Final Report of the Interlaboratorial Comparison to Characterize a Certified Reference Material Candidate for Nitrofuran Metabolites in Chicken Meat

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SUMMARY

1. Introduction

Nitrofurans are veterinary drugs used to treat infections in the gastrointestinal tract of animals, such as pig and poultry. Because of their carcinogenic and mutagenic characteristics, they are included in Annex IV to EU Regulation 2377/90 [1], where the prohibited substances are listed. In Brazil, the manufacture, manipulation, fractionation, marketing, import and use of nitrofurans and products containing these active ingredients for veterinary use and susceptible to use in the animal and insect feed was prohibited by the Ministry of Agriculture, Livestock and Food Supply (MAPA) through Normative Instruction nº. 09 of June 27, 2003 [2]. When administered, nitrofurans are rapidly and extensively metabolized, and their main metabolites are 3-amino-2-oxazolidinone (AOZ), 3-amino-5-morpholinomethyl-2-oxazolidinone (AMOZ), semicarbazide (SEM) and 1-aminohydantoin (AHD). Thus, these metabolites are used as markers for detection purposes in the monitoring of residues of these compounds in products of animal origin [3].

Issues related to food safety, as well as the search for improvement through the enhancement of international trade relations, have stimulated nations in the search for quality of measurements in the food area. Certified Reference Materials (CRMs) play a key role in this improvement because they give these measures traceability to the International System (SI). The important role of the CRM in face of the problems of trade restrictions in some countries, such as the measures adopted by the European Union with Directive 96/23 / EC [4], to control residues in the field of veterinary drugs in products of origin animal. In April 2002, a new testing methodology detected the presence of nitrofuran residues in batches of poultry meat exported by Brazil, banned in the EU. Some estimates suggest a loss of around US $ 40 million per year with additional controls [5]. According to data from the United States Department of Agriculture (USDA), in 2017 Brazil became the second largest producer of chicken meat in the world and remained the largest exporter.

Given this scenery, it is necessary to maintain and improve measures to guarantee the quality of this product, since its embargo by other countries would compromise the Brazilian trade balance. In order to carry out the control, it is necessary to use analytical methods that are applicable and that meet the national and international provisions. The use of CRM is recommended in guidelines on performance of analytical methods for unambiguous detection of chemical residues in foods of animal origin, such as the European Union's Commission Decision 2002/657 / EC [6] and the Analytical Quality Assurance Manual of the Ministry of Agriculture, Livestock and Food Supply [7].

This IC aimed to support the characterization value of a Certified Reference Material (CRM) candidate of nitrofurans metabolites in freeze-dried chicken muscle. The mass fraction values of the metabolites and their respective uncertainties were determined, and the measurements of residual moisture content of the freeze-dried material were optional.

2. Materials and Methods

2.1. Preparation of the Comparison Material

The CRM candidate was produced from the matrix blank mixture with chicken breast samples naturally contaminated with the nitrofuran metabolites. Matrix blank samples were obtained from commercially purchased chicken breasts, whose absence of metabolites was confirmed by High Performance Liquid Chromatography coupled to Tandem Mass Spectrometry (HPLC-MS/MS). Chicken breast samples containing the incurred metabolites (AOZ, AMOZ, SEM and AHD) were obtained in a previous work in which these substances were incorporated into the matrix through the metabolic process of the precursor drugs administered to the animals. The breeding of animals until slaughter was carried out at the Ildefonso Bastos Borges Agricultural Technical College (CTAIBB), located in the municipality of Bom Jesus do Itabapoana, Rio de Janeiro, Brazil [8].

The following procedure was performed individually for each tissue group (chicken breast) contaminated with the metabolites and for the matrix blank. The chicken breasts were cut into small pieces and ground in a blender with stainless steel container (7011S, Waring, Torrington, USA) and spread on trays in quantities of approximately 150 g in each tray. The samples were frozen at -80 °C for 24 h and then freeze-dried (LIOTOP, Model L101). The mass of material in each tray was determined before and after the freeze-drying process. The freeze-dried chicken breasts were ground in a knife mill for 3 min at 10,000 RPM, sieved at 420 μm, with the aid of a sieve shaker (Bertel, CIAL158) and stored separately in bottles of borosilicate amber glass at -20 °C. Each group of freeze-dried starting material (chicken breasts) was individually analyzed by HPLC-MS/MS in order to determine the mass fractions of the metabolites in each of these materials.

Based on the mass fraction results for each tissue contaminated with its respective nitrofuran metabolites, the required amount of each of these tissues together with a matrix blank was calculated to obtain the target mass fraction of the metabolites AOZ, AMOZ, SEM and AHD in the CRM candidate. The mixing of the tissues with each metabolite and matrix blank was performed in a Y powder homogenizer for 2 h. The obtained material was packed in 10 mL borosilicate amber glass bottles containing (1.30 ± 0.05) g of chicken muscle in each unit, totaling 256 bottles of CRM candidate. After the packaging, the whole batch was freeze-dried in the bottles, which were sealed under an inert atmosphere with N_2 gas. For this

purpose, upon completion of drying, prior to the withdrawal of the vials from the freeze-dryer chamber, a flask containing N_2 gas was connected to the air inlet of this chamber, so that the inert gas enters the chamber instead of the air, after the vacuum is interrupted.

Subsequently the batch was submitted to a gamma irradiation treatment at 7 kGy, performed at the Army Technology Center (CTEx, Guaratiba, Rio de Janeiro, Brazil). This treatment is important for the reduction of the microbiological load, which should guarantee a greater stability to the product. The batch was also submitted to microbiological analysis before and after irradiation.

The material preparation procedure was developed based on a previous viability study for AOZ and AMOZ analytes, carried out in the same matrix [9].

The IC material consists of freeze-dried incurred chicken breast muscle, containing the 4 nitrofuran metabolites: 3-amino-2-oxazolidinone (AOZ), 3-amino-5-morpholinomethyl-2-oxazolidinone (AMOZ), semicarbazide (SEM), 1-aminohydantoin (AHD). The material is contained in amber glass bottles with rubber cap and a flip-off aluminum sealing. Each bottle contains approximately 1.3 g of freeze-dried material.

The properties to be determined as well as their mass fraction ranges are shown in table 1. The ranges of nitrofuran metabolite mass fraction values are presented on a wet basis (in the reconstituted material). Determination of residual moisture was optional for participants who are able to perform this analysis according to the instructions given in the protocol.

Table 1 – Properties to be analyzed and indicative mass fractions.

Property	Mass fraction ranges
Residual moisture content (optional)	0.5 g/100 g to 10 g/100 g
AOZ mass fraction	0.5 µg/kg to 10 µg/kg
AMOZ mass fraction	0.5 µg/kg to 10 µg/kg
AHD mass fraction	0.5 µg/kg to 10 µg/kg
SEM mass fraction	0.5 µg/kg to 10 µg/kg

2.2. IC Material Homogeneity

For the homogeneity study, eleven samples were randomly selected and analyzed in duplicate for residual moisture content and for the mass fractions of nitrofuran metabolites.

The mass fractions of the four metabolites of nitrofurans, AOZ, AMOZ, SEM and AHD were determined by Isotope Dilution Mass Spectrometry (IDMS) using HPLC-MS/MS technique. The extraction procedure involved the acidic hydrolysis of the metabolites with 1 M HCl solution, derivatization for 16 h at 37 °C with 50 nM 2-nitrobenzaldehyde (2-NBA) solution, and two liquid-liquid extraction steps with ethyl acetate. A liquid chromatography system (Agilent 1200 Series, Agilent Technologies, Santa Clara, USA) coupled to a triple-quadrupole mass spectrometer (model 4000TM QTrap LC-MS/MS System, Applied Biosystems, Foster City, USA) was used. This same system was used in the stability studies and characterization for the CRM candidate.

A Karl Fischer Coulometric automatic titrator (Model 831, Metrohm, Herisau, Switzerland) equipped with a diaphragm generator electrode and a sample heating oven (Model 774, Metrohm, Herisau, Switzerland) was used to determine residual moisture.

Statistical procedures were performed according to the requirements of ABNT ISO Guide 35 [10], using single factor variance analysis (ANOVA). Before proceeding with ANOVA, the Cochran test verified the possible existence of discrepant variances of results within the same bottle. The heterogeneity uncertainty of the material obtained from this homogeneity study (u_{hom}), as well as the degree of heterogeneity (%) were calculated from the ANOVA data, as a function of the mean square values between the units ($MS_{between}$) and inside of the units (MS_{within}).

The results of the homogeneity study for the parameters studied in the CRM candidate batch are described in table 2, including ANOVA data (F values).

Table 2 - Results of the homogeneity study of residual moisture and mass fractions of the nitrofuran metabolites, AOZ, AMOZ, SEM and AHD, in the MRC candidate batch.

Parameter	**F_{calc}**	**F_{tab}**	**u_{hom}**	**Level of heterogeneity (%)**
Residual moisture	2.96	3.02	0.086 g/100 g	6.21
AOZ	1.93	2.85	0.14 µg/kg	2.66
AMOZ	0.72	2.85	0.092 µg/kg	1.79
AHD	1.18	2.85	0.065 µg/kg	1.47
SEM	1.08	2.85	0.046 µg/kg	0.94

The residual moisture content in the CRM candidate batch presented a level of heterogeneity of 6.21%, while the mass fractions of the metabolites presented between 0.94% and 2.66%. Based on the target uncertainties of the CRM property values established in the planning step, a maximum level of heterogeneity of 10% was considered to be acceptable for the purpose. Thus, all property values were considered homogeneous.

2.3. IC Material Stability

The analytical method used in the stability studies were the same as those used in the homogeneity studies. The statistical analysis of the results was performed by linear regression, following the principles of the ABNT ISO Guide 35 [10]. The short-term stability study (transport conditions) was performed for the residual moisture of the material and for the mass fraction of the nitrofuran metabolites, at two temperatures: 20 ° C (ambient temperature) and 50 ° C (temperature at which a material can arrive on a land transport). The study time covered 63 days divided into 6 periods (05, 13, 32, 48, 54 and 63 days). Two samples were analyzed on each day. Table 3 summarizes the results of short-term stability studies for the CRM candidate batch.

Table 3 - Results of the homogeneity study of residual moisture and mass fractions of nitrofuran metabolites, AOZ, AMOZ, SEM and AHD, in the candidate batch of CRM.

Parameter	Temp. (°C)	b_1	$s(b_1)$	*p value	Uncertainty	Conclusion
Residual moisture	20	0.00239	0.000920	0.0234	0.058 g/100 g	**unstable**
	50	0.0102	0.00170	0.0000628	0.11 µg/kg	**unstable**
AOZ	20	-0.00255	0.00168	0.157	0.11 µg/kg	stable
	50	-0.00971	0.00305	0.00785	0.19 µg/kg	**unstable**
AMOZ	20	-0.00399	0.00516	0.456	0.33 µg/kg	stable
	50	-0.00516	0.004599	0.283	0.29 µg/kg	stable
AHD	20	-0.00328	0.00282	0.274	0.18 µg/kg	stable
	50	-0.00511	0.00435	0.267	0.27 µg/kg	stable
SEM	20	0.000245	0.00272	0.930	0.17 µg/kg	stable
	50	0.000856	0.00390	0.830	0.25 µg/kg	stable

* The property is considered stable when p value > 0.05.

In the presented results, p value of the linear coefficient (b_1) of the plotted curve for the mass fraction of the studied property as a function of time should be observed. Values of p greater than 0.05 mean that, in the statistical test t for slope of the curve, the calculated Student t-value did not exceed the two-tailed critical value for n - 2 degrees of freedom and 95% confidence level. In this case, slopes can be considered significantly equal to zero for a 95% confidence level and the evaluated parameters considered stable during the studied period. For the residual moisture content, the p values found in the studies at both temperatures were less than 0.05 and therefore considered unstable at these temperatures during the study period. As for the mass fraction of the nitrofuran metabolites, only the AOZ presented instability at the study temperature of 50 ºC, with a p value less than 0.05. The other analytes were considered stable at both studied temperatures.

The long-term stability study (storage conditions) was also performed for the residual moisture of the material and for the mass fraction of the nitrofuran metabolites at storage temperature of -20 ° C. The study time was 360 days divided into 6 periods (28, 81, 136, 189, 252 and 360 days). Reference samples were maintained at -80 ° C and analyzed along with the samples stored at -20 ° C. Two samples were analyzed in duplicate on each day. Table 4 presents the summary of the long-term stability study (at -20 ° C) for the CRM candidate batch.

Table 4 - Results for the 360-day stability study at -20 ° C of residual moisture and mass fractions of nitrofuran metabolites, AOZ, AMOZ, AHD and SEM, in the CRM candidate batch.

Parameter	b_1	$s(b_1)$	*p value	Uncertainty	Conclusion
Residual moisture	-0.0000520	0.000244	0.833	0.088 g/100 g	stable
AOZ	0.000168	0.000705	0.813	0.25 µg/kg	stable
AMOZ	0.000183	0.000477	0.704	0.17 µg/kg	stable
AHD	0.000267	0.000527	0.617	0.19 µg/kg	stable
SEM	0.000492	0.000512	0.345	0.18 µg/kg	stable

All the studied properties presented stability at -20 ° C for 360 days, according to linear regression data, since all *p* values were greater than 0.05.

Although the results showed instability for the residual moisture content under the conditions of the short-term stability study, in the majority of cases the mass fractions of the metabolites were not affected. For example, at 20 ° C for 63 days the residual moisture content showed instability while the mass fractions of all the metabolites remained stable.

2.4. Characterization of the Comparison Material by Inmetro

Due to the results of the short-term studies for the residual moisture content, it was decided not to characterize this parameter. However, in order to adopt a more conservative procedure, the material was transported on dry ice to ensure that the temperature did not exceed -20 ° C, where all properties are stable, including the residual moisture content.

For the characterization of the candidate CRM of nitrofurans metabolites in chicken muscle, Inmetro used a "single reference measurement procedure (as defined in the ABNT ISO/IEC Guide 99) in a single

laboratory", which is one of the approaches of characterization presented in ABNT NBR ISO 17034 [11].

For extraction of the metabolites, 0.25 g ± 0.01 g of freeze-dried samples were first reconstituted with 0.75 g ± 0.01 g of ultrapure water and fortified with the working solution of internal standard (IS). In the hydrolysis and derivatization steps, 4 mL of ultrapure water, 500 µL of 1 mol.L^{-1} hydrochloric acid solution and 150 µL of 50 mM 2-NBA solution in DMSO were sequentially added. Samples were shaken for 16 h at 37 °C in an incubator shaker (NT 712, Nova Técnica, Piracicaba, Brazil). After cooling to room temperature, the pH of the samples was adjusted to 7 by the addition of 5 mL of 0.1 mol.L^{-1} solution of dibasic potassium phosphate and 400 µL of 1 mol.L^{-1} sodium hydroxide solution. 5 mL of 0.2 g.mL^{-1} NaCl solution was added and the free metabolite residues were extracted twice by addition of 5 mL of ethyl acetate, stirred for 20 min and centrifuged at 4 °C, 2000 rpm for 15 min (Z300 K, Hermle Wehingen, Germany). The organic phases from the two extractions were combined in 15 mL glass tubes and evaporated under nitrogen flow (Hurricane-Eagle, Younglin Instrument, South Korea). The extracts were reconstituted in 300 µL of water: methanol (50:50 v / v) and transferred to eppendorf tubes and centrifuged at 4 °C and 10,000 rpm for 15 min. The reconstituted extracts were filtered through a 0.22 µm PVDF filter (Merck Millipore Ltd., Ireland) and transferred to 2 ml vial bottles with volume reducer ("insert vial") and analyzed by HPLC-MS/MS.

The HPLC-MS/MS technique was employed with the Isotope Dilution Mass Spectrometry (IDMS) method, using the analogous and isotopically labeled IS. The exact matching calibration method was used.

The calculation of the mass fractions of the nitrofuran metabolites (w_x) by exact matching calibration was performed through equation 1.

$$w_x = w_z \times \frac{m_z}{m_{yc}} \times \frac{m_y}{m_x} \times \frac{R_B}{R_{BC}} \quad (1)$$

Where,

w_x is the mass fraction of the analyte in the CRM candidate; w_z is the mass fraction of the analyte in the working solution; m_z is the mass of analyte solution added to the calibration blend; m_{yc} is the mass of IS solution added to the calibration blend; m_y is the mass of IS solution added to the sample; m_x is the sample mass; R_B is the ratio of the intensities of the analyte/IS signals in the sample; and R_{BC} is the ratio of the intensities of the analyte/IS signals in the calibration blend.

Regarding the residual moisture content in the freeze-dried chicken, due to the obtained results in the studies of homogeneity and stability, the CRM candidate was not characterized for this parameter.

Although the analytical method used by Inmetro is considered a reference method, with potential to be a primary method, there is no similar CRM in a similar matrix available to check its level of accuracy. Because it is a complex matrix (chicken muscle), where the interferences are very common, the characterization of this CRM candidate was performed with support of this interlaboratory comparison (IC) results, which are presented in this report.

2.5. Guidance to Participants

Participants were advised on the storage, handling and reconstitution of samples, as well as on the number of replicates and results to be reported.

Each participating laboratory received four (4) comparison materials, i.e. four (4) 10 mL amber glass bottles, containing 1.3 g of material in freeze-dried form. The determinations of the mass fractions of the nitrofuran metabolites were performed for 3 (three) of the 4 (four) bottles received, two aliquots being taken from each bottle (two true replicates from each bottle), totaling 6 (six) measurements. The minimum amount of analyzed sample per aliquot was 250 mg of freeze-dried material, in order to guarantee that a possible heterogeneity of the material, at aliquots lower than those studied, did not influence the results. An additional bottle, other than those required for the analyzes, was provided for use in initial tests. The determinations were performed by the analytical method implemented in the routine of the participating laboratory.

Participants were instructed to report all results of mass fraction measurements of nitrofuran metabolites, in terms of mass of the reconstituted sample. The individual results for each replicate of each analyzed bottle were reported on a reporting results form, as well as the combined final result, standard uncertainty and expanded uncertainty (with 95% confidence level), the main uncertainty sources, and details of the analytical method and standards used.

Measurement of the residual moisture content of the freeze-dried sample was optional in this IC. When performed, participants were instructed to use the Karl Fischer coulometric titration technique. The minimum amount of sample for determination of residual moisture was 100 mg.

2.6. Processing of Participants' Results

Participating laboratories were asked to report their results on wet basis, that is, for the metabolites mass fractions in the reconstituted sample. The results were reported by analyzed aliquot, the masses of the aliquots and water of the reconstitution, as well as the average final result in wet basis, the uncertainty associated to this result and the coverage factor "*k*" for conversion in expanded uncertainty.

The reconstitution procedure was contemplated in the comparison protocol, which contained all relevant information for the participants, such as how to store and handle the samples, how to report the results and the instructions for completing the forms. According to the reconstitution procedure, participants were instructed to use a quantity of water determined with accuracy (five decimal places), according to the mass of the aliquot of the sample used, according to equation 2:

$$m_w = 2{,}9 \times m_s \tag{2}$$

Where, m_w is the water mass to be added and m_s the mass of the aliquot of freeze-dried material taken for analysis.

However, due to differences in the quantities of water used, the reported results for reconstituted sample mass are not comparable, since the metabolite mass fraction varies as a function of the water mass that the laboratory used for reconstitution. For this reason, the reported results were normalized to a single water proportion of 74%, using the reported masses of the aliquots and reconstitution water masses. This proportion of 74% was estimated based on an approximation of the results of experiments where the amount of water in the real samples of chicken breast used in the RM production was calculated.

To do this normalization of the reported results by each laboratory, the result of each aliquot reported in wet basis by the laboratory "i" was converted in mass fraction of the metabolite in the freeze-dried material (as supplied), using equation 3. These results are comparable, since the mass fractions are expressed for the mass of the material in the form in which it was supplied.

$$x_{i_L} = x_{i_R} \times \frac{\left(m_{aliquot} + m_{water}\right)}{m_{aliquot}} \tag{3}$$

Where, x_{i_L} is the mass fraction of the metabolite in the freeze-dried material, calculated for the aliquot analyzed by laboratory "*i*"; x_{i_R} is the mass fraction of the metabolite in the wet basis material, as reported for the aliquot analyzed by laboratory "*i*"; $m_{aliquot}$ is the mass of the aliquot; and m_{water} the reconstitution water mass.

Each result per aliquot was then converted into a result expressed as a normalized wet basis, multiplying those results by 0.26 (equation 4), which corresponds to a water content of 74%.

$$x_{i_N} = x_{i_L} \times 0{,}26 \quad (4)$$

Where, x_{i_N} is the mass fraction of the metabolite in normalized wet basis, calculated for the aliquot analyzed by laboratory "i".

After this conversion, which was performed for the result of each aliquot, the average result of the laboratory expressed in normalized wet basis was calculated. The reported uncertainties were also converted into a normalized wet basis.

Thus, the statistical treatment of the participants' results for the calculation of the consensus value was performed for the participants' results and uncertainties transformed to a normalized wet basis.

2.7. Statistical Analysis of the Participants' Results

The statistical analysis of the results was performed according to the CCQM Guide for estimating consensus value: "CCQM Guidance note: Estimation of a KCRV consensus and associated Degrees of Equivalence" [12]. It was included a descriptive analysis of the results, from the representation and the evaluation of the results consistency, besides an inferential analysis, with the estimation of consensus values through a combination of results and the calculation of the measurement uncertainty of the estimators.

3. Results and Discussion

3.1. Characterization performed by Inmetro

In the analysis for characterization, a greater metrological rigor in comparison with the analysis of the homogeneity and stability studies was adopted to obtain more accurate results, contemplating all possible sources of uncertainty. In addition to the exact matching calibration procedure, which tends to provide results with smaller uncertainties, two calibration solutions were prepared independently. These solutions were used in the preparation of two also independent calibration blends (CB1 and CB2) also independent, that is, matrix blank samples fortified with these solutions, which were used for calibration. The final mean of the results obtained from only one of the calibration blend was considered as the final result. However, the results obtained from the two calibration blends were compared using the t-Student test for comparison of means, with a confidence level of 95%. All analytes, with the exception of

semicarbazide (SEM), presented equal means of their results obtained by the two calibration blends, CB1 and CB2. In the case of SEM, where there was a difference between means, this results variation obtained "between calibration blends " was included as an additional source of uncertainty.

Table 5 shows the results per replicate, the mean result, and the standard and expanded uncertainties obtained by Inmetro for the characterization of each CRM candidate property value.

Table 5 - Results per replicate, mean result and standard and expanded uncertainties obtained by Inmetro for the characterization of the CRM candidate.

Bottle	Replicate	AOZ (µg/kg)	AMOZ (µg/kg)	AHD (µg/kg)	SEM (µg/kg)
1	1	5.07	5.29	4.97	6.02
	2	5.61	5.53	5.12	6.01
2	1	5.49	5.81	5.67	5.98
	2	5.28	5.44	5.30	6.04
3	1	5.28	5.38	4.41	5.97
	2	5.65	5.47	5.01	6.06
average		5.40	5.49	5.08	6.01
standard uncertainty (u)		0.28	0.23	0.45	0.19
expanded uncertainty (U, k=2)		0.56	0.45	0.90	0.37

The metrological traceability of the obtained results was achieved by the use of the IDMS method, which is a potentially primary measurement procedure of ratio, associated to the gravimetric method for the preparation of the calibration blends. In addition, the purity of the analyte standards used in the preparation of the calibration solutions was determined by ^{1}H and ^{13}C qNMR using CRM dimethyl terephthalate (TraceCERT®, Sigma-Aldrich, St. Louis, USA) as an internal standard.

Figure 1 shows a column chart with the contributions of the main sources of uncertainty associated with the values of Inmetro's measurements for each analyte, in the characterization of the CRM candidate.

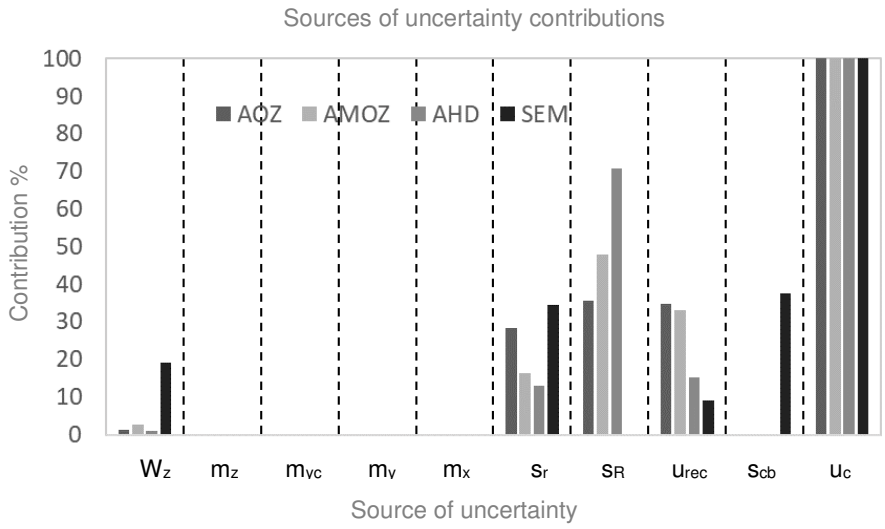

Figure 1 - Column graph showing the contribution (%) of the main sources of uncertainty in the characterization of the CRM candidate, for the AOZ, AMOZ, AHD and SEM metabolites.

In figure 1, u_{rec} is the uncertainty of recovery; s_R is the standard-deviation between analyzed aliquots; s_r is the standard-deviation of repeatability; s_{cb} is the standard-deviation corresponding to the variation of the results obtained from different calibration blends; and m_y, m_{yc}, m_z, m_x and w_z, are the sources of uncertainty from equation 1 of exact matching. The source w_z is composed of the masses used in the preparation of the calibration blend solution and includes the purity of the standard.

3.2. Participants' Results

Only one participant reported the result for the residual moisture content of the provided samples. In view of that and due to the instability issues of this parameter in some studied temperatures, the material was not characterized for the residual moisture content and the results will not be presented in this report.

For the mass fraction of the metabolites, as already mentioned, laboratories were instructed to report the values on a wet basis, that is, for the reconstituted material. However, since the proportions between sample mass and reconstitution water mass used by each participant are different, then the results cannot be compared directly. Therefore, the results were previously converted into normalized wet basis, according to item 2.6, so that they could be treated. Tables 6, 7, 8 and 9 present the results as reported by participants and the same results after conversion to normalized wet basis.

Table 6 - Participants' results for the AOZ mass fraction as reported and in normalized wet basis.

		Results as reported				**Results in wet basis, normalized**			
Laboratory Code	**Bottle**	**Result per aliquot (µg/kg)**	**Average (µg/kg)**	***k***	**U (µg/kg)**	**Result per aliquot (µg/kg)**	**Average (µg/kg)**	**U (µg/kg)**	**u (µg/kg)**
16	237	3.84	4.247*	2	0.12	3.88	4.29	0.12	0.06
		4.12				4.18			
	098	4.04				4.08			
		4.62				4.66			
	028	4.09				4.12			
		4.77				4.79			
06	52	1.899	1.914	2	0.107	1.91	1.93	0.11	0.05
		1.95				1.96			
	114	1.874				1.88			
		1.918				1.93			
	216	1.94				1.96			
		1.906				1.91			
26	131	158	1.6	2	0.12	1.60	1.62	0.12	0.06
		1.54				1.56			
	159	1.65				1.68			
		1.59				1.62			
	194	1.66				1.69			
		1.58				1.60			
56	89	1.773	1.87	0.99**	0.3	1.36	1.42	0.23	0.09
		1.869				1.39			
	177	1.923				1.47			
		1.918				1.42			
	228	1.872				1.42			
		1.856				1.48			
Inmetro	62	-	-	-	-	1.32	1.40	0.15	0.07
		-				1.46			
	136	-				1.43			
		-				1.37			
	233	-				1.37			
		-				1.47			

* The value reported by laboratory 16 does not correspond to the mean values reported per replicate (4.78 µg/kg). Therefore, the mean was recalculated.

** Laboratory 56 reported the *k* value of 0.99 which was considered as a 99% confidence level. The value of *k* was therefore calculated on the basis of this confidence level.

Table 7 - Participants' results for the AMOZ mass fraction as reported and in normalized wet basis.

		Results as reported				**Results in wet basis, normalized**			
Laboratory Code	**Bottle**	**Result per aliquot (µg/kg)**	**Average (µg/kg)**	***k***	**U (µg/kg)**	**Result per aliquot (µg/kg)**	**Average (µg/kg)**	**U (µg/kg)**	**u (µg/kg)**
41*	237	NR	NR	NR	NR	NR	NR	NR	NR
		NR				NR			
	098	NR				NR			
		NR				NR			
	028	NR				NR			
		NR				NR			
39	52	2.216	2.136	2	0.15	2.23	2.15	0.15	0.08
		2.218				2.23			
	114	2.105				2.12			
		2.119				2.13			
	216	2.046				2.06			
		2.115				2.12			
11	131	1.57	1.61	2	0.11	1.58	1.63	0.11	0.06
		1.55				1.57			
	159	1.53				1.56			
		1.58				1.61			
	194	1.74				1.77			
		1.67				1.69			
32	89	2.033	2.12	0,99*	0.3	1.55	1.62	0.23	0.09
		2.179				1.63			
	177	2.043				1.56			
		2.24				1.65			
	228	2.131				1.61			
		2.12				1.69			
Inmetro	62	-	-	-	-	1.37	1.43	0.12	0.06
		-				1.44			
	136	-				1.51			
		-				1.41			
	233	-				1.40			
		-				1.42			

* Laboratory 32 reported the *k* value of 0.99, which was considered as a 99% confidence level. The value of *k* was therefore calculated on the basis of this confidence level.

Table 8 - Participants' results for the AHD mass fraction as reported, and in normalized wet basis.

		Results as reported				**Results in wet basis, normalized**			
Laboratory Code	**Bottle**	**Result per aliquot (µg/kg)**	**Average (µg/kg)**	***k***	**U (µg/kg)**	**Result per aliquot (µg/kg)**	**Average (µg/kg)**	**U (µg/kg)**	**u (µg/kg)**
10	237	2.46	1.97	2	0.12	2.48	1.99	0.12	0.06
		2.29				2.32			
	098	1.75				1.77			
		2.08				2.10			
	028	1.66				1.67			
		1.6				1.61			
18	52	1.875	1.875	2	0.116	1.89	1.89	0.12	0.06
		1.896				1.90			
	114	1.823				1.83			
		1.874				1.89			
	216	1.882				1.90			
		1.897				1.90			
42	131	1.4	1.36	2	0.16	1.41	1.38	0.16	0.08
		1.24				1.26			
	159	1.41				1.43			
		1.38				1.40			
	194	1.33				1.35			
		1.4				1.42			
53	89	1.823	1.51	99*	0.45	1.39	1.14	0.34	0.13
		1.32				0.98			
	177	1.57				1.20			
		1.621				1.20			
	228	1.161				0.88			
		1.509				1.20			
Inmetro	62	-	-	-	-	1.29	1.32	0.24	0.12
		-				1.33			
	136	-				1.47			
		-				1.38			
	233	-				1.15			
		-				1.30			

* Lab 53 reported the *k* value of 99 that was considered as a 99% confidence level. The value of *k* was therefore calculated on the basis of this confidence level.

Table 9 - Participants' results for the SEM mass fraction as reported and in normalized wet basis.

		Results as reported				**Results in wet basis, normalized**			
Laboratory Code	**Bottle**	**Result per aliquot (µg/kg)**	**Average (µg/kg)**	***k***	**U (µg/kg)**	**Result per aliquot (µg/kg)**	**Average (µg/kg)**	**U (µg/kg)**	**u (µg/kg)**
01	237	6.4	4.78	2	0.12	6.46	4.82	0.12	0.06
		5.06				5.14			
	098	3.86				3.90			
		4.7				4.74			
	028	4.24				4.27			
		4.42				4.44			
38	52	2.016	1.999	2	0.131	2.03	2.06	0.14	0.07
		2.079				2.09			
	114	2.068				2.08			
		2.111				2.13			
	216	2.039				2.06			
		1.999				2.00			
08	131	1.65	1.56	2	0.13	1.68	1.59	0.13	0.07
		1.49				1.51			
	159	1.59				1.62			
		1.54				1.56			
	194	1.62				1.64			
		1.49				1.51			
50	89	2.552	2.4	99*	0.5	1.95	1.83	0.38	0.15
		2.173				1.62			
	177	2.267				1.73			
		2.596				1.92			
	228	2.414				1.83			
		2.397				1.91			
Inmetro	62	-	-	-	-	1.57	1.56	0.10	0.05
		-				1.56			
	136	-				1.56			
		-				1.57			
	233	-				1.55			
		-				1.58			

* Laboratory 50 reported the *k* value of 99, which was considered as a 99% confidence level. The value of *k* was therefore calculated on the basis of this confidence level.

In addition to the quantitative results, participants also reported information on the sample extraction and clean - up procedures, as well as the internal standards (IS) and standards used in the analysis, as shown in table 10.

Table 10 - Participants' information on analytical techniques, extraction and clean-up methods, internal standards and standards used in IC.

Analytical and calibration technique	**Extraction and clean-up procedures***	**Standards (supplier, purity)**	**Origin of purity value**	**Internal standards (supplier, purity)**
HPLC -MS/MS, internal standardization	• Addition of IS and equilibration for 15 min; • Acid hydrolysis and derivatization with NBA for 16 h to 37 ± 2 ° C; • Adjustment to pH 7 ± 0.5; • Extraction with ethyl acetate (2x); • Evaporation with N_2 flow; • Solubilization with 200 µL of methanol and 800 µL of ultrapure water; • Extract filtration with 0.45 µm membrane.	AOZ (Dr Ehrenstorfer, 99.3 %) AHD (Dr Ehrenstorfer, 99.38 %) SEM (Dr Ehrenstorfer, 99.5 %)	Supplier Certificate	AOZ-D_4 (Sigma-Aldrich, 99.3 %)
HPLC -MS/MS, internal standardization with matrix-matched calibration	• Acid hydrolysis and derivatization with 2-NBA; • Extract with ethyl acetate; • Evaporation; • Solubilization of extracts with ACN/water.	AOZ (Witega, 99.7%); AMOZ (Witega, 99.4 %); AHD (Witega, 99.3 %); SEM·HCl (Sigma-Aldrich, 99 %)	Supplier Certificate	AOZ-D_4 (Witega, > 99.3 %) AMOZ-D_5 (Witega, > 99.1 %) AHD-^{13}C (Witega, > 99 %) Hydrochloride SEM-[^{13}C,$^{15}N2$] (Witega, > 99 %)
HPLC -MS/MS, internal standardization with matrix-matched calibration	• Acid hydrolysis and simultaneous derivatization with 2-NBA overnight; • pH adjustment; • Extract with ethyl acetate; • Solubilization of extracts with methanol: water; • cleaning with n-hexane (2x).	AOZ (Dr. Ehrenstorfer, 99.32 %) AMOZ (Sigma-Aldrich, 99.5 %) AHD (Dr. Ehrenstorfer, 99.38 %) SEM·HCl (Dr. Ehrenstorfer, 99.80 %)	Supplier Certificate	AOZ-D_4 (Dr. Ehrenstorfer, 97.92 %) AMOZ-D_5 (Dr. Ehrenstorfer, 97.59%) AHD-^{13}C (Sigma-Aldrich, 99.0%) Hydrochloride SEM-[^{13}C,$^{15}N2$] (Sigma-Aldrich, 99.0 %)
HPLC -MS/MS, internal standardization with matrix-matched calibration	• IS addition and equilibration time; • Acid hydrolysis and simultaneous derivatization with 2-NBA overnight; • Adjust to pH 7; Extraction with ethyl acetate (2x); • Evaporation; • Solubilization with methanol/amph (?) (15:85); Extraction with hexane (2x).	AOZ (Sigma, 99.7 %) AMOZ (Witega, 99.6 %); AHD (Witega, 99.6 %); SEM·HCl (Witega, 99.0 %)	Supplier Certificate	AOZ-D_4 (Witega, > 99.3 %) AMOZ-D_5 (Witega, > 99 %) AHD-^{13}C (Witega, > 99 %) Hydrochloride SEM-[^{13}C,$^{15}N2$] (Witega, > 99 %)

* Information on extraction methods is summarized and some steps may have been omitted by the participant himself. Therefore, this information does not depict the entire procedure, but provides only a general information on the principle of the method used for analyte extraction.

The information presented in the table is not necessarily in the same order as the quantitative results presented in the previous tables. Regarding the information presented, it should be noted that one participant reported that only one of the isotopically-labeled analytes (AOZ-D_4) was used as an internal standard. The same participant did not report whether used solution or matrix-matched calibration. All laboratories used the HPLC-MS/MS technique.

3.3. Consensus values

The mean values of the mass fractions of the nitrofuran metabolites obtained by each laboratory, converted into a standardized wet basis, as well as their associated uncertainties, were used in the calculation of the consensus value using the MM-estimation statistical method [13]. This estimator consists of a robust regression method that is recommended by the CCQM for the calculation of a consensus value when there is no mutual consistency between the results and the uncertainties also differ significantly [12].

Tables 11 to 14 summarize the average results of the participating laboratories and Inmetro for the mass fractions of nitrofuran metabolites AOZ, AMOZ, AHD and SEM in the CRM candidate, expressed as normalized wet basis. The results of the consensus values for each analyte and their respective standard and expanded uncertainties are also presented.

Table 11 - AOZ mass fraction results in the CRM candidate, consensus value and uncertainties associated to the results and to the consensus value, expressed as normalized wet basis.

AOZ				
Laboratory Code	**x_i (µg/kg)**	**$u(x_i)$ (µg/kg)**	***k***	**$U(x_i)$ (µg/kg)**
16	4.29	0.061	2.00	0.12
06	1.93	0.054	2.00	0.11
26	1.62	0.061	2.00	0.12
56	1.42	0.089	2.58*	0.23
Inmetro	1.40	0.073	2.00	0.15
Consensus value, X	1.66			
u(X)	0.13			
***k* (*DoFl*=4; 95 %)**	2.78			
U(X)	0.37			

*Value of *k* calculated based on a confidence level of 99%.

Table 12 - Results of AMOZ mass fraction in the CRM candidate, consensus value and uncertainties associated to the results and to the consensus value, expressed in normalized wet basis.

AMOZ				
Laboratory Code	**x_i (µg/kg)**	**$u(x_i)$ (µg/kg)**	***k***	**$U(x_i)$ (µg/kg)**
41	NR	NR	NR	NR
39	2.15	0.075	2.00	0.15
11	1.63	0.056	2.00	0.11
32	1.62	0.089	2.58*	0.23
Inmetro	1.43	0.059	2.00	0.12
Consensus value, X	1.65			
u(X)	0.16			
***k* (*DoF*=3; 95 %)**	3.18			
U(X)	0.51			

*Value of *k* calculated based on a confidence level of 99%.

Table 13 - Results of AHD mass fraction in the CRM candidate, consensus value and uncertainties associated to the results and to the consensus value, expressed in normalized wet basis.

AHD				
Laboratory Code	**x_i (µg/kg)**	**$u(x_i)$ (µg/kg)**	***k***	**$U(x_i)$ (µg/kg)**
10	1.99	0.061	2.00	0.12
18	1.89	0.058	2.00	0.12
42	1.38	0.081	2.00	0.16
53	1.14	0.13	2.58*	0.34
Inmetro	1.32	0.12	2.00	0.24
Consensus value, X	1.73			
u(X)	0.16			
***k* (*DoF*=4; 95 %)**	2.78			
U(X)	0.45			

*Value of *k* calculated based on a confidence level of 99%.

Table 14 - Results of SEM mass fraction in the CRM candidate, consensus value and uncertainties associated to the results and to the consensus value, expressed in normalized wet basis.

SEM				
Laboratory Code	**x_i (µg/kg)**	**$u(x_i)$ (µg/kg)**	***k***	**$U(x_i)$ (µg/kg)**
01	4.82	0.061	2.00	0.12
38	2.06	0.068	2.00	0.14
08	1.59	0.066	2.00	0.13
50	1.83	0.15	2.58*	0.38
Inmetro	1.56	0.048	2.00	0.10
Consensus value, X	1.70			
u(X)	0.13			
***k* (*DoF*=4; 95 %)**	2.78			
U(X)	0.35			

*Value of *k* calculated based on a confidence level of 99%.

Figures 2 to 5 present the results of laboratories participating in IC for the characterization of the CRM candidate of nitrofurans metabolites in chicken muscle, for each property value.

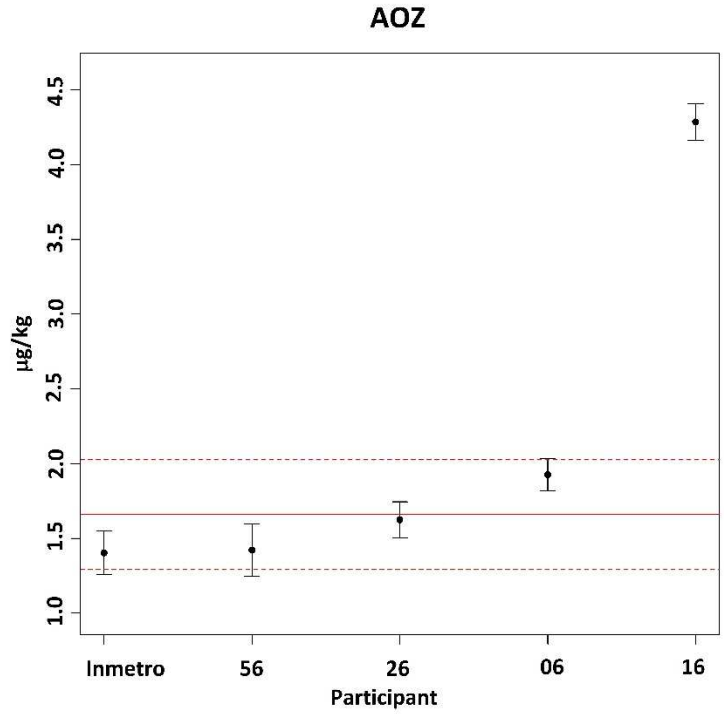

Figure 2 - Graph of participants' results for AOZ mass fraction with their expanded uncertainties and MM estimate value (consensus value) represented by the continuous line. The dashed line represents the expanded uncertainty of the consensus value.

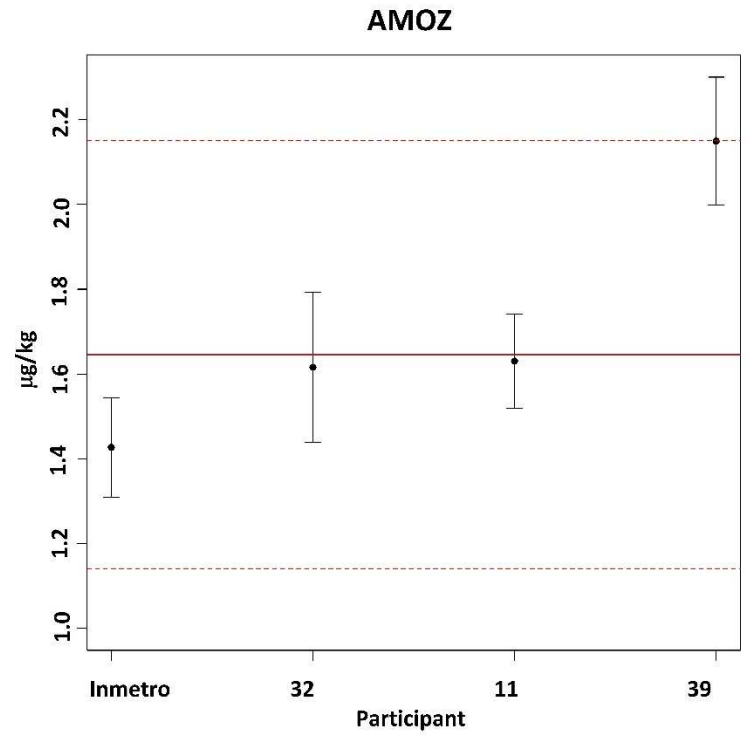

Figure 3 - Graph of participants' results for AMOZ mass fraction with their expanded uncertainties and MM estimate value (consensus value) represented by the continuous line. The dashed line represents the expanded uncertainty of the consensus value.

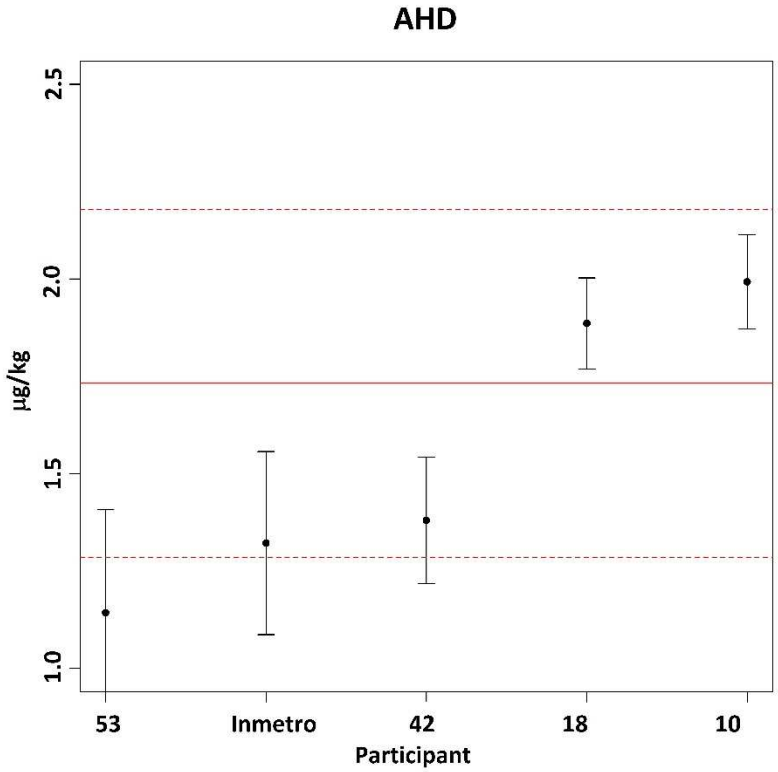

Figure 4 - Graph of participants' results for AHD mass fraction with their expanded uncertainties and MM estimate value (consensus value) represented by the continuous line. The dashed line represents the expanded uncertainty of the consensus value.

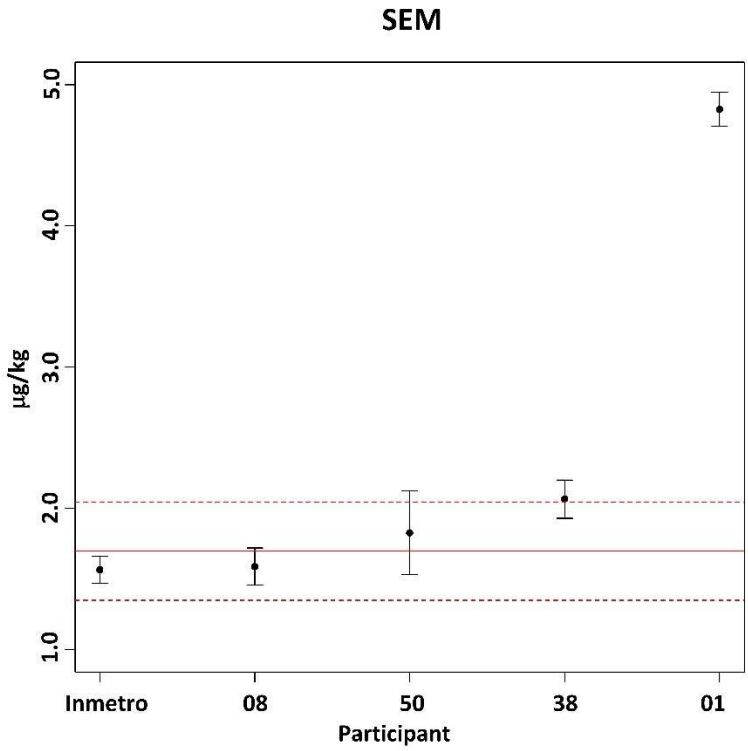

Figure 5 - Graph of participants' results for SEM mass fraction with their expanded uncertainties and MM estimate value (consensus value) represented by the continuous line. The dashed line represents the expanded uncertainty of the consensus value.

When the expanded uncertainties of the participating laboratories are within the range of the consensus value, there is no statistical evidence of difference between such measurements. Thus, the results of laboratories coded as 56, 06 and 26 (AOZ); 32, 11 and 39 (AMOZ); 53, 42, 18 and 10 (AHD); and, 08, 50 and 38 (SEM), are statistically equivalent to the consensus value represented by the solid line in the figures because their expanded uncertainties cross the confidence interval of this value, delimited by dashed lines.

The confidence intervals correspond to the expanded uncertainties, U(X), for $k = 2$ and a confidence level of 95%. The results of codes **16** for AOZ and **01** for SEM were out of the confidence interval and thus are not compatible with the consensus value. These results were considered suspect to be aberrant by the visual analysis of Figures 2 and 5, since they are well above the other results and the confidence interval. The suspicion of these aberrant values was confirmed by the Grubbs test. However, these aberrant results were not removed from the data set, since the statistical method used for the calculation of the MM-estimation is a robust method. The robust methods are based on calculations of position measurements and therefore are not influenced by extreme values and usually these values are not removed from the data set for statistical treatment.

The results of Inmetro can be considered equivalent to the consensus values for all analytes since they are within the confidence interval, or their uncertainty crosses the confidence interval, as in the case of AHD.

Inmetro is a National Metrology Institute (NMI) and is the producer of the CRM candidate in question. In order to the measurements and values provided by a National Metrology Institute (NMI) or a Designated Institute (DI) be recognized by BIPM (International Bureau of Weights and Measures) and, consequently, the Institute have its Calibration and Measurement Capabilities (CMC) published in the CIPM Key Comparison Database (KCDB) [14], metrological traceability to the SI needs to be established, either via the primary realization or representation of the measurement unit in question or via another NMI or DI that has a relevant CMC with appropriate uncertainty published in the KCDB. Therefore, Inmetro, as producer of this CRM and National Metrology Institute, needs to follow this additional criterion.

In this interlaboratory comparison, Inmetro could combine its results only with another NMI or Designated Institute, with CMC published in the KCDB. However, not all participants met these requirements in order for the consensus value to be used as the characterization value. In addition, most participants did not provide requested information, the sources of uncertainty of their results and their respective contributions. None of the participants provided information on the origin of the purity values of the standards used in order to minimally evidence the metrological traceability of these values to the SI.

Therefore, the final value of the CRM candidate characterization was established as the value provided only by Inmetro and the IC consensus value was compared to this characterization value in order to reinforce its reliability.

4. Confidenciality

Each participant was identified by individual code that is known only by the participant and by the coordination of the IC. As established in the registration form, the identification of the accredited laboratories and in the stage of accreditation will be sent to the General Accreditation Coordination (Cgcre). The participant received, via e-mail, his identification code corresponding to its participation in the IC. This code was used as identification of the participant in the completion of the record of results. The results can be used in works and publications by Inmetro respecting the confidentiality of each participant.

As established in item 4.10.4 of ABNT ISO/IEC 17043:2011, under exceptional circumstances, a regulatory authority may request the results and identification of IC participants to the provider. If this occurs, the IC provider will notify this action to participants.

5. Conclusions

This interlaboratory comparison reached its goal to establish consensus values for the AOZ, AMOZ, AHD and SEM metabolites mass fractions in the chicken muscle CRM candidate.

Most of the reported results showed agreement with the consensus values. Inmetro's characterization results were all considered compatible with the consensus values and will be used as characterization values of the CRM candidate for the mass fractions of the four nitrofuran metabolites.

It is important to emphasize that it was not possible to establish the metrological traceability of the consensus value, since there was no way to prove the metrological traceability of the results of each participant. Therefore, the traceability of Inmetro's characterization value was established, not by comparison with the consensus value, but by the use of potentially primary measurement procedures, the use of standards with determined purity with traceability and by inclusion of possible sources of error as sources of uncertainty.

The laboratories invited to participate in this IC have, in some way, recognized competence in the analysis of nitrofuran metabolites in food matrices. However, results with systematic errors can occur occasionally. The purpose of this IC was not to evaluate the performance of laboratories, however, it is up to each of the participants who obtained results that are not compatible with the consensus value, to perform a critical evaluation of these results in order to seek a better understanding of possible sources of error in the laboratories methods of analysis and to implement improvement actions.

Finally, the technical and organizational committee of this IC, on behalf of Inmetro, thanks the laboratories that participated in this interlaboratory comparison, which made it possible to complete the characterization phase of the certification project of this CRM candidate.

6. Participants

We received four (4) registrations in the Interlaboratory Comparison to Characterize a Candidate for Certified Reference Materials for Nitrofuran Metabolites in Chicken and all submitted their results. The list of participants who submitted the results to the coordination of this IC is presented in Table 15. It is

important to note that the numbering of the table is only indicative of the number of participants in the IC, and is not, in any way, associated with the identification of participants in the presentation results.

Table 15 – Participants.

	Organização
1.	Bundesamt für Verbraucherschutz (BVL), Germany.
2.	Finnish Food Authority, Finland.
3.	Laboratório Federal de Defesa Agropecuária de São Paulo (LFDA/SP), Brazil.
4.	NSF Bioensaios, Brazil.

Total participants: 4.

7. References

[1] EUROPEAN COMMISSION. Council Regulation 2901/93 of 18 October 1993, amending Annexes I, II, III and IV to Regulation (EEC) No 2377/90 laying down a Community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin. Official Journal of the European Communities, L264, 1993.

[2] BRASIL. Ministério da Agricultura, Pecuária e Abastecimento. Instrução Normativa nº 9 de 27 de junho de 2003. Proíbe a fabricação, a manipulação, o fracionamento, a comercialização, a importação e o uso dos princípios ativos cloranfenicol e nitrofuranos e os produtos que contenham estes princípios ativos, para uso veterinário e suscetível de emprego na alimentação de todos os animais e insetos. *Diário Oficial da União*, Brasília, DF, 30 de junho de 2003, Seção 1, p. 4.

[3] McCRACKEN, R. J.; SPENCE, D. E.; FLOYD, S. D.; KENNEDY, D. G. Evaluation of the residues of furazolidone and its metabolite, 3-amino-2-oxazolidinone (AOZ), in eggs. *Food Addit Contam,* v. 18, n. 11, p. 954-959, 2001.

[4] EUROPEAN COMMISSION. Diretiva 96/23/CE. On measures to monitor certain substances and residues thereof in live animals and animal products and repealing Directives 85/358/EEC and 86/469/EEC and Decisions 89/187/EEC and 91/664/EEC. Official Journal of the European Communities. nº L 125 de 23/05/1996 p. 0010 – 0032.

[5]CONTINI, E.; TALAMINI, D. J. D. Barreiras da União Européia para a importação de produtos de suínos e aves do Brasil. In: SEMINÁRIO INTERNACIONAL DE AVES E SUÍNOS, Florianópolis, SC. Anais eletrônicos...Florianópolis, 2004. Available in <http://www.cnpsa.embrapa.br/down.php?tipo=artigos&cod_artigo=28>. Accessed in June 2008.

[6] EUROPEAN COMMISSION. European Commission Decision 2002/657/EC of 12 August 2002, implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. Official Journal of the European Communities, L22, 2002a.

[7] Brasil. Ministério da Agricultura Pecuária e Abastecimento.Manual de garantia da qualidade analítica. / Ministério da Agricultura Pecuária e Abastecimento. Secretaria de Defesa Agropecuária. – Brasília: MAPA/ACS, 2011.227 p.

[8] ZÚNIGA, N. O. C. *Desenvolvimento de criação experimental de frango de corte para viabilização de estudo de metabolização de nitrofuranos*. 2010. 211 f. Dissertação (Mestrado em Ciência de Alimentos) – Universidade Federal do Rio de Janeiro, Instituto de Química. Rio de Janeiro, RJ, 2010.

[9] VIOLANTE, F. G. M. et al. Feasibility study for the development of a certified reference material of nitrofuran metabolites in chicken breast muscle from incurred samples. *Measurement*, v. 129, p. 368–374, 2018.

[10] ASSOCIAÇÃO BRASILEIRA DE NORMAS TÉCNICAS. ABNT ISO GUIA 35: Materiais de referência – Princípios gerais e estatísticos para certificação. Rio de Janeiro, 2012.

[11] ABNT. ASSOCIAÇÃO BRASILEIRA DE NORMAS TÉCNICAS. NBR ISO 17034:2017: requisitos gerais para a competência de produtores de material de referência: São Paulo, 2017a. 28 p.

[12] CCQM Guidance note: Estimation of a consensus KCRV and associated Degrees of Equivalence. Version 10, 2013. Available in: <https://www.bipm.org/cc/CCQM/ Allowed/19/CCQM13-22_Consensus_KCRV_v10.pdf>. Accessed in January 2019.

[13] SUSANTI, Y. et al. M estimation, S estimation, and MM estimation in robust regression. *International Journal of Pure and Applied Mathematics*, v. 91, n. 3, p. 349-360, 2014.

[14] CIPM. *Decision CIPM/2009-24*. Traceability in the CIPM MRA. Sèvres: BIPM, 2009. Available in: https://www.bipm.org/cc/CIPM/Allowed/98/CIPM2009_24_ TRAC_MRA_REV_13_OCT _2009.pdf . Accessed in December 2018.

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