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CERTIFICATION REPORT

Certification of mass fractions of nitromidazoles in pork meat

Certified Reference Materials ERM®-BB124

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Certification of mass fractions of nitroimidazoles in pork meat

Certified Reference Materials ERM®-BB124

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Summary

This report describes the preparation of the pork meat matrix reference material ERM-BB124 and the certification of the content (mass fraction) of six nitroimidazole parent drugs and hydroxy metabolites.

The preparation and processing of the material, homogeneity and stability studies, and the characterisation are described hereafter and the results are discussed. Uncertainties were calculated in compliance with the Guide to the Expression of Uncertainty in Measurement (GUM) [1] and include uncertainties due to possible heterogeneity, instability, characterisation, and calibrant uncertainty. The certified values and their uncertainties are listed below:

Nitroimidazole in the reconstituted material	Certified value [1] [µg/kg]	Uncertainty [2] [µg/kg]	Number of accepted sets of results
Ronidazole (RNZ)	2.09	0.25	11
Metronidazole (MNZ)	1.93	0.15	12
2-hydroxymethyl-1-methyl-5-nitroimidazole (HMMNI)	0.69	0.09	11
Hydroxymetronidazole (MNZOH)	6.2	0.9	11
Hydroxyipronidazole (IPZOH)	1.67	0.12	11

1) These values are the mass fractions based on the unweighted mean of accepted results.
2) The uncertainties are the expanded uncertainties (k = 2) of the values defined in 1).

Additionally, the following certified value has been assigned:

Nitroimidazole in the reconstituted material	Certified value [1] [µg/kg]
Dimetridazole (DMZ)	<0.25

1) This value corresponds to the limit of quantification (LOQ) of the most sensitive method in the characterisation study. With a probability of 95% the certified value is below 0.25 µg/kg.

The assigned values and their uncertainties are based on a minimum sample intake of 5 g reconstituted material (corresponding to 1.25 g powder).

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1 Glossary

ANOVA	Analysis of variances
APCI	Atmospheric pressure chemical ionisation
C_{18}	Octadecyl silica
C_8	Octyl silica
CAS	Chemical Abstracts Services
CCα	Decision limit
CRM	Certified reference material
d_3-	Tri-deuterated compound
DAD	Diode-array detector
DMZ	Dimetridazole
ERM	European Reference Material
ESI	Electrospray ionisation
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry
GUM	Guide to the Expression of Uncertainty in Measurement
HMMNI	2-hydroxymethyl-1-methyl-5-nitroimidazole
HPLC	High-performance liquid chromatography
IPZ	Ipronidazole
IPZOH	Hydroxy ipronidazole
IRMM	Institute for Reference Materials and Measurements
IUPAC	International Union for Pure and Applied Chemistry
LC	Liquid chromatography
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LLE	Liquid liquid extraction
LOD	Limit of detection
LOQ	Limit of quantification
m/m	Mass-to-mass
MNZ	Metronidazole
MNZOH	Hydroxy metronidazole
MRM	Multiple reaction monitoring
MS	Mass spectrometry
$MS_{between}$	Mean of squares between groups (ANOVA)
MS_{within}	Mean of squares within groups (ANOVA)
MW	Molecular mass
n	Number of replicates
n.m.	Not measured
p	Level of significance
PSA	Particle size analysis
QC	Quality control
RNZ	Ronidazole
rpm	Rounds per minute
RSD	Relative standard deviation
RSD_{stab}	Relative standard deviation of all results of the stability study
s	Standard deviation
s_{bb}	Between-bottle standard deviation
SI	International Systems of Units
S/N	Signal-to-noise ratio
SPE	Solid phase extraction
s_{wb}	Within-bottle standard deviation
tBME	Tert-Butyl methyl ether

u^*_{bb} ..... Relative standard uncertainty due to the heterogeneity that can be hidden by the method repeatability
u_{bb} ..... Relative standard uncertainty due to between-bottle heterogeneity
u_{cal} ..... Relative uncertainty of common calibrant
u_{char} ..... Relative uncertainty of the characterisation exercise
$u_{CRM, rel}$ ..... Combined relative uncertainty of certified value
U_{CRM} ..... Expanded uncertainty of certified value
$U_{CRM, rel}$ ..... Expanded, relative uncertainty of certified value
u_{lts} ..... Relative uncertainty of long-term stability
u_{meas} ..... Uncertainty of measurement result
UPLC ..... Ultra-performance liquid chromatography
u_{pur} ..... Relative uncertainty of the calibrant purity
u_{sts} ..... Relative uncertainty of short-term stability
u_{weigh} ..... Relative uncertainty of the weighing steps for calibrant preparation
u_{Δ} ..... Combined uncertainty of certified value and measured value
U_{Δ} ..... Expanded uncertainty of certified value and measured value
x ..... Pre-defined shelf life
x_i ..... Time point i in an isochronous stability study
Δ ..... Difference between two measurement results
Δ_m ..... Difference between measured and certified value
$v_{MSwithin}$ ..... Degrees of freedom of MS_{within}

2 Introduction

2.1 Background

5-Nitroimidazoles have a long history in the treatment of diseases caused by protozoans (e.g. *Giardia lamblia*, *Entamoeba histolitica*) and for combating bacterial infections (coccidiosis, haemorrhagic enteritis) and have mainly been applied to poultry and pigs [2]. The most widely used nitroimidazole drugs were dimetridazole (DMZ), metronidazole (MNZ), ronidazole (RNZ), and ipronidazole (IPZ), depicted in Figure 1 together with their hydroxy metabolites. Several toxicological studies underpinned the suspicion that nitroimidazoles reveal mutagenic and carcinogenic properties [3,4]. In consequence, application of this class of veterinary medicines to food-producing animals has been prohibited in the EU [5-8]. Minimum required performance limits (MRPLs) for analytical methods used for detection and quantification of these forbidden substances have been discussed for several years but have never been implemented in legislation; instead, so-called "recommended concentrations" [9] of 3 µg/kg per nitroimidazole analyte have recently been issued by the Bundesinstitut für Verbraucherschutz und Lebensmittelsicherheit (Berlin), the responsible Community Reference Laboratory (CRL) for these veterinary drug substances. The recommended concentrations shall be taken into consideration for the implementation and validation of analytical methods with appropriate sensitivity. Nowadays, the majority of methods for nitroimidazoles applied in testing laboratories comprise chromatographic separation - using either gas chromatography (GC) or liquid chromatography (LC) - coupled to a mass spectrometer, which is the most suitable detector for confirmatory methods to comply with Commission Decision 2002/657/EC [10]. A clear trend towards LC-MS/MS has been observed in the past years.

Low or sub µg/kg analyte levels in complex food matrices such as meat or egg require highly accurate and sensitive methods for obtaining reliable results. An inter-comparison on nitroimidazoles in turkey and pig samples organized by the CRL in Berlin clearly demonstrated the necessity of improving the quality of the analytical data [11] and resulted in the request for having available a certified reference material (CRM) for method validation purposes (trueness determination) as well as for verifying the performance of analytical methods.

2.2 Choice of the material

An incurred material which closely resembles a typical sample analysed in the laboratory in terms of comparable analyte extractability was considered necessary. In a preliminary feasibility study, it was concluded that lyophilised pig muscle would be a suitable reference material format (combination of animal species, matrix, and matrix condition) [12]. Other aspects taken into account were raw material availability (cost, ethical issues related to animal experiments), and CRM-inherent considerations such as material homogeneity and stability [13]. The relevant target analytes have to be present in the reference material, which, in some cases, are the parent drugs (RNZ, MNZ), and in other cases the respective hydroxy metabolites (IPZOH, HMMNI). Concentrations of analytes in the tissue depend on withdrawal period, animal species, tissue, and the drug itself. Four separate incurred raw materials were prepared by administering one of the four parent drugs DMZ, RNZ, MNZ, or IPZ, respectively to pigs. These materials where then merged in an appropriate ratio and further blended with blank material to obtain a multi-analyte reference material with the envisaged target concentrations of 3 µg/kg per analyte.

Figure 1 depicts the parent drugs and metabolites to which a certified value was assigned in the CRM. An exception is IPZ, as this analyte is rapidly metabolised to IPZOH in the tissue and could not be detected anymore in the incurred raw material.

2.3 Definition of analytes and chemical structures

Table 1. Definition of nitroimidazole analytes comprised in ERM-BB124.

Trivial name and abbreviation	IUPAC name	CAS number	Chemical formula	Molecular mass (g/mol)
Dimetridazole (DMZ)	1,2-dimethyl-5-nitroimidazole	551-92-8	$C_5H_7N_3O_2$	141.13
Ronidazole (RNZ)	(1-methyl-5-nitroimidazol-2-yl)methyl carbamate	7681-76-7	$C_6H_8N_4O_4$	200.15
Metronidazole (MNZ)	2-(2-methyl-5-nitroimidazol-1-yl)ethanol	443-48-1	$C_6H_9N_3O_3$	171.15
2-hydroxymethyl-1-methyl-5-nitroimidazole (HMMNI)	(1-methyl-5-nitroimidazol-2-yl)methanol	936-05-0	$C_5H_7N_3O_3$	157.13
Hydroxy metronidazole (MNZOH)	2-(2-hydroxymethyl-5-nitroimidazol-1-yl)-ethanol	4812-40-2	$C_6H_9N_3O_4$	187.15
Hydroxy ipronidazole (IPZOH)	2-(1-methyl-5-nitroimidazol-2-yl)propan-2-ol	35175-14-5	$C_7H_{11}N_3O_3$	185.18

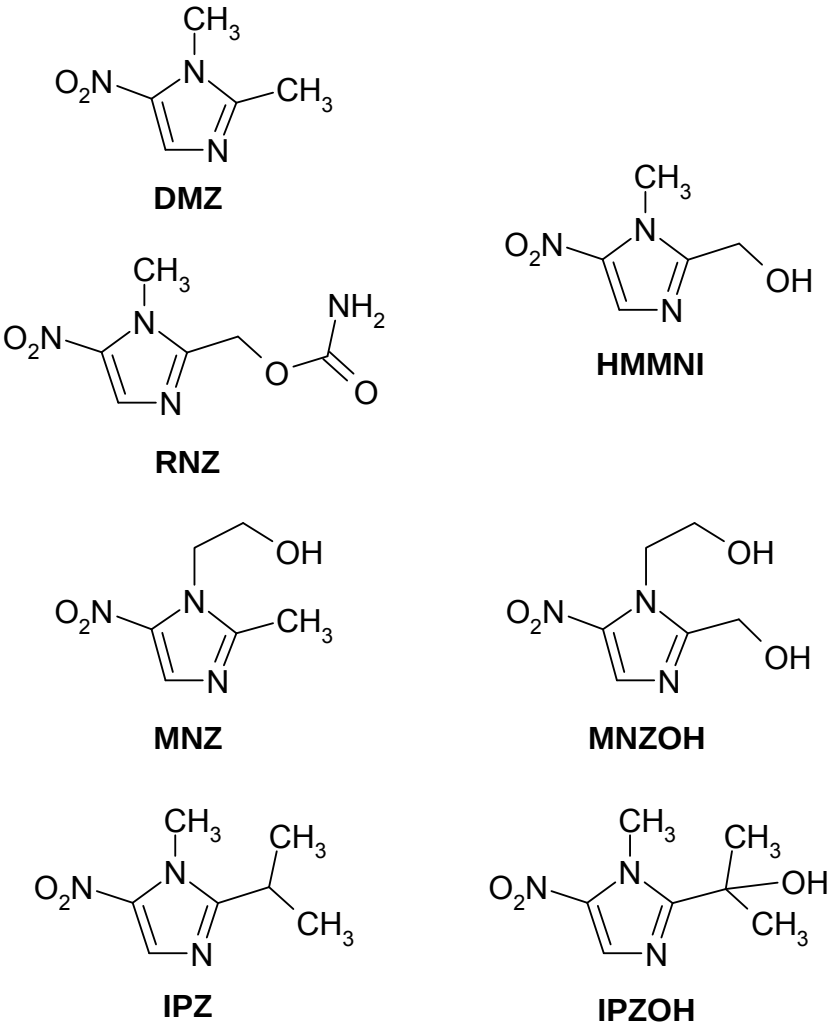

Fig. 1: Chemical structures of 5-nitroimidazoles parent drugs and their hydroxy metabolites. DMZ and RNZ have the same metabolite (HMMNI).

3 Participants

Project management and evaluation:
European Commission, Joint Research Centre, Institute for Reference Materials and Measurements, Reference Materials Unit, BE
(Work performed under ISO Guide 34 accreditation; Belac-268-Test)

Raw material provision:
Bundesamt für Verbraucherschutz und Lebensmittelsicherheit, DE

Processing:
European Commission, Joint Research Centre, Institute for Reference Materials and Measurements, Reference Materials Unit, BE
(Processing performed under ISO Guide 34 accreditation; Belac-268-Test)

Homogeneity and stability measurements:
Bundesamt für Verbraucherschutz und Lebensmittelsicherheit, DE
(Measurements performed under ISO/IEC 17025 accreditation; AKS-PL-12005)

Characterisation analysis:
Agence Française de Sécurité Sanitaire des Aliments, Laboratoire d'Etudes et de Recherches sur les Médicaments Vétérinaires et les Désinfectants, FR
(Measurements performed under ISO/IEC 17025 accreditation; COFRAC 1-0247)

Agri-Food and Biosciences Institute, Veterinary Sciences Division, GB
(Measurements performed under ISO/IEC 17025 accreditation; UKAS 2632)

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RIKILT – Institute of Food Safety, NL
(Measurements performed under ISO/IEC 17025 accreditation; RvA L014)

4 Processing of the material

Incurred raw materials were delivered in the frozen state from the Bundesamt für Verbraucherschutz und Lebensmittelsicherheit, Berlin (DE). Samples consisted of four separate batches 3 - 8 kg batches of pork muscle tissue (each having been produced by administration of one the four parent drugs DMZ, RNZ, MNZ, IPZ to a pig).

First, a feasibility study with a small portion of the delivered material was carried out to establish a suitable processing procedure which minimizes possible analyte decomposition due to thermal instability of some nitroimidazoles in meat at temperatures of 4 ºC or higher [14]. The conditions and order of individual processing steps described in the following were found to be appropriate and were applied in the production of the candidate reference material.

The delivered muscle tissue portions were cut in the frozen state and lyophilised in an Epsilon 2-85D freeze dryer (Martin Christ, Osterode, DE). The yield (mass ratio of freeze-dried matter to meat tissue) was determined gravimetrically on calibrated balances and calculated to be 25.1 m/m % (weighted mean of 4 individual incurred materials and blank material). The freeze-dried matter was then pre-crushed using a Retch heavy duty mill (Haan, DE) with a 6 mm sieve insert. The nitroimidazole content of each of the 4 portions was determined at IRMM using the in-house liquid chromatography tandem mass spectrometry (LC-MS/MS) method [Zeleny et al., J. Chromatogr. A, manuscript accepted]. Appropriate amounts of the four portions were merged to a blend, which was homogenized for 1 hour in a Turbula mixer (WAB, Basel, CH), immersed in liquid nitrogen overnight, and milled to a powder in a Palla VM-KT vibrating cryogenic mill (KHD Humboldt Wedag, Köln, DE). The powder was blended with a blank pork powder (processed in the same way) to achieve the envisaged analyte target concentrations around 3 µg/kg. Again, the material was Turbula mixed and cryogenically milled as described above. The resulting powder was sieved through a 710 µm stainless steel sieve (Model 17300, Russel Finex Industrial sieve, London, GB) and Turbula mixed. 10 g portions of powder were filled into amber glass bottles (100 mL) using a vibrating Laborette feeder (Fritsch, Idar-Oberstein, DE). Bottles were closed in the freeze-dryer under inert gas atmosphere after manual insertion of lyo-inserts. Capping and labelling was performed using a Bausch & Ströbel device (Ilshofen, DE). In total, 897 bottles of ERM-BB124 were processed; they were stored after production at -70 ºC.

5 Material characterisation measurements

5.1 Water content

The water content in the final material was measured by Karl Fischer titration [15]. Five bottles of the batch were chosen using a random stratified sample picking scheme and analysed in duplicate. The results are summarized in Table 2.

Table 2. Water content

Material	mean ± s [g/kg]
ERM-BB124	32.6 ± 0.2

5.2 Sieve analysis and particle size measurements

Before filling, a representative 10 g portion of the material was subjected to sieve analysis using a Hosokawa Alpine Sieve Analyser (Augsburg, DE) and 12 sieves with mesh sizes

between 20 and 1000 µm. The sieve fractions were measured on a calibrated Mettler PM1200 balance with a resolution of 1 mg. No particles were obtained below 40 and above 1000 µm. The median showed to be between 90 and 125 µm, and 90% of the material passed through the 250 µm sieve.

Particles size analysis (PSA) on the final material was performed using laser diffraction spectrometry. 5 samples of the batch were chosen using a random stratified sample picking scheme and analysed over a range of 0.5 to 875 µm using a Helos laser light scattering instrument (Sympatec, Clausthal-Zellerfeld, DE). A representative graph of the particle size distribution for ERM-BB124 is shown in Figure 2. Despite careful processing, some long fibres were found in the final material (micrograph analysis) which might impair PSA measurements. Nevertheless, the obtained results corroborate those from the sieve analysis presented above.

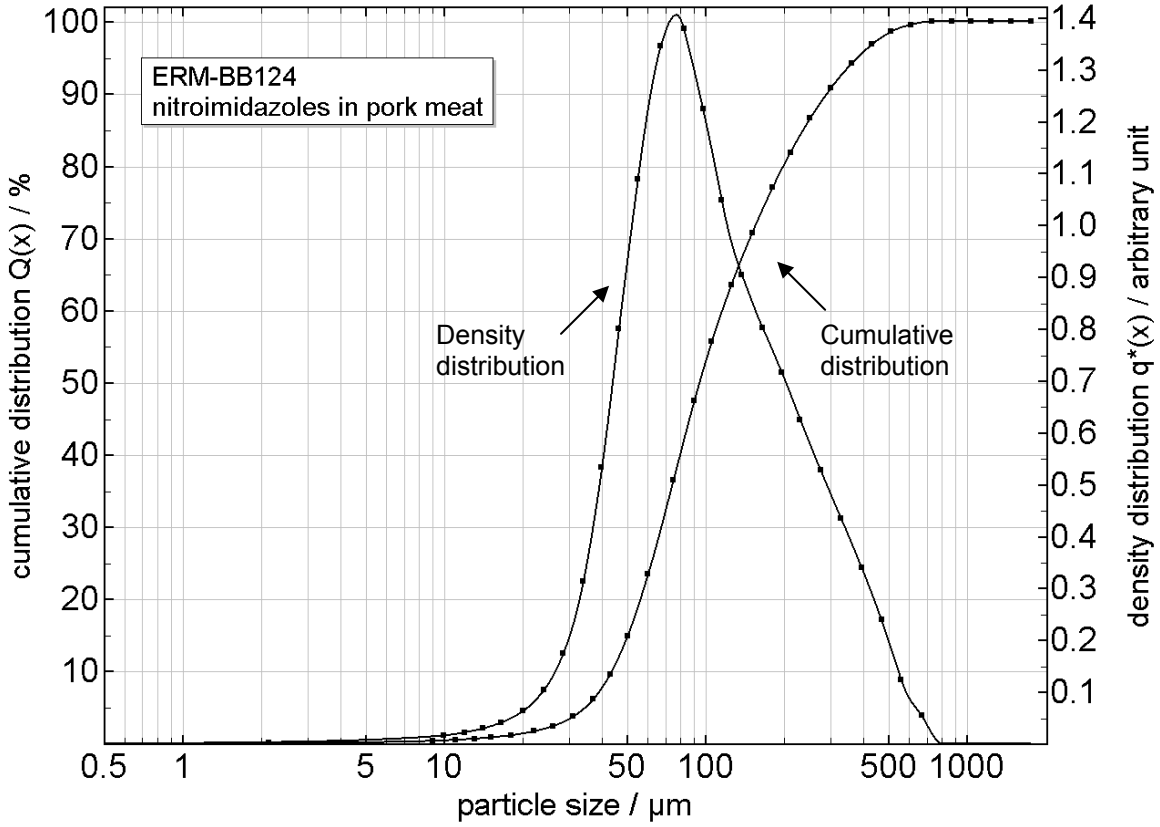

Figure 2. Particle size distribution of ERM-BB124

6 Homogeneity study

For the homogeneity study, 10 samples (~ 1.1 % of the total batch) of ERM-BB124 were chosen using a random stratified sample picking scheme and analysed in duplicate for their nitroimidazoles content. Measurements were performed by an in-house validated LC-MS/MS in the multiple reaction monitoring mode (MRM). Calibration was done with neat standard solutions, and deuterated internal standards were spiked to the samples in the beginning of the extraction procedure (except for d_3-MNZOH which was not available at the time of the study). MNZOH results were not corrected for recovery.

Samples were measured in a random order (predefined at IRMM and communicated to the laboratory) to allow distinction between an analytical trend and a trend in the filling sequence. Measurements were performed under repeatability conditions.

In all ERM-BB124 samples, five nitroimidazole compounds could be identified and quantified: RNZ, MNZ, HMMNI, MNZOH, and IPZOH. For DMZ, a tiny peak at the retention time window of DMZ was observed, however, the estimated concentration was far below the decision limit (CCα) of the method and thus results were indicated as "<CCα". The result thus points at the possible presence of trace levels of DMZ.

Data were checked for single and double outliers by applying the Grubbs test at a confidence level of 95% and 99%. Two outliers were detected (HMMNI, analytical sequence, Double Grubbs test, 95% level) which were scrutinised and retained as no technical reason was found to eliminate them. Regression analysis was performed to detect possible trends regarding the filling sequence or analytical sequence.

No significant slopes were found except for one case (MNZOH, analytical sequence, 95% confidence level, t value 2.24, t_{crit} 2.10; no significant slope at 99% level). Taking into account the somewhat poor method repeatability for this analyte, it can however be concluded that this trend is technically irrelevant. - In conclusion, the distribution of all five nitroimidazole analytes in the material can be regarded as homogeneous. Furthermore it was checked whether the data followed a normal or unimodal distribution using normal probability plots and histograms, respectively. Individual data and sample averages showed a unimodal distribution for all analytes, although some deviations from normality were observed. Finally, the uncertainty contribution from possible heterogeneity was estimated by a one-way analysis of variance (ANOVA) [16]:

Method repeatability (s_{wb}) expressed as a relative standard deviation is given as follows:

$$s_{wb} = \frac{\sqrt{MS_{within}}}{\bar{y}}$$

MS_{within}: mean square within a bottle from an ANOVA
$\bar{y}$: average of all results of the homogeneity study

Between-unit variability (s_{bb}) expressed as a relative standard deviation is given by the following equation:

$$s_{bb} = \frac{\sqrt{\frac{MS_{between} - MS_{within}}{n}}}{\bar{y}}$$

$MS_{between}$: mean square among bottles from an ANOVA
n: average number of replicates per bottle

The heterogeneity that can be hidden by method repeatability is defined as follows:

$$u^*_{bb} = \frac{s_{wb}}{\sqrt{n}} \sqrt[4]{\frac{2}{v_{MSwithin}}}$$

$v_{MSwithin}$: degrees of freedom of MS_{within}

The larger value of s_{bb} or u^*_{bb} was used as uncertainty contribution for homogeneity, u_{bb} (see Table 3 for a summary of results, values were converted into relative uncertainties).

Table 3. Homogeneity study results for ERM-BB124

	RNZ	**MNZ**	**HMMNI**	**MNZOH**	**IPZOH**
Average[1]	1.977	1.730	0.662	4.4200	1.636
RSD [%]	1.608	0.931	2.339	3.052	1.706
MS_{within}	0.0019164	0.0005909	0.0002316	0.0242762	0.0006424
$MS_{between}$	0.0020213	0.0005183	0.0004790	0.0363998	0.0015582
s_{wb} [%]	2.214	1.405	2.300	3.525	1.550
s_{bb} [%]	0.366	n.c.[2]	1.681	1.761	1.308
u^*_{bb} [%]	1.047	0.664	1.088	1.667	0.733
u_{bb} [%]	**1.047**	**0.664**	**1.681**	**1.761**	**1.308**

[1] Average values are given in µg/kg
[2] n.c. = not calculable because $MS_{between} < MS_{within}$

6.1 Minimum sample intake

The minimum sample intake is 5 g of reconstituted material (corresponding to 1.25 g of powder). Homogeneity and stability studies were performed using 5 g of material after reconstitution, proving that the samples are homogeneous at least at this level.

7 Stability studies

7.1 Short-term stability study

A four weeks isochronous study [17] was performed to evaluate stability of ERM-BB124 during transport. Fourteen samples were selected from the produced batch using a random stratified sample picking scheme. Samples were dispatched to the testing laboratory on dry ice.

Samples were stored at +4 °C and +18 °C and at a reference temperature of -70 °C. High temperatures usually employed in short-term stability studies (40 ºC, 60 ºC) were not investigated in this study. Two ampoules were stored at each temperature for 0, 1, 2, and 4 weeks. After the indicated storage periods, the samples were transferred to storage at -70 °C until analysis. Samples were analysed in duplicate under intermediate precision conditions in the order predefined at IRMM (randomised sample order) using the same LC-MS/MS method as for the homogeneity study. Again, results for MNZOH were not corrected for recovery.

Data (Annex B) were first checked for single and double outliers by applying the Grubbs test at confidence levels of 95% and 99%, respectively. A few outliers were detected, scrutinised, and not excluded as no technical reason was found to do so. Data points were plotted against time and the regression lines were calculated (see Table 4 for a summary). The observed slopes were tested for significance using a t-test, with $t_{\alpha,df}$ being the critical t-value (two-tailed) for a confidence level α = 0.05 (95 % confidence interval). The slope was considered as statistically significant when $b/s_b > t_{\alpha,df}$. In two cases (RNZ, MNZOH), slopes significantly different from 0 were found for a storage temperature at 4 ºC, whereas no significant slopes were found for neither analyte at 18 ºC. It was concluded that the uncertainty of the short-term stability (u_{sts}) can be assumed to be negligible if sample shipment is carried out with cooling elements, which therefore shall be the dispatch condition for sample shipment to the customer.

Table 4. Evaluation of the short-term stability study

	RNZ		MNZ		HMMNI		MNZOH		IPZOH	
Statistical parameters	4 ºC	18 ºC	4 ºC	18 ºC	4 ºC	18 ºC	4 ºC	18 ºC	4ºC	18 ºC
Slope (b) [%/week]	-0.89	-0.14	-0.21	0.11	-0.46	-0.91	-2.18	-0.40	-0.48	-0.18
$\lvert b\rvert/s_b$	2.49	0.30	0.66	0.23	1.85	1.77	2.49	0.75	1.26	0.72
Statistical significance (95% conf. interval) [1]	Yes	No	No	No	No	No	Yes	No	No	No
u_{sts} [%/week]	0.63	0.44	0.30	0.45	0.27	0.55	1.54	0.53	0.37	0.28

[1] $t_{0.05;14}$= 2.14

7.2 Long-term stability study

A 16 months isochronous study [17] was performed to evaluate the stability of ERM-BB124 during storage. The chosen study duration was a suitable compromise between data for sound statistics and considering preliminary stability data from BVL indicating suitable analyte stability for at least 1 year at -20 ºC.

Fourteen samples were picked from the produced batch using a random stratified sample picking scheme. Samples were stored at +4 °C and -20 °C, and at a reference temperature

of -70 °C. 2 ampoules were stored at each temperature for 0, 8, 12, and 16 months, respectively. After the indicated periods, the samples were transferred to -70 °C until analysis. Samples were dispatched on dry ice and kept at -30 ºC in the laboratory until analysis (two weeks). Samples were analysed in duplicate under intermediate precision conditions in the order predefined at IRMM (randomised sample order) using the same LC-MS/MS method as for the homogeneity study. In contrast to the homogeneity and short-term stability study, d_2-MNZOH was used as internal standard for MNZOH. Moreover, results were corrected for recovery.

Two of the 28 individual results (one result each of the -20 ºC and the 4 ºC sample series) had to be eliminated due to reported technical problems during analysis.

Data (Annex C) were checked for single and double outliers by applying the Grubbs test at confidence levels of 95% and 99%, respectively. No outliers were detected.

Data points were plotted against time and the regression lines were calculated to check for significant trends (degradation, enrichment) due to storage conditions. The observed slopes were tested for significance using a t-test, with $t_{\alpha,df}$ being the critical t-value (two-tailed) for a confidence level α = 0.05 (95 % confidence interval). The slope was considered as statistically significant when $b/s_b > t_{\alpha,df}$.

Finally, the uncertainty of stability u_{lts} [18] was calculated for a pre-defined shelf life of 2 years as:

$$u_{lts} = \frac{RSD_{stab}}{\sqrt{\sum (x_i - \bar{x})^2}} \cdot x$$

with RSD_{stab} being the relative standard deviation of all 16 individual results of the relevant stability study, x_i being the time point for each replicate, $\bar{x}$ being the average of all time points and x being the pre-defined shelf life. Results are summarized in Table 5.

Table 5. Evaluation of the long-term stability study

	RNZ		MNZ		HMMNI		MNZOH		IPZOH	
Statistical parameters	-20 ºC	4 ºC	-20 ºC	4 ºC	-20 ºC	4 ºC	-20 ºC	4 ºC	-20 ºC	4 ºC
Slope (b) [%/year]	1.13	1.64	-0.34	-1.06	-3.00	-0.97	1.87	0.92	0.84	-1.98
$\lvert b \rvert / s_b$	0.41	0.66	0.22	0.57	1.40	0.49	0.75	0.35	0.60	1.26
Statistical significance (95% conf. interval) [1]	No	No	No	No	No	No	No	No	No	No
u_{lts} [%/2 years]	5.394	5.003	3.053	3.640	4.560	3.867	4.891	5.137	2.763	3.211

[1] $t_{0.05;13}$= 2.16

At both tested temperatures, no significant slopes at the 95% level of confidence were detected, demonstrating stability of the material under these conditions. Nevertheless, -20 ºC was chosen as the storage temperature for the batch.

8 Characterisation

8.1 Design of the study

The decision was made to restrict the analytical methods used for the characterisation of the reference material to LC-MS/MS due to the finding of a possible bias in results when applying GC-MS and LC-MS/MS on given samples [12], but also considering the fact that the vast majority of testing laboratories are applying LC-MS/MS methods for confirmatory nitroimidazole analysis.

Eleven laboratories were carefully selected to perform the analytical measurements (one laboratory employed two methods which differed in the sample preparation part). Validated methods were an indispensable requirement for participation; an accredited method was considered an asset. The laboratories had to prove their measurement capabilities and had to demonstrate previous experience in nitroimidazole analysis in comparable matrices. In several cases, the laboratory's measurement capability was demonstrated by successful participation in a preliminary inter-comparison jointly organized by BVL and IRMM [19] where four lyophilised turkey muscle samples had to be analysed using LC-MS/MS methodology.

A common calibrant was used, which was prepared at and provided by IRMM. DMZ, RNZ, and MNZ were purchased from Dr. Ehrensdorfer (Augsburg, Germany). MNZOH, IPZOH, d_3-IPZOH, HMMNI, d_3-HMMNI, d_3-DMZ, and d_3-RNZ were obtained from Witega Laboratories (Berlin, Germany). d_3-MNZ was purchased from Chimete Srl (Rivalta Scrivia, Italy). Calibrant purities as indicated on the certificate of analyses of the providers have been verified at IRMM using LC-DAD, LC-MS (scan mode) and LC-MS/MS and were found to agree with the values on the certificates. First, individual stock solutions were prepared by weighing in the crystalline substances on a calibrated balance followed by addition of LC-grade methanol. Thereafter, these solutions were pooled to yield two stock solutions comprising 6 nitroimidazole analytes and 5 deuterated analogues, respectively. All dilution steps were controlled gravimetrically. Uncertainty of the common calibrant solution was evaluated and comprised contributions from purity and all weighing operations. Table 6 summarizes the obtained u_{cal} values, calculated as the square root of the sum of squares of the relative uncertainties of the three individual contributions.

Table 6. Calibration solution uncertainties

	RNZ	MNZ	HMMNI	MNZOH	IPZOH
u_{pur} [%] [1]	0.380	0.233	0.380	0.233	0.380
u_{weigh} [%][2]	0.078	0.079	0.078	0.081	0.078
u_{cal} [%]	0.388	0.246	0.388	0.246	0.388

[1] calculated from indicated and verified purity values, rectangular distribution
[2] weighing steps: crystalline substance, solvent, aliquots of individual solutions, solvent addition to combined stock solution with concentration of 0.25 mg/mL

For the characterisation of ERM-BB124, each laboratory was provided with the following samples:

- 2 units of ERM-BB124
- 1 bottle (20 gram) of blank pork meat powder
- 2 ampoules of calibration stock solution (6 analytes à 0.25 mg/mL)
- 2 ampoules of internal standard stock solution (5 d_3-analytes à 0.25 mg/mL)

Laboratories had to apply their validated LC-MS/MS methods and had to use the provided calibration solution. Dilutions to the appropriate levels for the calibration curves were done at the participant laboratories. Preparation of calibration curves was done according the laboratories' method working instructions (neat standard solution calibration or matrix-matched calibration). Measurements had to be performed on two different days with independent calibrations on each day. Each of the two samples had to be measured four times, whereby duplicate measurements for each sample had to be done on both days (example: samples 15 and 28 received; day 1: 15 1st and 2nd sub-sample, 28 1st and 2nd subsample; day 2: 15 3rd and 4th subsample, 28 3rd and 4th subsample). Reconstitution of the samples was prescribed as follows: to 1.25 g powder, 3.75 g of distilled water had to be added. Higher amounts could be used if required by the laboratory's working instruction, whereby the 1:3 m/m ratio of powder to water had to be maintained. The blank pork meat powder provided was used for the preparation of quality control (QC) samples (blank matrix sample, sample spiked at low μg/kg level), and for the preparation of matrix-matched calibration when applicable.

8.2 Results and technical evaluation

The individual methods employed by the laboratories are summarised in Tables 7 - 9 (sample preparation and calibration, overview LC-MS, transitions used for quantification). It can be seen that the laboratory methods varied substantially in terms of employed extraction solution and clean-up procedure. Some differences were also seen in the applied calibration curves, the internal standard used for MNZOH, the LC solvent systems, and the reversed-phase LC columns used (column dimension, particle size). All but one lab operated the ion source of the mass spectrometer in the positive electro-spray ionisation (ESI+) mode, and the transitions used for quantification were the same or very similar in all the laboratories.

Table 7. Methods in the characterisation study – sample preparation and calibration

Lab code	Sample intake[1] [g]	Extraction solution	Clean-up	Calibration[2]	Internal standard for MNZOH
1	4	Phosphate buffer/ethyl acetate	Iso-octane defatting	Matrix-matched (powder)	d_3-MNZ
2	5	Acetonitrile	SPE (strong cation exchanger)	Neat standards	n.m.[3]
3	5	Dichloromethane	SPE (silica)	Matrix-matched (powder)	d_3-MNZ
4	5	Phosphate/ wolframate buffer	Iso-octane defatting	Neat standards	-[4]
5	5	Ethyl acetate	-	Matrix-matched (powder)	d_3-MNZ
6	5	Ammonium acetate buffer/acetonitrile	LLE (diatomaceous earth)	Matrix-matched (powder)	d_3-MNZ
7	6	McIlvaine buffer/acetonitrile	LLE (tBME/hexane)	Matrix-matched (meat)	d_3-MNZ
8	5	Water	SPE (lipophilic-hydrophilic copolymer)	Matrix-matched (powder)	d_3-RNZ
9	5	Phosphate buffer	Hexane defatting, LLE (diatomaceous earth)	Neat standards	d_3-MNZ
10	5	Phosphate buffer with protease	Hexane defatting, LLE (diatomaceous earth)	Neat standards	d_3-MNZ
11	5	Acetonitrile	Hexane defatting, SPE (strong cation exchanger)	Matrix-matched (meat)	d_3-MNZ
12	5	Ethyl acetate	-	Matrix-matched (powder)	d_3-MNZ

[1] reconstituted material; labs 1 and 7 used the sample intake according to their working instructions
[2] powder: blank provided by IRMM; meat: verified blank meat from laboratory
[3] not measured
[4] external calibration; obtained value corrected for pure extraction recovery and for ionisation (matrix effect)

Deuterated MNZOH was not commercially available at the time of calibrant solution preparation and therefore not included in the internal standard stock solution. For all analytes except MNZOH, labs used the respective d_3-analogue as internal standard; for MNZOH, three different internal standards were applied in total by the laboratories (see last column of Table 7). The individual results as obtained are listed in Annex D.

Table 8. Methods in the characterisation study – separation and quantification

Lab code	LC column	Solvent system[1]	HPLC system	Mass spectrometer[2]
1	Symmetry® C_{18}, 150 x 3.9 mm, 5 µm (Waters)	Methanol/acetonitrile /formic acid	Agilent 1100	Quattro LCZ (Micromass)
2	Acquity UPLC BEH C_{18}, 100 x 2.1 mm, 1.7 µm (Waters)	Ammonium acetate/methanol	Waters Acquity UPLC	Quattro Premier XE (Micromass)
3	Luna® C_8, 150 x 4.6 mm, 3 µm (Phenomenex)	Formic acid/ acetonitrile	Waters 2695 Alliance	Quattro Ultima (Micromass)
4	Atlantis® dC_{18}, 100 x 2.1 mm, 3 µm (Waters)	Formic acid/ acetonitrile	Waters Acquity UPLC	Quattro Micro (Micromass)
5	XTerra® MS C_{18}, 100 x 2.1 mm, 3.5 µm (Phenomenex)	Formic acid/ methanol	Waters 2690	Quattro Ultima (Micromass)
6	YMC-Pack™ ODS-AM C_{18}, 150 x 3 mm, 5 µm (YMC)	Ammonium acetate/ acetonitrile	Agilent 1100	Q-trap 4000 (Applied Biosystems)
7	Luna® $C_{18\ (2)}$, 150 x 2mm, 3 µm (Phenomenex)	Formic acid/ methanol	Agilent 1100	Quattro LC (Waters)
8	Alltima® C_{18}, 150 x 3.2 mm, 5 µm (Alltech)	Formic acid/ methanol	Waters 2690 Alliance	Quattro Ultima (Micromass)
9	Acquity UPLC BEH C_{18}, 100 x 1 mm, 1.7 µm (Waters)	Formic acid/ acetonitrile	Waters Acquity UPLC	Quattro Premier XE (Micromass)
10	Acquity UPLC BEH C_{18}, 100 x 1 mm, 1.7 µm (Waters)	Formic acid/ acetonitrile	Waters Acquity UPLC	Quattro Premier XE (Micromass)
11	Gensis® C_{18}, 250 x 3 mm, 4 µm (Jones Chromatography)	Acetic acid/ acetonitrile	Agilent 1100	Quattro 2 (Waters)
12	Gemini® C_{18}, 150 x 3 mm, 5 µm (Phenomenex)	Ammonium formate/ acetonitrile	Agilent 1100	Q-trap 4000 (Applied Biosystems)

[1] all labs used an elution gradient except lab 1 (isocratic conditions)
[2] all labs operated the ion source on the mass spectrometer in the ESI+ mode except lab 11 which used APCI+

Table 9. Methods in the characterization study - MRM transitions used for quantification[1]

Lab code	DMZ	RNZ	MNZ	HMMNI	MNZOH	IPZOH
1	142>96	201>140	172>128	158>140	188>126	n.m. [2]
2	142>96	201>140	172>128	158>140	n.m. [2]	186>168
3	142>96	201>140	172>128	158>140	188>126	186>168
4	142>96	201>140	172>128	158>140	188>126	186>168
5	142>96	201>140	172>128	158>140	188>126	186>122
6 [3]	142>96	201>55	172>128 172>81 172>45	158>140	188>126	186>168
7	142>96	201>140	172>128	158>140	188>126	186>168
8	142>96	201>140	172>128	158>140	188>126	186>168
9	142>96	201>140	172>128	158>140	188>123	186>168
10	142>96	201>140	172>128	158>140	188>123	186>168
11	142>96	201>140	172>128	158>140	188>126	186>168
12	142>96	201>140	172>128	158>140	188>123	186>168

[1] Values represent the parent (molecular ion) and the daugther ion, respectively
[2] not measured
[3] MNZOH: mean of the results from the 3 transitions used

All data were recovery-corrected, either intrinsically by having employed matrix-matched calibration and the d_3-labelled internal standards, or, in case of external calibration, by correcting the obtained value by total recovery afterwards (extraction recovery and ionisation/matrix effect, which have been assessed with appropriate QC samples in the analytical series; laboratory 4 for MNZOH). For quantification, labs either directly used the output of the validated instrument software (calibration line calculated by regression analysis), or copied the obtained areas from the instrument software into a validated excel sheet for further calculation of the analyte concentrations in the samples.

After receipt of the data sets, the results were subjected to technical evaluation. In two cases, the scopes of the laboratory methods did not include all study analytes: IPZOH was not included in the scope of the method applied in laboratory 1, and MNZOH was not included in the scope of the method employed by laboratory 2. Furthermore, the obtained data set for RNZ in laboratory 3 was rejected after scrutiny due to large day-to-day variation in results which also held for the QC samples checking recovery. Finally, the data set of laboratory 4 for HMMNI could not be included in further calculations as results were reported as "<CCα".

In total, 88 results each for RNZ, HMMNI, MNZOH and IPZOH from 11 laboratories, and 96 results for MNZ from 12 laboratories were accepted after technical scrutiny and subjected to statistical data assessment.

The accepted sets of results were submitted to the following statistical tests:

- Scheffe's multiple t-test to check if the means of two labs are significantly different
- Dixon's test to detect outlying lab means
- Nalimov t-test to detect outlying lab means
- Grubb's test to detect single and double outliers
- Cochran test to check for outlying lab variances
- Bartlett test to check for homogeneity of lab variances
- ANOVA to assess between lab and within lab variances and test their significance employing the SNEDECOR F-test
- Skewness and Kurtosis test to assess the normality of the lab means distribution. The later tests are only used if seven or more datasets have been accepted; otherwise normal probability plots have been used.

Datasets were first subjected to the Cochran test to identify outlying laboratory variances. No outlying variances were detected. The results of the statistical tests of the finally considered data for ERM-BB124 are summarized in Table 10. It shall be noted that the mean of means (certified value) hold for the reconstituted material.

The outlying means of RNZ (lab 8), MNZ (lab 4), MNZOH (lab 4) and IPZOH (lab 5) lie within two standard deviations of the mean of means and are therefore not significantly different to the mean value. By contrast, the outlying means of HMMNI (lab 11) and MNZOH (lab 5) lie outside two standard deviations of the mean of means and were thus scrutinized further. The confidence interval (p=0.05) of the distribution of individual laboratory means around the mean of means was calculated for the two respective cases.

HMMNI lab 11: lab mean 0.49 µg/kg, confidence interval 0.21 µg/kg, means of means 0.69 µg/kg;
MNZOH lab 5: lab mean 4.13 µg/kg, confidence interval 2.21 µg/kg, mean of means 6.23 µg/kg

In both cases, the respective laboratory means lie within the calculated confidence intervals and can therefore be considered as not significantly different from the mean of means.

Table 10. Summary of statistical evaluation for ERM-BB124

Analyte	RNZ	MNZ	HMMNI	MNZOH	IPZOH
Number of data sets	11	12	11	11	11
Number of replicate measurements	88	96	88	88	88
Mean of means [µg/kg]	2.09	1.93	0.69	6.23	1.67
Relative standard deviation of mean of means [%]	6.15	7.77	13.30	15.86	5.41
Relative standard error of mean of means [%]	1.85	2.24	4.01	4.78	1.63
All data sets compatible two by two? (Scheffe's test)	No	No	No	No	No
Outlying means? (Dixon test, Nalimov t-test, Grubbs test)	Lab 8 Nalimov (p=0.05)	Lab4 Nalimov (p=0.05)	Lab 11 Dixon and Nalimov (p=0.05)	Lab 5 Dixon (p=0.05) Labs 4 and 5 Nalimov (p=0.05)	Lab 5 Nalimov (p=0.05)
Outlying lab variances? (Cochran test)	No	No	No	No	No
Lab variances homogeneous? (Bartlett test)	No	No	No	No	No
Distribution of means normal? (Skewness & kurtosis, normal probability plot)	Yes	Yes	Yes	Yes	Yes
Variances between labs significantly different? (SNEDECOR)	Yes	Yes	Yes	Yes	Yes

The results for DMZ were reported as "not detected" by all laboratories. By inspection of the individual chromatograms, a tiny peak at the retention time window of DMZ could be detected in a few data sets, whereas in most of the data sets no peak was detected.

Table 11. CCα and LOD values for DMZ in the methods of the characterization study

Lab	CCα [µg/kg]	Basis of value (v= validation data, e = estimation)	LOD [µg/kg]	Basis of value (v= validation data, e = estimation)
1	1.31	v	~ 0.05	e
2	0.89	v	~ 0.1	e
3	2.2 [1]	v	~ 0.2	e
4	-	-	0.133 (0.4) [2]	v
5	0.44	v	~ 0.1	
6	-	-	0.075 (0.125) [3]	v
7	0.38	v	~ 0.1	e
8	0.5 [4]	-	~ 0.1	e
9	1.39	v	~ 0.05	e
10	1.39	v	~ 0.05	e
11	<0.25	v	~ 0.1	e
12	0.39	v	~ 0.1	e

[1] preliminary validation data with wide-range calibration curve
[2] Values represent LOD and LOQ, which were determined as S/N of 3:1 and 9:1, respectively
[3] Values represent LOD and LOQ, which were determined as S/N of 3:1 and 5:1, respectively
[4] reporting limit (CCα not determined)

Table 11 lists the CCα values of the laboratory methods for DMZ (method validation data) and, if available, the respective limit of detection (LOD) and/or limit of quantification (LOQ) values (which have mostly not been determined in the labs during validation as these parameters are not required by 2002/657/EC). The two LODs indicated for labs 4 and 6 were available from method validation data and were calculated as a S/N of 3:1 by analysing matrix blank samples. In cases where LODs were not available (i.e. not included in the scope of validation of the laboratory's method), estimations were made at IRMM from provided results and chromatograms (matrix-matched samples and calibration curve, blank matrix samples). Values are indicating that the applied methods are showing comparable LODs for DMZ. For calculating the certified value for DMZ however, only LODs which have been included in the scope of validation of a laboratory's method were taken into account. The lowest of these LOD values (0.075 µg/kg) was multiplied by 3.3 (more conservative estimation, [20]) to obtain the corresponding LOQ, yielding 0.25 µg/kg. This value corresponds to the lowest reported CCα value (laboratory 11).

9 Certified values and uncertainties

The certified values for ERM-BB124 are calculated as the mean of means of the accepted data sets. The standard error of the mean of means was used as an estimation of the uncertainty contribution of the characterisation exercise. The standard error is calculated as the standard deviation divided by the square root of the number of accepted data sets.
The combined uncertainty of the certified value includes contributions from the between-bottle heterogeneity, long-term storage, the characterisation study, and the contribution of the common calibration solution. The relative combined uncertainty is calculated as the square root of the sum of squares of the relative uncertainties of the individual contributions, according to:

$$u_{CRM} = \sqrt{u_{bb}^2 + u_{lts}^2 + u_{char}^2 + u_{cal}^2}$$

Table 12 summarizes the individual uncertainty contributions and the resulting expanded uncertainties, and indicates the certified values and their uncertainties after rounding.

Table 12. Certified values and uncertainties for ERM-BB124

	RNZ	MNZ	HMMNI	MNZOH	IPZOH
u_{bb} [%]	1.047	0.664	1.681	1.761	1.308
u_{lts} [%][1)]	5.394	3.054	4.560	4.891	2.763
u_{char} [%]	1.854	2.244	4.010	4.782	1.631
u_{cal} [%]	0.389	0.246	0.389	0.246	0.389
$u_{CRM, rel}$ [%]	5.812	3.855	6.313	7.068	3.487
$U_{CRM,rel}$ (k=2) [%]	11.624	7.710	12.626	14.136	6.974
Certified value [µg/kg]	**2.09**	**1.93**	**0.69**	**6.2**	**1.67**
U_{CRM} (k=2) [µg/kg]	**0.25**	**0.15**	**0.09**	**0.9**	**0.12**

[1)] Shelf life 24 months

In addition, a certified value (mass fraction) for DMZ has been assigned as "< 0.25 µg/kg with a 95% level of confidence". This value corresponds to the recalculated LOQ of the most sensitive method in the characterisation study, whereby only methods were taken into account which included the LOD in the scope of validation.

10 Metrological traceability

The measurements results for assigning nitroimidazole mass fraction values to the material were obtained by employing methods with different sample preparation procedures (from extraction with organic solvent without any clean-up, up to extensive sample preparation involving matrix digestion with protease, defatting step and liquid-liquid extraction (LLE)). All methods utilised LC-MS/MS methodology for analyte separation and quantification. The liquid chromatography parts of the methods mainly differed in type of eluents used, the type of reversed phase columns applied (C_8 or C_{18} phase, particle size, column dimension), and LC system differences (UPLC or HPLC systems, flow rate, column temperature, injected sample amount). The mass spectrometry parts generally used positive electro-spray ionisation and exclusively utilised the instruments in the triple quadrupole configuration by applying tandem mass spectrometry in the multiple reaction monitoring mode. The same or similar transitions (parent ions, daughter ions) were used for quantification. Nevertheless, MS methods differed in some compound-dependent parameters (dwell times, collision energies) as well as in source/gas-related MS-settings (temperature at ionisation point, ion spray voltage, curtain gas, etc.)

For the obtained data, independence of the results from the extraction technique can be concluded. Data in a preliminary study [12] pointed to a possible result bias for some nitroimidazole analytes when applying either GC-MS or LC-MS/MS respectively, which led to the decision to exclusively apply liquid chromatography isotope dilution mass spectrometry methods in the characterisation exercise for the reference material. As a consequence, certified values only hold when liquid chromatography isotope dilution mass spectrometry is employed for quantification.

The certified values are traceable to the common calibrants used. The common calibration solution was prepared gravimetrically at IRMM using pure crystalline substances. Purities indicated by the providers were experimentally verified at IRMM using LC-DAD, LC-MS, and LC-MS/MS. The indicated amount-of-substance concentration values for the calibrant solution are therefore traceable to the SI.

Consequently, the certified mass fractions for RNZ, MNZ, HMMNI, MNZOH, IPZOH, and DMZ are traceable to the International System of Units (SI).

11 Instructions for use

11.1 Safety precautions

The usual laboratory safety precautions apply.

11.2 Reconstitution of the material

- Allow the bottle to warm up to ambient temperature before opening.
- Weigh accurately an aliquot of 1.25 ± 0.01 g. The weighing should be performed immediately after opening of the vial to minimise water uptake by the lyophilised powder.
- Add an accurately weighed amount of 3.75 ± 0.01 g of distilled water to the powder.
- In case the working instruction of the laboratory's method foresees a higher sample intake than 5 g of reconstituted material, the 1:3 m/m ratio of powder to distilled water has to be maintained.
- Mix to a homogeneous sample, for instance by vortexing the powder-water mixture for at least 1 min at maximum speed.

11.3 Intended use

This material is intended to be used for method performance control and validation purposes (trueness determination). For assessing the method performance, the measured values of the CRMs are compared with the certified values following a procedure described by Linsinger [21]. The procedure is described here in brief:

- Calculate the absolute difference between mean measured value and the certified value (Δ_m).
- Combine measurement uncertainty (u_{meas}) with the uncertainty of the certified value (u_{CRM}): $u_{\Delta} = \sqrt{u_{meas}^2 + u_{CRM}^2}$
- Calculate the expanded uncertainty (U_{Δ}) from the combined uncertainty (u_{Δ}) using a coverage factor of two (k = 2), corresponding to a confidence interval of approximately 95 %
- If $\Delta_m \leq U_{\Delta}$ then there is no significant difference between the measurement result and the certified value, at a confidence level of about 95 %.

11.4 Storage conditions

The materials should be stored at a temperature of -20 ± 2°C. However, the European Commission cannot be held responsible for changes that happen during storage of the material at the customer's premises, especially of open samples.

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Annex A. Homogeneity data

Table A1. Results of homogeneity study

Bottle number	Replicate 1 (µg/kg)	Replicate 2 (µg/kg)	Average (µg/kg)	Replicate 1 (µg/kg)	Replicate 2 (µg/kg)	Average (µg/kg)
	RNZ			**MNZ**		
45	2.076	1.987	2.032	1.764	1.720	1.742
105	1.953	1.891	1.922	1.739	1.754	1.747
263	2.006	2.024	2.015	1.730	1.712	1.721
299	2.025	1.937	1.981	1.761	1.731	1.746
358	1.985	1.892	1.939	1.702	1.772	1.737
520	1.960	1.989	1.975	1.702	1.713	1.708
544	1.945	2.025	1.985	1.708	1.729	1.719
639	1.951	2.001	1.976	1.706	1.705	1.706
788	1.983	1.981	1.982	1.767	1.727	1.747
876	1.974	1.964	1.969	1.747	1.710	1.729
	HMMNI			**MNZOH**		
45	0.702	0.674	0.688	4.509	4.409	4.459
105	0.704	0.668	0.686	4.250	4.544	4.397
263	0.629	0.663	0.646	4.210	4.180	4.195
299	0.663	0.666	0.665	4.222	4.635	4.429
358	0.665	0.657	0.661	4.303	4.428	4.366
520	0.639	0.650	0.645	4.395	4.579	4.487
544	0.643	0.655	0.649	4.459	4.501	4.480
639	0.664	0.666	0.665	4.725	4.539	4.632
788	0.654	0.672	0.663	4.540	4.537	4.539
876	0.636	0.663	0.650	4.036	4.399	4.218
	IPZOH					
45	1.633	1.651	1.642			
105	1.643	1.674	1.659			
263	1.713	1.665	1.689			
299	1.600	1.666	1.633			
358	1.676	1.642	1.659			
520	1.588	1.637	1.613			
544	1.624	1.616	1.620			
639	1.582	1.605	1.594			
788	1.600	1.627	1.614			
876	1.640	1.635	1.638			

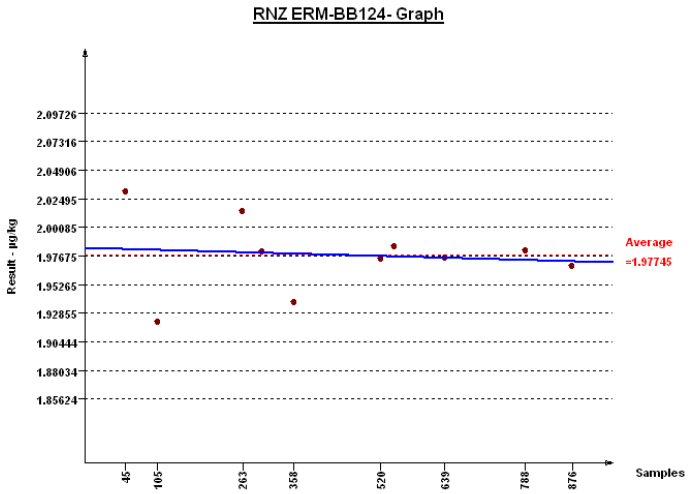

Figure A1. Homogeneity of RNZ in ERM-BB124. The x axis depicts the sample numbers (filling sequence). The indicated points are mean values of duplicate measurements.

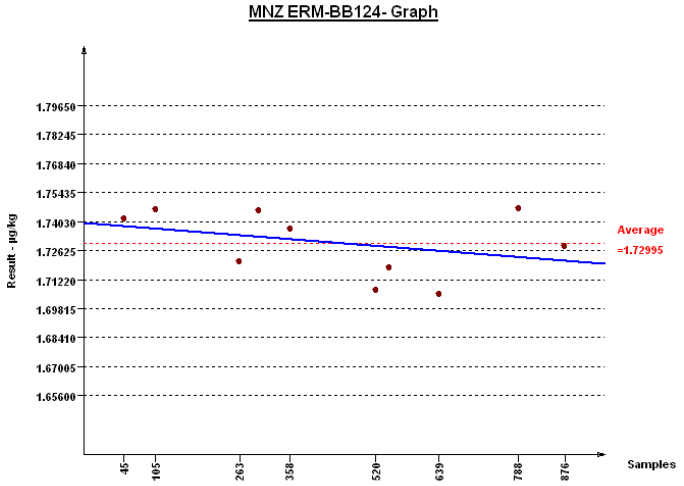

Figure A2. Homogeneity of MNZ in ERM-BB124. The x axis depicts the sample numbers (filling sequence). The indicated points are mean values of duplicate measurements.

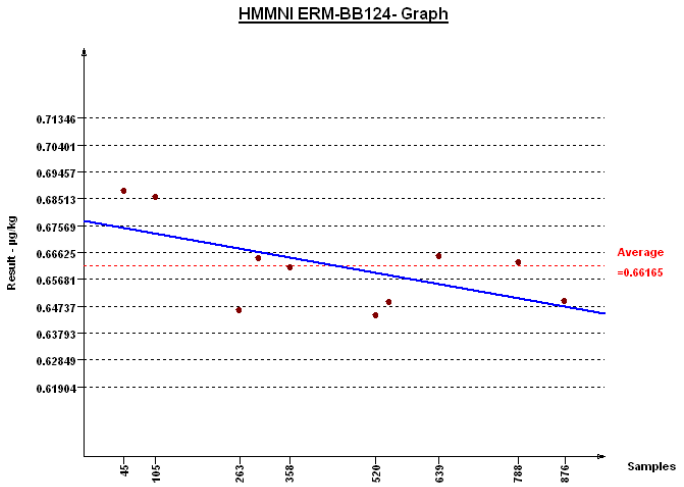

Figure A3. Homogeneity of HMMNI in ERM-BB124. The x axis depicts the sample numbers (filling sequence). The indicated points are mean values of duplicate measurements.

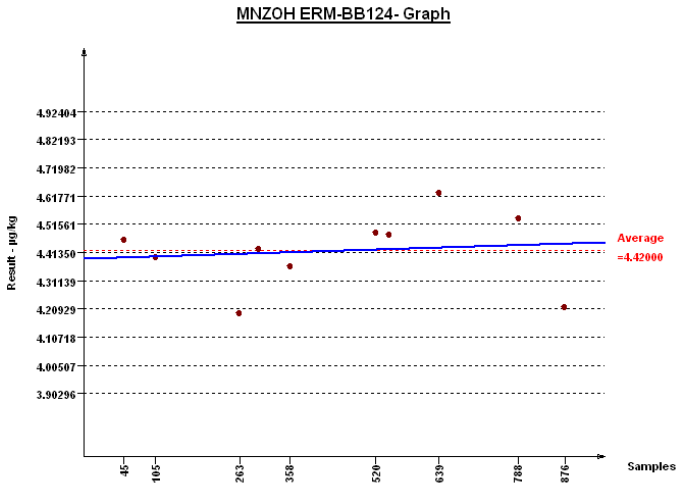

Figure A4. Homogeneity of MNZOH in ERM-BB124. The x axis depicts the sample numbers (filling sequence). The indicated points are mean values of duplicate measurements.

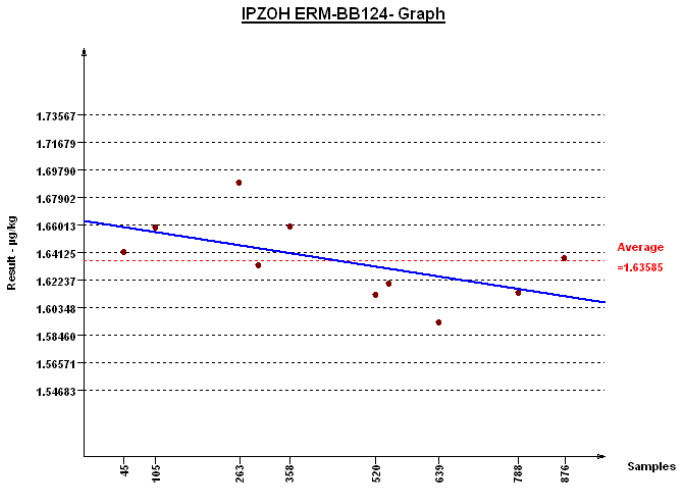

Figure A5. Homogeneity of IPZOH in ERM-BB124. The x axis depicts the sample numbers (filling sequence). The indicated points are mean values of duplicate measurements.

Annex B. Short-term stability data

Table B1. Results of the short-term stability study

Time (weeks)	4 ºC	18 ºC	4 ºC	18 ºC	4 ºC	18 ºC
	RNZ		**MNZ**		**HMMNI**	
0	2.180	2.180	1.921	1.921	0.836	0.836
0	2.142	2.142	1.855	1.855	0.825	0.825
0	2.087	2.087	1.865	1.865	0.825	0.825
0	2.065	2.065	1.927	1.927	0.814	0.814
1	2.030	2.126	1.870	1.907	0.792	0.82
1	2.017	2.069	1.859	1.842	0.818	0.837
1	2.114	2.085	1.885	1.816	0.832	0.831
1	2.114	2.108	1.891	1.890	0.820	0.791
2	2.040	2.063	1.858	1.856	0.798	0.843
2	2.066	2.08	1.924	1.913	0.803	0.781
2	2.092	2.233	1.904	1.846	0.802	0.814
2	2.046	2.006	1.925	1.898	0.821	0.853
4	1.988	2.165	1.899	1.939	0.801	0.816
4	2.094	2.083	1.892	1.775	0.806	0.790
4	2.011	2.099	1.799	1.971	0.821	0.747
4	2.053	2.064	1.892	1.882	0.810	0.825
	MNZOH		**IPZOH**			
0	4.872	4.872	1.690	1.690		
0	4.922	4.922	1.731	1.731		
0	4.880	4.880	1.640	1.640		
0	4.659	4.659	1.643	1.643		
1	4.822	4.797	1.631	1.673		
1	4.493	4.615	1.654	1.679		
1	5.576	4.507	1.672	1.679		
1	4.785	4.875	1.665	1.649		
2	4.594	4.627	1.660	1.655		
2	4.665	4.784	1.739	1.625		
2	4.627	4.891	1.669	1.676		
2	4.689	4.900	1.669	1.644		
4	4.574	4.588	1.586	1.705		
4	4.633	4.615	1.662	1.641		
4	4.317	4.995	1.661	1.656		
4	4.404	4.673	1.647	1.652		

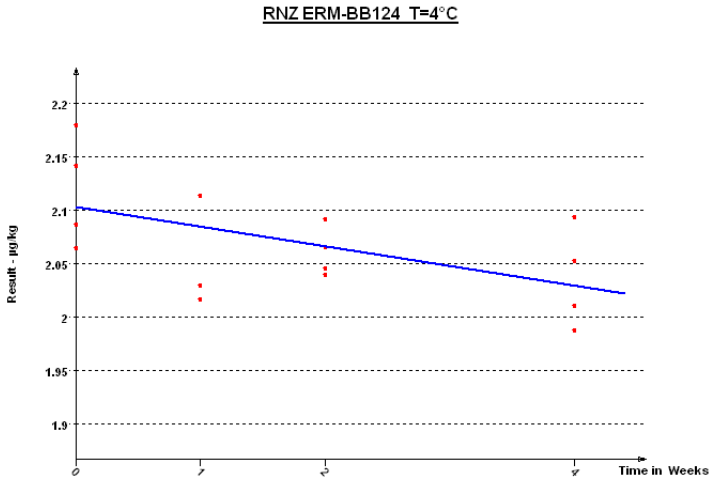

Figure B1. Short-term stability for RNZ at 4 ºC.

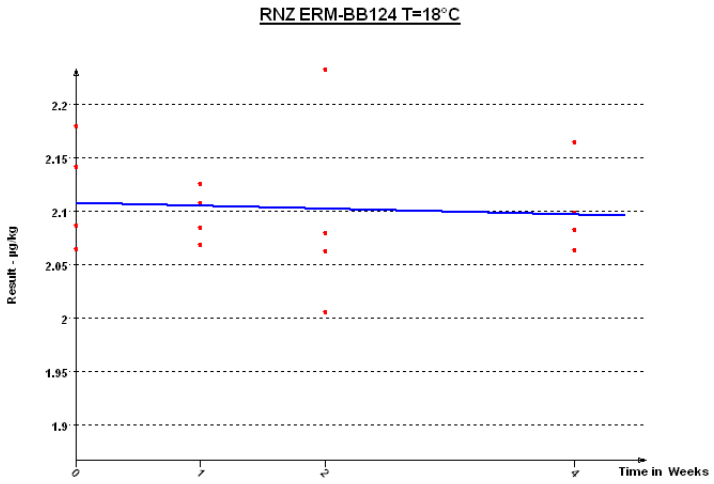

Figure B2. Short-term stability study for RNZ at 18 ºC.

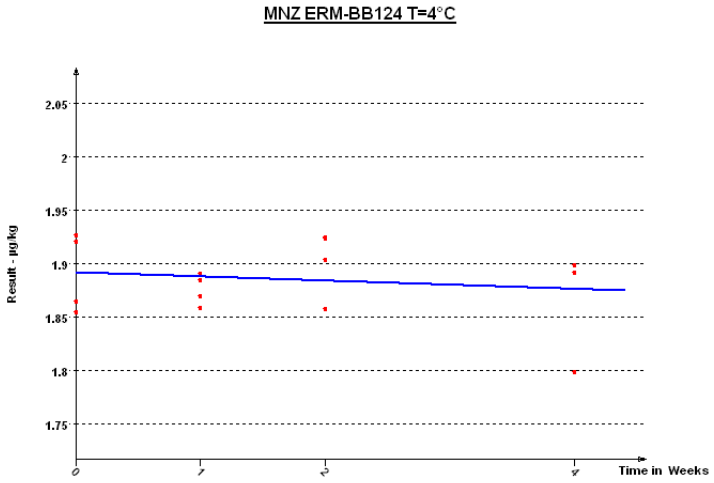

Figure B3. Short-term stability for MNZ at 4 ºC.

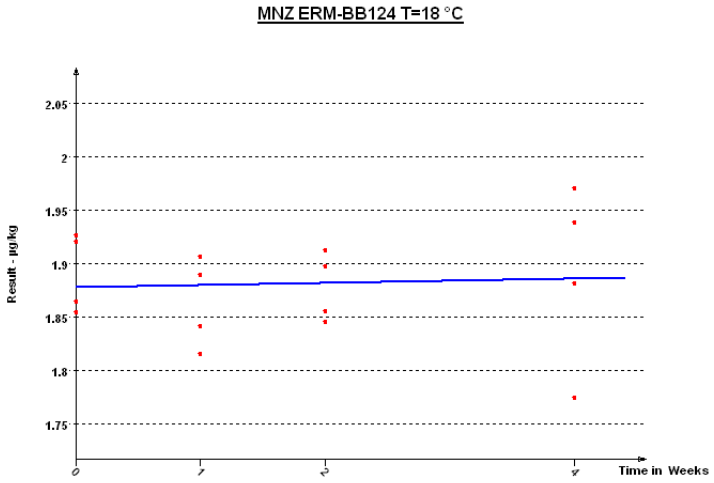

Figure B4. Short-term stability for MNZ at 18 ºC.

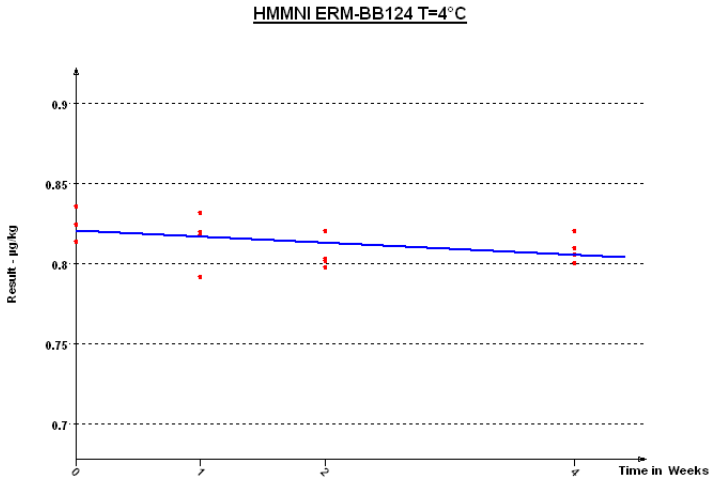

Figure B5. Short-term stability for HMMNI at 4 ºC.

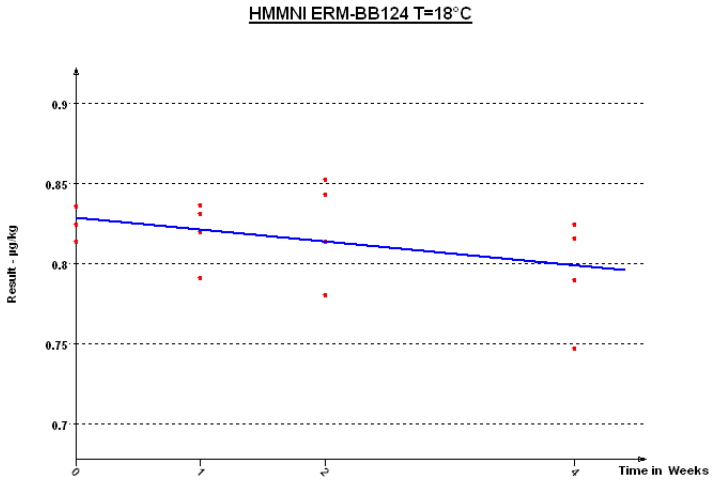

Figure 6. Short-term stability for HMMNI at 18 ºC.

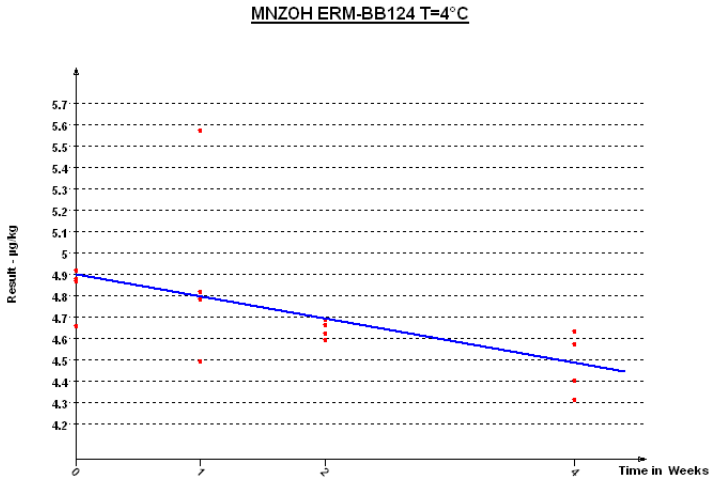

Figure B7. Short-term stability for MNZOH at 4 ºC.

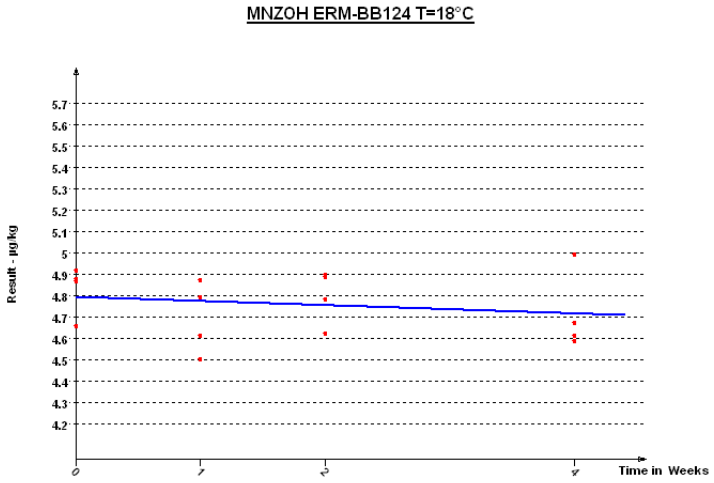

Figure B8. Short-term stability for MNZOH at 18 ºC.

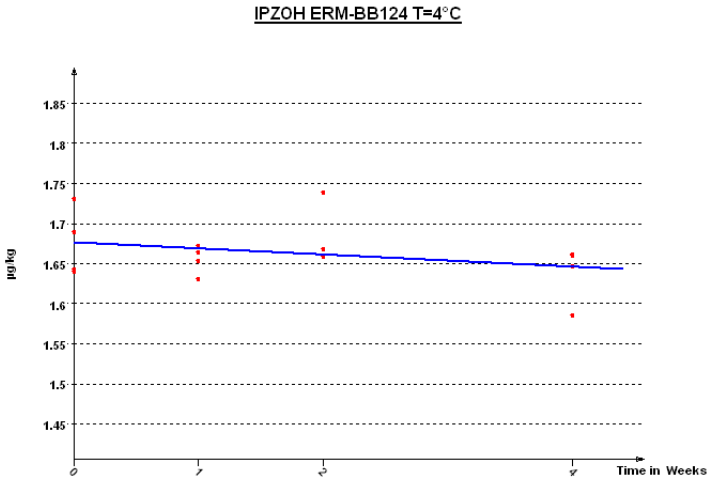

Figure B9. Short-term stability for IPZOH at 4 ºC.

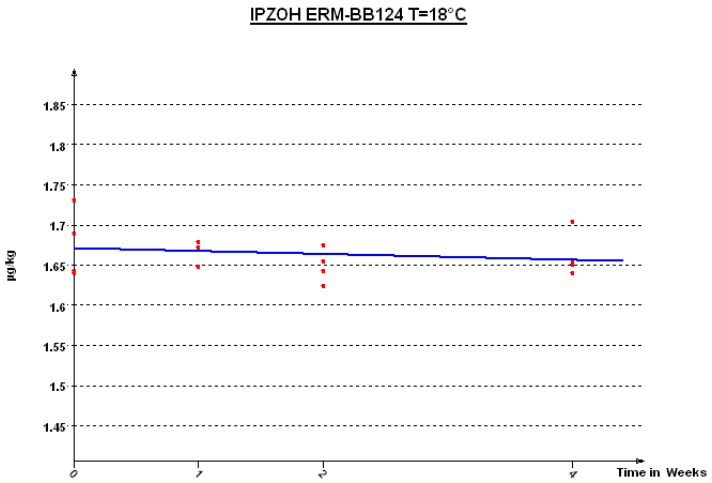

Figure B10. Short-term stability for IPZOH at 18 ºC.

Annex C. Long-term stability data

Table C1. Results of the long-term stability study

Time (months)	-20 ºC	4 ºC	-20 ºC	4 ºC	-20 ºC	4 ºC
	RNZ		**MNZ**		**HMMNI**	
0	2.219	2.219	1.963	1.963	0.804	0.804
0	2.001	2.001	1.918	1.918	0.772	0.772
0	1.944	1.944	1.978	1.978	0.770	0.770
0	2.251	2.251	1.962	1.962	0.745	0.745
8	2.268	2.261	1.926	1.916	0.704	0.746
8	2.266	2.093	2.003	1.899	0.790	0.770
8	2.375	2.117	2.070	1.807	0.787	0.761
8	2.200	2.170	1.967	2.048	0.733	0.821
12	2.170	1.997	2.022	1.913	0.770	0.760
12	2.167	2.193	2.001	1.924	0.770	0.759
12	2.097	2.154	1.926	1.927	0.745	0.769
12	2.139	2.247	1.839	1.970	0.743	0.750
16	2.167	2.163	1.914	1.879	0.756	0.752
16	2.483	2.179	1.969	1.847	0.784	0.725
16	2.193	2.102	1.939	2.053	0.744	0.824
16	2.067	n.d.	2.001	*1.932*	0.761	0.742
	MNZOH		**IPZOH**			
0	6.690	6.690	1.865	1.865		
0	6.648	6.648	1.748	1.748		
0	6.468	6.468	1.769	1.769		
0	6.744	6.744	1.865	1.865		
8	7.337	7.161	1.802	1.846		
8	6.759	6.924	1.875	1.828		
8	7.053	6.383	1.861	1.766		
8	7.262	6.614	1.819	1.700		
12	6.805	6.842	1.873	1.795		
12	7.420	6.458	1.797	1.718		
12	6.636	7.039	1.767	1.805		
12	6.815	7.473	1.823	1.752		
16	6.517	6.911	1.776	1.828		
16	6.651	6.585	1.708	1.765		
16	7.226	6.220	1.886	1.717		
16	6.508	6.644	1.862	1.772		

Underlined values: results not considered for evaluation (reported technical problems); n.d., not detected

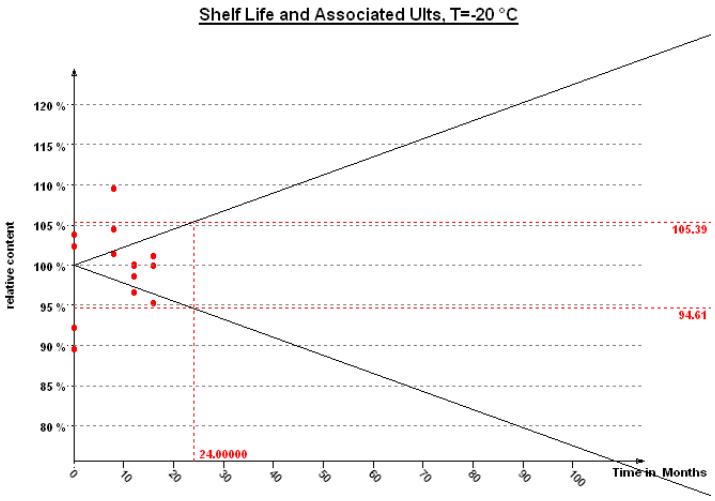

Figure C1. Long-term stability for RNZ at -20 ºC with associated u_{lts} for storage period of 24 months

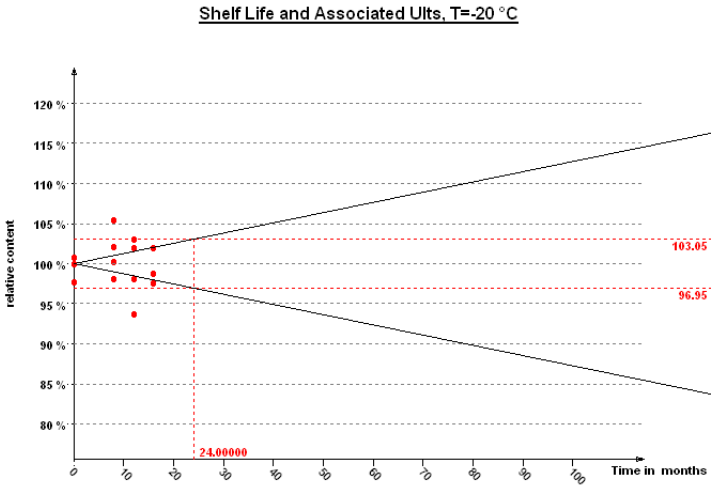

Figure C2. Long-term stability for MNZ at -20 ºC with associated u_{lts} for storage period of 24 months

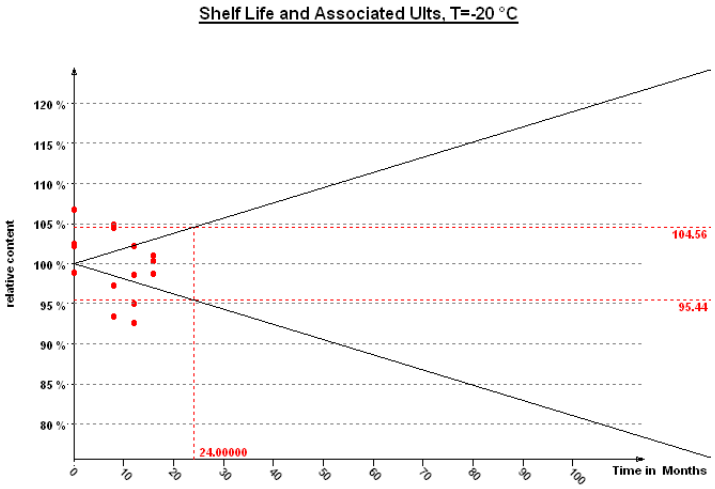

Figure C3. Long-term stability for HMMNI at -20 ºC with associated u_{lts} for storage period of 24 months

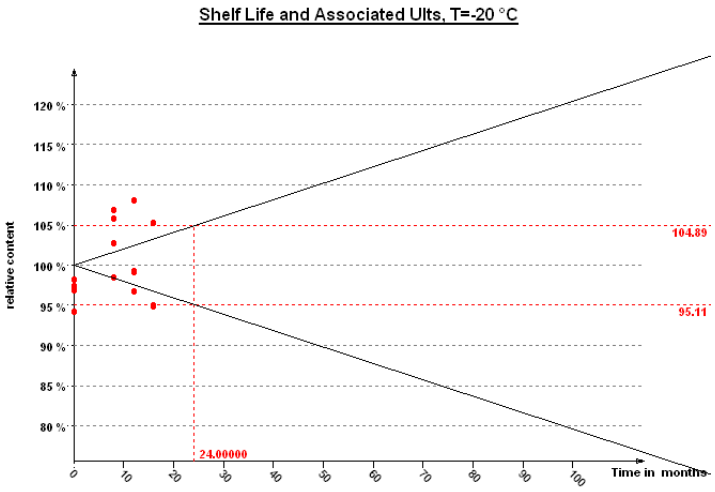

Figure C4. Long-term stability for MNZOH at -20 ºC with associated u_{lts} for storage period of 24 months

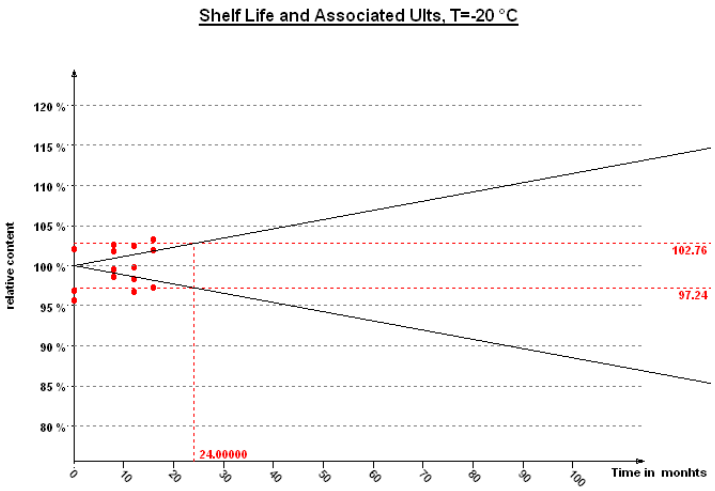

Figure C5. Long-term stability for IPZOH at -20 ºC with associated u_{lts} for storage period of 24 months

Annex D. Characterisation data

Table D1. Results of characterisation measurements for RNZ

RNZ mass fraction in ERM-BB124[µg/kg]								
Lab code	Day 1				Day 2			
1	2.21	2.16	2.20	2.11	2.18	2.20	2.16	2.26
2	2.04	1.85	2.20	1.92	2.11	1.86	1.98	1.99
4	2.09	1.89	1.94	2.14	2.14	2.63	2.11	2.21
5	1.85	1.94	1.83	1.88	1.87	1.94	1.86	1.91
6	2.29	2.27	2.28	2.23	2.14	2.20	2.10	2.06
7	2.07	2.13	2.02	2.07	2.22	2.27	2.18	2.14
8	2.48	2.26	2.18	2.27	2.50	2.49	2.32	2.24
9	2.07	2.12	2.05	2.10	2.05	2.08	1.98	2.04
10	2.08	2.06	2.08	2.10	2.02	2.12	2.03	2.12
11	2.19	1.87	2.54	2.03	1.70	1.84	1.87	1.93
12	1.98	1.95	1.96	1.93	1.97	1.86	2.07	2.04

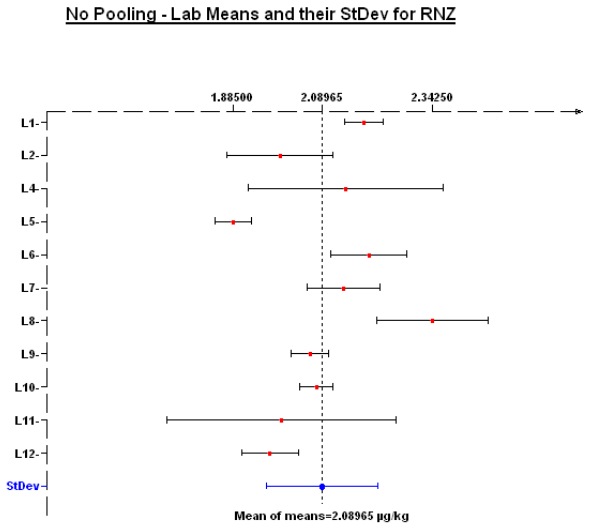

Figure D1. Laboratory means, mean of means and their standard deviation for RNZ

Table D2. Results of characterisation measurements for MNZ

MNZ mass fraction in ERM-BB124 [µg/kg]								
Lab code	Day 1				Day 2			
1	1.91	1.82	1.79	1.81	2.00	1.99	1.93	1.85
2	1.71	1.68	2.88	2.33	1.60	1.62	2.52	2.79
3	1.85	1.76	1.77	1.69	2.08	1.94	2.26	2.11
4	2.12	2.54	2.07	2.21	1.96	2.31	2.13	2.39
5	1.69	1.71	1.66	1.67	1.66	1.71	1.64	1.68
6	1.92	1.98	2.07	2.03	1.95	1.98	1.96	1.91
7	1.91	2.00	1.98	1.92	2.03	2.04	1.91	2.00
8	1.96	1.94	1.88	1.86	2.01	2.00	1.89	1.93
9	1.94	1.94	1.91	1.93	1.81	1.86	1.89	1.92
10	1.95	1.96	1.92	1.95	1.96	1.93	1.91	1.96
11	1.87	2.02	1.87	1.97	1.64	1.61	1.76	1.70
12	1.79	1.71	1.76	1.64	1.71	1.65	1.86	1.83

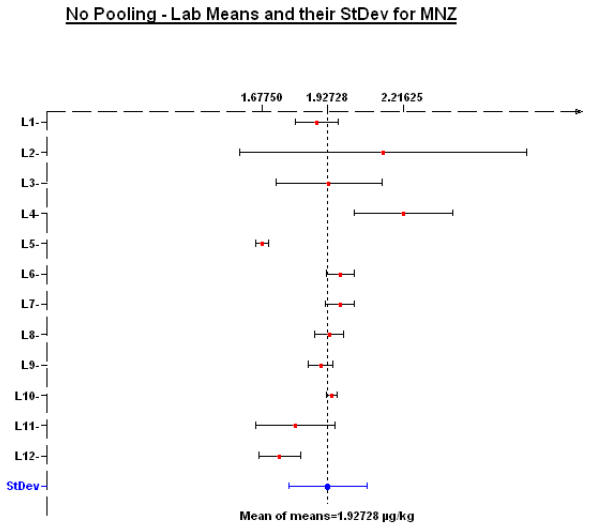

Figure D2. Laboratory means, mean of means and their standard deviation for MNZ

Table D3. Results of characterisation measurements for HMMNI

HMMNI mass fraction in ERM-BB124 [µg/kg]								
Lab code	Day 1				Day 2			
1	0.68	0.73	0.71	0.69	0.78	0.79	0.81	0.77
2	0.61	0.60	0.68	0.63	0.59	0.53	0.82	0.70
3	0.50	0.59	0.55	0.51	0.92	0.93	0.61	0.86
4*	< CCα	< CCα	< CCα	< CCα	< CCα	< CCα	< CCα	< CCα
5	0.60	0.53	0.60	0.56	0.62	0.63	0.62	0.62
6	0.71	0.69	0.72	0.72	0.73	0.74	0.72	0.71
7	0.68	0.63	0.63	0.69	0.91	0.81	0.82	0.83
8	0.72	0.72	0.69	0.71	0.69	0.67	0.71	0.64
9	0.78	0.72	0.74	0.77	0.66	0.66	0.71	0.64
10	0.88	0.89	0.88	0.88	0.80	0.80	0.81	0.84
11	0.58	0.59	0.56	0.61	0.44	0.42	0.41	0.33
12	0.76	0.67	0.67	0.64	0.63	0.61	0.59	0.68

* CCα: 0.6 µg/kg

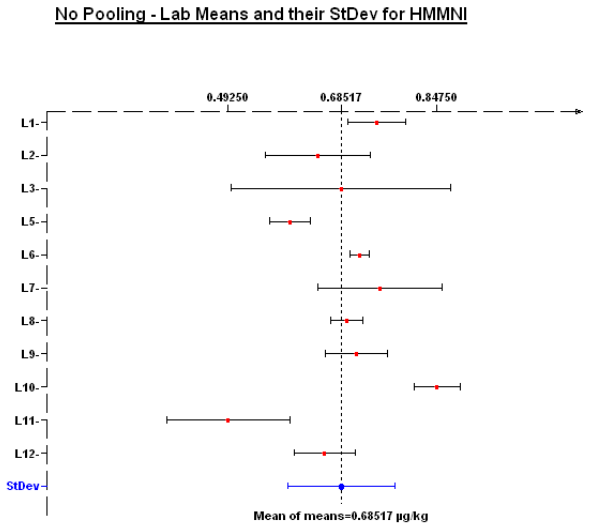

Figure D3. Laboratory means, mean of means and their standard deviation for HMMNI

Table D4. Results of characterisation measurements for MNZOH

MNZOH mass fraction in ERM-BB124 [µg/kg]								
Lab code	Day 1				Day 2			
1	7.07	5.84	7.28	6.13	8.72	7.21	7.81	6.25
2	n.m.	n.m.	n.m.	n.m.	n.m.	n.m.	n.m.	n.m.
3	6.93	6.49	6.91	7.13	6.05	6.65	7.31	7.59
4	6.50	7.74	6.23	6.32	8.30	9.82	9.87	10.09
5	4.07	4.09	4.33	4.02	4.04	4.37	4.28	3.86
6	6.13	5.52	6.12	6.62	7.02	6.90	6.78	5.84
7	5.98	5.96	5.76	6.05	6.29	6.16	6.39	6.34
8	5.93	6.32	6.32	6.16	6.46	6.65	6.23	6.66
9	6.08	5.80	5.49	5.66	5.59	5.51	5.71	6.73
10	6.31	5.99	5.92	6.01	5.84	5.90	5.60	5.93
11	7.45	6.78	6.17	5.82	6.80	6.06	5.14	5.56
12	6.22	5.08	5.59	4.95	5.01	5.12	6.02	6.45

n.m., not measured

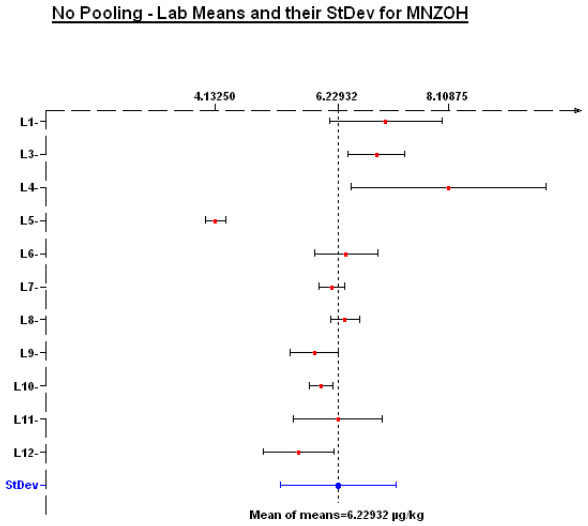

Figure D4. Laboratory means, mean of means and their standard deviation for MNZOH

Table D5. Results of characterisation measurements for IPZOH

IPZOH mass fraction in ERM-BB124 [µg/kg]								
Lab code	Day 1				Day 2			
1	n.m.	n.m.	n.m.	n.m.	n.m.	n.m.	n.m.	n.m.
2	1.64	1.60	2.01	1.78	1.43	1.52	1.90	1.81
3	1.67	1.59	1.53	1.51	1.87	1.75	1.84	1.90
4	1.88	1.76	1.82	1.91	1.60	1.62	1.60	1.39
5	1.50	1.50	1.49	1.47	1.57	1.55	1.45	1.49
6	1.67	1.76	1.71	1.78	1.70	1.64	1.61	1.52
7	1.66	1.69	1.69	1.71	1.84	1.87	1.83	1.79
8	1.69	1.78	1.79	1.78	1.68	1.74	1.62	1.70
9	1.67	1.67	1.63	1.69	1.76	1.71	1.66	1.66
10	1.61	1.62	1.57	1.60	1.67	1.67	1.70	1.70
11	1.35	1.99	1.74	1.64	2.47	1.95	1.67	1.46
12	1.62	1.55	1.48	1.48	1.55	1.52	1.66	1.23

n.m., not measured

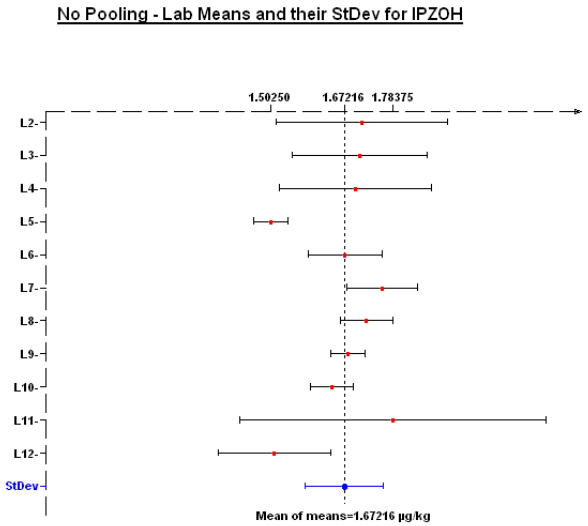

Figure D5. Laboratory means, mean of means and their standard deviation for IPZOH

European Commission

EUR 23574 EN – Joint Research Centre – Institute for Reference Materials and Measurements
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Abstract

This report describes the preparation of the pork meat matrix reference material ERM-BB124 and the certification of the content (mass fraction) of six nitroimidazole parent drugs and hydroxy metabolites.

The preparation and processing of the material, homogeneity and stability studies, and the characterisation are described hereafter and the results are discussed. Uncertainties were calculated in compliance with the Guide to the Expression of Uncertainty in Measurement (GUM) [1] and include uncertainties due to possible heterogeneity, instability, characterisation, and calibrant uncertainty. The certified values and their uncertainties are listed below:

Nitroimidazole in the reconstituted material	Certified value [1)] [µg/kg]	Uncertainty [2)] [µg/kg]	Number of accepted sets of results
Ronidazole (RNZ)	2.09	0.25	11
Metronidazole (MNZ)	1.93	0.15	12
2-hydroxymethyl-1-methyl-5-nitroimidazole (HMMNI)	0.69	0.09	11
Hydroxymetronidazole (MNZOH)	6.2	0.9	11
Hydroxyipronidazole (IPZOH)	1.67	0.12	11

1) These values are the mass fractions based on the unweighted mean of accepted results.
2) The uncertainties are the expanded uncertainties (k = 2) of the values defined in 1).

Additionally, the following certified value has been assigned:

Nitroimidazole in the reconstituted material	Certified value [1)] [µg/kg]
Dimetridazole (DMZ)	<0.25

1) This value corresponds to the limit of quantification (LOQ) of the most sensitive method in the characterisation study. With a probability of 95% the certified value is below 0.25 µg/kg.

The assigned values and their uncertainties are based on a minimum sample intake of 5 g reconstituted material (corresponding to 1.25 g powder).

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