CCQM-K141
High Polarity Analytes in Food - Enrofloxacin and Sulfadiazine in Bovine Tissue

Track A Key Comparison

Final Report
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Table of Contents

1. Introduction 3

2. Measurands, Indicative Ranges and Reference Standards 3

3. Study Material 4

3.1 Homogeneity 4

3.2 Stability 7

3.3 Freeze thaw stability 8

3.4 Shipping, sample handling, moisture content and reporting results 10

3.5 Study schedule and sample distribution 11

4. Calibration Materials 12

5. Methods Used by Participants 14

6. Participant Results for Enrofloxacin, Sulfadiazine and Moisture 17

7. Preliminary Assessment of Results 21

8. Follow-up Work Conducted by NRC 22

9. Measurement Equations and Uncertainty Estimation 25

10. Determination of the Key Comparison Reference Values (KCRV) and Degrees of Equivalence (DoEs) 26

11. How Far Does the Light Shine? 30

12. Conclusions 30

13. Acknowledgements 31

14. Literature cited 31

Appendix I. Sample amounts, pre-treatments, extraction and clean up methods, all participants 32

Appendix II. Participants methods: Calibration, instrumentation and MS/MS transitions 38

Appendix III. Measurement Equations and Uncertainty Budgets 44

Appendix IV. Other Information Reported 73

Appendix V. Core Competency Tables 79

Appendix VI. Information Tables 94

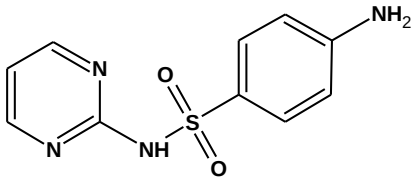
1. Introduction

Analysis of veterinary drug residues in bovine muscle is a topic of great importance due to potential health risks, trade and export issues. The ability to provide assurance to both consumers and import/export countries of the absence, presence and quantification of these residues in bovine muscle relies on the implementation of precise and accurate methods of analysis. An international comparison study based on the analysis of veterinary drugs in bovine muscle would satisfy the need to address chemical measurement-related issues important for international trade, environmental, health and food safety-related decision making and provide evidence for the establishment of the equivalence of measurement results among NMI/DIs.

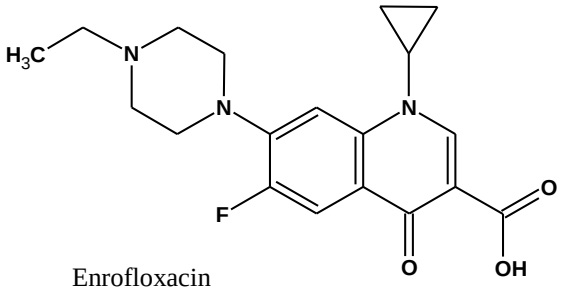
At the October 2014 meeting of the OAWG in Tsukuba Japan it was agreed to conduct a Track A Key comparison in mid to late 2016 to test the core competencies of laboratories that deliver measurement services for polar analytes in a food matrix. At the following meeting in Paris in 2015 the OAWG voted to study two polar veterinary drugs: enrofloxacin and sulfadiazine in a bovine muscle tissue matrix which is currently under development by the National Research Council Canada (NRCC) and the Canadian Food Inspection Agency (CFIA) as a multi-drug residue CRM (BOTS-1). As a Track A study, it was expected that all NMIs or DIs with relevant claims would participate; a parallel pilot study, CCQM-P178, was also conducted with the same material for interested parties. With only two pilot study participants, a separate pilot study report was not prepared, but their results are listed separately in this report with their explicit permission.

2. Measurands, Indicative Ranges and Reference Standards

The two analytes are the broad-spectrum sulfonamide and fluoroquinolone antibiotics: sulfadiazine and enrofloxacin (below) for which maximum residue limits are enforced in many countries. The measurands are the mass fractions of these analytes in beef muscle determined on a dry mass basis.



Sulfadiazine
$C_{10}H_{10}N_4O_2S$
MW=250 pK_{ow} = 0.3
CAS: 68-35-9



Enrofloxacin
$C_{19}H_{22}FN_3O_3$
MW=359 pK_{ow} = - 0.7
CAS: 93106-60-6

The study requires extraction, clean-up, analytical separation, and selective detection of the analytes in a food matrix. Three ≥10 g bottles of freeze dried powdered muscle tissue were supplied. NRC also provided isotopically labelled solutions of the two analytes: $^{13}C_6$ sulfadiazine and enrofloxacin-d_5 (HI Salt) to those interested in using IDMS methodologies. Procurement and purity assignment with appropriate metrological traceability of native calibrants are the responsibility of individual participants. The indicative ranges for the mass fractions of the analytes are provided in Table 1.

Table 1. Indicative ranges

Measurand	Mass Fraction Range (µg/kg)
sulfadiazine	500-5000
enrofloxacin	20-200

3. Study Material

The matrix, bovine muscle tissue, was a high fat and high protein product that falls within Sector 4 of the AOAC International food triangle. The bovine muscle was derived from a single animal (bovine heifer RFID# 124000230337331) that was administered with chemical based pharmaceutical agents prior to processing. Following processing at Drake Meat Processors Inc. (Drake, Saskatchewan) the muscle tissue was sent for further processing (wet homogenisation, freeze drying and grinding) at NSF International's Guelph Food Technology Centre, Ontario, Canada before shipment to the NRCC Ottawa where it was further homogenised and bottled in ≥10 g amounts in glass bottles under argon and further sealed in tri-laminate foil envelopes. Long term storage of the material at NRCC is at -80°C.

3.1 Homogeneity

Fourteen bottles of bovine muscle tissue were selected in a random stratified design across the bottling run. 0.5 g sub-samples were analysed in duplicate for enrofloxacin and sulfadiazine using an LC-IDMS method and the absolute values were transformed relative to the mean. The results are shown in Figures 1 and 2. A one-way analysis of variance was used to evaluate homogeneity using an *F*-test ($P = 0.05$) and the results tabulated in Tables 2 and 3.

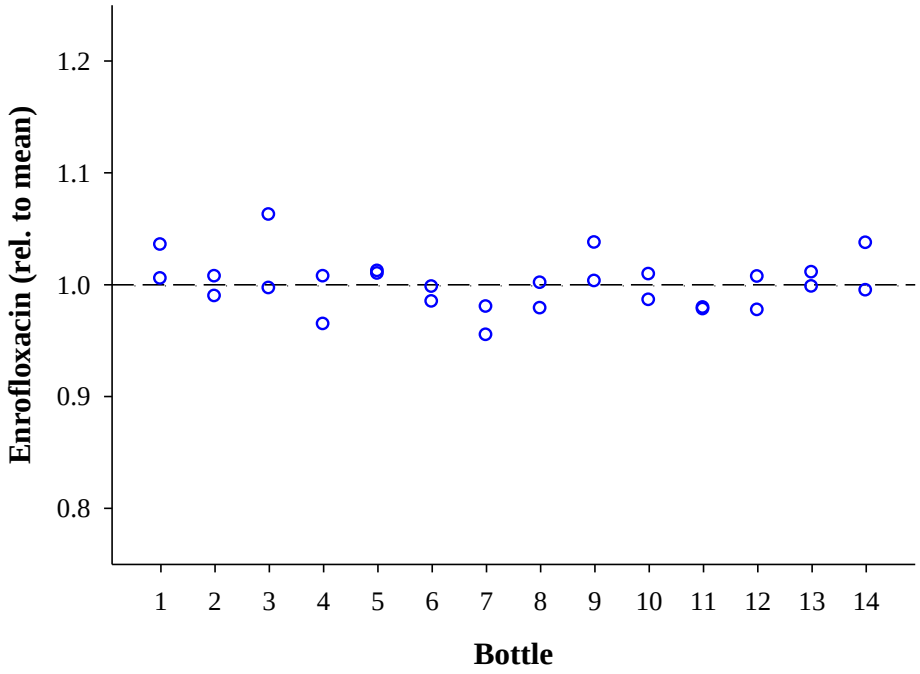


Figure 1. Homogeneity enrofloxacin

Table 2. ANOVA: Enrofloxacin homogeneity

Source of Variation	*SS*	*df*	*MS*	*F*	*P*	F_{crit}
Between Groups	0.008071	13	0.000621	1.314	0.308	2.507
Within Groups	0.006615	14	0.000473			
Total	0.014686	27				

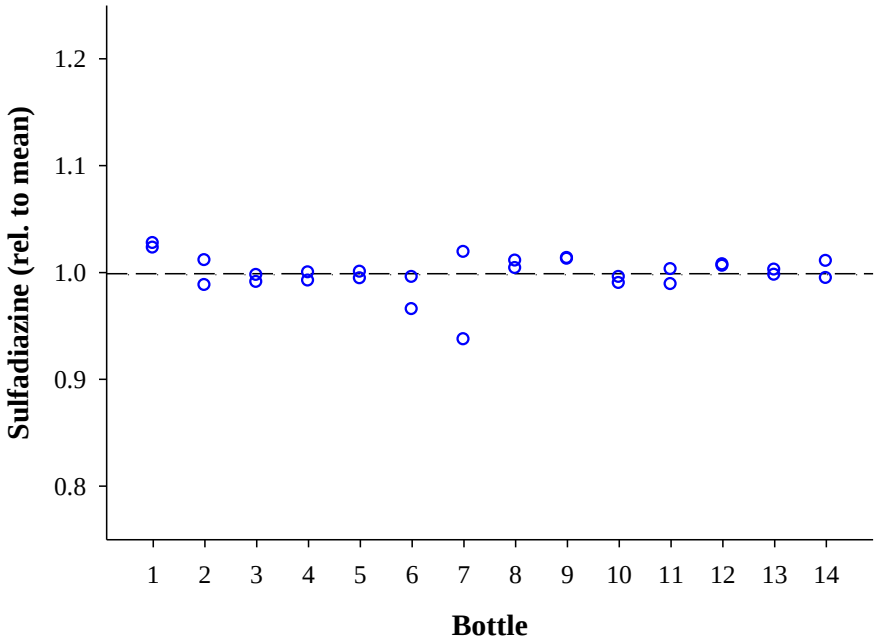


Figure 2. Homogeneity Sulfadiazine

Table 3. ANOVA: Sulfadiazine homogeneity

Source of Variation	*SS*	*df*	*MS*	*F*	*P*	F_{crit}
Between Groups	0.003693	13	0.000284	0.916	0.559	2.507
Within Groups	0.004341	14	0.00031			
Total	0.008034	27				

For both enrofloxacin and sulfadiazine the found values of *F* were less than the critical values, therefore there was no statistically significant difference between bottles for either analyte ($P > 0.05$). For enrofloxacin, MS_{within} was less than $MS_{between}$ therefore, u_{bb} was calculated as:

$$u_{bb} = \sqrt{\frac{MS_{between} - MS_{within}}{n}}$$

giving a relative standard deviation of 0.86%. However, for sulfadiazine the MS_{within} was greater than $MS_{between}$ and therefore a more conservative estimate u^*_{bb} was calculated as:

$$u^*_{bb} = \sqrt{\frac{MS_{within}}{n}} \cdot \sqrt[4]{\frac{2}{v_{MS_{within}}}}$$

giving a relative standard deviation of 1.25% (Linsinger et al., 2001).

3.2 Stability

Five, 2 g sub-samples were taken from each of 3 randomly selected bottles from the bottling run and re-sealed under argon in bottles and placed in tri-laminate envelopes and incubated at -80ºC, -20ºC, 6ºC, 20ºC and 37ºC temperatures. After 14 d, three 0.5 g samples were taken from each bottle and analysed using an LC-IDMS method and the absolute values were transformed relative to the mean. The results are shown in Figures 3 and 4. A one-way analysis of variance was used to evaluate differences between temperature treatments using an *F*-test (P = 0.05) and the results tabulated in Tables 4 and 5.

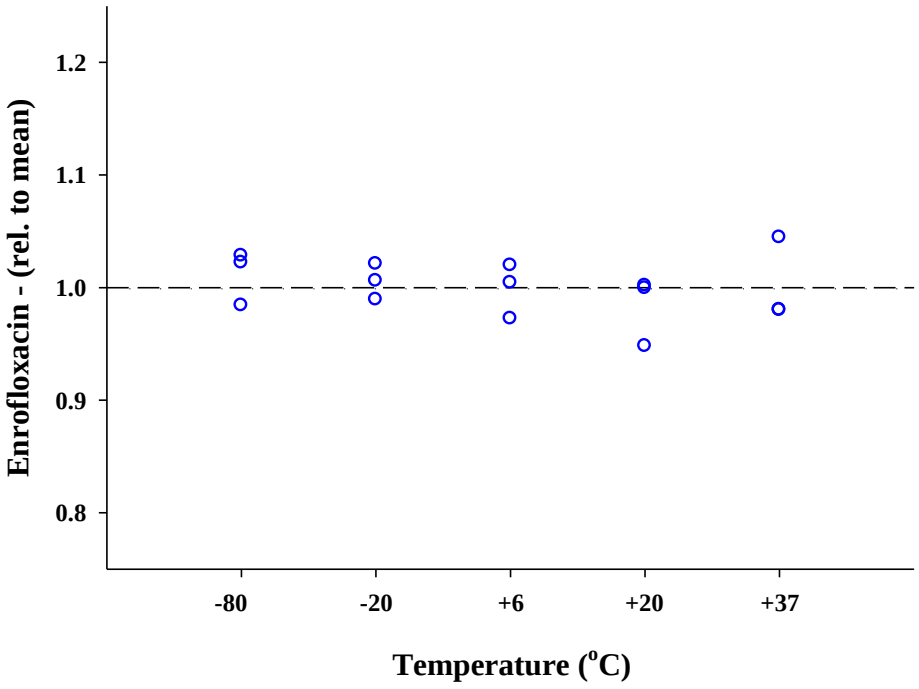


Figure 3. Enrofloxacin short-term stability

Table 4. ANOVA: Enrofloxacin, short-term stability

Source of Variation	*SS*	*df*	*MS*	*F*	*P*	F_{crit}
Between Groups	0.00135	4	0.000337	0.455	0.767	3.478
Within Groups	0.007416	10	0.000742			
Total	0.008766	14				

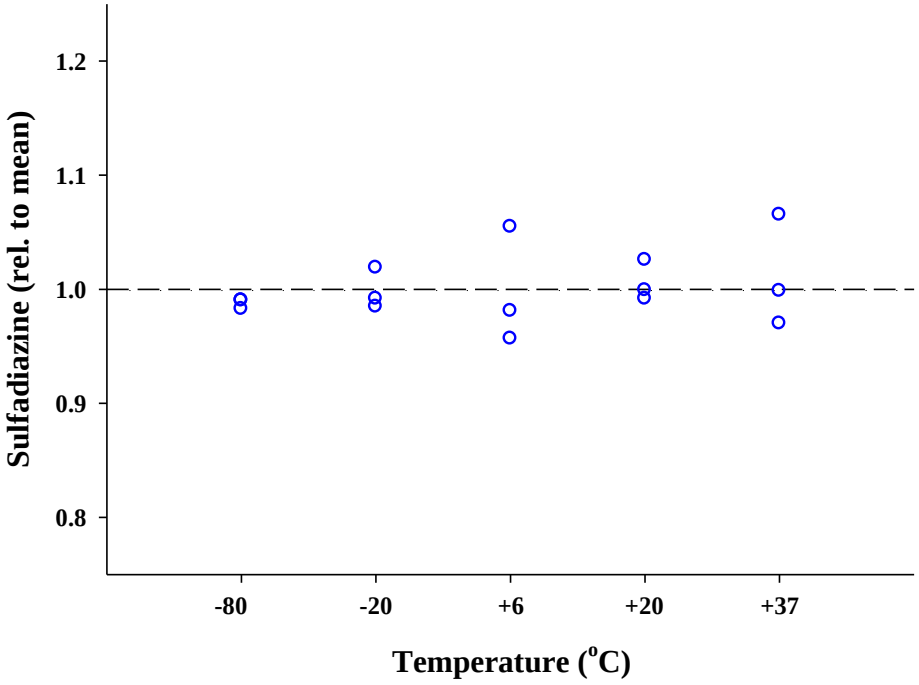


Figure 4. Sulfadiazine short-term stability

Table 5. ANOVA: Sulfadiazine short-term stability

Source of Variation	*SS*	*df*	*MS*	*F*	*P*	F_{crit}
Between Groups	0.000955	4	0.000239	0.211	0.926	3.478
Within Groups	0.01132	10	0.001132			
Total	0.012275	14				

For both enrofloxacin and sulfadiazine the found values of *F* were less than the critical values, therefore there was no statistically significant difference between bottles for either analyte ($P > 0.05$) due to incubation temperature.

3.3 Freeze thaw stability

The stability of enrofloxacin and sulfadiazine in the bovine tissue was measured following multiple freeze thaw cycles using an isochronous study design. A single bottle of the bovine tissue stored at -80ºC was removed and equilibrated to 20ºC for one hour, mixed by rolling and inversion by hand and two replicate 0.5 g samples (cycle 1) removed and samples and the bottle returned to the -80ºC freezer. This procedure was repeated 19 more times with duplicate samples taken at cycles 5 and 10 and five replicate samples taken at cycle 20. Sham sampling was conducted at all other times by simply mixing and opening the bottle and stirring with a spatula before returning the bottle to the freezer. After the final samples were taken all the samples were

removed from the freezer and subjected to analysis using an IDMS procedure. Note as all samples were refrozen after sampling and thawed again for the analysis actual freeze thaw cycles were all incremented by one. The results are given in Figures 5 and 6 and a one-way analysis of variance was used to evaluate differences between freeze thaw cycles using an *F*-test ($P = 0.05$) and the results tabulated in Tables 6 and 7. The results for both enrofloxacin and sulfadiazine clearly indicate no treatment effects due to freeze thaw cycling up to 21 times - which indicates that repetitive sampling from bottles held at -80ºC will not adversely affect the amount content of the study analytes.

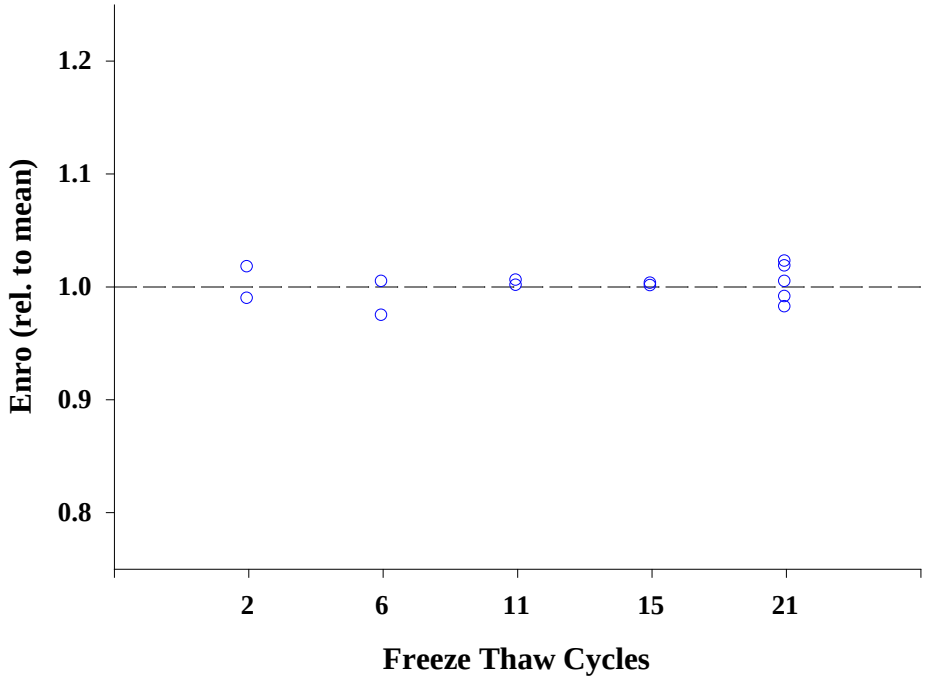


Figure 5. Enrofloxacin freeze-thaw stability

Table 6: ANOVA enrofloxacin freeze-thaw stability

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.000324	4	8.10E-05	0.315913	0.859684	3.837854
Within Groups	0.002052	8	0.000256			
Total	0.002376	12				

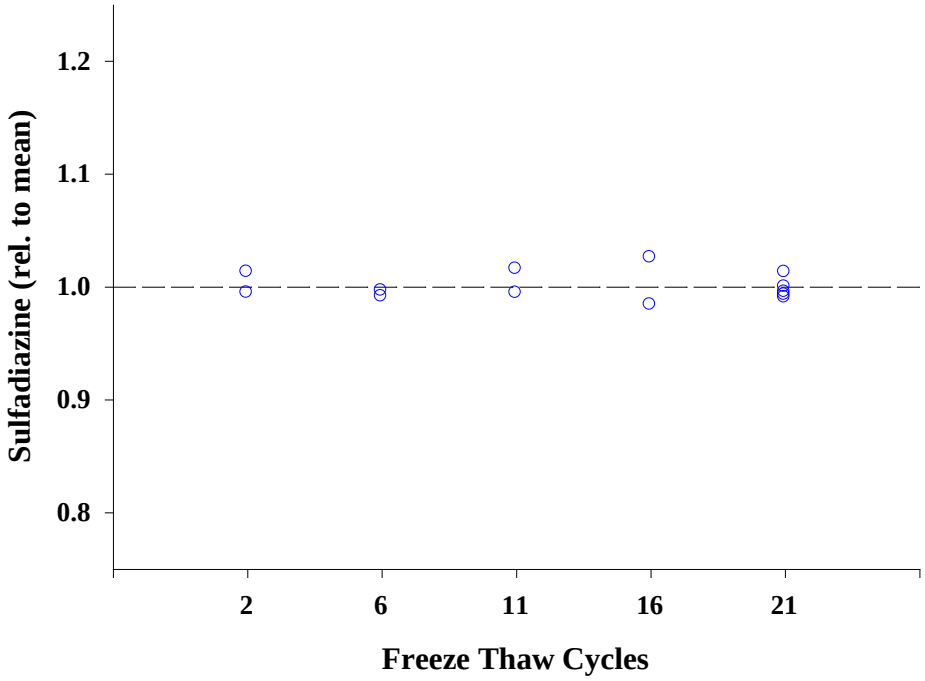


Figure 6. Sulfadiazine freeze-thaw stability

Table 7. ANOVA sulfadiazine freeze-thaw stability

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.000217	4	5.44E-05	0.272551	0.887661	3.837854
Within Groups	0.001596	8	0.000199			
Total	0.001813	12				

3.4 Shipping, sample handling, moisture content and reporting results

Each participant received three bottles of the study sample each containing ≥10g of freeze dried bovine tissue, shipped on dry ice and two flame sealed ampules of enrofloxacin-d_5 (HI Salt) containing 1.2 mL at a concentration of ~13.5 µg/mL in 50:50 MeOH : 5mM NaOH and two flame sealed ampules of $^{13}C_6$ sulfadiazine containing 1.2 mL at a concentration of ~100 µg/mL

in MeOH shipped on wet ice. On receipt, the recommended sample storage temperature was -80ºC and that for internal standards -20ºC. Participants were instructed that stock and working solutions of the internal standards should be equilibrated to room temperature and thoroughly vortex mixed before opening and use (sulfadiazine may crystallise out from solution at -20ºC). Similarly, sample bottles should be equilibrated to room temperature, mixed by rolling and inversion by hand before opening and sampling. Two sample bottles were intended for method development and one bottle was to be used for the final results. Following sampling the bottles were to be carefully resealed and returned to the -80ºC storage freezer. Given that the material is freeze dried from wet muscle tissue with a moisture content of ~ 65% w/w it was recommended that method development and validation examine sample reconstitution as a pre-treatment. Participants were requested to report results for each measurand (µg/kg) from a single bottle on a dry mass basis using their method of choice. A minimum sample intake of 0.5 g was recommended. Dry mass corrections were to be determined from the same bottle as used for the reported results and initiated at the same time as the sampling for the definitive analyses. Dry mass corrections were to be done based on mass change of three replicate (1 g recommended sample size) sub-samples placed over anhydrous calcium sulphate in a desiccator, under continuous vacuum, at room temperature for a minimum of 21 days until a constant mass was reached.

3.5 Study schedule and sample distribution

Sample Preparation	February 2015
Homogeneity and Stability testing	March 2016
Sample Distribution	June 2016
Deadline for Submission of Results	January 31st, 2017
Extended Deadline	March 17th, 2017*
Preliminary Discussion of Results	April 2017

*The deadline was extended by two weeks for VNIIM who, due to shipping and permit issues, only received their study samples in February.

Thirteen laboratories registered and participated in the Key Comparison for both enrofloxacin and sulfadiazine, two laboratories participated in the pilot NRC-Halifax and INTI (enrofloxacin only), one laboratory, INRAP, registered for the pilot, but was unable to submit results due to instrumentation issues.

4. Calibration Materials

Five of the thirteen K141 and one of the P178 participant laboratories utilised native sulfadiazine and enrofloxacin CRMs produced by NMIA (Table 8a) with the remaining opting to make their own purity assignments using qNMR and/or mass balance approaches to commercially sourced materials (Table 8b). All materials were assigned high purities. The distribution of results for either the enrofloxacin or sulfadiazine comparison shows no correlation with calibration standard source. In particular, participants using the NMIA materials reported results for both analytes that were evenly distributed across the result sets (See Figures 7 and 8 below).

Table 8a. NMI/DI use of NMIA CRMs for Native Sulfadiazine and Enrofloxacin

NMI/DI	Source(s)	Purities and Uncertainties (95% CI)	In-house Methods
NMIA	NMIA	enrofloxacin M747b 98.5 ± 0.6% sulfadiazine M317 99.7 ± 0.4%	NMIA CRM
HSA	NMIA	as above	N/A
GLHK	NMIA	as above	
NIMT	NMIA	as above	LC/MS and KF
BVL	NMIA	as above	
NRC-Halifax (P178)	NMIA	as above	qNMR and KF

Table 8b. NMI/DI Sources of Standards and Reference Materials and In-house Methods of Mass Fraction Assignment and Uncertainties

NMI/DI	Source(s)	Purities and Uncertainties (95% CI)	In-house Methods
EXHM	enrofloxacin – Fluka (17849) sulfadiazine – Sigma (35033)	998.4 ± 1,8 mg/g 997.1 ± 1,7 mg/g	qNMR via NIST350b
LGC	enrofloxacin Sigma. ref. 17849, BN 115M4889V sulfadiazine Sigma S8626, BN 056M4795V	99.60±0.25% (k=2.78) 99.48±0.20% (k=2)	qNMR
VNIIM	enrofloxacin: Sigma Aldrich no. 33699, batch: SZBE199XV sulfadiazine Sigma Aldrich No.35055, batch:	99.8 ± 0,5 % 99.8 ± 0,5 %	ID: LC/MS Mass balance: KF oven, ICP/MS for inorganic impurities GC/MS/TD for residual solvent

	BCBS4650V Vetranal		determination; LC/UV for related impurities
INMETRO	Sigma-Aldrich	Values not provided	qNMR/NMR
KRISS	sulfadiazine Dr. Ehrenstorfer enrofloxacin Dr. Ehrenstorfer	99.90 % ± 0.24 % (95%, k=2.45) 99.91 % ± 0.29 (95%, k=2.78)	Mass balance LC/UV, TGA, Karl Fischer Coulometry, HS-GC/MS)
UME	sulfadiazine Vetranal, Sigma Aldrich enrofloxacin, Dr. Ehrenstorfer	99.93%, ± 0.19% (k=2) and 95% confidence level 99.52%, ± 0.23% (k=2) and 95% confidence level	qNMR traceable
NRC-Ottawa	enrofloxacin: Sigma Lot BCBK3650V sulfadiazine: Sigma Lot BCBK1734V	997.7 mg/g, u_c: 4.7, U_c, k=2: 9.4 996.9 mg/g, u_c: 1.7, U_c, k=2: 3.5	qNMR traceable
NIM	enrofloxacin Sigma-Aldrich sulfadiazine: GBW(E)060901	99.7%±0.4% (k=2) 99.6%±0.4% (k=2).	Mass balance: LC-UV, LC/MS/MS, Karl-Fischer Titration, ICP-MS, GC-FID, TGA qNMR
INTI (P178)	enrofloxacin – Sigma Aldrich (17849) Lot 1369030V	98.7%	Not stated

Seven of the K141 and one of the P178 NMI/DI's sourced isotopically labelled internal standards from a variety of sources (Table 9) with the remaining laboratories using those supplied by NRC which were Enrofloxacin-d_5 (HI Salt) CDN Isotopes D-6993 stated chemical purity of 98.8% and > 99% isotopic enrichment and Sulfadiazine-$^{13}C_6$, Sigma Aldrich 32518 with stated chemical purity of 99.4% and > 99% isotopic enrichment. These were supplied as: two flame sealed ampules of enrofloxacin-d_5 (HI Salt) containing 1.2 mL at a concentration of ~13.5 µg/mL in 50:50 MeOH : 5mM NaOH and two flame sealed ampules of $^{13}C_6$ sulfadiazine containing 1.2 mL at a concentration of ~100 µg/mL in MeOH.

Table 9. Sources, Chemical and Isotopic Purities of Internal Standards of Participants not utilising NRC supplied materials

NMI/DI	Source(s)	Chemical (CP) and Isotopic Purities (IP)
HSA	Enrofloxacin-d_5 (ethyl-d_5) hydroiodic acid, Medical Isotopes Inc., NH, USA Sulfadiazine-$^{13}C_6$, Toronto Research Chemicals Inc., ON, CAN	CP 98.8%, IP 99% CP 98%, IP 99.8%
NMIA	Enrofloxacin-D_5 hydrochloride (D_5-ENR) Witega, Germany and NRC materials as supplied Sulfadiazine-$^{13}C_6$ ($^{13}C_6$-SDZ) NRC material as supplied	CP 99.0 ± 0.2%, IP > 99.0 CP 99.4 ± 0.2%, IP > 99.0 (Corrected for EtOH 0.2%)
GLHK	Enrofloxacin-d5 HCl Dr. Ehrenstorfer. Sulfadiazine-$^{13}C_6$ ($^{13}C_6$-SDZ) Witega, Germany	CP 99%, IP > 99% CP 99.6% ± 0.2 %, IP > 99.0
NMIT	Enrofloxacin-D_5 hydrochloride (D_5-ENR) Witega, Germany Sulfadiazine-$^{13}C_6$ ($^{13}C_6$-SDZ) Witega, Germany.	CP 99.0 ± 0.2%, IP > 99.0 CP 99.6% ± 0.2 %, IP > 99.0
UME	Sulfadiazine-phenyl-$^{13}C_6$ Vetranal, 10 mg Neat, Sigma Aldrich Enrofloxacin-d_5-hydrochloride Vetranal, 10 mg Neat, Sigma Aldrich	Not stated
NIM	Enrofloxacin-D_5·HCl (Witega CH005) Sulfadiazine-$^{13}C_6$ (TRC S699052):	CP 99.0%±0.2%, IP >99% CP 98%, IP 99.8%
LGC	Sulfadiazine-phenyl-$^{13}C_6$, Sigma ref. 32518, batch number SZBE310XV Enrofloxacin-D_5 hydrochloride, Sigma ref. 32983, batch number SZBF344XV	Not stated
INTI (P178)	Enrofloxacin d5: Sigma – Lot SZBF126XV	CP 99.7% IP > 99%

5. Methods Used by Participants

A summary of the sample intakes, pre-treatment, and IS spiking and equilibration times are given in Table 10 with full details in Appendix 1. Sample amounts varied from 0.5 g to 2.0 g. Except for INMETRO, all participants reconstituted the freeze-dried beef with ~1 to 3 mL of water or in the case of KRISS 0.1 % formic acid. Some laboratories added IS spikes prior to wetting the sample though most did so after reconstitution. Equilibration times for re-hydrating the sample varied considerably: from 10m to 16h and a similar wide range of equilibration times after IS spiking were used: from 30 min to 46 h.

Table 10. Summary of sample pre-treatment and internal standard spiking (add. data rqd.)

NMI/DI	Sample Intake (g)	Sample Reconst'd with Water	Amount of Water (g or mL)	Reconst. time, temp other	IS Spikes Before or After reconstitution	IS Equilibration time, temp
EXHM	0.7	Y	1.3 g	30 m, Rt, dark	After	30 m, Rt
HSA	0.5	Y	1 mL	16 h, 4ºC	After	16 h, 4ºC**
NMIA	0.5	Y	1 mL	1 h, Rt	After	12 h, 4ºC
LGC	1.0	Y	2 mL	2 h, Rt	After	46 h, Rt
VNIIM	0.5	Y	1.5 mL	2 x 30 m, S, Rt	Before	1 h, Rt
GLHK	0.5	Y	3 mL	12 h, 4ºC	Before	12 h, 4ºC**
INMETRO	0.75	N	ND	NR	NR	NR
KRISS	0.5	N	1.5 mL 0.1% FA	30 m, Rt	Before	30 m, Rt**
NIMT	0.5	Y	2.5 mL	12 h, 4ºC	After	1 h, Rt
UME	0.5	Y	0.92 g	15 m, Rt	After	2 h, 4ºC
BVL	0.5	Y	0.93 g	2 h, Rt	After	15 h, Rt
NRC-Ottawa	0.5	Y	1 mL	10 m, Rt	After	12 h, 4ºC
NIM	0.5	Y	1.5 g	30 m, Rt	After	30 m, Rt
INTI (enro) (P178)	2.0	Y	3.7 g	20 m, Rt	After	45 m, Rt
NRC-Halifax (P178)	0.7	Y	1.2 mL	30 m, Rt	Before	30 m, Rt

"FA" = formic acid, ** same as reconstitution time, "S" = sonication, "ND" = not detected, "NR" = Not reported

A summary of extraction methods, solvent systems and clean-up techniques are provided in Table 11 with full details in Appendix I. Except for VNIIM, a single extraction system was used for both analytes, though EXHM used three different methods with reportedly equivalent results. All laboratories used LC MS/MS instrumentation (triple quadrupole or quadrupole trap configurations) with isotope dilution methods. These ranged from single one way IDMS (INTI-P178) to hybrid standard addition IDMS methods (KRISS and NRC Ottawa); however, most employed double isotope dilution with single or multiple point calibrations (Appendix II). Notably, NIMT, EXHM and BVL used blank bovine tissue to prepare matrix matched calibration blends. A variety of reverse phase separations with C8/C-18/PFP or bi-phenyl columns were used and developed with either acetonitrile or methanol as the organic solvent and water. Formic acid was most commonly used as a modifier, although TES, oxalic acid and EDTA were also used. NMIA also used both 1D and 2D LC separations. All used positive ion MS detection although NMIA also used a negative ion method. Typical ion transitions used for sulfadiazine were *m/z* 251-156 and *m/z* 257-162 for the $^{13}C_6$ labelled compound and for enrofloxacin *m/z* 360-316 and *m/z* 365-321 for the d5 labelled compound, specific transitions used for quantitation and qualification are given in Table 6. No participants reported difficulties in chromatographic separation or interferences therefore it is not likely these issues would have contributed to disparate results.

Table 11. Summary of extraction and clean-up methods – all participants

NMI/DI	No. ext. steps	ext. vol (ml)	extraction time total, temperature	Extraction Solvent	SPE	De-Fat	Ext. dried	Final Solvent
EXHM	1 1 2	20 20 20	8h, 55ºC 20m, Rt 20m, 70ºC	1. 5 mL Tris buffer/Pronase – 15 mL ACN 5% FA 2. 5 mL TRIS + 15 mL ACN, 5% FA blend sonicate 3. ACN 5% FA PLE 2 x 10 mL	Y	N	N	ACN 5% FA
HSA	4	40	84m, precool ice bath first, Rt	1x 10 mL 0.1 M HCl in ACN 3x 10 mL 0.01 M HCl in ACN	Y	N	Y	0.01 mol/L HCl (85:15, H2O/ACN *v/v*)
NMIA	4	20	2.25 hr Rt	ACN/H2O 70/30 v/v	Y	Y	Y	ACN /H2O (10:90) 1 mM NaOH
LGC	1	28	48h Rt	ACN/H2O/AA 20/8/0.2 v/v/v	N	N	Y	ACN/H2O/AA 20/8/0.2 v/v/v ?
VNIIM	3	9	45m Rt	Sdz: ACN 0.1% FA Enro: ACN	N	Y		ACN 0.1% FA
GLHK	2	30	3.5h Rt	ACN 1% AA	Y	Y		ACN 1% AA?
INMETRO	2	10	40m	MeOH	N	N	Y	MeOH/H2O (80:20 v/v) 5% AA
KRISS	1	10	60m	ACN	Y	Y	Y	MeOH 0.2 mol/L HCl
NIMT	2	13	2h	1) 0.5mL EDTA, 5mL ACN, 2) ACN	Y	N	Y	90% ACN/H2O 0.1%FA
UME	1	30	4m +15m centrifugation	ACN 1% FA	N	Y	N	H2O/MeOH 80/20
BVL	3	20	?	Aq. Buffer pH4, citric acid/NaH_2PO_4/EDTA	Y	N	Y	ACN/H2O 10/90 0.1% FA
NRC Ottawa	2	9	80 min, Rt 2x30m + 10m Centrifugation	ACN/IPA/H2O 80/10/10 v/v/v	N	Y	N	MeOH/H2O 50/50
NIM	2	20	62 m	5% Trichloroacetic acid	Y	N	N	0.1% FA H2O/MeOH 90:10 v/v
INTI (enro) (P178)	2	30	20m Rt	EtOH 1% AA	Y	N	Y	ACN 0.1% FA
NRC Halifax (P178)	3	12	48m Rt 3 x 1m +15m centrifugation	ACN	N	N	N	ACN

6. Participant Results for Enrofloxacin, Sulfadiazine and Moisture

The results submitted by the participating laboratories for enrofloxacin, sulfadiazine and moisture are provided in Tables 12 and 13 and 14 respectively and corresponding plots in Figures 7 and 8.

Table 12. Summary of all participants' results for enrofloxacin

NMI/DI	Box-Bottle Number	Mass Fraction (µg/kg)	Combined Standard uncertainty u (µg/kg)	Coverage factor (k)	Expanded uncertainty U (µg/kg)	No. of ind. replicates (n)
NRC-OTT	1-10-136	52	1.2	2	2.5	20
NMIA	1-121002	53.3	0.8	3.2	2.4	15
LGC	1-121004	53.66	1.66	2	3.32	4
KRISS	1-007006	53.9	1.8	2.78	5	4
VNIIM	1-007024	54.98	1.54	2	3.08	5
GLHK	1-071009	59.1	2.4	2	4.8	4
UME	1-007004	59.3	3.3	2	6.6	4
INMETRO	1-121013	59.3	2.7	2	5.4	3
NIMT	1-071023	62	1.89	2.04	3.9	20
EXHM	1-071014	62.56	2.17	2.31	6.35	6
NIM	1-007021	65.1	2.7	2	5.4	6
HSA	1-071019	65.8	3.8	2	7.6	8
BVL	1-121022	96.6	6.95	2	13.9	3
INTI (P178)	1-121011	58	3	2	7	4
NRC-HFX (P178)	1-007002	52.1	3.3	2	6.6	3

Table 13. Summary of all participants' results for sulfadiazine

NMI/DI	Box-Bottle Number	Mass Fraction (µg/kg)	Combined Standard uncertainty u (µg/kg)	Coverage factor (k)	Expanded uncertainty U (µg/kg)	No. of independent replicates (n)
NRC-OTT	1-10-136	2085	46	2	92	20
NIMT	1-071023	2138	69.51	2.06	144	20
NMIA	1-121002	2218	24	2.6	63	15
LGC	1-121004	2246	69	2	138	4
UME	1-007004	2246.5	128	2	255.9	4
INMETRO	1-121013	2280	100	2	200	3
BVL	1-121022	2304	200	2	400	3
EXHM	1-071014	2324.6	57.8	2.2	127.2	6
NIM	1-007021	2349	78.7	2	157.4	6
VNIIM	1-007024	2373	75.9	2	152	5
KRISS	1-007006	2376	36	2.45	88	4
GLHK	1-071009	2410	96	2	192	4
HSA	1-071019	2534	119	2	239	8
NRC-HFX (P178)	1-007002	2254	128.5	2	257	3

Table 14. Summary of all participants' results for moisture

NMI/DI	Box-Bottle Number	Moisture Content (g/g)	Standard deviation (g/g)
INMETRO	1-121013	N/D	N/D
BVL	1-121022	0.00129	0.0009
VNIIM	1-007024	0.002	0.00005
LGC	1-121004	0.00204	0.00097
NIM	1-007021	0.0027	0.0003
NIMT	1-071023	0.00291	0.00024
EXHM	1-071014	0.0031	0.000318
GLHK	1-071009	0.0043	0.000054
NMIA	1-121002	0.00436	0.00027
NRC-OTT	1-10-136	0.0049	0.0008
KRISS	1-007006	0.00502	0.000142
HSA	1-071019	0.01055	0.000076
UME	1-007004	0.013	0.001
INTI (P178)	1-121011	ND	ND
NRC-HFX (P178)	1-007002	0.00955	0.00058

The median result for all participants (K141 and P178) for enrofloxacin was 59 µg/kg with a range of 45 µg/kg from 11% below to 64% above the median with a RSD of 18%. Without the one high value reported by BVL, the median result for all participants (K141 and P178) was 59 µg/kg with a range of 14 µg/kg from 11% below to 12% above the median with a RSD of 8%. The median result for K141 participants only, with the BVL result withdrawn, was 59 µg/kg with a range of 14 µg/kg from 12% below to 11% above the median with a RSD of 8%.

For sulfadiazine, the median result for all participants (K141 and P178) was 2292 µg/kg with a range of 449 µg/kg from 9% below to 11% above the median with a RSD of 5%. The median result for K141 participants only was 2304 µg/kg with a range of 449 µg/kg from 10% below to 10% above the median with a RSD of 5%.

These RSD values (sulfadizine 5%, enrofloxacin 8%) are not unexpected given their respective concentrations. Even so, it is useful to look at the spread of results for both analytes to determine if any methodologies are linked to the observed distribution of the results.

As noted above (Section 4) there is no indication the source of standards has had any influence on the reported results – noting the distribution of results with those using NMIA standards (Figures

7 and 8). The median moisture value reported was 0.003 g/g and although the overall distribution of values was relatively large (RSD = 23%) the actual amounts were very low and thus also the corresponding corrections for the measurements on a dry weight basis. It is difficult to draw any correlations with high or low results with the preconditioning and spiking procedures or the use of SPE clean-up or hexane de-fatting steps. However, it is noted (Figure 7) that highest reported values for sulfadiazine (HSA) were those extracted under relatively strongly acidic conditions (0.1 M HCl/ACN).

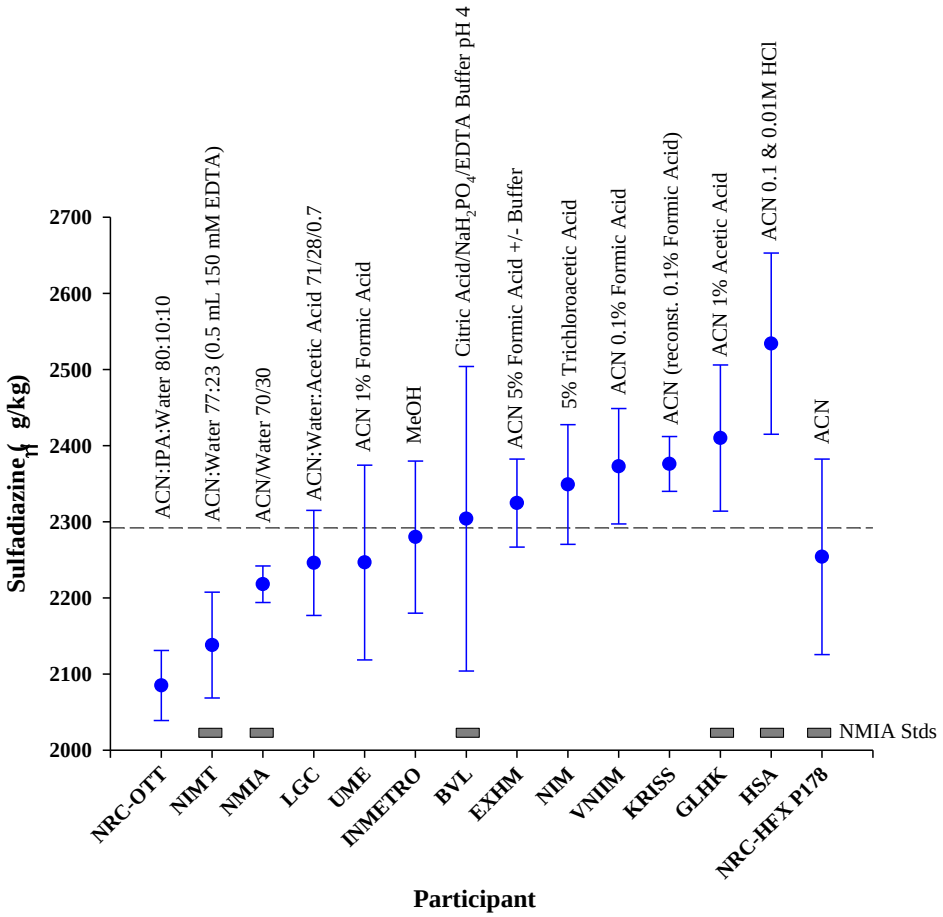


Figure 7. Sulfadiazine, extraction solvents – dotted line is the median: including pilot study participants (labelled P178)

Further, of the 7 values above the median for sulfadiazine all used acidic extraction solvents – or in the case of KRISS preconditioned with 0.1% formic acid. Similarly, of the seven values falling below the median, only two methods used acidic extraction and the three lowest values were from neutral or basic extraction solvent systems. The potential influence of acid or pH on extraction is worth investigating further, and it is noted that VNIIM chose to develop their method for sulfadiazine using ACN with 0.1% formic acid but used only ACN for enrofloxacin. Even so, a similar pattern is also seen with enrofloxacin (Figure 8) where the values falling below the median, with the exception of LGC and KRISS, did not employ acidic solvents or buffers and those at or above the median (with the exception of INMETRO) did.

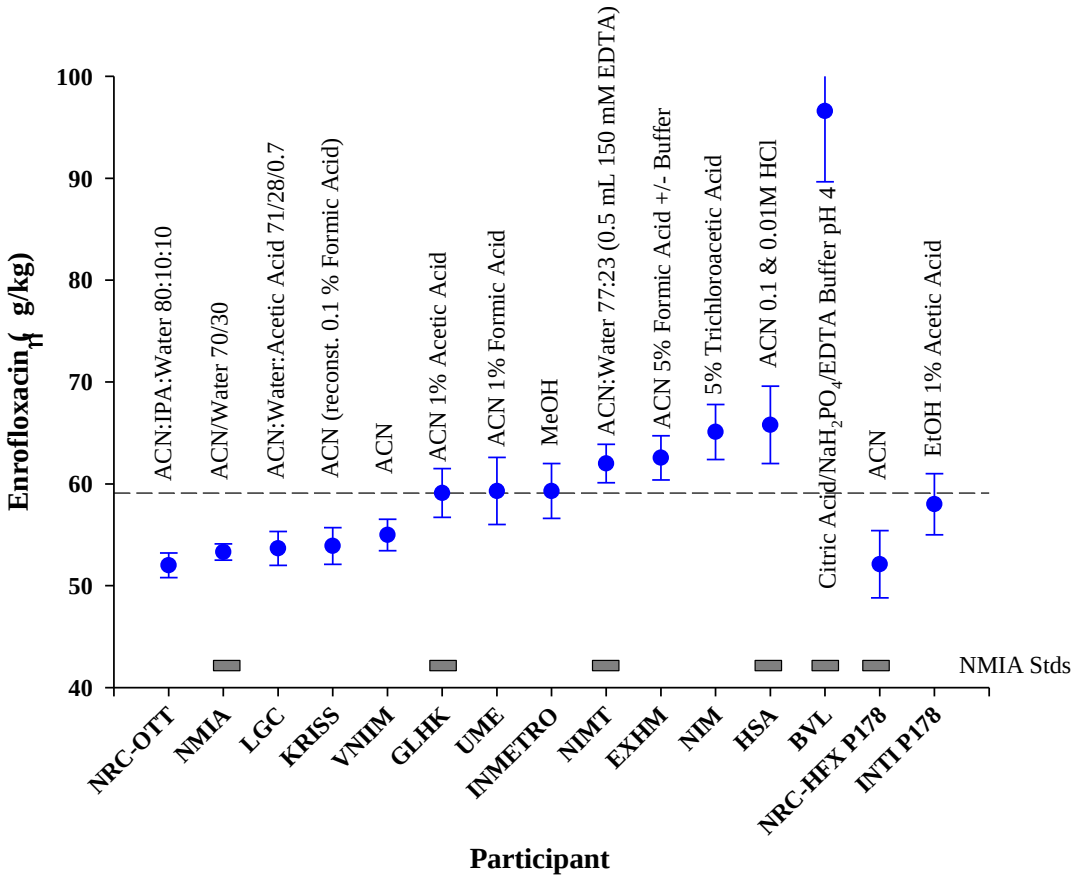


Figure 8. Enrofloxacin extraction solvents and standards – dotted line is the median: including pilot study participants (labelled P178)

7. Preliminary Assessment of Results

At the April 2017 meeting in Paris, presentations were made by BVL, NMIA, NIM, HSA, GLHK and UME which provided some further information and insight. BVL determined, post study, that the high value obtained for enrofloxacin was not a result of the extraction procedure, but due to an error in their preparation of the standard solution. NMIA noted some correlations in the results with IS equilibration time, potentially due to IS stability. HSA and GLHK noted the ampholytic nature of both enrofloxacin and sulfadiazine which reduces their solubility in neutral aqueous and some organic solvents. This influence of pH on the solubilities of the analytes provides a rational explanation for their choice of acidic extraction conditions and, in part, explains some of the variation in the study results with the pH of the extraction solvents. NIM presented extensive experimental data investigating different extraction solvent systems and pH which supported this hypothesis.

8. Follow-up Work Conducted by NRC

Prior to the OAWG meeting September 2017 in Ottawa, additional investigation into the solvents used for the preparation of primary standards and intermediate solutions as well as the extraction of enrofloxacin and sulfadiazine from matrix was performed by NRC and are described below.

The preparation of enrofloxacin solutions and extraction of enrofloxacin from bovine muscle tissue using acidified and neutral solvents was evaluated. An evaluation of the effect of solvents used in the preparation of primary standard solutions, spiking solutions and calibration solutions for the analysis of enrofloxacin by LC-MS/MS was performed. The solvents used were suspected to have an effect on the peak area ratio results for calibrations solutions, which in turn would affect the final mass fraction result in matrix.

Primary standards for enrofloxacin were prepared in methanol and 0.01N HCl:acetonitrile;85:15. Spiking solutions for enrofloxacin were prepared from the primary standards in 0.01N HCl:acetonitrile;85:15 and MeOH:water;50:50. The spiking solution for enrofloxacin-d5 was prepared in 0.01N HCl:acetonitrile;85:15 and used for all evaluations. Calibration solutions were prepared at concentrations matching post-extraction from BOTS-1 in 0.01N HCl:acetonitrile;95: 5 and water:MeOH:formic acid;90:10:0.1.

In total, eight calibration solutions were prepared to evaluate the different combinations of solvents used to prepare primary standards, spiking solutions and calibration solutions. The internal standard was prepared in a single solvent, allowing it to be used as a control. The solvents above were based on solvents used at NRC and other NMI's participating in the CCQM study. The calibration solutions were injected on the LC-MS/MS method for enrofloxacin to determine peak area results. The results indicated that: 1) the primary standard solvent did not have an effect on the results, 2) the spiking solution solvent had a significant effect on the results and 3) the calibration solution solvent did not have an effect on the results. Further analysis of the data indicated that enrofloxacin peak areas were lower by 4-11% when spiking solutions were prepared in MeOH:water;50:50 compared to preparation in 0.01N HCl:acetonitrile;85:15. This effect resulted in lower peak area ratios for the calibration solutions prepared from spiking solutions in MeOH:water;50:50. The fact that the calibration solution solvents, both of which contained acids (0.01N HCl or 0.1% formic acid), appeared to have no impact on peak areas indicates that presence of acid is more critical than solvent type and composition. Presumably an acid must be present to ensure no effects due to solubility and/or non-specific binding. It was also noted that the solubilities of enrofloxacin were dramatically different for different forms, i.e. enrofloxacin was a free base and was readily soluble in MeOH, while enrofloxacin-d5 was an HI salt and required either acidic or basic conditions for solubility to be achieved.

A stability evaluation was performed for enrofloxacin and enrofloxacin-d5 spiking solutions prepared in: 1) MeOH:water;50:50 and 2) 0.01N HCl:acetonitrile;85:15 and also for calibration solutions containing enrofloxacin and enrofloxacin-d5 in: 1) water:MeOH:formic acid;90:10:0.1 and 0.01N HCl:acetonitrile;85:15. The results indicated that no degradation of either enrofloxacin or enrofloxacin-d5 was observed for solutions stored at +37ºC (compared to -20ºC) for 24 hours.

An exhaustive extraction (4 x 10 mL) was performed with 0.1N HCl in acetonitrile (once) and 0.01N HCl in acetonitrile (3 times) with all supernatants combined. The result of 52.1 ng/g (NRC-OTT*) indicated that using the new acidic spiking solutions and extraction solvent did not

produce significantly higher results than the original NRC-OTT result of 52 ng/g.

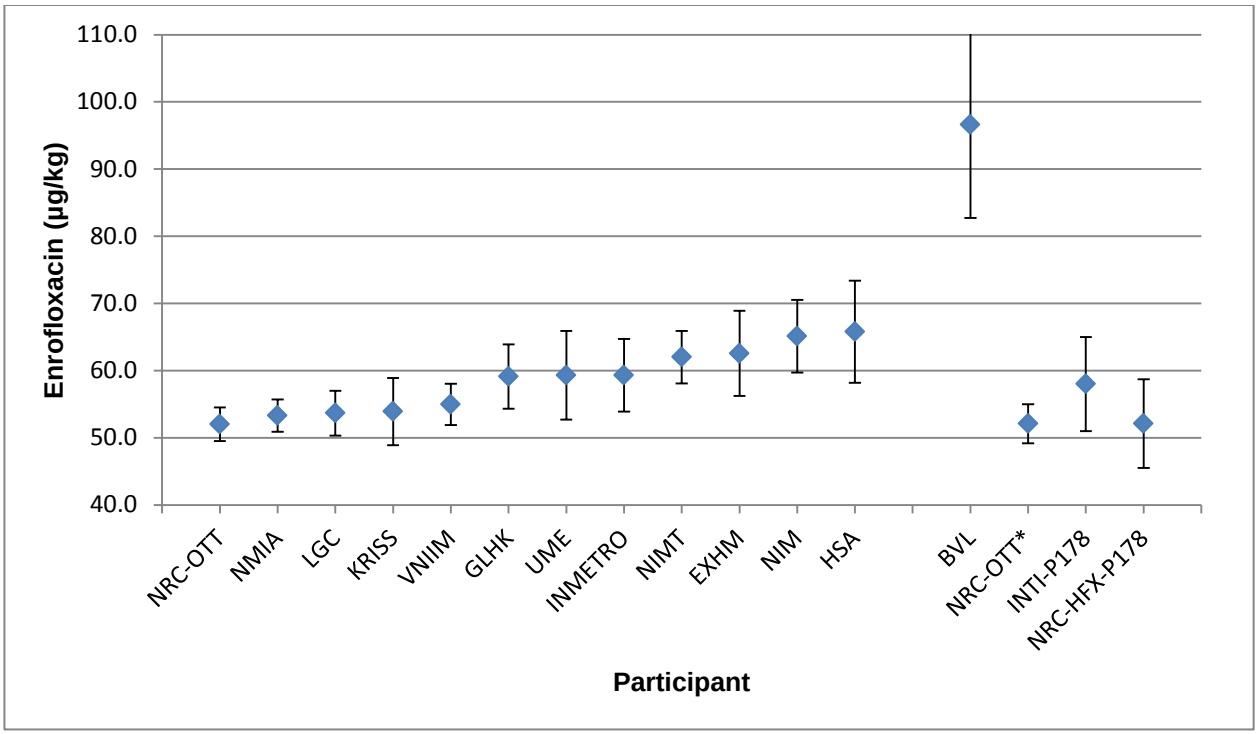


Figure 9. Reported results from all participants for enrofloxacin mass fraction in bovine tissue including pilot study participants (labelled –P178) and follow-up work result for NRC-Ottawa (NRC-OTT*). Error bars represent expanded uncertainties.

Overall, the results indicate that great care must be taken with amphoteric analytes such as enrofloxacin. The form (free base vs salt form) must be noted and appropriate solvents used in the preparation of all solutions.

For sulfadiazine, an evaluation of the combined effect of solvents used in the preparation of spiking solutions and extraction solvent was performed. The solvents used were suspected to have an effect on the peak area ratio results for calibrations solutions and the peak area ratio results for extracted samples. The first method below is the original NRC method, while the second method is an adaptation of the HSA method.

1) Spiking solutions for sulfadiazine and sulfadiazine-$^{13}C_6$ were prepared in MeOH:water;50:50. Bovine muscle tissue samples spiked with this solution were extracted twice with 4 mL of acetonitrile:isopropanol:water;80:10:10, with all supernatants combined (8 mL). The supernatants were diluted 10-fold in MeOH:water;50:50 prior to injection onto the LC-MS system.

2) Spiking solutions for sulfadiazine and sulfadiazine-$^{13}C_6$ were prepared in 0.01N HCl in water:acetonitrile;85:15. BOTS samples spiked with this solution were extracted once with 10 mL of 0.1 N HCl in acetonitrile and 3 times with 10 mL of 0.01N HCl in acetonitrile, with all supernatants combined (40 mL). The supernatants were diluted 2-fold in 0.01N HCl in water prior to injection onto the LC-MS system.

The results indicate that method 1 provided mass fraction results of 2194 ng/g while method 2 provided mass fraction results of 2376 ng/g (NRC-OTT*). Method 1 result is below the average CCQM result and in a similar range as the original NRC-OTT result of 2085 ng/g while method 2 result is slightly higher than the average CCQM result but still below the result obtained by HSA using a similar method.

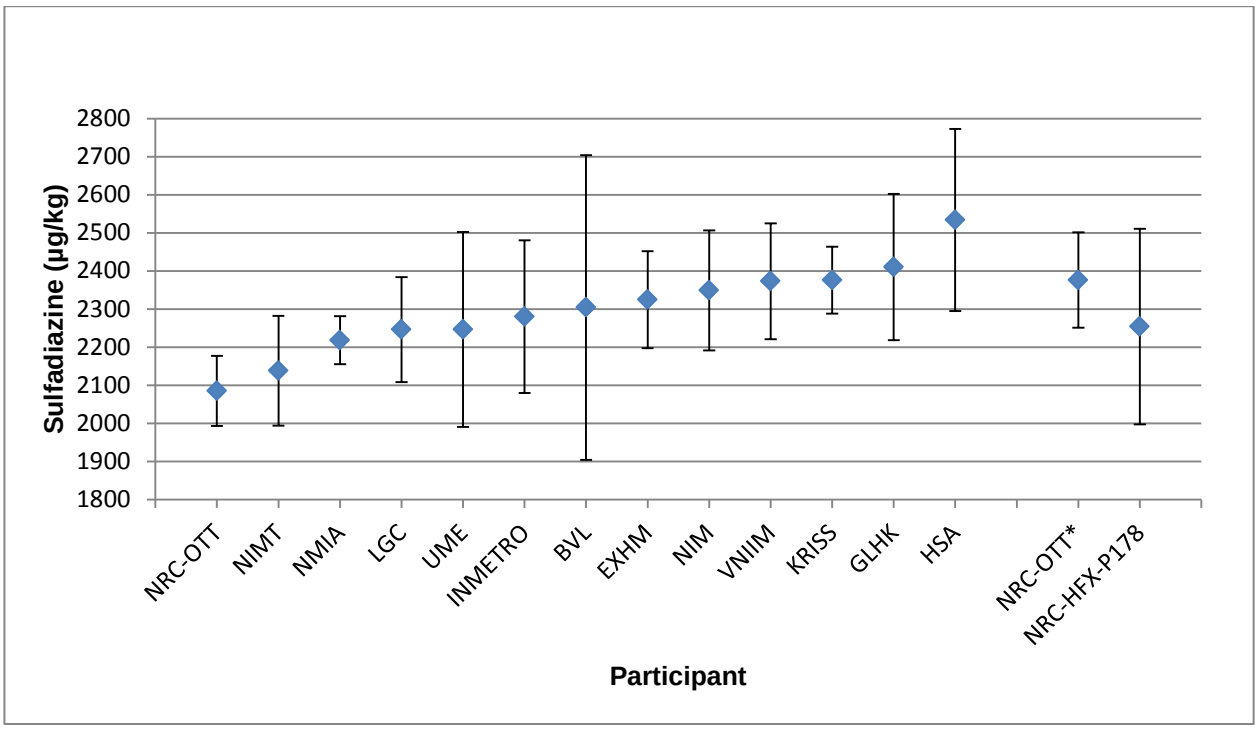


Figure 10. Reported results from all participants for sulfadiazine mass fraction in bovine tissue including pilot study participants (labelled –P178) and original and follow-up work result for NRC-Ottawa (NRC-OTT*). Error bars represent expanded uncertainties.

Further analysis of the data indicated that a combination of lower Cal peak area ratio and slightly higher peak area ratio for extracted samples using method 2 contributed to the higher result. Method 2 Cal solution showed that using 0.01N HCl in water:acetonitrile;85:15 as the solvent for the spiking solutions resulted in a 12% increase in peak area for sulfadiazine and a 20% increase in peak area for sulfadiazine-$^{13}C_6$, resulting in a lower Cal peak area ratio. Method 2 extracted bovine muscle tissue samples showed a 5% increase in peak area for sulfadiazine and a 3% increase in peak area for sulfadiazine-$^{13}C_6$ indicating that the acidified extraction solvent had a very small effect on the final result.

A stability evaluation was performed for sulfadiazine and sulfadiazine-$^{13}C_6$ spiking solutions prepared in: 1) MeOH:water;50:50 and 2) 0.01N HCl in water:acetonitrile;85:15 and for calibration solutions containing sulfadiazine and sulfadiazine-$^{13}C_6$ in: 1) water:MeOH:50:50 and 2) 0.01N HCl in water:acetonitrile;85:15. The results indicated that no degradation of either sulfadiazine or sulfadiazine-$^{13}C_6$ was observed for solutions stored at +37ºC (compared to -20ºC) for 24 hours.

Overall, the results indicated that the spiking solution and Cal solution preparation solvents have a greater effect on the final result than the extraction.

Following the September 2017 CCQM OAWG meeting in Ottawa, a request for additional information on the techniques and solvents used to prepare and handle primary standards, intermediate standards, spiking solutions and calibration solutions, was sent to study participants. Analysis of the information for trends may provide insight into the spread of the data. The Information Template for Analytes in Matrix Forms are contained in Appendix VI.

Several parameters were scrutinized for trends;
-Reference standard: form (salt/free base etc), preparation solvent, concentration, storage temperature, time before use, treatment before use
-Intermediate solutions: preparation solvent, concentrations
-Working solutions: preparation solvent, concentrations
-Internal standards: compound used, form, preparation solvent, concentrations
-Calibration solutions: preparation solvent, native concentration, IS concentration
-Final tissue extract solvent

Enrofloxacin: The reference standard concentration showed a weak correlation to mass fraction and there was a slight correlation for basic intermediate and working solution solvents with lower mass fraction and acidic solutions with higher mass fractions. There was no correlation however for neutral solutions to mass fraction. The solvent used to dissolve the final extract prior to injection also showed a weak correlation with neutral or basic solvents showing slightly lower mass fractions compared to acidic solvents.

Sulfadiazine: The solvent used to prepare the reference standard appeared to have a slight effect on mass fraction result as the two highest mass fraction results were determined with methods using 2% NH_3 or 0.01M HCl in solvent for reference standard preparation while all other methods used solvents (methanol or in one case, acetone) with no additives. The preparation solvent for internal standards (IS) and IS spiking solutions showed a general trend to higher mass fractions when acidic solvents were used. The solvents used to prepare calibration samples and to dissolve the final extract prior to injection also showed a trend to lower mass fractions with neutral or basic solvents and higher mass fractions with acidic solvents.

In summary, there is a general trend for both enrofloxacin and sulfadiazine to yield higher mass fraction results when acidic solvents are used to prepare reference standard solutions and calibration solutions and to extract and dissolve or dilute the final tissue extracts prior to injection. There are several dynamics involved in these processes and higher or lower final mass fraction results may be a result of effects on the measurand and/or the internal standard in the extraction process and/or the calibration solution preparation process. Given these dynamics, there is insufficient evidence to make a conclusion on whether the true mass fraction values are at the lower or higher ends of the reported results.

9. Measurement Equations and Uncertainty Estimation

Full reports by all the participants on their measurement equations and uncertainty estimates are provided in Appendix III, and any additional information is provided in Appendix IV.

10. Determination of the Key Comparison Reference Values (KCRV) and Degrees of Equivalence (DoEs)

All pilot study participants were excluded from the KCRV calculations and BVL voluntarily withdrew their value for enrofloxacin, citing that an error was made due to improper sample preparation or handling of their reference standard. Therefore, twelve results were used for the KCRV calculations for enrofloxacin, while thirteen were used for that of sulfadiazine. Listed in Table 15 are consensus estimators based on arithmetic mean, uncertainty-weighted mean, uncertainty-weighted mean corrected for over-dispersion, median, and DerSimonian-Laird mean. These values are proposed in accordance with CCQM/13-22 *Guidance note: Estimation of a consensus KCRV and associated Degrees of Equivalence*[2]. As agreed upon by participants, the DerSimonian-Laird (DSL) mean was chosen as the KCRV value in both cases as it takes into account the uncertainties from participants' results and it handles excess variance given the suspected influence of random effects. The DSL means were calculated in-house according to CCQM/13-22[2] and confirmed with the NIST Consensus Builder[3]. Participant results are shown relative to the KCRVs in Figure 11.

Table 15. Consensus estimators for enrofloxacin and sulfadiazine

	Enrofloxacin, µg/kg			**Sulfadiazine, µg/kg**		
Consensus estimator	X	$u(X)$	$U_{95}(X)$	X	$u(X)$	$U_{95}(X)$
Arithmetic mean	58.42	1.39	2.77	2299	33	65
Uncertainty-weighted mean	55.41	0.47	0.95	2259	15	30
Uncertainty-weighted mean (corrected for over-dispersion)	55.41	1.16	2.32	2259	29	58
Median	59.20	2.55	5.10	2304	36	71
DerSimonian-Laird mean	**57.81**	**1.28**	**2.57**	**2285**	**34**	**68**

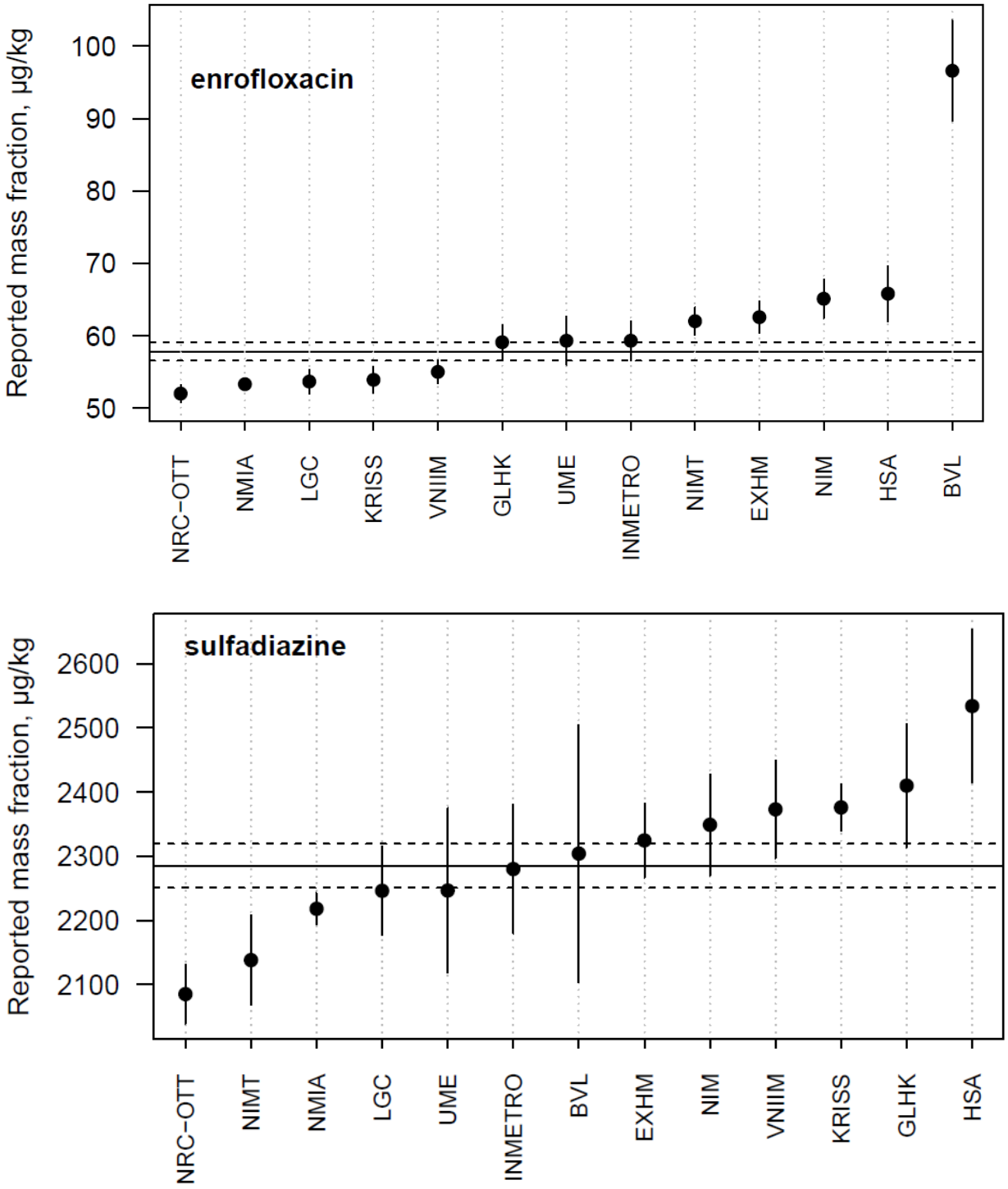


Figure 11. Plots of paricipants' results relative to the DSL-mean KCRV values for enrofloxacin (top) and sulfadiazine (bottom), uncertainties are standard uncertainties.

Degrees of equivalence for CCQM-K141 were calculated as $d_i = x_i$ – KCRV and their expanded uncertainties are expressed using the following equation, solved according to CCQM/13-22[2]:

$$U_{k=2}(d_i) = 2\sqrt{u^2(x_i) + u^2(\text{KCRV}) - 2\text{cov}(x_i, \text{KCRV})}$$

Relative degrees of equivalence were then calculated as $\%d_i = 100 \cdot d_i/\text{KCRV}$ with expanded uncertainties as $U_{k=2}(\%d_i) = 100 \cdot U_{k=2}(d_i)/\text{KCRV}$. These values are plotted in Figures 12 and 13, and listed in Tables 16 and 17 for enrofloxacin and sulfadiazine, respectively.

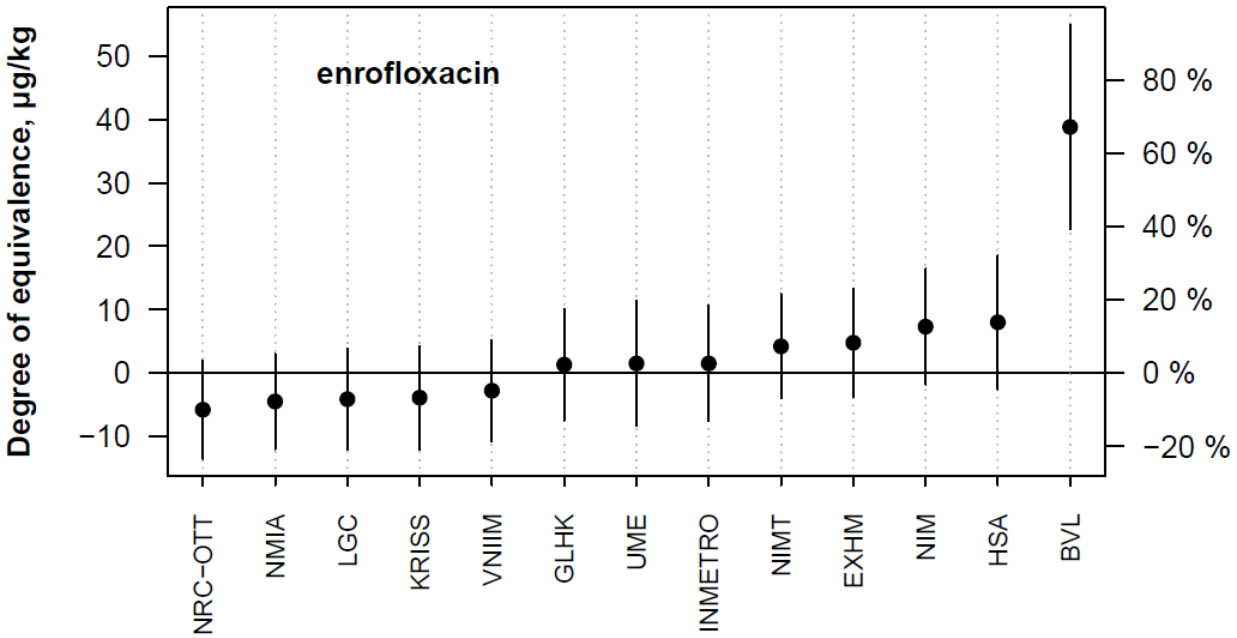


Figure 12. Degrees of equivalence estimates and 95% coverage intervals for enrofloxacin.

Table 16. Degrees of equivalence and their uncertainties (95% CI) for enrofloxacin.

Participant	d_E	$U(d_E)$	$\%d_E$	$U(\%d_E)$
NRC-OTT	-5.81	7.71	-10.05	13.34
NMIA	-4.51	7.50	-7.80	12.97
LGC	-4.15	8.04	-7.18	13.91
KRISS	-3.91	8.16	-6.77	14.12
VNIIM	-2.81	7.95	-4.86	13.75
GLHK	1.29	8.76	2.23	15.15
UME	1.49	9.86	2.57	17.06
INMETRO	1.49	9.10	2.57	15.74
NIMT	4.19	8.24	7.24	14.26
EXHM	4.75	8.52	8.21	14.73
NIM	7.29	9.10	12.61	15.74
HSA	7.99	10.56	13.82	18.26
BVL	38.8	16.13	67.09	27.90

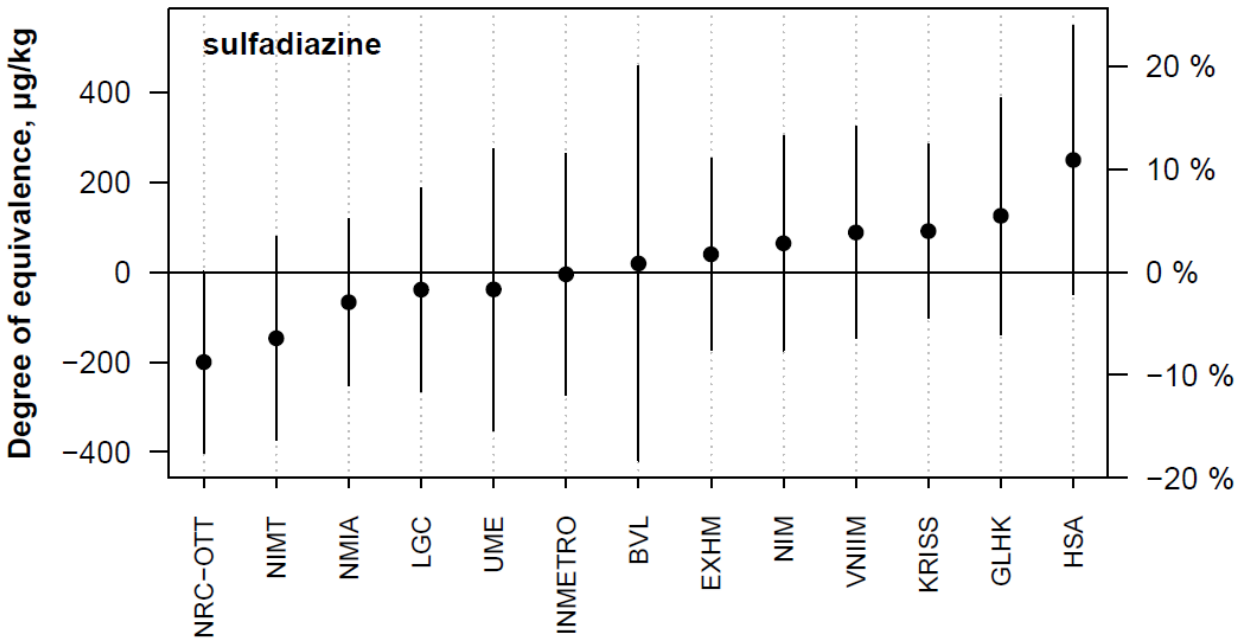


Figure 13. Degrees of equivalence estimates and 95% coverage intervals for sulfadiazine.

Table 17. Degrees of equivalence and their uncertainties (95% CI) for sulfadiazine.

Participant	d_E	$U(d_E)$	$\%d_E$	$U(\%d_E)$
NRC-OTT	-200.1	200.7	-8.76	8.78
NIMT	-147.1	226.2	-6.44	9.90
NMIA	-67.10	184.7	-2.94	8.08
LGC	-39.10	225.5	-1.71	9.87
UME	-38.60	312.0	-1.69	13.66
INMETRO	-5.10	268.0	-0.22	11.73
BVL	18.90	438.0	0.83	19.17
EXHM	39.50	212.6	1.73	9.30
NIM	63.90	237.9	2.80	10.41
VNIIM	87.90	234.2	3.85	10.25
KRISS	90.90	192.4	3.98	8.42
GLHK	124.9	262.1	5.47	11.47
HSA	248.9	297.4	10.89	13.02

11. How Far Does the Light Shine?

The study has tested the capabilities of participants for assigning mass fractions of high-polarity analytes (pK_{ow} > -2) with the molecular mass range from 200 to 500 g/mol at 20-5000 µg/kg levels in a high fat, high protein food matrix. Core competency tables for each participant can be found in Appendix 5.

12. Conclusions

This study demonstrated capabilities for measuring high-polarity analytes in a high fat and high protein matrix, namely enrofloxacin and sulfadiazine in bovine tissue. The level of agreement was reasonable given the measurands and matrix were new for most laboratories. The KCRV values and their uncertainties at the 95% confidence level of 57.81 ± 2.57 µg/kg for enrofloxacin and 2285 ± 68 µg/kg for sulfadiazine were calculated using the DSL means. While one participant's value was voluntarily excluded from the KCRV calculations for enrofloxacin, all other participants demonstrated equivalence for both measurands.

Significant effort was undertaken post-study to identify the major sources of variability between results. In particular, the various extraction conditions used by participants were investigated thoroughly. While there appeared to be a correlation between highly acidic conditions and higher

recovery, this was not definitive and could not be confirmed. The form of standards employed (ie. free base vs salts) and potential differential solubility between forms was also a suspected source of variability. Biases could also have been introduced with the choice of solvents used for standard preparation, with some solvents better able to minimize adsorption of the analytes to glass surfaces. Ultimately, it was difficult to identify one main parameter that caused the majority of the variability, and the effects of multiple parameters in some cases were off-setting.

Finally, it should be noted that shipping bovine tissue internationally, with the added complication of dry ice shipments, proved to be a significant challenge and a strain on resources. Therefore, careful consideration should be undertaken prior to planning similar future studies.

13. Acknowledgements

The study coordinators wish to thank the participating laboratories for providing results and additional information used in this study, including pilot study participants Pearse McCarron and Krista Thomas of NRC Halifax and Virginia Uchitel of the National Institute of Industrial Technology, Argentina (INTI). The contributions of NRC staff members Jennifer Bates and Patricia Grinberg are also acknowledged. Financial support from the NRC Scientific Support for the National Measurement System program is also acknowledged.

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2. https://www.bipm.org/cc/CCQM/Restricted/19/CCQM13-22_Consensus_KCRV_v10.pdf
3. Calculated using the NIST Consensus Builder online application available at https://consensus.nist.gov

Appendix I. Sample amounts, pre-treatments, extraction and clean up methods, all participants

NMI/DI	Sample Amt. (g)	Pre-treatment	Extraction	Cleanup
EXHM	0.7	The bottle was thoroughly shaken and six different (0.7g) samples were taken. Each sample was reconstituted with ultrapure water (1.3 g) in centrifuge tubes and was left to equilibrate in the dark at room temperature for 30 min.	The samples were spiked with internal standard solutions and were left to equilibrate for 30 min. (a) 5 mL of Tris buffer (8.0 pH) and 5 mg of Pronase were added and the mixtures were incubated for 8 h in a shaking water bath maintained at 55 °C. Then, 15 mL of acetonitrile containing 5% formic acid were added to the tubes that were subjected to intense blending for 3 min using and UltraTurrax T25. (b) 5 mL of Tris buffer (8.0 pH) were added and the mixtures were vortexed for 30 s at room temperature. Then, 15 mL of acetonitrile containing 5% formic acid were added to the tubes that were subjected to intense blending for 3 min using and UltraTurrax T25. In either case, the tubes were then sonicated for a further 15 min. (c) PLE was performed using (acetonitrile+5%formic acid):water 80:20 as an extraction solven. The procedure was carried out in a ASE 350 Dionex accelerated solvent extraction system using two 10 min static cycles at 1500 psi and 70 °C. Procedures (a), (b) and (c) were found to be equivalent	The dispersions were centrifuged at x 5000 rpm for 10 min at 4 °C and then transferred to a freezer at -20 °C for 1 h. The samples were then cleaned by dSPE using zirconium-based adsorbents, filtered through 0,22 µm PVDF filters and then injected in the LC-MS/MS system
HSA	0.5	Sample bottle was equilibrated to room temperature, and mixed by rolling and inversing before opening and sampling. About 0.5 g of the sample was weighed into a 50-mL centrifuge tube, and 1 mL of water was added. The mixture (sample blend) was vortexed after gravimetrically spiking with appropriate amounts of isotope labelled	The sample blend was first cooled in an ice bath, and 10 mL of 0.1 mol/L HCl in acetonitrile was added. After removing from the ice bath, the mixture was vortexed for 1 min, sonicated for 5 min, and shakened vigorously for 10 min using an orbital shaker. The mixture was then centrifuged at 4,000 rpm for 5 min. The supernatant was transferred to a 50 mL centrifuge tube. The extraction was repeated for three more times using 0.01 mol/L HCl in acetonitrile instead of 0.1 mol/L HCl in acetonitrile without applying ice bath. The supernatants were combined.	The combined supernatant from each sample blend was evaporated to dryness under nitrogen flow at 35 ^{o}C. The residue was reconstituted with 1 mL of 0.01 mol/L HCl in water:acetonitrile (85:15, *v/v*). The reconstituted solution was transferred into two Amicon Ultra-0.5 centrifugal filter units with Ultracel-3 membrance (0.5 mL each filter), and was centrifuged at 13,000 rpm for 10 min. The clear solution was combined and analysed using LC-MS/MS

		internal standard solutions.	Calibration blends prepared from weighing of native and isotope-labelled analytes into 50-mL centrifuge tubes were extracted once with 10 mL of 0.1 mol/L HCl.	for enrofloxacin. For sulfadiazine, the combined solution was diluted to about 50 ng/g before analysis. The extract from each calibration blend was subjected to the same post extraction procedure as that of each sample blend, except that filtration was not required.
NMIA	0.5	Reagent-grade water (1 mL) added and samples gently vortexed at room temperature 1 h. Added internal standard solutions in acetonitrile/water (10:90) containing 1 mM sodium hydroxide (~0.5 mL), samples gently vortexed for a few minutes and stored overnight at 4 °C.	Liquid/solid extraction using 4 x 5 mL acetonitrile /water (70:30) with end-over-end rotation, combined extracts evaporated to approximately 3 mL.	Liquid/liquid extraction with 2 x 3 mL hexane to remove fats. Solid-phase extraction of aqueous phase using Oasis HLB (3 mL, 60 mg, Waters), washing with methanol/water (20:80, 2 x 3 mL) and eluting with methanol/water (70:30, 2 x 3 mL), evaporate to dryness. Reconstitution solvent was acetonitrile /water (10:90) containing 1 mM sodium hydroxide. Extracts were reconstituted to 1mL. Reconstituted extracts were injected undiluted for negative-ion LCMS analysis. Portions of the extracts were diluted one-in-five with reconstitution solvent for positive-ion LCMS analysis.
LGC	1	Sample dispersed with 2 mL water and left to equilibrate for 2 h.	Sample and extracting solvent were placed in 50-mL polypropylene tubes with two ceramic homogenisers and kept rotating in a head-over-heels mixer for 48 h at room temperature. Solvent: 8 mL water + 20 mL acetonitrile + 200 µL acetic acid.	Centrifugation at 4000 rpm for 5 min. Temperature-induced phase separation (supernatants frozen for >2 h until two phases appear. Evaporation of supernatant and reconstitution. Filtration.
VNIIM	0.5	1,5 ml of water was added per sample before extraction	liquid/solid, sonication 3x15 min at room temperature solvent : AcN for Enrofloxacin extraction (3x3 ml); AcN + 0,1% HCOOH for Sulfadiazine extraction (3x3 ml)	Extract was defatted by 3 ml of hexane

GLHK	0.5	The sample (0.5g) is re-constituted with 3 mL of (purified) water for at least 12 hours at 4 ℃ before extraction.	Extracted with 2 × 15 mL 1% acetic acid in ACN. Each extraction was performed sequentially by the following methods: i) Ultrasonic agitation for 30 minutes, ii) Vertical shaking for 15 minutes, iii) Vortex mixing for 1 hour.	Defatting with n-hexane saturated with ACN and SPE Clean-up on Waters Oasis MCX SPE cartridge (150mg, 6mL).
INMETRO	0.75	Not Applicable.	Two steps of liquid/solid extraction with 5 mL methanol. The samples were shaken at room temperature for 20 min.	The extract was evaporated to dryness under N_2 steam and re-suspended in 500 µL of acetic acid 5 % : methanol (80:20 v/v).
KRISS	0.5	1. Liquid-liquid extraction using acetonitrile and n-hexane. 2. Clean-up of sample: solid phase extraction using an Oasis MAX SPE cartridge	Sample was weighed in about 0.5 g (x 6) unit with 50 mL tube and 10 mL of acetonitrile was added for a mechanical shaking for 1 hour. The acetonitrile layer was recovered after centrifugation at 1,520 x *g* for 5 minutes and followed by mixing with n-hexane. Another mechanical shaking was performed for 20 minutes for the mixture and then it was centrifuged for 5 minutes at 3,420 x *g* to recover acetonitrile layer which was followed by dryness with N_2.	Oasis MAX SPE cartrige (3 cc): 1. Reconstitution of LLE sample with 2 mL of 50 mmol/L NaH_2PO_4. 2. SPE conditioning with 1 mL methanol, 5 mol/L NaOH, and 1 mL ultra pure water. 3. Sample loading. 4. Washing of sample loaded cartridge with 5% ammonia in water. 5. Washing of cartridge with 1 mL of methanol. Analyte elution with 2 mL of 0.2 mol/L HCl in methanol.
NIMT	0.5	Sample blend was prepared by accurately weighing 0.5 g of test material. The amount of 2.5 mL of Milli-Q water was added. The isotopically labelled internal standard was then added to create the sample blends. Calibration blend was prepared by using 0.5 g of matrix-matched sample blank (freeze dried beef). The same amount of Milli-Q water as in the sample blend was added. Standards and internal standards	The amount of 0.5 mL of Na_2EDTA (150mM) was added, vortex mixed and stand for 10 min. This was followed by the addition of 5 mL of acetonitrile vortex mixed and shaken by a mechanical shaker for 40 min. The sample was then centrifuged at 4000 rpm for 10 min (4 °C). The supernatant (8 mL) was filtered through a 1 µm glass fiber filter and collected to a glass tube. The 5 mL amount of acetonitrile was added for a second extraction. The extract was collected, combined and evaporated to approx. 1.5 mL at 45 °C under N_2. The 1.5 mL residue was then carried on the SPE clean-up step.	Solid phase extraction (SPE) was performed by using Oasis HLB SPE cartridges (3 mL, 60 mg). The SPE cartridges were pre-conditioned with methanol (3 mL) and equilibrated with Milli-Q water (3 mL). The sample solutions obtained from the liquid-solid extraction after drying step (1.5 mL) were loaded onto the cartridges. The wash solvent of 5% methanol in Milli-Q water (2 mL) was applied, followed by 2 mL of a second wash solvent of hexane. The cartridges were dried by forcing air through each cartridge. Eluting solvent (methanol:

		were then added. Sample blend and calibration blend were let to soak and equilibrate for 1 hour prior to the extraction and clean-up steps.		acetonitrile, 50:50, 9 mL) was added to elute the analytes from the cartridges. The eluates were carefully evaporated to dryness under a stream of nitrogen at 45 °C and reconstituted in 0.8 mL of 0.1% (v/v) formic acid in Milli-Q water/0.1% formic acid in acetonitrile (9:1) by vigorous vortex-mixing. The reconstituted samples were filtered through 0.2 µm micro filter disk. The samples were transferred to sample vials for LC-MS/MS analysis.
UME	0.5	Freeze dried sample was reconstituted by adding water at 65 % w/w level.	0.5 g of sample was weighted into falcon tube, 0.92 g of water and then isotopically labelled standard solution was added gravimetrically. 30 mL of Acetonitrile:Formic Acid (99:1) % was added and vortex was applied for 4 minutes.	Centrifugation was applied at 14239*g* and 4 ˚C for 15 minutes and 15 mL of supernatant was transferred to another falcon tube and was evaporated under nitrogen stream until approximately 1 mL yellow part was remained. Then 2mL of *n*-hexane and 2 mL Water:Methanol (80:20)% mixture was added and mixed by vortex for one minute then centrifugation was applied at 4280*g* and 4 ˚C for 15 minutes. Lower phase was collected and filtered by 0.2 µm whatman filter and measured by LC/MS-MS
BVL	0.5	reconstitution of 0.5 g of freeze-dried sample with 0.93 g of water - treatment of samples in vortexer and with ultra-sonic equipment - fortification of the reconstituted samples by internal standards sulfadiazine $^{13}C_6$ and enrofloxacin D5 hydroiodide	addition of 10 ml of buffer solution to reconstituted samples - buffer: mix of McIlvaine buffer (citric acid/sodium dihydrogen phosphate) and ethylenediaminetetraacetic acid disodium salt (Na_2EDTA), pH=4.0 - centrifugation and filtration of supernatant - repetition of extraction of remaining particle phase with buffer, twice with 5 ml each - combination of collected supernatants	clean up by SPE cartridge (Oasis HLB, 6 ml, 200 mg) - conditioning of SPE cartridge, giving up of combined extract, washing of cartridge with water, drying of cartridge with air, elution of analytes with methanol - evaporation of eluate to dryness with nitrogen at 40 °C - reconstitution of residue with 1 ml of mix of components of mobile LC

				phase
NRC Ottawa	0.5	0.5g BOTS was reconstituted with 1.0 mL water and allowed to stand for a minimum of 10 minutes before further processing.	Liquid Solid Extraction: -Accurately weigh 0.5 g BOTS into a 15 mL tube -Add 1 mL water, vortex and allow to sit 10 min -Spike primary standard and/or internal standard -add 4 mL 80:10:10;ACN:IPA:water and shake 30 min -Centrifuge 10 min at 3000 RPM and remove supernatant -Repeat extraction one more time, combining supernatants (~ 8 mL)	Further cleanup/dilution-concentration: -Add 2 ml hexane to combined supernatant and shake for 5 min -Centrifuge 10 min at 3000 RPM and remove hexane layer -Sulfadiazine: -dilute 50 µL of supernatant with 450 µL 50:50;MeOH:water -filter through a 0.2 µm PTFE filter vial -Enrofloxacin: -Concentrate 4 mL supernatant to ~ 450 µL under vacuum (Sorvall centrifuge) -Add 50 µl MeOH and mix -filter through a 0.2 µm PTFE filter vial
NIM	0.5	Sample bottle was equilibrated to room temperature, mixed by rolling and inversion by hand. 1.5 g water was used for sample reconstitution	liquid/solid extraction 10.0 mL of 5% trichloroacetic acid solution was added in sample at room temperature. Then, homogenized for 60 s, shaked vigorously for 20 min and sonicated for 10 minutes. Repeated extraction once and combined the extraction solution	OASIS HLB cartridge (6 mL, 150 mg, Waters) was used for SPE clean-up step. For enrofloxacin, 10 mL of the extra999ct (without dilution) was transferred to the cartridge which was initially loaded with methanol and water. Then, sequentially washed with 6 mL of 5% methanol solution. Finally, the analyte was eluted with 8 mL methanol.For sulfadiazine, 400 µL of the extract was diluted with 6 mL of water. The dilution was transferred to the cartridge which was initially loaded with methanol and water. Then, sequentially washed with 6 mL of 5% methanol solution. Finally, the analyte was eluted with 8 mL methanol. The eluate was evaporated to dryness under nitrogen at 40 °C and reconstituted with 1 mL of 0.1% formic acid in water/methanol (90:10 v/v). The sample was centrifuged at 14,000 rpm for 10 min

				before analysis.
INTI P178	2 reconst.	Reconstitution of the sample with purified water, taking into account 65% of humidity	Extraction with 15 ml AcH 1% in EtOH. Shaker 5 min – Centrifugation 5 min 7500 rpm Re-extraction with 15 ml AcH 1% in EtOH Shaker 5 min– Centrifugation 5 min 7500 rpm	SPE: SCX – Elution with NH4OH in MeOH Evaporation Dilution to 2 ml with FM Filtration
NRC Halifax P178	0.7	BOTS-1 weighed into a falcon tube (minimum intake 0.7g). ISWS spike added using syringes with gravimetry. Sample allowed to sit ½ hour to absorb spike. Deionized water added to reconstitute moisture content to 65%. Sample allowed to sit ½ hour to absorb moisture.	Liquid/solid extraction using acetonitrile: 4mL acetonitrile added to wet sample, vortexed 1min. Centrifuged 15min, 7200 rpm. Solvent decanted into a volumetric flask (20mL). Extraction repeated twice more in the same manner and extracts combined. Final volume made to 20mL with deionized water.	Samples mixed well, 400 µL portion filtered through 0.45 µm PTFE for analyses

Appendix II. Participants methods: Calibration, instrumentation and MS/MS transitions

	Calibration	Instumentation/Chromatography	MS/MS transitions
EXHM	Matrix-matched calibrators were prepared using fresh blank bovine meat, by spiking the blank material with suitable amounts of the analytes and the internal standards, that were left to equilibrate for 1 h in the dark in a refrigerator	LC-MS/MS: Thermo Finnigan, TSQ Quantum Ultra AM Column: Zorbax Eclipse XDB-C8 (150 mm x 4.6 mm, 5 µm), flow 400 µL/min gradient (A: 0.1% formic acid, B: Acetonitrile + 0.1 % formic acid) 0 min: A 95%, 2 min A 95%, 15 min A 50%, 18 min A 0%, 21 min A 0%, 22 min A 95% 25 min: A 95%	SFZ: 251 (parent) to 156 (quantification), 108 and 92 (identification) SFZ-$^{13}C_6$ 257 (parent) to 162 (quantification) EFX: 360 (parent) to 316 (quantification), 245 and 204 (identification) EFX-d_5 365 (parent) to 321 (quantification)
HSA	IDMS with four-point calibration was used. The isotope mass ratio of the calibration blends were controlled to be within the range of 0.75 to 1.3. The isotope mass ratio of the sample blends were controlled to be close to 1.0 with an acceptable range of 0.85 to 1.15.	LC-MS/MS (Shimadzu 8040 mass spectrometer coupled with a Prominence UFLC LC20AD system) was used for the measurement. The LC method was as follows: Column: Phenomenex Luna PFP(2) column, 2.0 × 150mm, 5µm. Mobile phase A: 0.1% formic acid in water. Mobile phase B: 0.1% formic acid in acetonitrile. Gradient: 15% to 90% mobile phase B.	MRM transitions (positive mode electrospray ionisation) were used for quantitation. The ion pairs (m/z) monitored were as follows: Enrofloxacin: 360.1→342.3 (quantifying ion), and 360.1→316.4 (qualifying ion) Enrofloxacin-d_5: 365.2→347.4 (quantifying ion), and 365.2→321.4 (qualifying ion) Sulfadiazine: 251.1→156.2 (quantifying ion), and 251.1→108.2 (qualifying ion) Sulfadiazine-$^{13}C_6$:257.1→162.2 (quantifying ion), and 257.1→114.2 (qualifying ion) Only the results from the quantifying ions were reported. The results from the qualifying ions were solely used in the estimation of the measurement uncertainty.
NMIA	Exact-matched double IDMS analysis, replicate bracketed injections.	Three LC-MS/MS methods - Positive electrospray with single UPLC column (1D) or heart-cutting (2D) UPLC cleanups - 1D UPLC negative electrospray. 10 µL injections. 1D and 2D analyses on Thermo Fisher Scientific TSQ Vantage AM/Transcend TLX1 using Waters Acquity BEH C18 column (2.1 x 100 mm, 1.7 µm) and Restek Pinnacle DB Biphenyl (2.1 x 100 mm, 1.9 µm). 1. 1D on BEH C18 using a gradient of acetonitrile (10 to 20% over 5 min) in aqueous 0.2% formic acid,	1D and 2D analyses use positive-ion electrospray ionisation (HESI interface), and three MRM transitions for each analyte and internal standard. Average result from all relevant transitions were used for reference values. Parent ion > Product ion (collision energy eV) SDZ 250.8 > 65.11 (38) $^{13}C_6$-SDZ 257.0 > 70.14 (41) 250.8 > 92.09 (23) 257.0 > 98.15 (27) 250.8 > 108.09 (18) 257.0 > 114.14 (24) ENR 359.8 > 204.11 (30) D_5-ENR 365.0 > 204.12 (32) 359.8 > 245.15 (25)

		with a flow rate of 0.3 mL/min. Retention time (r.t.) for enrofloxacin (ENR) was 5.4 min, sulfadiazine (SDZ) r.t. 2.5 min. 2. 2D – first dimension as for 1D, ENR eluted to MS from first column with r.t. 6.0 min; SDZ peak transferred to Biphenyl column and eluted with a gradient of methanol (15 – 29% over 7 min) in aqueous 0.2% formic acid with a flow rate of 0.3 mL/min. SDZ r.t. 7.1 min. Negative-ion analysis (for ENR only) on Waters Acquity UPLC system and Waters Quattro Micro triple quadrupole MS using Waters Acquity BEH C18 column (1.0 x 100 mm, 1.7 µm). Gradient of acetonitrile/water (90:10) containing 25 mM triethylamine (10 – 20% over 6.7 min) in water with a flow rate of 0.1 mL/min. ENR r.t. 4.7 min.	365.0 > 245.17 (26) 359.8 > 316.24 (17) 365.0 > 321.3 (18) Negative-ion analysis uses negative-ion electrospray ionisation, and two MRM transitions for each of ENR and D_5-ENR. Cone 23.0 V for all. ENR 358.1 > 202.4 (16) D_5-ENR 363.1 > 202.4 (17) 358.1 > 245.15 (25) 363.1 > 245.17 (26)
LGC	Bracketed double exact matched IDMS	LC-MS/MS: An Agilent 1100 LC system (quaternary delivery pump, online degasser, refrigerated autosampler, and thermostatic column compartment) coupled to a Qtrap 4000 MS from Sciex (used as triple quadruple). Column: ACE Excel 2 C18-PFP, 150 mm × 3.0 mm, 2 µm, part no. EXL-1010-1503U Mobile phases: A) water 0.1% formic acid. B) acetonitrile 0.1 % formic acid. Gradient: 5% B for 1 min. Linear gradient until 55% B at 10 min. Flow rate: 0.4 mL/min Injection: 10 µL Temperature: 40 °C	• Sulfadiazine: 251/156 (qualifier MRM's: 251/92, 251/108, 251/65) • Sulfadiazine $^{13}C_6$: 257/162 (qualifier MRM's: 257/98, 257/166, 257/114, 251/60) • Enrofloxacin: 360/316 (qualifier MRM´s: 360/245) Enrofloxacin D_5: 365/321 (qualifier MRM´s: 365/326)
VNIIM	IDMS, single point	LC-MS/MS Column ZorbaxEclipcePlusC18 NarrowBoreRR 2.1x100mm 3.5 micron Solvent A: H2O + 0,05% HCOOH Solvent B: AcN + 0,05% HCOOH	MRM for ENR 360 →316 ENR IS 365 →347 MRM for SDZ 251 →108 SDZ IS 257 →114
GLHK	IDMS, bracketing	Agilent 1290 UPLC system with AB Sciex 6500 QTRAP mass spectrometer. Column: Phenomenex XB-C18 column (150mm × 2.1mm, 1.7µm)	MRM transitions for Enrofloxacin: 360>203 (Quantitation), 360>316 (Confirmation), 360>245 (Confirmation)

		preceded by Phenomenx SecurityGuard™ ULTRA Cartridge UHPLC C18 for 2.1mm I.D. Column. Column Temperature: 45°C. Mobile phase: Solvent A - 0.1% formic acid in H2O and Solvent B - 0.1% formic acid in MeOH. Flow rate: 350 µL/min. Gradient elution program: 95% A for 2 min; decreasing to 85% A from 2 – 5 min; decreasing to 10% A from 5 – 9 min and kept constant at 10% A from 9 – 13 min. The system was then conditioned at 95% A for 4 min before the next injection	MRM transitions for Enrofloxacin-d5: 365>203 (Quantitation), 365>321 (Confirmation), 365>245 (Confirmation) MRM transitions for Sulfadiazine: 251>156 (Quantitation), 251>108 (Confirmation), 251>96 (Confirmation) MRM transitions for Sulfadiazine-13C6: 257>162 (Quantitation), 257>114 (Confirmation), 257>96 (Confirmation) 3.8
INMETRO	LC-IDMS, bracketed exact matching calibration.	LC-MS/MS, column: Acquity UPLC BEH C18 (1.7 µm, 2.1 x 50mm), injection 5 µL, gradient, (A: 0.2 % formic acid containing 0.1 mM oxalic acid, B: 100 % acetonitrile), flow rate 0.3 mL/min. 0 min: 90%A 10%B, 1.5-5.0 m 15% B, 6-7 m 75% B	Enrofloxacin: 360>316 Sulfadiazine: 251>108 For Internal standards: enrofloxacin-d_5: 365>321 $^{13}C_6$ sulfadiazine: 257>114
KRISS	Standard Addition Isotope Dilution Mass Spectrometry (SA-IDMS) was used (Kim et al, Anal Chim Acta V787, p132-139, 2013) to construct a calibration curve for matrix-matching calibration. IDMS measurement was calibrated against the curve.	1. LC-MS/MS: Waters Xevo TQ-S/Acquity I class UPLC system 2. Column: Zorbax Eclipse XDB-Phenyl column (150 x3.0 mm i.d., 3.5-µmparticle size, Agilent) 3. Chromatographic conditions Mobile phase A: 0.1% formic acid in water + 10 µmol/L EDTA Mobile phase B: 0.1% formic acid in acetonitrile 4. Gradient	*m/z* 360 → 316 for enrofloxacin *m/z* 365 → 321 for enrofloxacin-d_5 *m/z* 251 → 156 for sulfadiazine *m/z* 257 → 162 for $^{13}C_6$-sulfadiazine
NMIT	A single point and bracketing IDMS calibration was used.	A LC-MS/MS system (Shimadzu LC system equipped with API 4000 MS/MS from AB Sciex) was used. ZORBAX SB-C_{18} HPLC column, 3.5µ, (150×4.6 mm) with Phenomenex C_{18} SecurityGuard column (4.0 × 2.0 mm) was utilized. The column temperature was maintained at 40 °C. The injection volume was 10 µL. The mobile phase was composed of solvent A (0.1 mM oxalic acid in 0.2 % formic acid in Milli-Q water) and solvent B (acetonitrile). The gradient program was: 0-8 min 2 % B; 8-10 min 98 % B; 15-17 min 2 % B (constant flow	Enrofloxacin 360.21 > 316.20 (primary ion for quantitation) Enrofloxacin 360.21 > 245.10 (secondary ion for confirmation) D_5-Enrofloxacin 365.22 > 321.19 (primary ion for quantitation) D_5-Enrofloxacin 365.22 > 245.09 (secondary ion for confirmation) Sulfadiazine 251.13 > 156.02 (primary ion for quantitation) Sulfadiazine 251.13 > 108.00 (secondary ion for confirmation) $^{13}C_6$-Sulfadiazine 257.20 > 161.98 (primary ion for quantitation)

		rate of 0.3 mL/min).The data were acquired in the positive multiple reaction monitoring (MRM) mode.	$^{13}C_6$-Sulfadiazine 257.20 > 114.05 (secondary ion for confirmation)
UME	IDMS, single point calibration was used	Zivak Tandem Gold LC-MS/MS, Luna PFP(2) 5µm 100 Å,150 mm x 2 mm i.d., Mobil Phase A: Water + MeOH + Formic acid (89.9:10.0:0.1)% , Mobil Phase B: MeOH + Formic acid (99.9:0.1)%, Gradient: Time A% , B% , Flow (mL/min) 0.00 100, 0, 300 5.00 22, 78, 300 6.00 22, 78, 300 6.01 100, 0, 300 12.00 100, 0, 300	Q1, Q3, Capillary, Collision Energy Sulfadiazine : 251; 156; 50; 15 Sulfadiazine $^{13}C_6$: 257; 162; 50; 15 Enrofloxacin : 360; 342; 70; 21 Enrofloxacin d_5 : 365; 347; 70; 21
BVL	calibration by external matrix calibration with internal standards using blank freeze-dried bovine muscle (0.5 g) - after reconstitution of blank samples, fortification on 6 concentration levels for each analyte (multi-point calibration) and fortification of internal standards sulfadiazine $^{13}C_6$ / enrofloxacin D5 hydroiodide on the same constant level as the samples of K-141 - concentrations of analytes and internal standards were defined after screening of samples - sample preparation and measurement in the same manner as the samples of K-141	measurement by LC-MS/MS (Agilent Technologies Infinity 1290 - SCIEX QTrap 6500) - LC column C18 with guard (150 x 2 mm, 3 µm, Phenomenex "Aqua") - mobile phase: A = water (0.1 % formic acid) and B = acetonitrile (0.1 % formic acid); gradient program: 0 min = 10 % B, 1 min = 10 % B, 12 min = 60 % B, 15 min = 60 % B, 16 min = 10 % B, 25 min = 10 % B; flow: 0.3 ml/min; oven temperature: 30 °C; injection volume: 10 µl;	SCIEX QTrap 6500 - MRM in positive ESI mode with two transitions for analytes and one transition for internal standard -sulfadiazine: SDZ 1: 251/156; SDZ 2: 251/108; $^{13}C_6$-SDZ: 257/162 -enrofloxacin: Enro 1: 360/316; Enro 2: 360/245; Enro-D5: 365/321
NRC-Ottawa	ID^{2}MS: Exact matching double isotope dilution mass spectrometry SA-ID^{2}MS: Exact matching standard	1) LC-MS/MS: HPLC: Agilent 1290 Infinity I 2) LC-HRAM-MS: HPLC: Agilent 1260	Enrofloxacin-1 360.2-316.2 Enrofloxacin-2 360.2/245.2 Enrofloxacin-d_5-1 365.2/321.2

	addition double isotope dilution mass spectrometry	Water:Formic Acid/ACN gradient Ace-3 C18, 50 x 2.1, 3µ	Enrofloxacin-d_5-2 365.2/245.2 Sulfadiazine-1 251.2/156.1 Sulfadiazine-2 251.2/108.1 Sulfadiazine-$^{13}C_6$-1 257.2/162.1 Sulfadiazine-$^{13}C_6$-2 257.2/114.1
NIM	Single point calibration, IDMS	HPLC-MS/MS system consisted of a Shimadzu LC30A HPLC and AB API 5500 MS/MS. X-Terra column (3.5 µm, 2.1 mm×100 mm, Waters). 0.1% formic acid in water (A) and methanol (B) were used as mobile phases. Flow rate was 0.15 mL/min. The dualistic gradient started at 10% B, held constant for 0.5 min; changed to 30% B by 3 min linearly, held constant by 7 min, changed to 90%B by 7.5 min; returned to 10% B by 11 min linearly, and then maintained for 4 min.	Enrofloxacin: 360.2/245.1*(quantitation), 360.2/316.2 D5-Enrofloxacin: 365.2/245.1*, 365.2/321.2 Sulfadiazine: 251.1/155.9*, 251.1/107.8 $^{13}C_6$-Sulfadiazine: 257.1/161.9*, 257.1/113.8
INTI P178	IDMS at one point, with three independent standards	LC MSMS Waters TQD Column: BEH C18 100mm x 2.1 mm 1,7 um Gradient with AcN and water with 0,1% formic acid	Enrofloxacin: 360.1>316.1 For Enrofloxacin-D5:365.1>321.1
NRC-Halifax P-178	Single point, exact matched double IDMS	*LC-MS/MS:* - Agilent 1290 HPLC with API5500 mass spectrometer - Column: Poroshell 120 SBC18, 2.7 µm, 2.1x150mm - Temperature: 40°C - Solvent: A= Deionised water with 0.2% HCOOH; B= MeCN with 0.2%HCOOH - Flow: 300µL/ min - Flow diversion used in all analyses (10- 14 min) - 7 min equilibration used in all analyses <u>Enrofloxacin</u>: 10-20% B/ 8 min, to 100%B @ 9 min, hold to 14 min; 2.5µL injections <u>Sulfadiazene</u>: 5-10% B/ 8 min, to 100%B @ 9 min, hold to 14 min; 1µL injections	Enrofloxacin: 360 / 342 Enrofloxacin d_5: 365 / 347 Sulfadiazene: 251 / 155 Sulfadiazene $^{13}C_6$: 257 / 161

Appendix III. Measurement Equations and Uncertainty Budgets

HSA

The mass fraction of the measurand (enrofloxacin or sulfadiazine) in the sample was calculated based on the IDMS calibration curve as follows:

$$C_X = (mR_B + b) \times \frac{W_Y}{M_X} = (mR_B + b) \times \frac{M_Y C_Y}{M_X} \quad (1)$$

where
C_X = mass fraction of the measurand in the sample
M_X = mass of sample (determined by weighing)
M_Y = mass of isotope labelled standard solution (determined by weighing)
W_Y = mass of the isotope labelled standard spiked into sample (equals to $M_Y \times C_Y$)
R_B = peak area ratio of sample blend (determined by LC-MS/MS measurements)
C_Y = concentration of isotope labelled standard solution (determined by weighing and from purity of the isotope labelled standard)
m = gradient of the slope of linear regression plot (determined by the linear fit of the isotope mass ratio from weighing and the peak area ratio from LC-MS/MS measurement of the calibration blends)
b = intercept on y axis of the linear regression plot (determined by the linear fit of the isotope mass ratio from weighing and the peak area ratio from LC-MS/MS measurement of the calibration blends)

As C_Y does not contribute to the measurement uncertainty of C_X, for the estimation of uncertainty, considering $R_M = mR_B + b$, and let $R_M = R_M' C_Y / C_Z$, Equation (1) is converted to:

$$C_X = R_M' \times \frac{M_Y C_Z}{M_X} \quad (2)$$

where
R_M = isotope mass ratio in sample blend
C_Z = concentration of the measurand in the calibration standard solution

A standard uncertainty was estimated for all components of the measurement in Equation (2), which were then combined using respective derived sensitivity coefficients to estimate a combined standard uncertainty in the reported result of enrofloxacin or sulfadiazine in the sample. A coverage factor k with a value of 2 was used to expand the combined standard uncertainty at a 95 % confidence interval. Possible sources of biases [method precision (F_P), choice of different ion pairs (F_I), choice of different calibration stock solutions (F_S), method recovery (F_R)] were accounted for in the final uncertainty budget with the use of the measurement equation:

$$C_X = F_P \times F_I \times F_S \times F_R \times R_M' \times \frac{M_Y C_Z}{M_X} \quad (3)$$

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

$$\frac{\partial C_X}{\partial R_M'} = \frac{C_X}{R_M'} \qquad \frac{\partial C_X}{\partial M_Y} = \frac{C_X}{M_Y} \qquad \frac{\partial C_X}{\partial M_X} = -\frac{C_X}{M_X} \qquad \frac{\partial C_X}{\partial C_Z} = \frac{C_X}{C_Z}$$

$$\frac{\partial C_X}{\partial F_P} = \frac{C_X}{F_P} \qquad \frac{\partial C_X}{\partial F_I} = \frac{C_X}{F_I} \qquad \frac{\partial C_X}{\partial F_S} = \frac{C_X}{F_S} \qquad \frac{\partial C_X}{\partial F_R} = \frac{C_X}{F_R}$$

The standard uncertainty of each component was calculated as follows:
(1) M_Y and M_X: The standard uncertainty was calculated based on the calibration report using the standard weights calibrated by the National Metrology Centre, A*STAR.
(2) F_P: The standard deviation of the results was used as the standard uncertainty of method precision.
(3) F_I: The standard deviation of the difference of the results using two ion pairs divided by the square root of the number of samples (for insignificant difference using t-test) or the average of the difference of the results using two ion pairs divided by 2 (for significant difference using t-test).
(4) C_Z: The certified purity value and associated uncertainty of enrofloxacin or sulfadiazine certified reference material from NMIA in combination with the uncertainty of weighing for preparation of the calibration stock solution.
(5) F_S: The standard deviation of the difference of the results from the use of two calibration stock solutions divided by the square root of the number of samples (for insignificant difference using t-test) or the average of the difference of the results from the use of two calibration stock solutions divided by 2 (for significant difference using t-test).
(6) F_R: Calculated from the deviation of the recovery from 100% and the uncertainty of the amount of enrofloxacin or sulfadiazine spiked in the sample.
(7) R_M' : Consider $R_M = R_M' \times C_Z/C_Y$, the conversion of equation $R_M = mR_B + b$ leads to:

$R_B = (C_Z \times R_M') / (C_Y \times m) - b/m$

Let $m' = C_Z/(C_Y \times m)$ and $b' = -b/m$, we have:

$R_B = m'R_M' + b'$

The standard uncertainty of R_M' was calculated using the following equation:

$$u_{R_M} = \frac{1}{m'} \times s_{y/x} \times \sqrt{\frac{1}{N} + \frac{1}{n} + \frac{\left(R_B - \overline{R_{Bc}}\right)^2}{m'^2 \sum_{i=1}^{n} (R_{Mci} - \overline{R_{Mc}})^2}} \tag{4}$$

where

$s_{y/x}$ = standard deviation of the regression
R_B = peak area ratio of sample blend
$\overline{R_{Bc}}$ = average peak area ratio of calibration blends
n = number of calibration blends used for the linear regression plot
N = injection time for each sample
R_{Mci} = isotope mass ratio in calibration blends
$\overline{R_{Mc}}$ = average isotope mass ratio in calibration blends

The combined standard uncertainty was calculated using the equation below:

$$u = \sqrt{\sum_i c_i^2 u_{xi}^2} \tag{5}$$

where
u = combined standard uncertainty
c_i = sensitivity coefficient of each component
u_{xi} = standard uncertainty of each component

The full uncertainty budget is given in Tables 1 and 2.

Table 1. Uncertainty budget for enrofloxacin.

	x	u_{xi}	u_{xi}/x	c_i	$c_i^2 \cdot u_{xi}^2$	**Contribution**
M_X (g)	0.4913	0.000092	0.02%	0.13	1.514E-10	0.0011%
M_Y (g)	0.3345	0.000092	0.03%	0.20	3.267E-10	0.0023%
C_Z (mg/kg)	2892.0	15.993	0.55%	0.00	1.323E-07	0.92%
R_M'	0.8371	0.005893	0.70%	0.08	2.143E-07	1.49%
F_P(mg/kg)	0.0658	0.002348	3.57%	1.00	5.515E-06	38.26%
F_I (mg/kg)	0.0658	0.001176	1.79%	1.00	1.383E-06	9.59%
F_S (mg/kg)	0.0658	0.001386	2.11%	1.00	1.922E-06	13.33%
F_R (mg/kg)	0.0658	0.002291	3.48%	1.00	5.247E-06	36.40%

Table 2. Uncertainty budget for sulfadiazine.

	x	u_{xi}	u_{xi}/x	c_i	$c_i^2 \cdot u_{xi}^2$	**Contribution**
M_X (g)	0.4913	0.000092	0.02%	5.16	2.247E-07	0.0016%
M_Y (g)	0.4036	0.000092	0.02%	6.28	3.331E-07	0.0023%
C_Z (mg/kg)	166.2	0.7392	0.44%	0.02	1.269E-04	0.89%
R_M'	1.056	0.002577	0.24%	2.40	3.826E-05	0.27%
F_P(mg/kg)	2.5338	0.1039	4.10%	1.00	0.01080	75.94%
F_I (mg/kg)	2.5338	0.02074	0.82%	1.00	4.303E-04	3.02%
F_S (mg/kg)	2.5338	0.00992	0.39%	1.00	9.850E-05	0.69%
F_R (mg/kg)	2.5338	0.05223	2.06%	1.00	0.00273	19.18%

NMIA

The measurement equation used for both analytes is

$$\omega_X = \overline{\left(\frac{R_B}{R_{Bc}} \bullet \frac{m_{Zc}}{m_{Yc}} \bullet \frac{m_Y}{m_X} \right)} \bullet \omega_Z \bullet (p+1) \bullet F_{matching} \bullet F_{matrix} \bullet T_{weighX4}$$

where
ω_x = mass fraction of analyte in sample
ω_z = mass fraction of analyte in the calibration standard solution used to prepare calibration blend
m_y = mass of internal standard solution added to sample blend
m_{yc} = mass of internal standard solution added to calibration blend
m_x = mass of sample added to sample blend
m_{zc} = mass of calibration standard solution added to calibration blend
R_b = observed isotope amount ratio in sample/internal standard blend
R_{bc} = observed isotope amount ratio in standard/internal standard calibration blend
(p+1) = moisture content correction factor (p = mass fraction of water in the dry mass of the sample)
F_{matrix} = term to account for uncertainty associated with discounting potential matrix effects or chromatographic interferences (value of 1)
$F_{ISequil}$ = term to account for uncertainty associated with discounting potential bias related to equilibration of labelled internal standard with analyte in the sample prior to extraction (value of 1)
$T_{weighx4}$ = term to account for weighing accuracy of masses of sample and standard solutions (value of 1)
Terms inside the brackets are included in the average of replicate determinations and their precision is incorporated in the measurement precision. Only estimates of accuracy are required for these terms.
Terms outside the brackets require estimates of both precision and accuracy in the MU budget.

Uncertainty estimates for each term in the measurement equation were combined as described in the GUM (JCGM 100) using sensitivity coefficients and the Welch-Satterthwaite equation to give the reported expanded uncertainties.

The values of terms in the measurement equation and their uncertainties with degrees of freedom (ν) in the uncertainty budget are summarised in the following table, which also summarises their derivation.

	Enrofloxacin			
Factor	*x*	*u(x)*	*ν*	*Source of uncertainty estimate*
Measurement Precision	0.0530	0.00008	14	Standard deviation of the mean of 15 independent analyses of the study material
p+1	1.00436	0.00012	11	Standard deviation of the mean of 12 measurements of the moisture content in 3 samples over 22-31 days
ω_z	0.1215	0.0016	2	Combined uncertainty in purity of reference material and observed reproducibility of preparation of reference standard solutions
Trueness Factors				
$T_{weighx4}$	1	3.2E-06	200	Maximum potential bias in weighing for sample and calibration blends from balance calibration certificates.
F_{matrix}	1	0.0013	7	Between group uncertainty from ANOVA of repeated measurements of the study material grouped by MRM transition used.
$F_{ISequil}$	1	0.0057	4	Between-group uncertainty from ANOVA of duplicate analyses of study material using internal standard equilibration times ranging from 2 – 26 hours.

	Sulfadiazine			
Factor	*x*	*u(x)*	*ν*	*Source of uncertainty estimate*

Measurement Precision	2.208	0.009	14	Standard deviation of the mean of 15 independent analyses of the study material
p+1	1.00436	0.00012	11	Standard deviation of the mean of 12 measurements of the moisture content in 3 samples over 22-31 days
ω_z	3.760	0.012	9	Combined uncertainty in purity of reference material and observed reproducibility of preparation of reference standard solutions
Trueness Factors				
$T_{weighx4}$	1	3.23E-06	200	As for enrofloxacin (above)
F_{matrix}	1	0.0022	1	Between group uncertainty from ANOVA of repeated measurements of the study material grouped by determination method.
$F_{ISequil}$	1	0.009	3	Between-group uncertainty from ANOVA of duplicate analyses of study material using internal standard equilibration times ranging from 2 – 24 hours

Measurement precision: the standard deviation of the mean of the results for 15 sub-samples (16 sub- samples were analysed, but one sub-sample gave an anomalous result for ENR and a different sub-sample gave an anomalous result for SDZ, and these results were excluded after being identified as outliers by Grubbs test).
p+1: the standard deviation of the mean of the results for four measurements made at 22, 24, 29 and 31 days on each of the three sub-samples for moisture analysis
ω_z: Uncertainty related to potential bias in the mass fraction of the calibration solution (ω_z) was estimated by combining the uncertainty for the purity of the reference material, a component related to the scale correction value from the balance used for standard preparation and a component for the observed reproducibility of standard preparation. Calibration blends made from standard solutions prepared from three stock solutions were compared. ANOVA was used to investigate whether there was a significant difference between the results and to estimate an uncertainty contribution.

LGC

Each individual sample blend was injected repeated times bracketed by its corresponding calibration blend. The amount of analyte was calculated for each of the last 5 injections using the reduced form of the IDMS equation:

$$W_{X_i} = \frac{1}{m_X} \times (m_Z \times W_Z) \times \frac{m_{Y,SB}}{m_{Y,CB}} \times \frac{R_{SB_i}}{R_{CB_i}}$$

Where:

- W_{Xi} is the mass fraction of the analyte in the sample calculated for injection *i*,
- m_X is the mass of the sample weighed,
- m_Z is the mass of the solution of the natural compound added to the calibration blend,
- W_Z is the mass fraction of the natural compound in the solution added to the calibration blend
- $m_{Y,CB}$ is the mass of the solution of the labelled compound added to the calibration blend,
- $m_{Y,SB}$ is the mass of the solution of the labelled compound added to the sample blend,
- R_{SBi} is the response ratio of each of the individual injection *i*.
- R_{CBi} is the average ratio of the responses of the 2 bracketing calibration blends of injection *i*.

The mass fraction of each individual sample was calculated as the average of the 5 calculated mass fractions of the individual injections multiplied by the calculated dry-mass correction factor (*D*) for the day of the analysis of the sample:

$$W_X = D \times \left(\frac{\sum_{i=1}^{5} W_{X_i}}{5} \right)$$

The standard uncertainty of each individual measurement was estimated using the following equation:

$$u_{W_X} = W_X \times \sqrt{\left(\frac{u_D}{D}\right)^2 + \left(\frac{u_{m_X}}{m_X}\right)^2 + \left(\frac{u_{m_Z}}{m_Z}\right)^2 + \left(\frac{u_{W_Z}}{W_Z}\right)^2 + \left(\frac{u_{m_{Y,SB}}}{m_{Y,SB}}\right)^2 + \left(\frac{u_{m_{Y,CB}}}{m_{Y,CB}}\right)^2 + \left(\frac{u_{\left(\frac{R_{SB}}{R_{CB}}\right)}}{\frac{R_{SB}}{R_{CB}}}\right)^2}$$

Where:

- $\frac{u_D}{D}$ is the relative uncertainty of the dry-basis conversion factor.
- $\frac{u_{m_X}}{m_X}$ is the relative uncertainty associated with the mass of sample used,
- $\frac{u_{m_Z}}{m_Z}$ is the relative uncertainty of the mass of natural solution added to the calibration blend.
- $\frac{u_{W_Z}}{W_Z}$ is the relative uncertainty associated with the mass fraction of the calibration solution.
- $\frac{u_{m_{Y,SB}}}{m_{Y,SB}}$ is the relative uncertainty of the mass of labelled solution added to the sample blend.
- $\frac{u_{m_{Y,CB}}}{m_{Y,CB}}$ is the relative uncertainty of the mass of labelled solution added to the calibration blend.
- $\frac{R_{SB}}{R_{CB}}$ is the averaged bracketed response ratio
- $u_{\left(\frac{R_{SB}}{R_{CB}}\right)}$ is the standard deviation of 5 bracketed response ratios.

Final mass fraction was calculated as the average of the 4 individual results. Total combined uncertainty was estimated by averaging the individual combined standard uncertainties.

VNIIM

$$w_{ан} = \frac{S_{ан} \cdot m_{IS}}{S_{IS} \cdot F \cdot m}$$

w- mass fraction of the ENR (SDZ) in the sample, mkg/kg;
m_{is} - mass of internal standard added to sample before sample preparation, mkg;
m - mass of sample (dry mass), kg;
F - response factor.
$F=(S_{ancal}*m_{is})/(S_{iscal}*m_{an})$
C_{an}- mass of ENR (SDZ) in calibration solution;
m_{is} - mass of internal standard in calibration solution;
S_{ancal} - peak area for the ENR (SDZ);
S_{iscal} - peak area for the internal standard
$m = m_1(100 – 0,18)$
m_1 –mass of sample before moisture determination; 0,18 – moisture content, %

Source of uncertainty	u, %	
	SDZ	**ENR**
mass of sample(m, dry mass)	0,012	0,012
purity of reference standard	0,29	0,29
preparation of reference standard solution	0,44	0,44
preparation of calibration solution	0,058	0,058
RSD of F determination	0,39	1,64
mass of internal standard added to sample before extraction (m_{IS})	0,48	0,14
RSD of results, %	3.1	2,2
comb.std uncertainty	**3.2**	**2,8**
expanded uncertainty (k=2)	**6.4**	**5,6**

INMETRO

The following equation was used to calculate the mass fraction of both analytes (W_x):

$$W_x = W_z \times \frac{m_z}{m_{yc}} \times \frac{m_y}{m_x} \times \frac{R_B}{R_{BC}}$$

Where,
W_z: mass fraction of the calibration standard solution
m_z: mass of standard solution added to calibration blend
m_{yc}: mass of internal standard solution added to calibration blend
m_y: mass of internal standard solution added to sample
m_x: mass of sample
R_B: analyte/internal standard area ratio in the sample blend
R_{BC}: analyte/internal standard area ratio in the calibration blend

Uncertainty budget:

		Sulfadiazine		**Enrofloxacin**	
Source		Uncertainty component (g/g)	Contribution (%)	Uncertainty component (g/g)	Contribution (%)
Mass fraction of standard solution (W_z)	mass of standard	2.0×10^{-8}	4.6	5.3×10^{-8}	11.4
	mass of standard stock solution	2.4×10^{-11}		7.3×10^{-8}	
	mass of stock solution aliquote	7.6×10^{-9}		7.4×10^{-10}	
	mass of work standard solution	3.1×10^{-11}		8.0×10^{-13}	
	standards purity	2.5×10^{-10}		6.3×10^{-12}	
Mass of standard solution added to calibration blend (m_z)		9.2×10^{-9}	0.9	2.4×10^{-10}	0.8
Mass of internal standard solution added to calibration blend (m_{yc})		2.8×10^{-8}	8.3	7.3×10^{-10}	7.4
Mass of internal standard solution added to sample blend (m_y)		3.1×10^{-8}	10.2	8.1×10^{-10}	9.0
Mass of sample (m_x)		3.2×10^{-10}	0.0	8.2×10^{-12}	0.0
Analyte/internal standard area ratio in the sample blend (R_B)		5.3×10^{-8}	29.0	1.5×10^{-9}	30.3
Analyte/internal standard area ratio in the calibration blend (R_{BC})		4.4×10^{-8}	20.2	1.5×10^{-9}	30.3
Repeatability		5.1×10^{-8}	26.9	8.9×10^{-10}	10.9
Overall		9.8×10^{-8}	100.0	2.7×10^{-09}	100.0

KRISS

<Standard addition experiment>

The concentration of each analyte was calculated using the following equation.

$$y = k(x + C_{sample})$$

where,

k is the response factor of the instrument;

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

$$y = \left(\frac{M_{is-sol,subsample,i} \times C_{is-sol}}{M_{subsample,i}} \right) AR_{subsample,i}$$

$$x = \left(\frac{M_{s-sol,subsample,i} \times C_{s-sol}}{M_{subsample,i}} \right)$$

where,

$M_{is\text{-}sol,subsample,i}$ is the mass of the internal standard solution added into the ith subsample;

$C_{is\text{-}sol}$ is the concentration of the internal standard in the internal standard solution;

$M_{subsample,i}$ is the mass of the ith subsample;

$AR_{subsample,i}$ is the observed area ratio of the target analyte and its isotope-labeled internal standard in the ith subsample;

$M_{s\text{-}sol,subsample,i}$ is the mass of the standard solution added into the ith subsample;

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

The standard uncertainty of the final measurement value C_{sample}, $u(C_{sample})$, was calculated by combining the standard uncertainty of C_{sample} from the least-square-fit line, $u_{lsf}(C_{sample})$, and the stadnard uncertainty of $C_{s\text{-}sol}$, $u(C_{s\text{-}sol})$, as following equation.

$$u(C_{sample}) = \sqrt{u_{lsf}^2(C_{sample}) + u^2(C_{s-sol})}$$

The $u_{lsf}(C_{sample})$ and $u(C_{s\text{-}sol})$ can be calucated using the following equations as the equation, $y=k(x+C_{sample})$, can be rewritten as $y=kx+a$.

$$u_{lsf}(C_{sample}) = \sqrt{\left(\frac{s_k}{k}\right)^2 + \left(\frac{s_a}{a}\right)^2}$$

$$u(C_{s-sol}) = \sqrt{u_{purity}^2 + u_{gravi.}^2}$$

where,

k is the slope of the least-square-fit line;

a is y-intercept which is the value of y when x is zero;

C_{sample} is a/k;

s_k is the standard deviation of k calculated from the least-square fitting of experimental results;

s_a is the standard deviation of a calculated from the least-square fitting of experimental results;

u_{purity} is the standard uncertainty for the purity analysis of the target analyte used for the preparation of the standard solution;

$u_{gravi.}$ is the standard uncertainty for the gravimetric preparation of the target analyte used for the preparation of the standard solution.

<Application of the result of standard addition experiment to IDMS experiment>

The plot of $AR_{subsample,\,i}$ versus $IR_{subsample,\,i}$ (target analyte/its isotope-labeled internal standard in the ith subsample) was made to draw a calibration curve by using the result of the standard addition experiment. The $IR_{subsample,\,i}$ can be calculated as follows.

$$IR_{subsample\,i} = \frac{M_{subsample\,i} \times C_{sample} + M_{s-sol,subsample\,i} \times C_{s-sol}}{M_{is-sol,subsample\,i} \times C_{is-sol}}$$

IDMS measurement was performed with 4 subsamples. From the area ratio $AR_{subsample}$ observed by LC/MS for each subsample, $IR_{subsample}$ was calculated using the reconstructed calibration curve. Then, the concentration of analytes, $C_{sample,IDMS}$, in each subsample was calculated using the following equation.

$$C_{sample,IDMS} = f \cdot \frac{IR_{subsample\,i} \times M_{is-sol,subsample\,i} \times C_{is-sol}}{M_{subsample\,i}}$$

Where f is the dry mass correction factor, f=1/(1-x), in which x is the moisture content of the KC sample.

The uncertainty of the mean, $u(C_{\text{mean}})$, for 4 subsamples was calculated by using the following equation.

$$u(C_{\text{mean}}) = \sqrt{u_{\text{char,sys}}^2 + \frac{SD_{bb}^2}{\sqrt{n}}}$$

Where $u_{\text{char,sys}}$ is the uncertainty caused by systematic effects, SD_{bb} is standard deviation of the measurement result of four subsamples, and n is the number of replicates (n=4).

NIMT

Measurement equation:

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

$$F_{drymass} = \left[1 - \frac{\%moisture}{100}\right]$$

Where:

w_x = mass fraction of enrofloxacin/sulfadiazine in bovine tissue

w_z = mass fraction of enrofloxacin/sulfadiazine in the calibration solution used to prepare the calibration blend

m_y = mass of spike solution added to sample blend

m_{yc} = mass of spike solution added to calibration blend

m_{zc}= mass of standard solution added to calibration blend

m_x = 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

Combined uncertainty equation:

$$\frac{u(x)}{x} = \sqrt{\left(\frac{u(w_{zc})}{w_{zc}}\right)^2 + \left(\frac{u(m_y)}{m_y}\right)^2 + \left(\frac{u(m_{yc})}{m_{yc}}\right)^2 + \left(\frac{u(m_{zc})}{m_{zc}}\right)^2 + \left(\frac{u(m_x)}{m_x}\right)^2 + \left(\frac{u(F_{drymass})}{F_{drymass}}\right)^2 + \left(\frac{u(F_I)}{F_I}\right)^2 + \left(\frac{u(F_E)}{F_E}\right)^2 + \left(\frac{u(F_P)}{F_P}\right)^2}$$

Where;

$u(w_{z,c})$ is the standard uncertainty of the mass fraction of analyte in the calibration solution used to prepare the calibration blend. The value was estimated from the purity of enrofloxacin/sulfadiazine standard, masses weighed for preparation of stock solutions and uncertainty using different standards (standard comparison).

$u(m_y)$, $u(m_{y,c})$, $u(m_x)$ and $u(m_{z,c})$ are standard uncertainties of the masses. These values were estimated from the bias and precision effect of the balance.

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

$u(F_I)$ is the standard uncertainty of the interference effect. This value was estimated 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.

$u(F_{drymass})$ is the standard uncertainty of the dry mass correction factor which was estimated from the moisture content analysis.

Note: For the uncertainty contributing to the R'_B and $R'_{B,C}$,the precision in measuring the isotope amount ratios of the analyte and the internal standard in the sample and calibration blends was assumed to be incorporated in the overall method precision. The effect of any biases on these ratios was assumed to be negligible as any systematic biases should cancel out since the calibration blends and sample blends were exact-matched for analyte concentration and isotope ratio. Other biases that may arise from interferences, extractions are captured in other factors.

Uncertainty budget: Enrofloxacin

Combination of Uncertainties			
Factor	**Values** **x**	**Uncertainties** **u(x)**	**u(x)/(x)**
Measurement equation factors			
Method Precision, F_P	1.0000	0.02009	2.009%
m_{zc}	0.29237	0.000044	0.0150%
m_y	0.29408	0.000044	0.0150%
m_{yc}	0.29496	0.000044	0.0149%
$F_{drymass}$	0.99709	0.000238	0.0239%
m_x	0.50750	0.000044	0.0087%
w_z	102.6775	0.869475	0.8468%
Additional Factors			
Extraction effects, F_E	1.000	0.0200	2.000%
Interference from two different ion pairs, F_I	1.000	0.0071	0.712%

Uncertainty Analysis Results		
w_x=	**62.05**	ng/g
u(x) =	1.888	ng/g
u(x)/x =	3.04%	
Veff(total) =	32.259	
***k*=**	2.04	(@ 95% level)
***U*(x) =**	**3.846**	
%*U*(x) =	**6.20%**	

Uncertainty budget: Sulfadiazine

Combination of Uncertainties			
Factor	**Values** **x**	**Uncertainties** **u(x)**	**u(x)/(x)**
Measurement equation factors			
Method Precision, F_P	1.0000	0.02976	2.976%

m_{zc}	0.33206	0.000044	0.0132%
m_{y}	0.33448	0.000044	0.0131%
m_{vc}	0.33406	0.000044	0.0132%
$F_{drvmass}$	0.99709	0.000238	0.0239%
m_{x}	0.50181	0.000044	0.0088%
w_{z}	3279.8871	26.067115	0.7948%
Additional Factors			
Extraction effects, F_E	1.000	0.0100	1.000%
Interference from two different ion pairs, F_I	1.000	0.0027	0.272%

Uncertainty Analysis Results		
w_x=	**2138.50**	ng/g
u(x) =	69.506	ng/g
u(x)/x =	3.25%	
Veff(total) =	24.906	
***k*=**	2.06	(@ 95% level)
***U*(x) =**	**143.453**	
%*U*(x) =	**6.71%**	

UME

Measurement Equation:

$$RF = \frac{A_N \cdot C_{IS}}{A_{IS} \cdot C_N}$$

RF: Response Factor
C_N : Concentration of native analyte (mg/kg)
A_N : Area of native analyte
C_{IS}: Concentration of labelled analyte (mg/kg)
A_{IS}: Area of labelled analyte

$$C_{Analyte} = \frac{A_N \cdot n_{IS} \cdot 1000}{A_{IS} \cdot RF \cdot M_{sample}}$$

RF: Response Factor
C_N : Concentration of analyte in unknown sample (µg/kg)
A_N : Area of native analyte in unknown sample
Aıs: Area of labelled analyte
n_{is}: Total amount of added internal Standard (µg)
M_{sample}: Sample intake (g)

Uncertainty Calculations CCQM-K141/P178

Bottom up approach was used

Sources:
1-Mass of sample intake
2-Spiking of labelled stock solution
3-Native stock solution
4-Calibration
5-Recovery
6-Repeatability
7-Water determination

1-Mass of sample intake

	Value	Standard Measurement Uncertainty
Mass of bovine tissue sample		
Calibration	m_{tissue} (g)	$um_{calibrationsample}$ (g)
Mass of Tare		
Calibration	m_{tare} (g)	$um_{calibrationtare}$

$$u(m_{SI}) = \sqrt{(u_{mcalibsample})^2 + (u_{mcalibtare})^2}$$

2-Spiking of Isotopic Labelled Compounds Stock Solution

Mass	Value	Standard Measurement Uncertainty
Mass of spiking of IS	$m_{solution}$ (g)	$u_{mspikeIS}$ (g)
Calibration		

$$u(m_{IS}) = \sqrt{(u_{mcalib})^2}$$

3-Native Stock Solution

$$u(C_{1ststocksol}) = \sqrt{(u_{purity})^2 + (u_m)^2}$$

$$u(C_{2ndstocksol}) = \sqrt{(u_{purity})^2 + (u_m)^2}$$

4-Calibration

$$u(RF) = SD$$

5-Uncertainty of Recovery

$$u(R_m) = R_m \sqrt{\left(\frac{\overline{u(C_{obs})}}{\overline{C_{obs}}}\right)^2 + \left(\frac{u(C_{cert})}{C_{cert}}\right)^2}$$

$$R_m = \frac{\overline{C_{obs}}}{C_{cert}}$$

uC_{obs}	standard measurement uncertainty of observed concentration of analyte observed
C_{obs}	concentration of

analyte

uC_{cert} standard measurement uncertainty of certified concentration of analyte

C_{cert} certified concentration of analyte

Mean

R_m recovery

5-Uncertainty of Repeatability

$$u(r) = \frac{SD}{\sqrt{n}}$$

6- Water Determination

Mass of Sample

$$u(m_{sample}) = \sqrt{2 * (u_{Cmcalibration})^2}$$

Repeatability of Water Determination

$$u(R) = \frac{SD}{\sqrt{n}}$$

$$u(Water) = \sqrt{u(R)^2 + (um_{sample})^2}$$

COMBINED STANDARD MEASUREMENT UNCERTAINTY

$$\frac{u_c(Analyte)}{c_{Analyte}} = \sqrt{\left(\frac{u(m_{SI})}{m_{SI}}\right)^2 + \left(\frac{u(m_{SLS})}{c_{SLS}}\right)^2 + +\left(\frac{u(C_{NSS})}{C_{NSS}}\right)^2 + \left(\frac{u(RF)}{RF}\right)^2 + \left(\frac{u(R_m)}{R_m}\right)^2 + \left(\frac{u(r)}{r}\right)^2 + \left(\frac{u(Water)}{Water}\right)^2}$$

Uncertainty Budget of Sulfadiazine

Parameters	Unit	Value (X)	u(x)	u(x)/X
Mass of sample intake	g	0.5	1.2621E-05	2.52E-05
Spiking Labelled stock solution	g	0.1	0.00000914	9.14E-05
Native stock solution	µg/kg	10	0.10	9.84E-03
Calibration		0.976	0.010	1.06E-02
Recovery		0.961	0.041	4.26E-02
Repeatability	µg/kg	2246.5	20.84	9.28E-03
Water determination	g/g	0.013	0.0004	3.37E-02
Relative Standard Measurement Uncertainty				0.057
Result (µg/kg)		2246.5		
Combined Standard Measurement Uncertainty			128.0	
Expanded Uncertainty (k=2)			255.9	
Relative Mesurement Uncertainty (%)			11.4	

Uncertainty Budget of Enrofloxacin

Parameters	Unit	Value (X)	u(x)
Mass of sample intake	g	0.5	1.2621E-05
Spiking Labelled stock solution	g	0.1	0.00000914
Native stock solution	µg/kg	0.5	0.0049
Calibration		1.191	0.007
Recovery		0.988	0.025
Repeatability	µg/kg	59.29	2.026
Water determination	g/g	0.013	0.0004
Relative Standard Measurement Uncertainty			
Result (µg/kg)		59.3	
Combined Standard Measurement Uncertainty			3.3
Expanded Uncertainty (k=2)			6.6
Relative Mesurement Uncertainty (%)			11.1

BVL

$$x = (y - a)/b$$

$$b = \left(n\sum x_i y_i - \sum x_i \sum y_i\right) \Big/ \sqrt{\left\{n\sum x_i^2 - \left(\sum x_i\right)^2\right\}}$$

$$a = \left(\sum y_i - b\sum x_i\right)/n$$

x: analyte concentration in the sample (µg/kg)
x_i: analyte concentration of the i-th standard (µg/kg)
a: intercept of the calibration curve
b: slope of the calibration curve
y: area of the analyte peak of the sample
y_i: area of the analyte peak of the i-th standard
n: number of analyses per concentration range

$$U = k * \sqrt{(u_{res})^2 + (u_{ss})^2 + (u_{sw})^2 + (u_{sp})^2 + (u_{dm})^2}$$

u_{res}: relative uncertainty of result as relative within-laboratory reproducibility
u_{ss}: uncertainty of calibration solution
u_{sw}: uncertainty of sample weight
u_{sp}: uncertainty of sample spike
u_{dm}: uncertainty of dry mass

Contributions to measurement uncertainty:
Enrofloxacin

	u		Target		u(x)/X [%]	
Calibration solution:	0.470542213	ng/g	10000	ng/g	0.005	2.2141E-05
Sample weight:	1.39425E-05	g	0.5	g	0.003	7.7757E-06
Sample spike:	1.21865E-05	g	0.0478	g	0.025	0.0006498
Reproducibility method:	6.95	ng/g	96.6	ng/g	7.20	51.8
Dry mass:	0.0009	g/g	0.99871	g/g	0.09	0.0080595

k= 2

u= [%] 7.2

U= [%] 14.40

Contributions to measurement uncertainty:
Sulfadiazin

	u		Target		u(x)/X [%]	
Calibration solution:	0.33	ng/g	10000	ng/g	0.003	0.0000109
Sample weight:	1.39425E-05	g	0.5	g	0.003	7.7757E-06
Sample spike:	1.21865E-05	g	0.0478	g	0.025	0.0006498
Reproducibility method:	200	ng/g	2304	ng/g	8.70	75.7
Dry mass:	0.0009	g/g	0.99871	g/g	0.09	0.0080595

k= 2

u= [%] 8.70

U= [%] 17.40

NRC Ottawa

ID^{2}MS:

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

ID^{2}MS	Double isotope dilution mass spectrometry
A	Analyte in the sample (natural isotopic composition)
A*	Analyte in the primary standard (natural isotopic composition)
B	Analyte in the isotopic standard (isotopically enriched composition)
AB	Blend of sample (A) and isotopic standard (B)
A*B	Blend of primary standard (A*) and isotopic standard (B)
AA*B	Blend of sample (A), primary standard (A*) and isotopic standard (B)
w_A	Mass fraction of A (natural) in the sample (unknown)
w_{A^*}	Mass fraction of A (natural) in the primary standard
$m_{A^*\text{-}1}$	Mass of A (natural) in blend-1 (A*B) (Cal)
$m_{B\text{-}1}$	Mass of B (Isotopic IS) in blend-1 (A*B) (Cal)
$m_{A\text{-}2}$	Mass of matrix sample in blend-2 (AB) (Spiked matrix)
$m_{B\text{-}2}$	Mass of B (Isotopic IS) in blend-2 (AB) (Spiked matrix)
R_1	Measured isotope ratio in blend-1 (A*B)
R_2	Measured isotope ratio in blend-2 (AB)
R_A	Measured isotope ratio in blend-3 (A)

R_{A^*}	Measured isotope ratio in blend-4 (A*)
R_B	Measured isotope ratio in blend-5 (B)

SA-ID^{2}MS:

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

SA-ID^{2}MS	Standard addition-double isotope dilution mass spectrometry
A	Analyte in the sample (natural isotopic composition)
A*	Analyte in the primary standard (natural isotopic composition)
B	Analyte in the isotopic standard (isotopically enriched composition)
AB	Blend of sample (A) and isotopic standard (B)
A*B	Blend of primary standard (A*) and isotopic standard (B)
AA*B	Blend of sample (A), primary standard (A*) and isotopic standard (B)
w_A	Mass fraction of A (natural) in the sample (unknown)
w_{A^*}	Mass fraction of A (natural) in the primary standard
$m_{A\text{-}1}$	Mass of matrix sample in blend-1 (AA*B) Note: (A* = 0 in blend 1)
$m_{A^*\text{-}1}$	Mass of A (natural) in blend-1 (AA*B)
$m_{B\text{-}1}$	Mass of B (Isotopic IS) in blend-1 (AA*B)
$m_{A\text{-}2}$	Mass of matrix sample in blend-2 (AA*B)
$m_{A^*\text{-}2}$	Mass of A (natural) in blend-2 (AA*B)
$m_{B\text{-}2}$	Mass of B (Isotopic IS) in blend-2 (AA*B)
R_1	Measured isotope ratio in blend-1 (AA*B) Note: (A* = 0 in blend 1)
R_2	Measured isotope ratio in blend-2 (AA*B)
R_A	Measured isotope ratio in blend-3 (A)
R_{A^*}	Measured isotope ratio in blend-4 (A*)
R_B	Measured isotope ratio in blend-5 (B)

Uncertainty Budget:

The combined uncertainty estimate (u_c) included uncertainties due to measurement (u_{char}), possible inconsistency between the various measurement methods (u_{method}) and possible uncertainties due to reference standard purity (u_{purity}). The combined uncertainty estimate (u_c) was calculated as the square root of the sum of squares of the individual uncertainty contributions. A coverage factor of 2 was used to calculate the expanded uncertainty (U_C).

NIM

The mass fraction (µg/kg) of analytes (Cx) in the sample was calculated as follows:

The expanded measurement equation given was used to calculate the mass fraction of the measurand. The additional factors (F) in the expanded measurement equation represent aspects of the measurement procedure that may influence the measured mass fraction value. They are given a value of 1 but they add an uncertainty component to the uncertainty budget.

Expanded measurement equation

$C_x = F_I \times F_P \times F_E \times (M_y \times M_z c \times Rb)/(M_x \times F_{drymass} \times M_y c \times Rbc)$

Where :

- Cx is the mass fraction of analytes in the sample (ng/g);
- F_I is the matrix effect interference factor
- F_P is the method precision factor
- F_E is the extraction efficiency factor
- M_y is mass of internal standard (isotopologue) added to the sample blend (g)
- $M_z c$ is mass of analyte added to the calibration blend (g)
- Rb is peak area ratio of analyte /isotopologue in sample blend
- M_x is mass of sample (g)
- $F_{drymass}$ is the drymass correction factor obtained from moisture content analysis
- $M_y c$ is mass of internal standard(isotopologue) added to the calibration blend (g)
- Rbc is peak area ratio of analyte /isotopologue in calibration blend

The detailed uncertainty budgets were listed as follows:

Uncertainty of Enrofloxacin			
Source of uncertainty	Parameter x	$u_{(x)}$	$u_{(x)}/(x)$
M_y (g)	0.12	0.44E-03	0.37%
M_x (g)	0.50	0.44E-03	0.09%
$M_y c$ (g)	0.45	0.44E-03	0.10%
$M_z c$(g)	0.51	0.16E-02	0.32%
$F_{drymass}$	0.9973	0.51E-03	0.05%
Extraction effects, F_E (1)	1	2.00E-02	2.00%
Interference from matrix effect , F_I (1)	1	0.70E-02	0.70%
Method Precision, F_P (1)	1	3.53E-02	3.53%
Relative combined standard uncertainty (uc)			4.15 %
Coverage factor, *k*			2
Relative expanded uncertainty (Uc)			8.3 %
Mass Fraction (µg/kg)	65.1		
Expanded uncertainty, U (µg/kg)	5.4		

Uncertainty of Sulfadiazine			
Source of uncertainty	Parameter x	$u_{(x)}$	$u_{(x)}/(x)$
M_y (g)	0.11	0.44E-03	0.40%
M_x (g)	0.50	0.44E-03	0.09%
$M_y c$ (g)	0.39	0.44E-03	0.12%

$M_z c(g)$	0.44	0.15E-02	0.34%
$F_{drymass}$	0.9973	0.51E-03	0.05%
Extraction effects, F_E (1)	1	2.00E-02	2.00%
Interference from matrix effect , F_I (1)	1	0.55E-02	0.55%
Method Precision, F_P (1)	1	2.58E-02	2.58%
Relative combined standard uncertainty (uc)			3.35 %
Coverage factor , *k*			2
Relative expanded uncertainty (Uc)			6.7 %
Mass Fraction (µg/kg)	2349.0		
Expanded uncertainty, U (µg/kg)	157.4		

EXHM

The measurement equation is:

$$w_{M,S} = w_{M,C} \frac{100}{Rec} \times \frac{1}{1 - H} \times \frac{m_{is,S}}{m_{M,S}} \times \frac{m_{M,C}}{m_{is,C}} \times \frac{R_S}{R_C}$$

where $w_{M,S}$ = dry mass fraction of the analyte (SDZ or EFX) in the sample, (*µg/kg*)

$w_{M,C}$ = mass fraction of the analyte (SDZ or EFX) in the calibration solution, (*µg/kg*)

H = sample moisture content (g/g)

Rec = recovery (%), assessed against other independent methods

$m_{is,S}$ = mass of internal standard solution added to sample blend, (*g*)

$m_{M,S}$ = mass of test material in sample blend, (*g*)

$m_{M,C}$ = mass of the analyte (SDZ or EFX) solution added to calibration blend, (*g*)

$m_{is,C}$ = mass of internal standard solution added to calibration blend, (*g*)

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:

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

where s_R is the standard deviation under reproducibility conditions, n the number of determinations 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 budgets for SDZ and EFX

Sulfadiazine

uncertainty component	value	sensitivity coefficient	standrard uncertainty	relative uncertainty	$C_i \times u_i$	$(C_i \times u_i)^2$
method precision	2324,55	1,0000	17,758	0,0076	17,7578	315
mass fraction of SDZ in the calibration solution, (*µg/kg*)	40043,59	0,0581	102,055	0,0025	5,9243	35,1
sample moisture content, (g/g)	0,0031	-2331,7486	0,0002	0,0596	-0,4288	0,184
recovery (%)	100,00	-23,2455	2,3450	0,0235	-54,51	2971
mass of SDZ-$^{13}C_6$ solution added to sample blend, (*g*)	0,03964	58641,5647	0,00007	0,0018	4,1049	16,85
mass of test material in sample blend, (*g*)	0,70000	-3320,7880	0,00003	0,0000	-0,1070	0,011
mass of SDZ solution added to calibration blend, (*g*)	0,03946	58909,0630	0,00001	0,0003	0,6795	0,462
mass of SDZ-$^{13}C_6$ solution added to calibration blend, (*g*)	0,03980	-58405,8197	0,00001	0,0003	-0,6743	0,455
measured peak area ratio of the selected ions in the sample blend	0,7240	3210,7067	considered to be included in the			
measured peak area ratio of the selected ions in the calibration blend	0,7024	-3309,4414	estimation of method precision			

result (µg/kg)	2324,55
combined standard uncertainty (µg/kg)	57,79
relative standard uncertainty (%)	2,49
effective degrees of freedom	11,1
coverage factor	2,20
expanded uncertainty (µg/kg)	127,2

Enrofloxacin

uncertainty component	value	sensitivity coefficient	standrard uncertainty	relative uncertainty	$C_i \times u_i$	$(C_i \times u_i)^2$
method precision	62,56	1,0000	2,171	0,0347	2,1710	4,713
mass fraction of EFX in the calibration solution, (µg/kg)	1000,80	0,0625	2,397	0,0024	0,1498	0,022
sample moisture content, (g/g)	0,0031	-62,7576	0,0002	0,0596	-0,0115	0,000
recovery (%)	100,00	-0,6256	2,6870	0,0269	-1,68	2,826
mass of EFX-d_5 solution added to sample blend, (g)	0,04142	1510,4755	0,00007	0,0017	0,1057	0,011
mass of test material in sample blend, (g)	0,70000	-89,3770	0,00003	0,0000	-0,0029	0,000
mass of EFX solution added to calibration blend, (g)	0,04550	1375,0306	0,00001	0,0003	0,0161	0,000
mass of EFX-d_5 solution added to calibration blend, (g)	0,04165	-1502,1343	0,00001	0,0003	-0,0174	0,000
measured peak area ratio of the selected ions in the sample blend	0,5963	104,9202	considered to be included in the			
measured peak area ratio of the selected ions in the calibration blend	0,6185	-101,1542	estimation of method precision			

result (µg/kg)	62,56
combined standard uncertainty (µg/kg)	2,75
relative standard uncertainty (%)	4,40
effective degrees of freedom	8,1
coverage factor	2,31
expanded uncertainty (µg/kg)	6,35

GLHK

1. Calculate the peak area ratio (R) of target analyte and its isotope labeled as follows:

$$R = \frac{A_X}{A_{IS}}$$

Where
A_X = peak area of target analyte (quantitative MRM transition)
A_{IS} = peak area of corresponding isotope labeled analyte (quantitative MRM transition)

2. Calculate the mass ratio of target analyte (AmtR) and its isotope labeled internal standard as follows:

$$AmtR = \frac{m_x}{m_{IS}}$$

Where
m_X = mass of target analyte (ng)
m_{IS} = mass of corresponding isotope labeled analyte (ng)

3. Establish a calibration bracket by plotting the peak area ratio (R) versus the mass ratio (AmtR) of the calibration brackets. Obtain the following linear equation from the graph.

$$R = (s)(Amt\,R) + b$$

Where
R = Area ratio of target analyte/isotope labeled analyte (y-axis)
s = slope of the linear equation
AmtR = mass ratio of target analyte/isotope labeled analyte (x-axis)
b = y-intercept

4. Calculate the mass of target analyte in sample (m_{Xspl}) using the following equation:

$$m_{Xspl} = \frac{\left(\frac{A_{Xspl}}{A_{ISspl}}\right) - b}{s} \times m_{ISspl}$$

Where

m_{Xspl} = mass of target analyte in sample (ng)
A_{Xspl} = peak area of target analyte in sample solution (quantitative MRM transition)
A_{ISspl} = peak area of isotope labeled analyte in sample solution (quantitative MRM transition) b = y-intercept of the linear equation as obtained in Clause 3.
s = slope of the linear equation as obtained in Clause 3.
m_{ISspl} = mass of isotope labeled analyte added in the sample (ng)

5. The moisture content (%M) in the sample is calculated as follows:

$$\%M = \frac{W2 - W3}{W2 - W1} \times 100\%$$

Where

W3 = weight of glass vial with sample after drying (g)
W2 = weight of glass vial sample before drying (g)
W1 = weight of glass vial (g)

6. The dry mass correction factor (F_{Dry}) is calculated as follows:

$$F_{Dry} = 1 - \frac{\%M}{100}$$

7. Calculate the moisture corrected mass fraction of target analyte (C_{Xspl}) in sample in ng/g as follows:

$$C_{Xspl} = \frac{m_{Xspl}}{m_{spl} \times F_{Dry}}$$

Where

m_{Xspl} = mass of target analyte in sample (ng) m_{spl} = mass of sample used (g)
F_{Dry} = dry mass correction factor

Uncertainties were estimated based on contribution from four factors: 1) purity of reference material, 2) method

precision, 3) method bias, 4) uncertainty from dried weight determination. Detailed breakdowns are given as follows:

Enrofloxacin (Enro)

Description	*Value x*	*Std. Unc.*	*Rel. Std. Unc.*
RM [u(S)]	1	0.003915	0.003915
Precision [u(P)]	1	0.034276	0.034276
Method Bias [u(B)]	1	0.021497	0.021497
Dried weight [u(D)]	1	0.000132	0.000132

Combined Std. Uncertainty, u(Enro), µg/kg = Dried mass fraction of Enro x$\sqrt{u(S)^2 + u(P)^2 + u(B)^2 + u(D)^2}$

= 59.13×0.040648

= **2.4**

Expanded Uncertainty. U(Enro), µg/kg = u(Enro) × k (where k = coverage factor of 2)

= **4.**

8. The moisture content (%M) in the sample is calculated as follows:

$$\%M = \frac{W2 - W3}{W2 - W1} \times 100\%$$

Where
W3 = weight of glass vial with sample after drying (g)
W2 = weight of glass vial sample before drying (g)
W1 = weight of glass vial (g)

9. The dry mass correction factor (F_{Dry}) is calculated as follows:

$$F_{Dry} = 1 - \frac{\%M}{100}$$

10. Calculate the moisture corrected mass fraction of target analyte (C_{Xspl}) in sample in ng/g as follows:

$$C_{Xspl} = \frac{m_{Xspl}}{m_{spl} \times F_{Dry}}$$

Where
m_{Xspl} = mass of target analyte in sample (ng)
m_{spl} = mass of sample used (g)
F_{Dry} = dry mass correction factor

Uncertainties were estimated based on contribution from four factors: 1) purity of reference material, 2) method precision, 3) method bias, 4) uncertainty from dried weight determination. Detailed breakdowns are given as follows:

Enrofloxacin (Enro)

Description	*Value x*	*Std. Unc.*	*Rel. Std. Unc.*
RM [u(S)]	1	0.003915	0.003915
Precision [u(P)]	1	0.034276	0.034276
Method Bias [u(B)]	1	0.021497	0.021497
Dried weight [u(D)]	1	0.000132	0.000132

Combined Std. Uncertainty, u(Enro), µg/kg = Dried mass fraction of Enro x $\sqrt{u(S)^2 + u(P)^2 + u(B)^2 + u(D)^2}$

= 59.13×0.040648

= **2.4**

Expanded Uncertainty. U(Enro), µg/kg = u(Enro) × k (where k = coverage factor of 2)

= **4.8**

Sulfadiazine (Sulf)

Description	*Value x*	*Std. Unc.*	*Rel. Std. Unc. u(x)*
RM [u(S)]	1	0.003817	0.003817
Precision [u(P)]	1	0.024286	0.024286
Method Bias [u(B)]	1	0.031242	0.031242
Dried weight [u(D)]	1	0.000132	0.000132

Combined Std. Uncertainty, u(Sulf), µg/kg = Dried mass fraction of Sulf x $Ju(S)^2 + u(P)^2 + u(B)^2 + u(D)^2$

= 2409.59×0.039755

= **96**

Expanded Uncertainty. U(Sulf), µg/kg = u(Sulf) × k (where k = coverage factor of 2)

= **192**

INTI (P178)

Rf: (Area enro in std * Concentration E d5 in std)/(Area enro d5 in std*concentration E in std)

Conc in extract (mg/g)= (Area in extract * concentration enro d5 in extract)/(Area enro d5 in extract*Rf)

Conc in CCQM (mg/g)= Conc in extract (mg/g)* massof extract/((mass of reconstituited*mass of CCQM)/(mass CCQM+mass of water added))

Conc in CCQM (ug/kg)= Conc in CCQM (mg/g)*1000(ug/mg)*1000(g/kg)

The components of uncertainty were mass, repetibility and recovery. The coverage factor 2.

NRC-Halifax (P178)

The concentrations of the analytes were determined using the following:

$$W_X = W_Z \times \frac{m_z}{m_{yc}} \times \frac{m_y}{m_x} \times \frac{R'_B}{R'_{BC}} \times F$$

Where:

W_x = mass fraction of the analyte in the sample
W_z = mass fraction of the CRM in the calibration blend
m_z = volume of final extract
m_{yc} = mass of isotope solution added to the calibration blend
m_y = mass of isotope solution added to the sample
m_x = mass of sample
R'_B = peak area ratio of analyte/isotope in sample blend
R'_{BC} = peak area ratio on isotope/analyte in calibration blend
F = dry mass correction factor

The following were used to determine the overall uncertainties:

μ_{std} = relative uncertainties of the certified values of the reference materials
μ_{ci} = relative uncertainties of the analyses of the samples
μ_{dm} = relative uncertainty from Karl Fischer

Relative uncertainties:	IDMS	Karl Fisher	NMIA CRM
Enrofloxacin	0.063	0.00058	0.006
Sulfadiazene	0.057	0.00058	0.004

These were combined using the following formula:

$$\mu = \sqrt{\mu_{std}^2 + \mu_{ci}^2 + \mu_{dm}^2}$$

Final uncertainties were expanded using k=2 (95% confidence)

Appendix IV. Other Information Reported

EXHM

Also analysed sucessfully FAPAS test material 02281 (pig kidney) for SDZ.

HSA

Enrofloxacin and sulfadiazine reference standards from Sigma-Aldrich were purity assessed in-house by quantitative ^{1}H NMR, and were used to spike into the comparison sample for quality control purpose. The quality control sample was measured together with the comparison sample. The recovery results obtained from the quality control samples ranged from 91.7% to 98.6% with an average of 93.9% for enrofloxacin, and from 93.4% to 102.4% with an average of 96.9% for sulfadiazine. The recovery results were found to be well within the measurement uncertainty ranges of the reported results for enrofloxacin (±11.5%) and sulfadiazine (±9.4%).

NMIA

In order to comply with the protocol and initiate moisture determination at the same time as the sampling for definitive analysis, the entire bottle no. 121002 was accurately sampled into 3 x 1 g sub-samples for drying and 16 x 0.5 g sub-samples for analysis. The 0.5 g sub-samples were stored at -80 °C and analysed in four batches over four weeks. Sub-samples were transferred to the fridge the day before analysis to make equilibration to room temperature for weighing easier.

The drying protocol specified continuous vacuum for 21 days. The vacuum pump attached to the desiccator was accidentally turned off for several days during the first two weeks as a result of electrical maintenance work. However, constant mass was observed in the dry weighings taken between 15 and 30 days.

LGC

Due to a low level of moisture determined with the specified protocol, moisture was checked using Karl Fischer and determined to be at (1.511±0.025)% (sealed vials heated to 140 °C)

Final results reported corrected for moisture using the specified protocol.

VNIIM

1) The moisture determination was made as suggested in the Protocol.
2) Evaluation of matrix effects (ion suppression) was carried out by method of post-extraction additions. Ion suppression effect for SDZ was reached 60%. Ion suppression effect for ENR was not observed
3) In the process of measuring the mass fraction of **Sulfadiazine (SDZ)** mixed results were obtained (see below).

For sample preparation and analysis method choosing the Sample № BOTS -1-121015 was taken.

The results of **SDZ** mass fraction in Sample No.BOTS -1-121015 (3 measurements for each of 3 sample aliquots) are given in Table 1

Table 1.

№	**Mass fraction of SDZ, µg/kg**	**Average value of mass fraction, µg/kg**
Sample 1	3666	3459
Sample 2	3680	
Sample 3	3330	

After choosing analysis conditions Sample No.BOTS -1-121009 was taken for determination. The results (3 measurements for each of 5 sample aliquots) are given in Table 2.

Table 2.

№	**Mass fraction of SDZ, µg/kg**	**Average value of mass fraction, µg/kg**
Sample 1	2311	2393
Sample 2	2609	
Sample 3	2653	
Sample 4	2351	
Sample 5	2340	

As you see, the results of Sample No.BOTS -1-121015 and Sample No.BOTS -1-121009 are different by 30%.

The measurements of **SDZ** mass fraction in Sample No.BOTS -1-121009 and Sample No.BOTS -1-121015 were repeated (3 measurements for each of 3 sample aliquots) using exactly the same conditions (scheme, time, hands). The results are in Table 3.

Table 3.

Sample №	**Mass fraction of SDZ, µg/kg**	**Average value of mass fraction, µg/kg**
BOTS -1-121009_1	2368	2380
BOTS -1-121009_2	2393	
BOTS -1-121009_3	2380	
BOTS -1-121015_1	3362	3307
BOTS -1-121015_2	3310	
BOTS -1-121015_3	3250	

After that the Sample NoBOTS -1-121021 was taken for determination. The results are in Table 4 (3 measurements for each of 5 sample aliquots).

Table 4.

№	**Mass fraction of SDZ, µg/kg**	**Average value of mass fraction, µg/kg**
Sample 1	2175	2400
Sample 2	2194	
Sample 3	2518	
Sample 4	2680	
Sample 5	2504	

Conclusion: the results of **SDZ** mass fraction in Sample No.BOTS -1-121021 and Sample No.BOTS -1-121009 **are equal between each other** (within the extended uncertainty of measurements), but both of them are **significantly different** from the result of Sample No.BOTS -1-121015.

GLHK

(i) Suggested protocol for moisture determination was used.

(ii) For reference, the moisture-content-uncorrected analyte contents are given as below:

Enrofloxacin : 58.9 µg/kg

Sulfadiazine : 2399 µg/kg

(iii) The mean moisture content of the sample from the bottle BOTS-1-071009 was found

to be 0.43% (w/w).

INMETRO

No loss of mass was observed after 21 days in desiccator when the method established in the Key Comparison Study Protocol was used. Therefore results expressed in item 2 assumed that the sample has no moisture. However, we have determined the moisture content by coulometric Karl Fischer titration for comparison and the average result was 0.0138 g/g (n=3, standard deviation=0.0005 g/g). Considering the Karl Fischer data for moisture content, the dry mass fractions are $(23.1 \times 10^2 \pm 2.0 \times 10^{2)}$ µg/kg for sulfadiazine and (60.1 ± 5.4) µg/kg for enrofloxacin (k=2).

NIMT

We found that the stock standard solution of enrofloxacin was not stable at 4 °C in a period of 3 months. The stock standard solution of this compound was therefore freshly prepared for each experiment and stored at -20 °C if needed.

BVL

The determination of moisture was performed as described in the Key Comparison Protocol of May 2016 described. The test sample portion of 1 g was placed over anhydrous calcium sulphate in a desiccator at room temperature for 21 days. The mean value for moisture was 0.129 % with a SD of 0.09 %.

The method was validated according to Commission Decision 2002/657/EC. The validation parameters fulfilled the requirements of the Decision.

NRC Ottawa

The moisture content of BOTS-1 was determined via loss on drying in a vacuum dessicator. Four samples (2 g each) were weighed, placed in the vacuum dessicator and re-weighed each week until a constant weight was achieved. The results indicated 0.0049 g/g moisture content in BOTS-1. All BOTS-1 measurand mass fraction results were adjusted to a dry weight basis using this correction factor.

NIM China

Two kinds of extraction solvent were compared during our method development. Method 1 was extracted with 1% formic acid in ACN. Method 2 was extracted with 5% trichloroacetic acid. The result was listed in Table 1. Method 2 was used in the subsequent experiment and the final report.

Table 1.

	BOTS-1-121010			
	Method 1		Method 2	
	enrofloxacin	Sulfadiazine	enrofloxacin	Sulfadiazine
1	61.89	2225.02	65.68	2303.00
2	61.08	2192.54	62.85	2301.29
3	61.35	2243.10	61.78	2371.39
Mean	61.44	2220.22	63.44	2325.23

INTI (P178)

For the moisture determination we used AOAC 950.46 B a)

Results: 0,00622 (g/g) desv std 0,0022

NRC Halifax (P178)

Moisture determination of BOTS-1 and enrofloxacin were both completed using Karl Fisher analyses.

Supporting data obtained through external calibration with native standards and dilutions to remove mitigate matrix effects in ESI.

Appendix V. Core Competency Tables

CCQM-K141	*EXHM*	High polarity analytes in food-Enrofloxacin and Sulfadiazine in Bovine Tissue
Scope of Measurement: Participation in this study would provide the opportunity to demonstrate measurement capabilities including: (a) value assignment of primary reference standards; (b) extraction of analytes of interest from the matrix; (c) cleanup and separation of analytes of interest from other interfering matrix or extract components; (d) separation and quantification using liquid chromatography/mass spectrometry (LC-MS/MS). The study will test the capabilities of participants for assigning mass fractions of high-polarity analytes (pK_{ow} > -2) with the molecular mass range from 200 to 500 from 20-5000 µg/kg in a high fat, high protein matrix (Sector 4 AOAC Int. food triangle).		
Competency	**Tick, cross, 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?		*enrofloxacin* *sulfadiazin*
Identity verification of analyte(s) in calibration material.#		
For calibrants which are a highly-pure substance: Value-Assignment / Purity Assessment method(s).#	✓	*qNMR*
For calibrants which are a calibration solution: Value-assignment method(s).#	✓	*gravimetric*
Sample Analysis Competencies		
Identification of analyte(s) in sample	✓	*rt, ion ratios*
Extraction of analyte(s) of interest from matrix	✓	*enzymatic hydrolysis,* *liquid/liquid extraction, ASE*
Cleanup - separation of analyte(s) of interest from other interfering matrix components (if used)	✓	*centrifugation, dSPE*
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	✓	*IDMS – exact matching*
Verification method(s) for value-assignment of analyte(s) in sample (if used)		
Other		*a QC material from FAPAS was analysed in parallel for SDZ*

CCQM-K141	*GLHK*	High polarity analytes in food-Enrofloxacin and Sulfadiazine in Bovine Tissue
Scope of Measurement: Participation in this study would provide the opportunity to demonstrate measurement capabilities including: (a) value assignment of primary reference standards; (b) extraction of analytes of interest from the matrix; (c) cleanup and separation of analytes of interest from other interfering matrix or extract components; (d) separation and quantification using liquid chromatography/mass spectrometry (LC-MS/MS). The study will test the capabilities of participants for assigning mass fractions of high-polarity analytes (pK_{ow} > -2) with the molecular mass range from 200 to 500 from 20-5000 µg/kg in a high fat, high protein matrix (Sector 4 AOAC Int. food triangle).		
Competency	**Tick, cross, 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?		**NMIA CRM (Sulfadiazine: M317, Enrofloxacin: M747b)**
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, LC-MS/MS with 3 MRM transitions**
Extraction of analyte(s) of interest from matrix	✓	**Liquid/solid extraction with ultrasonic, vertical shaking and vortex mixing**
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	✓	**IDMS – bracketing**
Verification method(s) for value-assignment of analyte(s) in sample (if used)	N/A	
Other		

CCQM-K141	*HSA*	High polarity analytes in food-Enrofloxacin and Sulfadiazine in Bovine Tissue
Scope of Measurement: Participation in this study would provide the opportunity to demonstrate measurement capabilities including: (a) value assignment of primary reference standards; (b) extraction of analytes of interest from the matrix; (c) cleanup and separation of analytes of interest from other interfering matrix or extract components; (d) separation and quantification using liquid chromatography/mass spectrometry (LC-MS/MS). The study will test the capabilities of participants for assigning mass fractions of high-polarity analytes (pK_{ow} > -2) with the molecular mass range from 200 to 500 from 20-5000 µg/kg in a high fat, high protein matrix (Sector 4 AOAC Int. food triangle).		
Competency	**Tick, cross, 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 enrofloxacin CRM (M747b) and pure sulfadiazine CRM (M317) from NMIA were used as calibrants. The certified purity values are traceable to the SI unit for mass (kg).
Identity verification of analyte(s) in calibration material.#	√	LC-MS/MS method was used to identify the analytes in the CRMs from NMIA by comparing the m/z of the parent and daughter ions.
For calibrants which are a highly-pure substance: Value-Assignment / Purity Assessment method(s).#	NA	*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).#	NA	*Indicate how you established analyte mass fraction in calibration solution*
Sample Analysis Competencies		
Identification of analyte(s) in sample	√	LC-MS/MS method was used to identify the analytes in the sample by comparing the retention time and the m/z of the parent and daughter ions with CRMs from NMIA.
Extraction of analyte(s) of interest from matrix	√	After adding 1 mL of water and isotope labelled internal standard solutions, the sample was cooled in an ice bath and 10 mL of 0.1 mol/L HCl in acetonitrile was added. The mixture was removed from the ice bath and was vortexed for 1 min, sonicated for 5 min, then shakened vigorously for 10 min using an orbital shaker. The mixture was then centrifuged at 4,000 rpm for 5 min. The supernatant was transferred to a 50 mL centrifuge tube. The extraction was repeated for three more times using 0.01 mol/L HCl in acetonitrile instead of 0.1 mol/L HCl in acetonitrile without applying ice bath. The supernatants were combined.
Cleanup - separation of analyte(s) of interest from other interfering matrix components (if used)	√	The combined supernatant was evaporated to dryness under nitrogen flow at 35 °C. The residue was reconstituted with 1 mL of 0.01 mol/L HCl in water:acetonitrile (85:15, v/v). The reconstituted solution was transferred into two Amicon Ultra-0.5 centrifugal filter units with Ultracel-3 membrance (0.5 mL each filter), and was centrifuged at 13,000 rpm for 10 min. The clear solution was combined

		andanalysed using LC-MS/MS for enrofloxacin. For sulfadiazine, the combined solution was diluted to about 50 ng/g before analysis.
Transformation - conversion of analyte(s) of interest to detectable/measurable form (if used)	NA	*Indicate chemical transformation method(s), if any, (i.e., hydrolysis, derivatization, other)*
Analytical system	√	LC-MS/MS
Calibration approach for value-assignment of analyte(s) in matrix	√	(a) IDMS method was used. (b) Four-point calibration curve was used.
Verification method(s) for value-assignment of analyte(s) in sample (if used)	√	Enrofloxacin and sulfadiazine reference standards from Sigma-Aldrich were purity assessed in-house by quantitative ^{1}H NMR, and were used to spike into the comparison sample for quality control purpose. The quality control sample was measured together with the comparison sample. The recovery results obtained from the quality control samples ranged from 91.7% to 98.6% with an average of 93.9% for enrofloxacin, and from 93.4% to 102.4% with an average of 96.9% for sulfadiazine. The recovery results were found to be well within the measurement uncertainty ranges of the reported results for enrofloxacin (±11.6%) and sulfadiazine (±10.9%).
Other	NA	*Indicate any other competencies demonstrated.*

CCQM-K141	*NIMT*	High polarity analytes in food-Enrofloxacin and Sulfadiazine in Bovine Tissue
Scope of Measurement: Participation in this study would provide the opportunity to demonstrate measurement capabilities including: (a) value assignment of primary reference standards; (b) extraction of analytes of interest from the matrix; (c) cleanup and separation of analytes of interest from other interfering matrix or extract components; (d) separation and quantification using liquid chromatography/mass spectrometry (LC-MS/MS). The study will test the capabilities of participants for assigning mass fractions of high-polarity analytes (pK_{ow} > -2) with the molecular mass range from 200 to 500 from 20-5000 µg/kg in a high fat, high protein matrix (Sector 4 AOAC Int. food triangle).		
Competency	**Tick, cross, 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 materials, certified reference materials (CRMs) from NMIA, M747b for enrofloxacin and M317 for sulfadiazine*
Identity verification of analyte(s) in calibration material.#	✓	*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*
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)	N/A	*Indicate chemical transformation method(s), if any, (i.e., hydrolysis, derivatization, other)*
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)	-	*Indicate any confirmative method(s) used, if any.*
Other	-	*Indicate any other competencies demonstrated.*

CCQM-K141	*BVL*	High polarity analytes in food-Enrofloxacin and Sulfadiazine in Bovine Tissue
Scope of Measurement: Participation in this study would provide the opportunity to demonstrate measurement capabilities including: (a) value assignment of primary reference standards; (b) extraction of analytes of interest from the matrix; (c) cleanup and separation of analytes of interest from other interfering matrix or extract components; (d) separation and quantification using liquid chromatography/mass spectrometry (LC-MS/MS). The study will test the capabilities of participants for assigning mass fractions of high-polarity analytes (pK_{ow} > -2) with the molecular mass range from 200 to 500 from 20-5000 µg/kg in a high fat, high protein matrix (Sector 4 AOAC Int. food triangle).		
Competency	**Tick, cross, 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 from NMI Australia
Identity verification of analyte(s) in calibration material.#	✓	Verification by LC-QToF
For calibrants which are a highly-pure substance: Value-Assignment / Purity Assessment method(s).#	x	Mass balance approach, In-house verification, organic impurities by LC-QToF
For calibrants which are a calibration solution: Value-assignment method(s).#	N/A	
Sample Analysis Competencies		
Identification of analyte(s) in sample	✓	*LC-MS/MS + LC-QToF (i.e., retention time, mass spec ion ratios by 2 transitions, exact mass)*
Extraction of analyte(s) of interest from matrix	✓	Vortexing, sonication, shaking
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) internal standard (isotopically labelled) b) multi-point matrix calibration curve
Verification method(s) for value-assignment of analyte(s) in sample (if used)	✓	Standard addition
Other	N/A	

BVL's result for enroflaxacin was withdrawn form the KCRV calculation and its DoE value did not cross zero. The cause for this was believed to be improper sample preparation or handling of the reference standard.

CCQM-K141	*UME*	High polarity analytes in food-Enrofloxacin and Sulfadiazine in Bovine Tissue
Scope of Measurement: Participation in this study would provide the opportunity to demonstrate measurement capabilities including: (a) value assignment of primary reference standards; (b) extraction of analytes of interest from the matrix; (c) cleanup and separation of analytes of interest from other interfering matrix or extract components; (d) separation and quantification using liquid chromatography/mass spectrometry (LC-MS/MS). The study will test the capabilities of participants for assigning mass fractions of high-polarity analytes (pK_{ow} > -2) with the molecular mass range from 200 to 500 from 20-5000 µg/kg in a high fat, high protein matrix (Sector 4 AOAC Int. food triangle).		
Competency	**Tick, cross, 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 substances were used* *Sulfadiazine Vetranal, Sigma Aldrich(USA), 100 mg neat* *Enrofloxacin, Dr. Ehrenstorfer (Germany), 0.1 g neat*
Identity verification of analyte(s) in calibration material.#	√	*LC-MS/MS*
For calibrants which are a highly-pure substance: Value-Assignment / Purity Assessment method(s).#	√	*The purity determination of Sulfadiazine (G3OK-K141-RM-1) was performed by qNMR with using 1,3,5-Trimethoxybenzene IS in traceability chain of UME-CRM-1301. The purity is 99.93%, uncertainty is 0.19% at k=2 and 95% confidence level*. *The purity determination of Enrofloxacin (G3OK-K141-RM-2) was performed by qNMR with using maleic acid IS in traceability chain of UME-CRM-1301. The purity is 99.52%, uncertainty is 0.23% at k=2 and 95% confidence level*
For calibrants which are a calibration solution: Value-assignment method(s).#	N/A	-
Sample Analysis Competencies		
Identification of analyte(s) in sample	√	*Retention time* *Parent/product ion*
Extraction of analyte(s) of interest from matrix	√	*Solid/liquid*
Cleanup - separation of analyte(s) of interest from other interfering matrix components (if used)	√	*Liquid/liquid clean-up with n-hexane, centrifugation, filter 0.2 µm*
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) single-point calibration*
CCQM-K141	***INMETRO***	**High polarity analytes in food-Enrofloxacin**

		and Sulfadiazine in Bovine Tissue
Scope of Measurement: Participation in this study would provide the opportunity to demonstrate measurement capabilities including: (a) value assignment of primary reference standards; (b) extraction of analytes of interest from the matrix; (c) cleanup and separation of analytes of interest from other interfering matrix or extract components; (d) separation and quantification using liquid chromatography/mass spectrometry (LC-MS/MS). The study will test the capabilities of participants for assigning mass fractions of high-polarity analytes (pK_{ow} > -2) with the molecular mass range from 200 to 500 from 20-5000 µg/kg in a high fat, high protein matrix (Sector 4 AOAC Int. food triangle).		
Competency	**Tick, cross, 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 substances (sulfadiazine Sigma-Aldrich batch 1448399V, enrofloxacin Fluka batch 1140438) with purity determined in-house*
Identity verification of analyte(s) in calibration material.#	√	*NMR*
For calibrants which are a highly-pure substance: Value-Assignment / Purity Assessment method(s).#	√	*Enrofloxaxin: qNMR using cholesterol Nist SRM 911c as internal standard. Sulfadiazine: combination of qNMR using dimethylsulfone Sigma TraceCERT as internal standard and mass balance using HPLC-DAD, Karl Fischer titration and TGA*
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	√	*Comparison of HPLC retention time with calibrant, mass spectrum ion ratios*
Extraction of analyte(s) of interest from matrix	√	*Two steps of liquid/solid extraction with methanol (room temperature shaking for 20 min)*
Cleanup - separation of analyte(s) of interest from other interfering matrix components (if used)	√	*After drying under N_2 steam, samples were re-suspended with acetic acid 5% and methanol and centrifuged in order to separate some of the interfering matrix components*
Transformation - conversion of analyte(s) of interest to detectable/measurable form (if used)	**X**	
Analytical system	√	*LC-MS/MS*
Calibration approach for value-assignment of analyte(s) in matrix	√	*a) IDMS* *b) bracketed exact matching calibration*
Verification method(s) for value-assignment of analyte(s) in sample (if used)	√	*Results were checked by an independent sample preparation quantified by IDMS with calibration curve rather than exact matching; at the same time, method recovery was assessed with a freeze-dried blank bovine tissue spiked with both sulfadiazine and enrofloxacin*
Other	**X**	

CCQM-K141	*KRISS*	High polarity analytes in food-Enrofloxacin and Sulfadiazine in Bovine Tissue
Scope of Measurement: Participation in this study would provide the opportunity to demonstrate measurement capabilities including: (a) value assignment of primary reference standards; (b) extraction of analytes of interest from the matrix; (c) cleanup and separation of analytes of interest from other interfering matrix or extract components; (d) separation and quantification using liquid chromatography/mass spectrometry (LC-MS/MS). The study will test the capabilities of participants for assigning mass fractions of high-polarity analytes (pK_{ow} > -2) with the molecular mass range from 200 to 500 from 20-5000 µg/kg in a high fat, high protein matrix (Sector 4 AOAC Int. food triangle).		
Competency	**Tick, cross, 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 substances for sulfadiazine and enrofloxacin were purchased from Dr. Ehrenstorfer. The purities for the two calibrants were assayed by KRISS.*
Identity verification of analyte(s) in calibration material.#	√	*LC-MS and LC/UV*
For calibrants which are a highly-pure substance: Value-Assignment / Purity Assessment method(s).#	√	*Mass balance: LC/UV analysis for structurally related impurities, thermo-gravimetric analysis for non-volatile impurities, Karl-Fischer Coulometry for water contents, headspace GC/MS for residual solvents*
For calibrants which are a calibration solution: Value-assignment method(s).#	√	*Gravimetrically prepared 4 mixtures of standard solution and isotope labeled internal standard solution were analyzed and cross checked by LC-MS/MS.*
Sample Analysis Competencies		
Identification of analyte(s) in sample	√	*LC-MS /MS*
Extraction of analyte(s) of interest from matrix	√	*Liquid/liquid extraction with acetonitrile and n-hexane*
Cleanup - separation of analyte(s) of interest from other interfering matrix components (if used)	√	*Oasis MAX SPE cartridge*
Transformation - conversion of analyte(s) of interest to detectable/measurable form (if used)	√	*No transformation*
Analytical system	√	*LC-MS/MS (Waters Acquity I class UPLC/Xevo-TQ-S)*
Calibration approach for value-assignment of analyte(s) in matrix	√	*a) Quantification mode: IDMS* *b) Calibration mode: Standard addition-ID MS method*
Verification method(s) for value-assignment of analyte(s) in sample (if used)	√	*No other method was used for the verification of the results. Instead the method used was validated with fortified blank beef. Beef was purchased from Korea local market and processed to make dried powder form followed by spiking with known amounts of sulfadiazine and enrofloxacin. This sample was used for the verification of the method.*
Other	N/A	

CCQM-K141	*NMIA*	High polarity analytes in food-Enrofloxacin and Sulfadiazine in Bovine Tissue
Scope of Measurement: Participation in this study would provide the opportunity to demonstrate measurement capabilities including: (a) value assignment of primary reference standards; (b) extraction of analytes of interest from the matrix; (c) cleanup and separation of analytes of interest from other interfering matrix or extract components; (d) separation and quantification using liquid chromatography/mass spectrometry (LC-MS/MS). The study will test the capabilities of participants for assigning mass fractions of high-polarity analytes (pK_{ow} > -2) with the molecular mass range from 200 to 500 from 20-5000 µg/kg in a high fat, high protein matrix (Sector 4 AOAC Int. food triangle).		
Competency	**Tick, cross, 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 substance certified reference materials used. – Enrofloxacin certified reference material, NMIA, report ID M747b.2016.01 – Sulfadiazine certified reference material, NMIA, report ID M317.2016.01
Identity verification of analyte(s) in calibration material.#	√	Electrospray LC-MS, ^{1}H-NMR, ^{13}C-NMR, ^{19}F-NMR, IR spectrometry and elemental composition
For calibrants which are a highly-pure substance: Value-Assignment / Purity Assessment method(s).#	√	Mass balance (HPLC/UV, Thermogravimetric analysis, Karl Fischer analysis, headspace GC-MS) and proton qNMR.
For calibrants which are a calibration solution: Value-assignment method(s).#	N/A	
Sample Analysis Competencies		
Identification of analyte(s) in sample	√	Sulfadiazine (SDZ): – Chromatographic retention time (1D and 2D modes). LCMSMS – three SRM transitions monitored in positive ion mode. Ion ratios agree with those in calibrant Enrofloxacin (ENR): – Chromatographic retention time (1D mode). LCMSMS – three SRM transitions monitored in positive ion mode and two transitions monitored negative ion. Ion ratios agree with those in calibrant
Extraction of analyte(s) of interest from matrix	√	Sample (0.5 g) reconstituted with 1mL water. Liquid/solid extraction using 4 x 5 mL acetonitrile / water (70:30) with end-over-end rotation.
Cleanup - separation of analyte(s) of interest from other interfering matrix components (if used)	√	Liquid/liquid extraction with hexane (2 x 3 mL) to remove fats. Solid-phase extraction clean-up of aqueous phase using Oasis HLB.
Transformation - conversion of analyte(s) of interest to detectable/measurable form (if used)	N/A	
Analytical system	√	Sulfadiazine

		- LC-MS/MS (reverse phase UPLC, positive ion electrospray, triple quadrupole with selected reaction monitoring) - LC-MS/MS (heart-cutting dual column reversed phase UPLC, positive ion electrospray, triple quadrupole MS with selected reaction monitoring) ENR - LC-MS/MS (reverse phase UPLC with positive ion electrospray, triple quadrupole MS with selected reaction monitoring) - LC-MS/MS (reverse phase UPLC with negative ion electrospray, triple quadrupole MS with selected reaction monitoring)
Calibration approach for value-assignment of analyte(s) in matrix	√	Exact-matching (single-point calibration) double isotope dilution mass spectrometry with replicate bracketed injections
Verification method(s) for value-assignment of analyte(s) in sample (if used)	√	Concordance within measurement uncertainty for values obtained using multiple different collisionally induced molecular transitions on two different chromatographic systems *Sulfadiazine:* LCMSMS: three SRM transitions monitored in positive ion mode for two different UPLC separation systems: System 1: Waters Acquity BEH C18 100x2 mm column with acetonitrile/aqueous formic acid mobile phase System 2: SDZ peak from System 1 transferred to Restek Pinnacle DB Biphenyl UPLC column and eluted with a methanol gradient. *Enrofloxacin:* LCMSMS three SRM transitions monitored in positive electrospray (ESI) mode and 2 SRM transitions monitored in negative ESI mode. System 1: Waters Acquity 1.7 um BEH C18 column (100 x 2.1 mm ID) column, with acetonitrile/aqueous formic acid mobile phase. Positive ESI. System 2: Waters Acquity 1.7 um BEHC18 column (100 x 1.0 mm ID) with gradient of acetonitrile/water containing 25 mM trimethylamine. Negative ESI.

CCQM-K141	*NRC-Ottawa*	**High polarity analytes in food-Enrofloxacin and Sulfadiazine in Bovine Tissue**
Scope of Measurement: Participation in this study would provide the opportunity to demonstrate measurement capabilities including: (a) value assignment of primary reference standards; (b) extraction of analytes of interest from the matrix; (c) cleanup and separation of analytes of interest from other interfering matrix or extract components; (d) separation and quantification using liquid chromatography/mass spectrometry (LC-MS/MS). The study will test the capabilities of participants for assigning mass fractions of high-polarity analytes (pK_{ow} > -2) with the molecular mass range from 200 to 500 from 20-5000 µg/kg in a high fat, high protein matrix (Sector 4 AOAC Int. food triangle).		
Competency	**Tick, cross, 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 materials were used to prepare calibration solutions for both enrofloxacin and sulfadiazine.* *Enrofloxacin:Sigma Lot BCBK3650V* *Sulfadiazine:Sigma Lot BCBK1734V*
Identity verification of analyte(s) in calibration material.[#]	✓	*LC-MS/MS and LC-HRAM-MS* *NMR*
For calibrants which are a highly-pure substance: Value-Assignment / Purity Assessment method(s).[#]	✓	*qNMR was used as the primary technique to assign mass fraction of the pure substances. Related impurities by HPLC-UV as well as volatiles and ash content by TGA were used as verification techniques.*
For calibrants which are a calibration solution: Value-assignment method(s).[#]	✓	*Analyte mass fraction in calibration solution was assigned via traceable gravimetric preparation of the solutions.*
Sample Analysis Competencies		
Identification of analyte(s) in sample	✓	*Identification of the analytes in the sample was carried out via HPLC retention time, MS/MS monitoring of 2 ion transitions and HRAM to select and monitor the exact mass of the analytes.*
Extraction of analyte(s) of interest from matrix	✓	*The analytes were extracted via a double liquid-solid extraction of the matrix.ACN:IPA:water:80:10:10*
Cleanup - separation of analyte(s) of interest from other interfering matrix components (if used)	✓	*An additional liquid-liquid cleanup was performed using a liquid-liquid extraction with hexane to remove non-polar compounds from the first extraction supernatant*
Transformation - conversion of analyte(s) of interest to detectable/measurable form (if used)	N/A	*No derivatization or any other chemical transformations were employed.*
Analytical system	✓	*1) LC-MS/MS* *2) LC-HRAM-MS*
Calibration approach for value-assignment of analyte(s) in matrix	✓	*a) Isotope dilution MS* *b) ID^{2}MS and SA-ID^{2}MS (2 point)*
Verification method(s) for value-assignment of analyte(s) in sample (if used)	✓	*LC-HRAM-MS was used as a confirmation technique and the data was combined with the LC-MS/MS data which was the primary technique.*
Other	N/A	*N/AP*

CCQM-K141	*LGC*	**High polarity analytes in food-Enrofloxacin and Sulfadiazine in Bovine Tissue**
Scope of Measurement: Participation in this study would provide the opportunity to demonstrate measurement capabilities including: (a) value assignment of primary reference standards; (b) extraction of analytes of interest from the matrix; (c) cleanup and separation of analytes of interest from other interfering matrix or extract components; (d) separation and quantification using liquid chromatography/mass spectrometry (LC-MS/MS). The study will test the capabilities of participants for assigning mass fractions of high-polarity analytes (pK_{ow} > -2) with the molecular mass range from 200 to 500 from 20-5000 µg/kg in a high fat, high protein matrix (Sector 4 AOAC Int. food triangle).		
Competency	**Tick, cross, 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 obtained in bulk from Sigma. In-house characterized by NMR and qNMR.*
Identity verification of analyte(s) in calibration material.#	✓	*NMR*
For calibrants which are a highly-pure substance: Value-Assignment / Purity Assessment method(s).#	✓	*qNMR*
For calibrants which are a calibration solution: Value-assignment method(s).#	✓	*Gravimetric preparation from highly-pure substance*
Sample Analysis Competencies		
Identification of analyte(s) in sample	✓	*Retention time + ion ratio of at least 2 product ions*
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)	✓	*Temperature-induced phase separation/Centrifugation*
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) EM-IDMS* *b) Bracketed double exact matching*
Verification method(s) for value-assignment of analyte(s) in sample (if used)	N/A	
Other	N/A	

CCQM-K141	NIM	High polarity analytes in food-Enrofloxacin and Sulfadiazine in Bovine Tissue
Scope of Measurement: Participation in this study would provide the opportunity to demonstrate measurement capabilities including: (a) value assignment of primary reference standards; (b) extraction of analytes of interest from the matrix; (c) cleanup and separation of analytes of interest from other interfering matrix or extract components; (d) separation and quantification using liquid chromatography/mass spectrometry (LC-MS/MS). The study will test the capabilities of participants for assigning mass fractions of high-polarity analytes (pK_{ow} > -2) with the molecular mass range from 200 to 500 from 20-5000 µg/kg in a high fat, high protein matrix (Sector 4 AOAC Int. food triangle).		
Competency	**Tick, cross, 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?		Enrofloxacin: Pure material, Sigma-Aldrich, 17849, 99.7%±0.4% （k=2） Sulfadiazine: Pure material, NIM, GBW(E)060901, 99.6%±0.4% （k=2）
Identity verification of analyte(s) in calibration material.#	✓	LC-MS/MS, comparison to independent reference material retention time and mass spectrum.
For calibrants which are a highly-pure substance: Value-Assignment / Purity Assessment method(s).#	✓	Mass balance approach and qNMR: LC-UV, LC/MS/MS, GC-FID, Karl-Fischer Titration, ICP-MS, and qNMR method was used for verification.
For calibrants which are a calibration solution: Value-assignment method(s).#	*N/A*	
Sample Analysis Competencies		
Identification of analyte(s) in sample	✓	Analytes identified through comparison against high purity calibrant retention time and mass spectrum ion ratios of 2 independent selected reaction monitoring (SRM) transitions by tandem ESI-MS/MS
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)	✓	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)* IDMS *b)* Single-point calibration
Verification method(s) for value-assignment of analyte(s) in sample (if used)	*N/A*	
Other	*N/A*	

CCQM-K141	*VNIIM*	– High polarity analytes in food- Enrofloxacin and Sulfadiazine in Bovine Tissue
Scope of Measurement: Participation in this study would provide the opportunity to demonstrate measurement capabilities including: (a) value assignment of primary reference standards; (b) extraction of analytes of interest from the matrix; (c) cleanup and separation of analytes of interest from other interfering matrix or extract components; (d) separation and quantification using liquid chromatography/mass spectrometry (LC-MS/MS). The study will test the capabilities of participants for assigning mass fractions of high-polarity analytes (pK_{ow} > -2) with the molecular mass range from 200 to 500 from 20-5000 µg/kg in a high fat, high protein matrix (Sector 4 AOAC Int. food triangle).		
Competency	**Tick, cross, 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 materials from Sigma:* *Sulfadiazine cat. # 35055* *Enrofloxacin cat.# 33699*
Identity verification of analyte(s) in calibration material.#	✓	*LC/MS*
For calibrants which are a highly-pure substance: Value-Assignment / Purity Assessment method(s).#	✓	*The purity of materials is determined in house by mass balance (KF titration with oven; ICP/MS; GC/MS/TD; LC/UV)*
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	✓	*Sonication - Liquid/solid sonication 3x15 min at room temperature* *- AcN for Enrofloxacin extraction (3x3 ml);* *- AcN + 0,1% HCOOH for Sulfadiazine extraction (3x3 ml)*
Cleanup - separation of analyte(s) of interest from other interfering matrix components (if used)	✓	*Defatted by 3 ml of Hexane*
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*
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	*Indicate any confirmative method(s) used, if any.*
Other	N/A	*Indicate any other competencies demonstrated.*

Appendix VI. Information Tables

CCQM-K141/P178	*BVL*	High polarity analytes in food-Enrofloxacin and Sulfadiazine in Bovine Tissue
Additional Information: We would like to collect additional information for enrofloxacin and sulfadiazine native and labelled solutions used for value assignment, including concentrations, dilutions and solvents used in their preparation. Please provide information in the fields below:		
Native Calibration Standard Information		
Reference standard forms		Enrofloxacin: *Free base* Sulfadiazine: *Free base*
Solvent used to prepare stock solution		Enrofloxacin: *MeOH/5 mM NaOH = 50%/50%* Sulfadiazine: *MeOH*
Concentration of stock solution		Enrofloxacin: *1080 µg/g* Sulfadiazine: *1270 µg/g*
Handling of stock solution		Storage conditions: *freezer* Time period between preparation and use: *1 week* Treatment before use: *equilibrate to room temperature and vortex*
Intermediate dilutions of stock solutions (solvent, concentration)		Enrofloxacin: *Water/MeOH = 90%/10%, 103.6 µg/g* Sulfadiazine: *Water/MeOH = 90%/10%, 105.9 µg/g*
Working solutions (solvent, concentration)		Enrofloxacin: *Water/MeOH = 90%/10%, 1.047 µg/g* Sulfadiazine: *Water/MeOH = 90%/10%, 10.66 µg/g*
Isotopically-labelled Internal Standard Information (modify first three rows if alternative internal standards used)		
Internal standard forms		Enrofloxacin-d5: *HI salt* Sulfadiazine-13C6: *Free base*
Solvent used to prepare Internal Standard solution		Enrofloxacin-d5: *MeOH/5 mM NaOH 50%/50%* Sulfadiazine-13C6: *MeOH,*
Concentration of Internal Standard solution		Enrofloxacin-d5: *14.6 µg/g (13.5 µg/ml) HI salt* Sulfadiazine-13C6: *126 µg/g (100 µg/ml) free base*
Intermediate dilutions of Internal standard solution (solvent, concentration)		Enrofloxacin-d5: *Water/MeOH = 90%/10%, 0.97 µg/g free base* Sulfadiazine-13C6*: Water/MeOH = 90%/10%, 9.62 µg/g free base*
Internal Standard Spiking solutions (solvent, concentration)		Enrofloxacin-d5: *Water/MeOH = 90%/10%, 0.97 µg/g free base* Sulfadiazine-13C6: *Water/MeOH = 90%/10%, 9.62 µg/g free base*
Final calibration solution and sample extract information		
Details of calibration solutions , i.e. native and internal standard blends, as injected into MS (concentrations, solvent)		Enrofloxacin: 9.86 - 155 *ng/0.5g lyoph. Sample (6 point matrix calibration curve)* Enrofloxacin-d5: 48.5 *ng/0.5g lyoph. sample* Solvent: e.g. *Water/ACN = 90%/10% (0.1 % formic acid)* Sulfadiazine: *101 – 1584 ng/0.5g lyoph. Sample (6 point*

		matrix calibration curve) Sulfadiazine-13C6: *481 ng/0.5g lyoph. sample* Solvent: *Water/ACN = 90%/10% (0.1 % formic acid)*
Solvent for bovine tissue sample extracts as injected into MS		Enrofloxacin: *Water/ACN = 90%/10% (0.1 % formic acid)* Sulfadiazine: *Water/ACN = 90%/10% (0.1 % formic acid)*

CCQM-K141/P178	***EXHM***	**High polarity analytes in food-Enrofloxacin and Sulfadiazine in Bovine Tissue**
Additional Information: We would like to collect additional information for enrofloxacin and sulfadiazinenative and labelled solutions used for value assignment, including concentrations, dilutions and solvents used in their preparation. Please provide information in the fields below:		
NativeCalibration Standard Information		
Reference standard forms		Enrofloxacin: *Free base* Sulfadiazine: *Free base*
Solvent used to prepare stock solution		Enrofloxacin: *in MeOH* Sulfadiazine: *in MeOH*
Concentration of stocksolution		Enrofloxacin: *3248 µg/g* Sulfadiazine: *2424 µg/g*
Handling of stock solution		Storage conditions: *freezer* Time period between preparation and use: *two (2) days* Treatment before use: *equilibrate to room temperature and vortex*
Intermediate dilutions of stock solutions (solvent, concentration)		Enrofloxacin: *MeCN, 188.6 µg/g, then dilution to 7.3 µg/g* Sulfadiazine: *MeCN, 151.7 µg/g*
Working solutions (solvent, concentration)		Enrofloxacin: *MeCN,1.0 µg/g* Sulfadiazine: *MeCN, 40.0 µg/g*
Isotopically-labelled Internal Standard Information (modify first three rows if alternative internal standards used)		
Internal standard forms		Enrofloxacin-d5: *provided by NRC* Sulfadiazine-13C6: *provided by NRC*
Solvent used to prepare Internal Standard solution		Enrofloxacin-d5: *provided by NRC* Sulfadiazine-13C6: *provided by NRC*
Concentration of Internal Standard solution		Enrofloxacin-d5: *~ 13.5 µg/mL (as provided by NRC)* Sulfadiazine-13C6: *~ 100 µg/mL (as provided by NRC)*
Intermediate dilutions of Internal standard solution (solvent, concentration)		Enrofloxacin-d5: Sulfadiazine-13C6:
Internal Standard Spiking solutions (solvent, concentration)		Enrofloxacin-d5: *~1 µg/mL MeOH/5 mM NaOH 90%/10%* Sulfadiazine-13C6: *~40 µg/mL MeOH,*
Final calibration solution and sample extract information		
Details of calibration solutions , i.e. native and internal standard blends, as injected into MS (concentrations,		Enrofloxacin: *~ 2 ng/mL* Enrofloxacin-d5: *~ 2 ng/mL*

solvent) *Calibration was done in matrix matched solutions from extracted blank samples*		Solvent: *extract from blank bovine meat [75% (MeCN with 5% formic acid, 25%(0,1 M tris buffer pH8)]* Sulfadiazine: *73.7 ng/mL* Sulfadiazine-13C6: *~ 80 ng/mL* Solvent: *extract from blank bovine meat [75% (MeCN with 5% formic acid, 25%(0,1 M tris buffer pH8)]*
Solvent for bovine tissue sample extracts as injected into MS		Enrofloxacin: *[75% (MeCN with 5% formic acid, 25%(0,1 M tris buffer pH8)]* Sulfadiazine: *[75% (MeCN with 5% formic acid, 25%(0,1 M tris buffer pH8)]*

CCQM-K141/P178	***GLHK***	**High polarity analytes in food-Enrofloxacin and Sulfadiazine in Bovine Tissue**
Additional Information: We would like to collect additional information for enrofloxacin and sulfadiazine native and labelled solutions used for value assignment, including concentrations, dilutions and solvents used in their preparation. Please provide information in the fields below:		
Native Calibration Standard Information		
Reference standard forms		Enrofloxacin: Enrofloxacin (free base) Sulfadiazine: Sulfadiazine (free base)
Solvent used to prepare stock solution		Enrofloxacin: 2% NH3 in MeOH Sulfadiazine: 2% NH3 in MeOH
Concentration of stock solution		Enrofloxacin: ~ 1.5 mg/g Sulfadiazine: ~ 1.2 mg/g
Handling of stock solution		Storage conditions: Refrigerator, 4 °C Time period between preparation and use: ~ 2 - 3 days Treatment before use: Equilibrate to room temperature for at least 3 – 4 hours. Vortex the solution thoroughly before use.
Intermediate dilutions of stock solutions (solvent, concentration)		Enrofloxacin: ~ 17, 54 and 275 μg/g in 50% MeOH in H2O Sulfadiazine: ~ 40 and 200 μg/g in 50% MeOH in H2O
Working solutions (solvent, concentration)		Enrofloxacin: 1600 ng/g in 10mM ammonium formate in 0.1% FA in MeOH : 0.1% FA in H2O (1:9) Sulfadiazine: 12500 ng/g in 10mM ammonium formate in 0.1% FA in MeOH : 0.1% FA in H2O (1:9)
Isotopically-labelled Internal Standard Information (modify first three rows if alternative internal standards used)		
Internal standard forms		Enrofloxacin-d5: Enrofloxacin-d5 HCl salt Sulfadiazine-13C6: Sulfadiazine-13C6 (free base)
Solvent used to prepare Internal Standard solution		Enrofloxacin-d5: 2% NH3 in MeOH Sulfadiazine-13C6: 2% NH3 in MeOH

Concentration of Internal Standard solution		Enrofloxacin-d5: ~ 0.7 mg/g Sulfadiazine-13C6: ~ 1.2 mg/g
Intermediate dilutions of Internal standard solution (solvent, concentration)		Enrofloxacin-d5: ~ 2.3, 12 and 60 μg/g in 50% MeOH in H2O Sulfadiazine-13C6: ~ 30 and 210 μg/g in 50% MeOH in H2O
Internal Standard Spiking solutions (solvent, concentration)		Enrofloxacin-d5: ~ 220 ng/g in 10mM ammonium formate in 0.1% FA in MeOH : 0.1% FA in H2O (1:9) Sulfadiazine-13C6: ~ 7330 ng/g in 10mM ammonium formate in 0.1% FA in MeOH : 0.1% FA in H2O (1:9)
Final calibration solution and sample extract information		
Details of calibration solutions , i.e. native and internal standard blends, as injected into MS (concentrations, solvent)		Enrofloxacin: 41, 51,57,62,72 and 82 ng/g Enrofloxacin-d5: 60 ng/g Solvent: 5mM EDTA and 10mM Ammonium formate in 0.1% FA in MeOH/0.1% FA in H2O (1:9) Sulfadiazine: 1650, 1962, 2273, 2582, 2999 and 3316 ng/g Sulfadiazine-13C6: 2572 ng/g Solvent: 5mM EDTA and 10mM Ammonium formate in 0.1% FA in MeOH/0.1% FA in H2O (1:9)
Solvent for bovine tissue sample extracts as injected into MS		Enrofloxacin: 5mM EDTA and 10mM Ammonium formate in 0.1% FA in MeOH/0.1% FA in H2O (1:9) Sulfadiazine: 5mM EDTA and 10mM Ammonium formate in 0.1% FA in MeOH/0.1% FA in H2O (1:9)

CCQM-K141/P178	***HSA***	**High polarity analytes in food-Enrofloxacin and Sulfadiazine in Bovine Tissue**
Additional Information: We would like to collect additional information for enrofloxacin and sulfadiazine native and labelled solutions used for value assignment, including concentrations, dilutions and solvents used in their preparation. Please provide information in the fields below:		
Native Calibration Standard Information		
Reference standard forms		Enrofloxacin: *Free base* Sulfadiazine: *Free base*
Solvent used to prepare stock solution		Enrofloxacin: *0.01 M HCl in water* Sulfadiazine: *0.01 M HCl in water:ACN=85:15*
Concentration of stock solution		Enrofloxacin: *~ 2500 μg/g* Sulfadiazine: *~ 160 μg/g*
Handling of stock solution		Storage conditions: *freezer, -20 ºC* Time period between preparation and use: one day Treatment before use: *equilibrate to room temperature and*

		vortex
Intermediate dilutions of stock solutions (solvent, concentration)		Enrofloxacin: *0.01 M HCl in* water, *~ 24 µg/g* Sulfadiazine: *NA*
Working solutions (solvent, concentration)		Enrofloxacin: *0.01 M HCl in* water, *0.1 µg/g* Sulfadiazine: *0.01 M HCl in water:ACN=85:15, 3 µg/g*
Isotopically-labelled Internal Standard Information (modify first three rows if alternative internal standards used)		
Internal standard forms		Enrofloxacin-d5: *HI salt* Sulfadiazine-13C6: *Free base*
Solvent used to prepare Internal Standard solution		Enrofloxacin-d5: *0.01 M HCl in water* Sulfadiazine-13C6: *0.01 M HCl in water:ACN=85:15*
Concentration of Internal Standard solution		Enrofloxacin-d5: *~120 µg/g* *Sulfadiazine-13C6: ~160 µg/g*
Intermediate dilutions of Internal standard solution (solvent, concentration)		Enrofloxacin-d5: *0.01 M HCl in water, ~5µg/g* Sulfadiazine-13C6*: NA*
Internal Standard Spiking solutions (solvent, concentration)		Enrofloxacin-d5: *0.01 M HCl in water, 0.092 µg/g ~ 0.1µg/g* Sulfadiazine-13C6: *0.01 M HCl in water:ACN=85:15, ~3µg/g*
Final calibration solution and sample extract information		
Details of calibration solutions , i.e. native and internal standard blends, as injected into MS (concentrations, solvent)		Enrofloxacin: ~ 0.05 *µg/g* Enrofloxacin-d5: *~ 0.05 µg/g* Solvent: *0.01 M HCl in water:ACN=85:15* Sulfadiazine: *~ 0.07 µg/g* Sulfadiazine-13C6: *~ 0.07 µg/g* Solvent: *0.01 M HCl in water:ACN=85:15*
Solvent for bovine tissue sample extracts as injected into MS		Enrofloxacin: *0.01 M HCl in water:ACN=85:15* Sulfadiazine: *0.01 M HCl in water:ACN=85:15*

CCQM-K141/P178	***Inmetro***	**High polarity analytes in food-Enrofloxacin and Sulfadiazine in Bovine Tissue**
Additional Information: We would like to collect additional information for enrofloxacin and sulfadiazine native and labelled solutions used for value assignment, including concentrations, dilutions and solvents used in their preparation. Please provide information in the fields below:		
Native Calibration Standard Information		
Reference standard forms		Enrofloxacin: *Free base* Sulfadiazine: *Free base*
Solvent used to prepare stock solution		Enrofloxacin: *1 mM NaOH in Methanol* Sulfadiazine: *Acetone*
Concentration of stock solution		Enrofloxacin: *0.136 mg/g* Sulfadiazine: *1.24 mg/g*

Handling of stock solution		Storage conditions: *freezer (-20 °C)* Time period between preparation and use: *2 weeks* Treatment before use: *equilibrate to room temperature and vortex*
Intermediate dilutions of stock solutions (solvent, concentration)		Enrofloxacin: *N/A* Sulfadiazine: *N/A*
Working solutions (solvent, concentration)		Enrofloxacin: *H_2O, 0.147 µg/g* Sulfadiazine: *H_2O, 5.05 µg/g*
Isotopically-labelled Internal Standard Information (modify first three rows if alternative internal standards used)		
Internal standard forms		Enrofloxacin-d5: *HI salt* Sulfadiazine-13C6: *Free base*
Solvent used to prepare Internal Standard solution		Enrofloxacin-d5: *MeOH/50 mM NaOH 50%/50%* Sulfadiazine-13C6: *MeOH,*
Concentration of Internal Standard solution		Enrofloxacin-d5: 10.8 µg/g Sulfadiazine-13C6: 126 µg/g
Intermediate dilutions of Internal standard solution (solvent, concentration)		Enrofloxacin-d5: *N/A* Sulfadiazine-13C6: *N/A*
Internal Standard Spiking solutions (solvent, concentration)		Enrofloxacin-d5: *H_2O, 0.459 µg/g* Sulfadiazine-13C6: *H_2O, 17.1 µg/g*
Final calibration solution and sample extract information		
Details of calibration solutions , i.e. native and internal standard blends, as injected into MS (concentrations, solvent)		Enrofloxacin: *0.0668 µg/g in freeze-dried bovine tissue* Enrofloxacin-d5: *0.0672 µg/g in freeze-dried bovine tissue* Solvent: *acetic acid 5 % in water: methanol (80:20 v/v)* Sulfadiazine: 2.29 *µg/g in freeze-dried bovine tissue* Sulfadiazine-13C6: 2.50 *µg/g in freeze-dried bovine tissue* Solvent: *acetic acid 5 % in water: methanol (80:20 v/v)*
Solvent for bovine tissue sample extracts as injected into MS		Enrofloxacin: *acetic acid 5 % in water: methanol (80:20 v/v)* Sulfadiazine: *acetic acid 5 % in water: methanol (80:20 v/v)*

CCQM-K141/P178	***LGC***	**High polarity analytes in food-Enrofloxacin and Sulfadiazine in Bovine Tissue**
Additional Information: We would like to collect additional information for enrofloxacin and sulfadiazine native and labelled solutions used for value assignment, including concentrations, dilutions and solvents used in their preparation. Please provide information in the fields below:		
Native Calibration Standard Information		
Reference standard forms		Enrofloxacin: free base Sulfadiazine: free base
Solvent used to prepare stock solution		Enrofloxacin: methanol with 0.1 % (v/v) NaOH 1 M

		Sulfadiazine: methanol
Concentration of stock solution		Enrofloxacin: 1195 mg/kg Sulfadiazine: 405 mg/kg
Handling of stock solution		Storage conditions: 4 °C, darkness. Time period between preparation and use: 6 days maximum for reported samples Treatment before use: equilibrate to room temperature
Intermediate dilutions of stock solutions (solvent, concentration)		Enrofloxacin: 0.14 mg/kg in acetonitrile Sulfadiazine: NA
Working solutions (solvent, concentration)		Enrofloxacin: 0.135 mg/kg in acetonitrile Sulfadiazine: 5.7 mg/kg in acetonitrile
Isotopically-labelled Internal Standard Information (modify first three rows if alternative internal standards used)		
Internal standard forms		Enrofloxacin-d5: hydrochloride Sulfadiazine-13C6: free base
Solvent used to prepare Internal Standard solution		Enrofloxacin-d5: methanol with 0.1 % (v/v) NaOH 1 M Sulfadiazine-13C6: methanol
Concentration of Internal Standard solution		Enrofloxacin-d5: 206 mg/kg Sulfadiazine-13C6: 270 mg/kg
Intermediate dilutions of Internal standard solution (solvent, concentration)		Enrofloxacin-d5: 5.5 mg/kg in acetonitrile Sulfadiazine-13C6: NA
Internal Standard Spiking solutions (solvent, concentration)		Enrofloxacin-d5: 0.135 mg/kg in acetonitrile Sulfadiazine-13C6: 5.7 mg/kg in acetonitrile
Final calibration solution and sample extract information		
Details of calibration solutions , i.e. native and internal standard blends, as injected into MS (concentrations, solvent)		Enrofloxacin: about 11 µg/L Enrofloxacin-d5: e.g. about 11 µg/L Solvent: methanol/water (2/8, v/v, matrix matched using blank beef muscle extracts) Sulfadiazine: about 450 µg/L Sulfadiazine-13C6: about 450 µg/L Solvent: methanol/water (2/8, v/v, matrix matched using blank beef muscle extracts)
Solvent for bovine tissue sample extracts as injected into MS		Enrofloxacin: methanol/water (2/8, v/v) Sulfadiazine: methanol/water (2/8, v/v)

CCQM-K141/P178	***NIM***	**High polarity analytes in food-Enrofloxacin and Sulfadiazine in Bovine Tissue**
Additional Information: We would like to collect additional information for enrofloxacin and sulfadiazine native and labelled solutions used for value assignment, including concentrations, dilutions and solvents used in their preparation. Please provide information in the fields below:		
Native Calibration Standard Information		

Reference standard forms		Enrofloxacin: *Free base* Sulfadiazine: *Free base*
Solvent used to prepare stock solution		Enrofloxacin: *MeOH,* Sulfadiazine: *MeOH*
Concentration of stock solution		Enrofloxacin: *633 µg/g* Sulfadiazine: *626 µg/g*
Handling of stock solution		Storage conditions: *freezer -20°C* Time period between preparation and use: *<1 month* Treatment before use: *equilibrate to room temperature and vortex*
Intermediate dilutions of stock solutions (solvent, concentration)		Enrofloxacin: *MeOH, 9.92 µg/g* Sulfadiazine: *MeOH, 9.90 µg/g*
Working solutions (solvent, concentration)		Enrofloxacin: *MeOH 0.279 µg/g* Sulfadiazine: *MeOH 0.500 µg/g*
Isotopically-labelled Internal Standard Information (modify first three rows if alternative internal standards used)		
Internal standard forms		Enrofloxacin-d5: *HCl salt* Sulfadiazine-13C6: *Free base*
Solvent used to prepare Internal Standard solution		Enrofloxacin-d5: *MeOH* Sulfadiazine-13C6: *MeOH,*
Concentration of Internal Standard solution		Enrofloxacin-d5: *654 µg/g* Sulfadiazine-13C6: *593 µg/g*
Intermediate dilutions of Internal standard solution (solvent, concentration)		Enrofloxacin-d5: *MeOH, 10.1 µg/g* Sulfadiazine-13C6: *MeOH, 10.1 µg/g*
Internal Standard Spiking solutions (solvent, concentration)		Enrofloxacin-d5: *MeOH, 0.292µg/g* Sulfadiazine-13C6: *MeOH, 10.1µg/g*
Final calibration solution and sample extract information		
Details of calibration solutions , i.e. native and internal standard blends, as injected into MS (concentrations, solvent)		Enrofloxacin: *0.0154 µg/g* Enrofloxacin-d5: *0.0162 µg/g* Solvent: *0.1% formic acid in Water/MeOH 90%/10%* Sulfadiazine: *0.0219 µg/g* Sulfadiazine-13C6: *0.0200µg/g* Solvent: *0.1% formic acid in Water/MeOH 90%/10%*
Solvent for bovine tissue sample extracts as injected into MS		Enrofloxacin: *0.1% formic acid in Water/MeOH 90%/10%* Sulfadiazine: *0.1% formic acid in Water/MeOH 90%/10%*

CCQM-K141/P178	***NIMT***	**High polarity analytes in food-Enrofloxacin and Sulfadiazine in Bovine Tissue**
Additional Information: We would like to collect additional information for enrofloxacin and sulfadiazinenative and labelled solutions used for value assignment, including concentrations, dilutions and solvents used in their preparation. Please provide information in the fields below:		
NativeCalibration Standard Information		

Reference standard forms		Enrofloxacin: *HCl salt* Sulfadiazine:
Solvent used to prepare stock solution		Methanol
Concentration of stocksolution		Enrofloxacin: *520µg/g* Sulfadiazine: 518 *µg/g*
Handling of stock solution		Storage conditions: *at -20 °C* Time period between preparation and use: *1 week* Treatment before use: *equilibrate to room temperature and vortex*
Intermediate dilutions of stock solutions (solvent, concentration)		Enrofloxacin: *MeOH, 4.0µg/g* Sulfadiazine: *MeOH, 3.4µg/g*
Working solutions (solvent, concentration)		Enrofloxacin: *MeOH, 0.150µg/g* Sulfadiazine: *MeOH, 3.4µg/g*
Isotopically-labelled Internal Standard Information (modify first three rows if alternative internal standards used)		
Internal standard forms		Enrofloxacin-d5: *HCl salt* Sulfadiazine-13C6:
Solvent used to prepare Internal Standard solution		Enrofloxacin-d5: *MeOH* Sulfadiazine-13C6: *MeOH,*
Concentration of Internal Standard solution		Enrofloxacin-d5: 140 µg/g Sulfadiazine-13C6: 170 µg/g
Intermediate dilutions of Internal standard solution (solvent, concentration)		Enrofloxacin-d5: *MeOH, 4.3 µg/g* Sulfadiazine-13C6: 3.2 µg/g
Internal Standard Spiking solutions (solvent, concentration)		Enrofloxacin-d5: *MeOH, 0.152 µg/g* Sulfadiazine-13C6: 3.2 µg/g
Final calibration solution and sample extract information		
Details of calibration solutions , i.e. native and internal standard blends, as injected into MS (concentrations, solvent)		Enrofloxacin: *0.150 µg/g* Enrofloxacin-d5: *0.152 µg/g* Solvent: 0.1% formic acid in water/0.1% formic acid in acetonitrile (9:1) 0.8 mL Sulfadiazine: 3.4 *µg/g* Sulfadiazine-13C6: 3.2 *µg/g* Solvent: 0.1% formic acid in water/0.1% formic acid in acetonitrile (9:1) 0.8 mL
Solvent for bovine tissue sample extracts as injected into MS		Enrofloxacin: 0.1% formic acid in water/0.1% formic acid in acetonitrile (9:1) 0.8 mL Sulfadiazine: 0.1% formic acid in water/0.1% formic acid in acetonitrile (9:1) 0.8 mL

CCQM-K141/P178	*NMIA*	High polarity analytes in food-Enrofloxacin and Sulfadiazine in Bovine Tissue

Additional Information: We would like to collect additional information for enrofloxacin and sulfadiazine native and labelled solutions used for value assignment, including concentrations, dilutions and solvents used in their preparation. Please provide information in the fields below:

Native Calibration Standard Information	
Reference standard forms	Enrofloxacin: Free base (NMIA, M747b.2016.01) Sulfadiazine: Free base (NMIA, M317.2016.01)
Solvent used to prepare stock solution	Enrofloxacin: 1mM NaOH in MeOH (prep: add 0.5 mL of 0.8 M NaOH (aq) to 400 mL MeOH) Sulfadiazine: MeOH
Concentration of stock solution	Enrofloxacin: 505.2 ug/g Sulfadiazine: 690.7 ug/g
Handling of stock solution	Storage conditions: Fridge 4 °C Time period between preparation and use: SDZ: 9 days (prepared 29/11/2016, diluted 7/12/2016) ENR: 8 days (prepared 30/11/2016, diluted 7/12/2016) Treatment before use: equilibrate to room temperature and vortex for 1 hour
Intermediate dilutions of stock solutions (solvent, concentration)	Enrofloxacin: 11.94 ug/g (diluent: 1mM NaOH in MeOH) Sulfadiazine: 57.22 ug/g (diluent: MeOH)
Working solutions (solvent, concentration)	Enrofloxacin: 0.122 ug/g (diluent: 10% MeOH in 1 mM aq NaOH) Sulfadiazine: 3.76 ug/g (diluent: 10% MeOH in 1 mM aq NaOH) Intermediate solutions equilibrated to room temperature and vortexed for 1 hour before dilution.

Isotopically-labelled Internal Standard Information (modify first three rows if alternative internal standards used)	
Internal standard forms	Enrofloxacin-d5: hydrochloride (Witega, CH005-25) Sulfadiazine-13C6: 100 ug/mL in MeOH, solution supplied by NRC
Solvent used to prepare Internal Standard solution	Enrofloxacin-d5: MeOH Sulfadiazine-13C6:100 ug/mL in MeOH (as received)
Concentration of Internal Standard solution	Enrofloxacin-d5: 117.5 ug/g Sulfadiazine-13C6: 100 ug/mL in MeOH (stored at -20°C as per study instructions until use, then equilibrated to Rt and vortexed before dilution)
Intermediate dilutions of Internal standard solution (solvent, concentration)	Enrofloxacin-d5: 4.99 ug/g (diluent: 1mM NaOH, 10% MeOH in H_2O) Sulfadiazine-13C6: No intermediate dilution
Internal Standard Spiking solutions (solvent, concentration)	Enrofloxacin-d5: 0.113 ug/g (diluent: 1mM NaOH, 10% ACN in H_2O) Sulfadiazine-13C6: 3.76 ug/g (diluent: 1mM NaOH, 10% ACN in H_2O)

Final calibration solution and sample extract information	
Details of calibration solutions , i.e. native and internal standard blends, as injected into MS (concentrations, solvent)	Enrofloxacin: 0.005 ug/g Enrofloxacin-d5: 0.005 ug/g Solvent: 1 mM NaOH, 0.9% MeOH, 9.1 % ACN in H_2O Sulfadiazine: 0.17 ug/g Sulfadiazine-13C6: 0.17 ug/g Solvent: 1 mM NaOH, 0.9% MeOH, 9.1 % ACN in H_2O
Solvent for bovine tissue sample extracts as injected into MS	Enrofloxacin: 10% ACN in 1 mM aqueous NaOH, Sulfadiazine: 10% ACN in 1 mM aqueous NaOH,

CCQM-K141/P178	***VNIIM***	**High polarity analytes in food-Enrofloxacin and Sulfadiazine in Bovine Tissue**
Additional Information: We would like to collect additional information for enrofloxacin and sulfadiazinenative and labelled solutions used for value assignment, including concentrations, dilutions and solvents used in their preparation. Please provide information in the fields below:		
NativeCalibration Standard Information		
Reference standard forms		Enrofloxacin: *e.g. Free base* Sulfadiazine: *Free base*
Solvent used to prepare stock solution		Enrofloxacin: *MeOH:H_2O:NaOH(0,1N)=50:50:0,25(v/v/v)* Sulfadiazine: *MeOH*
Concentration of stocksolution		Enrofloxacin: *146µg/g* Sulfadiazine: *126µg/g*
Handling of stock solution		Storage conditions: *+4ºC* Time period between preparation and use: *24 h* Treatment before use: *equilibrating to room temperature*
Intermediate dilutions of stock solutions (solvent, concentration)		
Working solutions (solvent, concentration)		Enrofloxacin: *MeOH:H_2O:NaOH(0,1N)=50:50:0,25(v/v/v)* *14,6 µg/g* Sulfadiazine: *MeOH, 12,6 µg/g*
Isotopically-labelled Internal Standard Information (modify first three rows if alternative internal standards used)		
Internal standard forms		Enrofloxacin-d5: *HI salt* Sulfadiazine-13C6: *Free base*

Solvent used to prepare Internal Standard solution		Enrofloxacin-d5: e.g. *MeOH/50 mM NaOH 50%/50%* Sulfadiazine-13C6:e.g. *MeOH*
Concentration of Internal Standard solution		Enrofloxacin-d5: e.g. 14,6 µg/g Sulfadiazine-13C6: 126 µg/g
Intermediate dilutions of Internal standard solution (solvent, concentration)		
Internal Standard Spiking solutions (solvent, concentration)		Enrofloxacin-d5: e.g. 14,6 µg/g Sulfadiazine-13C6: 126 µg/g
Final calibration solution and sample extract information		
Details of calibration solutions , i.e. native and internal standard blends, as injected into MS (concentrations, solvent)		Enrofloxacin: *0.03 µg/g* Enrofloxacin-d5: *0.035 µg/g* Solvent: *MeOH:H_2O:NaOH(0,1N)=50:50:0,25(v/v/v)* Sulfadiazine: *5 µg/g* Sulfadiazine-13C6: *5 µg/g* Solvent: *MeOH*
Solvent for bovine tissue sample extracts as injected into MS		Enrofloxacin: *ACN** Sulfadiazine: *ACN:HCOOH = 1000:1 (v/v)**

CCQM-K141/P178	***NRC-Ottawa***	**High polarity analytes in food-Enrofloxacin and Sulfadiazine in Bovine Tissue**
Additional Information: We would like to collect additional information for enrofloxacin and sulfadiazine native and labelled solutions used for value assignment, including concentrations, dilutions and solvents used in their preparation. Please provide information in the fields below:		
Native Calibration Standard Information		
Reference standard forms		Enrofloxacin: *Free base* Sulfadiazine: *Free base*
Solvent used to prepare stock solution		Enrofloxacin: *MeOH* Sulfadiazine: *MeOH*
Concentration of stock solution		Enrofloxacin: *126 µg/g* Sulfadiazine: *630 µg/g*
Handling of stock solution		Storage conditions: *-20°C freezer* Time period between preparation and use: *350 days* Treatment before use: *equilibrate to room temperature and vortex*
Intermediate dilutions of stock solutions (solvent, concentration)		Enrofloxacin: *N/AP* Sulfadiazine: *N/AP*
Working solutions (solvent, concentration)		Enrofloxacin: *MeOH:water ; 50:50, 0.332 µg/g* Sulfadiazine: *MeOH:water ; 50:50, 11.9 µg/g*
Isotopically-labelled Internal Standard Information (modify first three rows if alternative internal standards used)		
Internal standard forms		Enrofloxacin-d5: *HI salt* Sulfadiazine-13C6: *Free base*

Solvent used to prepare Internal Standard solution		Enrofloxacin-d5: *MeOH:50 mM NaOH ; 50:50* Sulfadiazine-13C6: *MeOH,*
Concentration of Internal Standard solution		Enrofloxacin-d5: *108 µg/g* Sulfadiazine-13C6: *632 µg/g*
Intermediate dilutions of Internal standard solution (solvent, concentration)		Enrofloxacin-d5: *N/AP* Sulfadiazine-13C6*: N/AP*
Internal Standard Spiking solutions (solvent, concentration)		Enrofloxacin-d5: *MeOH:water ; 50:50, 0.363 µg/g* Sulfadiazine-13C6: *MeOH:water ; 50:50, 11.4 µg/g*
Final calibration solution and sample extract information		
Details of calibration solutions , i.e. native and internal standard blends, as injected into MS (concentrations, solvent)		Enrofloxacin: 0.032 µg/g Enrofloxacin-d5: 0.035 µg/g Solvent: Water:MeOH:formic acid ; 90:10:0.1 Sulfadiazine: *0.011 µg/g* Sulfadiazine-13C6: *0.011 µg/g* Solvent: *MeOH:water ; 50:50*
Solvent for bovine tissue sample extracts as injected into MS		Enrofloxacin: Water:MeOH ; 90:10 Sulfadiazine: *MeOH:water ; 50:50*

CCQM-K141/P178	*NRC-Hfx-P178*	High polarity analytes in food- Enrofloxacin and Sulfadiazine in Bovine Tissue
Additional Information: We would like to collect additional information for enrofloxacin and sulfadiazine native and labelled solutions used for value assignment, including concentrations, dilutions and solvents used in their preparation. Please provide information in the fields below:		
Native Calibration Standard Information		
Reference standard forms		Enrofloxacin: HI salt Sulfadiazine: free base
Solvent used to prepare stock solution		Enrofloxacin: 50% MeOH/ 5mM NaOH Sulfadiazine: 100% MeOH
Concentration of stock solution		Enrofloxacin: 27 µg/mL Sulfadiazine: 104 µg/mL
Handling of stock solution		<u>Enrofloxacin</u> Storage conditions: Stock solutions stored under argon in ampoules @ -80°C Time period between preparation and use: 1 month Treatment before use: equilibrate to room temperature and vortex <u>Sulfadiazine</u> Storage conditions: Stock solutions stored under argon in ampoules @ -12°C Time period between preparation and use: 2 months Treatment before use: equilibrate to room temperature and vortex
Intermediate dilutions of stock solutions (solvent, concentration)		Enrofloxacin: 275 ng/mL in 50% MeOH/ 5mM NaOH Sulfadiazine: 10.4 µg/mL in100% MeOH
Working solutions (solvent, concentration)		Enrofloxacin: 1.4 ng/mL in 50% MeOH/ 5mM NaOH Sulfadiazine: 65 ng/mL in100% MeOH
Isotopically-labelled Internal Standard Information (modify first three rows if alternative internal standards used)		
Internal standard forms		Enrofloxacin-d5: HI salt Sulfadiazine-13C6: Free base
Solvent used to prepare Internal Standard solution		Enrofloxacin: 50% MeOH/ 5mM NaOH Sulfadiazine: 100% MeOH
Concentration of Internal Standard solution		Enrofloxacin: ~13.5 µg/mL in 50% MeOH/ 5mM NaOH (as supplied by NRC-OTT) Sulfadiazine: ~ 100 µg/mL in 100% MeOH (as supplied by NRC-OTT)

Intermediate dilutions of Internal standard solution (solvent, concentration)		Used IS materials as supplied
Internal Standard Spiking solutions (solvent, concentration)		Enrofloxacin: ~290 ng/mL in 50% MeOH/ 5mM NaOH Sulfadiazine: ~ 12 µg/mL in 100% MeOH
Final calibration solution and sample extract information		
Details of calibration solutions , i.e. native and internal standard blends, as injected into MS (concentrations, solvent)		Enrofloxacin: 1.4 ng/mL Enrofloxacin-d5: 1.2 ng/mL Solvent: 50% MeOH/ 5mM NaOH Sulfadiazine: 64 ng/mL Sulfadiazine-13C6: 64 ng/mL Solvent: 100% MeOH
Solvent for bovine tissue sample extracts as injected into MS		Enrofloxacin: ~ 40% H_2O in % MeCN Sulfadiazine: ~ 40% H_2O in % MeCN