mass fraction (µg/kg) dry mass basis	Combined Standard uncertainty u (µg/kg)	Coverage factor (k)	Expanded uncertainty U (µg/kg)	Number of replicates (n)
metronidazole	8.78	0.31	2	0.63	6

Quantitative Results of HSA

Part II: Results:					
Sample No. used for reporting	K180 - NIIM P220048 27.1.2022 Item: 176				
Moisture content (w/w %):	1.85				
Analyte/ Mass Fraction	Mass fraction (µg/kg) dry mass basis	Combined Standard uncertainty u (µg/kg)	Coverage factor (k)	Expanded uncertainty U (µg/kg)	Number of replicates (n)
metronidazole	8.16	0.42	2	0.85	4

Quantitative Results of INM Colombia

Part II: Results:					
Sample No. used for reporting	P220048 Item: 66				
Moisture content (w/w %):	0.80				
Analyte/ Mass Fraction	Mass fraction (µg/kg) dry mass basis	Combined Standard uncertainty u (µg/kg)	Coverage factor (k)	Expanded uncertainty U (µg/kg)	Number of replicates (n)
metronidazole	8.02	0.22	2	0.44	3

Quantitative Results of INMETRO

Part II: Results:					
Sample No. used for reporting	K180 - 224				
Moisture content (w/w %):	**2.07**				
Analyte/ Mass Fraction	Mass fraction (µg/kg) dry mass basis	Combined Standard uncertainty u (µg/kg)	Coverage factor (k)	Expanded uncertainty U (µg/kg)	Number of replicates (n)
metronidazole	8.61	0.19	2	0.39	5

Quantitative Results of INRAP

Part II: Results:					
Sample No. used for reporting	K180 - 126/33/190				
Moisture content (w/w %):	1.03%				
Analyte/ Mass Fraction	Mass fraction (µg/kg) dry mass basis	Combined Standard uncertainty u (µg/kg)	Coverage factor (k)	Expanded uncertainty U (µg/kg)	Number of replicates (n)
metronidazole	7.68	0.29	2	0.57	6

Quantitative Results of KRISS

Part II: Results:					
Sample No. used for reporting	K180 - Item: 41				
Moisture content (w/w %):	**1.20%**				
Analyte/ Mass Fraction	Mass fraction (µg/kg) dry mass basis	Combined Standard uncertainty u (µg/kg)	Coverage factor (k)	Expanded uncertainty U (µg/kg)	Number of replicates (n)
metronidazole	8.71	0.10	2.31	0.24	5

Quantitative Results of LGC

Part II: Results:					
Sample No. used for reporting	K180 - 273, 249, 129				
Moisture content (w/w %):	**0.996 ± 0.0003**				
Analyte/ Mass Fraction	Mass fraction (µg/kg) dry mass basis	Combined Standard uncertainty u (µg/kg)	Coverage factor (k)	Expanded uncertainty U (µg/kg)	Number of replicates (n)
metronidazole	8.55	0.15	2	0.3	9

Quantitative Results of NIM China

Part II: Results:					
Sample No. used for reporting	item 133				
Moisture content (w/w %):	**1.63%**				
Analyte/ Mass Fraction	Mass fraction (µg/kg) dry mass basis	Combined Standard uncertainty u (µg/kg)	Coverage factor (k)	Expanded uncertainty U (µg/kg)	Number of replicates (n)
metronidazole	8.13	0.20	2	0.40	6

Quantitative Results of NIMT

Part II: Results:					
Sample No. used for reporting	243				
Moisture content (w/w %):	**1.738**				
Analyte/ Mass Fraction	Mass fraction (µg/kg) dry mass basis	Combined Standard uncertainty u (µg/kg)	Coverage factor (k)	Expanded uncertainty U (µg/kg)	Number of replicates (n)
metronidazole	8.33	0.175	2.0	0.35	6

Quantitative Results of NMIA

Part II: Results:					
Sample No. used for reporting	K180 - 135				
Moisture content (w/w %):					
Analyte/ Mass Fraction	Mass fraction (µg/kg) dry mass basis	Combined Standard uncertainty u (µg/kg)	Coverage factor (k)	Expanded uncertainty U (µg/kg)	Number of replicates (n)
metronidazole	8.42	0.064	2.02	0.13	7

Quantitative Results of NRC Canada

Part II: Results:					
Sample No. used for reporting	P22048 Bottle - 167				
Moisture content (w/w %):	1.13				
Analyte/ Mass Fraction	Mass fraction (µg/kg) dry mass basis	Combined Standard uncertainty u (µg/kg)	Coverage factor (k)	Expanded uncertainty U (µg/kg)	Number of replicates (n)
metronidazole (MNZ)	8.45	0.30	2	0.60	6

Quantitative Results of SASO-NMCC

Part II: Results:					
Sample No. used for reporting	K180 - 260				
Moisture content (w/w %):	**0.57%**				
Analyte/ Mass Fraction	Mass fraction (µg/kg) dry mass basis	Combined Standard uncertainty u (µg/kg)	Coverage factor (k)	Expanded uncertainty U (µg/kg)	Number of replicates (n)
metronidazole	4.76	0.12	2	0.25	10

Quantitative Results of UME

Part II: Results:					
Sample No. used for reporting	K180 - 180				
Moisture content (w/w %):	1.30				
Analyte/ Mass Fraction	Mass fraction (µg/kg) dry mass basis	Combined Standard uncertainty u (µg/kg)	Coverage factor (k)	Expanded uncertainty U (µg/kg)	Number of replicates (n)
metronidazole	9.16	0.52	2	1.03	4

Quantitative Results of VNIIM

Part II: Results:					
Sample No. used for reporting	49				
Moisture content (w/w %):	**1.2**				
Analyte/ Mass Fraction	Mass fraction (µg/kg) dry mass basis	Combined Standard uncertainty u (µg/kg)	Coverage factor (k)	Expanded uncertainty U (µg/kg)	Number of replicates (n)
metronidazole	10.48	0.47	2	0.94	5

APPENDIX I: Participants' comments

The following are pictures of the comments as provided by the participants in the "Results" worksheet of the "Reporting Form" Excel workbook. Information is grouped by participant and presented in alphabetized acronym order.

Comments submitted by BVL

Part V: Comments / additional information

Other information, observations or evidences, if any, that can further support your results.

contributions to measurement uncertainty:

	u	(unit)	target	(unit)	u(x)/X [%]	(u(x)/X)^2
u calibration solution:	0,01923	ng/g	12,766	ng/g	0,1506	0,02269
u sample weight:	0,02444	mg	1000,000	mg	0,0024	0,00001
u sample spike:	0,02105	mg	15,470	mg	0,1361	0,01852
u dry mass:	0,03299	g	0,793	g	4,1602	17,30686
reproducibility method:	0,15264	ng/g	2,120	ng/g	7,2000	51,84000

Comments submitted by CENAM

N/A

Comments submitted by EXHM

Part V: Comments / additional information

Other information, observations or evidences, if any, that can further support your results.

The result (8,128 ng/g) is provided on a dry basis for the lyofilized material.
IRMM ERM-BB124 was analysed in parallel to the BVL material with a recovery of 102% (within the uncertainty of the reference values).
The result has also been confirmed by standard additions.
An alternative analytical procedure was also followed for the analysis (the material was spiked with the internal standard, then subjected to enzymatic hydrolysis with Flavourzyme (Novozymes A/S). The resulting mixture was centrifuged, MNZ was isolated by solid phase extraction using Extrelut columns) and quantified by LC-HRMS (SCIEX X500R), providing equivalent results.

Comments submitted by GLHK

N/A

Comments submitted by HSA

Part V: Comments / additional information
Other information, observations or evidences, if any, that can further support your results.
Method recovery was investigated using ERM®-BB124 (certified mass fraction of metronidazole in pig muscle (reconstituted material) = 1.93 ± 0.15 µg/kg). The recovery results fall within the expanded uncertainty range of the certified mass fraction of metronidazole in pig muscle (ranged between 97.3 % to 101.5 %) and were used to estimate the uncertainty of method recovery (Frec). Besides the reported method recovery evaluation, method recovery was also investigated using the comparison study material spiked gravimetrically with metronidazole solution prepared from GBW(E)060908 (NIM). The spiked material was analysed in parallel with the SBs for quality control (QC). Each QC was subjected to the same extraction and clean-up as the study sample. The recovery results ranged between 97.5% to 99.2%. Subsamples of the study samples were analysed using another column, i.e. Kinetex Polar C18 (100 x 2.1 mm, 2.6 µm). The results were used to estimate the uncertainty in the use of different columns (Fcol) to account for the possible largest bias in results from using different column. Extraction using ethyl acetate, methanol and 0.1% formic acid in ethyl acetate were studied. The results were used to estimate the uncertainty in the use of different sample extraction solvent (Fext) to account for the possible largest bias in results from using different extraction solvent. 0.1% formic acid in ethyl acetate was used as the extraction solvent in view of the pKa of metronidazole. The number of extraction cycles was studied. No significant difference in the results obtained from the SB extracted three times vs SB extracted two times was found. Hence, SB was extracted two times for optimum extraction.

Comments submitted by INM Colombia

Part V: Comments / additional information
Other information, observations or evidences, if any, that can further support your results.
An In-house quality control was used for the method and extraction procedures development.

Comments submitted by INMETRO

Part V: Comments / additional information
Other information, observations or evidences, if any, that can further support your results.
Two calibration solutions were prepared completely independently and used for cross-confirmation of the results. Control samples were prepared by spiking blank matrix samples with metronidazole solution prepared from other standard than the used for calibration solutions. The mean recovery obtained for the control samples was very close to 100 %. The metronidazole standard used to prepare the control samples was: United States Pharmacopeia Reference Standard (USP), lot: JOC316. Purity was determined by ^{1}H-qNMR at Inmetro (1000.33 mg/g ± 1.86 mg/g, K=2, 95%). Maleic Acid, CRM 8792.0001 - INMETRO, used as internal standard in qNMR purity determination.

Comments submitted by INRAP

N/A

Comments submitted by KRISS

N/A

Comments submitted by LGC

N/A

Comments submitted by NIM China

N/A

Comments submitted by NIMT

Part V: Comments / additional information
Other information, observations or evidences, if any, that can further support your results.
Two sources of the pure standard (NIM- GBW(E)060908 and INMETRO-8365) were compared. The measurement results were comparable. The test material was soaked under two different conditions (1 hour vs overnight) before the extraction step. The results obtained from two conditions were not significantly different.

Comments submitted by NMIA

Part V: Comments / additional information
Other information, observations or evidences, if any, that can further support your results.
ERM BB124, certified value for reconstituted material 1.93 ± 0.15 ug/kg equivalent to 7.72 ± 0.60 ug/kg, was analysed with each batch of K180 samples. Multiple results obtained in each batch (from different SPE and/or LCMS methods) were averaged, and the average of three batch results was 7.72 ± 0.04 ug/kg (standard u - standard deviation of the mean)

Comments submitted by NRC Canada

Part V: Comments / additional information
Other information, observations or evidences, if any, that can further support your results.
Uncertainties due to weighing of samples was considered negligible.

Comments submitted by SASO-NMCC

N/A

Comments submitted by UME

N/A

Comments submitted by VNIIM

N/A