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International Collaborative Trial of a Real-time PCR Approach for the Relative Quantitation of Horse DNA

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Abstract

Following the UK/EU Horse-meat issue of 2013, a real-time PCR approach for the relative quantitation of horse DNA was published. An international collaborative trial was subsequently organised to evaluate the repeatability and reproducibility of the method in line with IUPAC guidance. Thirteen international laboratories participated in the trial, representing laboratories from EU member states and the United States of America. Participants analysed five blindly labelled test samples, representing DNA extracted from 0.1, 0.5, 1, 5, and 20% (w/w) horse meat in a beef meat background. Each test sample was represented by four units in the experimental design, and each unit represented by triplicate PCR technical replicates. Values for the PCR efficiency and r-squared associated with the calibration curves from the collaborative trial showed acceptance with EC published acceptance criteria. A mixed effects model, based on maximum likelihood, was used to analyse the data. The relative repeatability standard deviation (RSD_r) was less than 15% and the relative reproducibility standard deviation (RSD_R) was less than 26% across all samples analysed, inclusive of the 0.1% (w/w) gravimetric sample. These estimates fulfil the acceptance criteria for the precision associated with a method subject to a collaborative trial as outlined in published IUPAC guidance. A small but consistent positive bias (less than 15%) was observed, thought to be mainly contributed by the sample preparation approach. The method is now being considered for international standardisation.

Keywords

Collaborative trial; Horse meat; Real-time PCR; Quantitation; Repeatability; Reproducibility

Introduction

Food authenticity and food fraud are becoming increasingly prevalent within the food industry, partially due to the pressures faced by producers within today's challenging financial climate and also due to the international nature of modern food production. The 2013 EU wide issue where the Food Safety Authority of Ireland (FSAI) reported a significant amount of horse DNA had been found in a beef burger product on sale to the public at a local supermarket [1], emphasised the need for the development of accurate analytical approaches for the quantitative detection of meat adulteration in a sample.

In 2013 the EU Reference Laboratory (EURL) for Animal Proteins in feeding stuffs published recommendations for detection of horse DNA [2,3]. These recommendations included expressing the amount of horse meat in relation to other meat species on a w/w basis using a DNA approach, as well as implementing a threshold level of 1% (w/w) for enforcement action. On the 27th March 2014, the European Commission published a recommendation regarding a second coordinated control plan with a view to establishing the prevalence of fraudulent practices in the marketing of certain foods [4].

The EU horse meat issue highlighted that there was a general lack of guidance and harmonisation on how the amount of meat adulteration in a sample should be expressed. This further reinforced the requirement for a quantitative approach to be developed to accurately measure the amount of horse DNA present in a sample. In response to this EU horse meat issue, a real-time PCR method for the quantitation of horse DNA was developed within the UK using funding from the Department for Environment, Food & Rural Affairs (Defra) and the method subsequently published (Defra project FA0135) [5]. The method used two nuclear DNA targets for quantitation, a horse specific target and a general reference target for any mammalian DNA. The method was demonstrated to be capable of detecting and accurately quantitating the amount of horse DNA present in samples of raw, lean beef muscle mixtures. The method had been validated through an objective

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assessment of performance characteristics using weight for weight (w/w) gravimetric raw horse-meat in raw beef (meat) materials. The quantitative approach for horse DNA was validated in terms of specificity, PCR efficiency and linearity, Limit of Detection (LOD), and trueness and precision on a set of raw meat samples. Additional method validation performed in a subsequent Defra project (Defra project FA0146) [6] showed the applicability of the method with different samples, evaluated the measurement uncertainty around the 1% (w/w) threshold level for enforcement action, and provided evidence of the method's suitability for use in complex foods. These two Defra projects [5,6] resulted in the generation of a standard operating procedure (SOP) for the method [7] and a peer reviewed paper describing the approach [8].

In order to independently assess the performance of the method between laboratories, an international collaborative trial of the method was funded by the UK Food Standards Agency [9]. The trial was designed and implemented in accordance with guidance available from the International Union of Pure and Applied Chemistry (IUPAC) for the design, conduct and interpretation of collaborative studies [10] and the European Network of GMO Laboratories (ENGL) [11].

The resulting international collaborative trial for the evaluation of the real-time PCR method involved the co-operation of thirteen laboratories from the United Kingdom (UK), additional European Union (EU) member states, and the United States of America (USA). This paper summarises the implementation of the trial, the results obtained, and the analysis of the performance of the method and subsequent interpretation.

Materials and Methods

Meat samples

Raw muscle tissue (horse and beef meat) which had been trimmed free of surface inter-muscular fat and connective tissue, was sourced from a supplier (Kezie Ltd, Duns, UK) and authenticated as to species identity using real-time PCR and DNA sequencing. For the real-time PCR, species specific genomic targets using PCR assays specific for horse [12] and beef [13] were used. For the DNA sequencing, two mitochondrial genes (12S rRNA and cytochrome b) were used for the species specific identification. Universal PCR primers which anneal to conserved regions of the 12S, and cytochrome b mitochondrial genes were used to synthesise PCR products, for which the DNA sequence was subsequently derived by Sanger sequencing. Species authenticity was then confirmed with use of the Basic Local Alignment Search Tool (BLAST) [14] available at the website of the National Centre for Bioinformatics (NCBI) [15] and species identity confirmed using sequence information available on GenBank [16].

Test samples were produced for mass-based ratio preparations of: 100%, 20%, 5%, 1%, 0.5% and 0.1% weight for weight (w/w) raw horse muscle tissue in a raw beef muscle tissue background. The raw horse meat muscle tissue was cubed, thoroughly homogenised in a food processor, combined, mixed and the resultant paste stored in polythene bags on ice until required. The same approach was taken for the preparation of the beef (meat) material. For the 20% and 5% w/w samples, an appropriate mass of the 100% homogenised raw horse meat was weighed using a United Kingdom Accreditation Service (UKAS) certified calibrated top-pan balance (accurate to two decimal places) and combined with an appropriate mass of the 100% w/w beef (meat). To ensure the accuracy of mass based ratio mixes the top-pan balance reading was allowed to stabilise and where necessary additional material was added or removed with the aid of a micro-spatula. In order to provide representative low levels for the 1.0%, 0.5% and 0.1% w/w samples, an appropriate mass of the homogenised 5% w/w raw horse was combined with an appropriate mass of the 100% w/w beef (meat). Each of the samples was thoroughly mixed in a clean food processor, three aliquots of 1 g set aside for use with DNA extraction, and the remaining materials each double bagged in zip lock polythene bags and stored at -80°C. Details of the sample composition for the test samples used are listed in Table 1.

Test Sample	100% Beef (g)	100% Horse (g)	5% Horse in Beef (g)	Total Mass (g)
100% w/w Beef	2000	0	0	2000
100% w/w Horse	0	2000	0	2000
20% w/w Horse in Beef	160	40	0	200
5.0% w/w Horse in Beef	190	10	0	200
1.0% w/w Horse in Beef	160	0	40	200
0.5% w/w Horse in Beef	180	0	20	200
0.1% w/w Horse in Beef	196	0	4	200

Table 1: Generation of gravimetrically prepared admixes (top pan balance accurate to two decimal places)

DNA Extraction

DNA extraction was performed on 1 g tissue samples using a modified CTAB extraction method of Binke et al. (2003) [17]. Purified DNA was suspended in 0.5 ml of nuclease free water and both DNA yield (A_{260}) and quality characteristics ($A_{260}:A_{230}$ and $A_{260}:A_{280}$) determined using a Nanodrop™ ND-1000 Spectrophotometer (Thermo Scientific, UK). Suitability of the extracted DNA for use with real-time PCR was performed using the horse specific (*EC-GHR1*) and universal mammalian (*MSTN*) real-time PCR assays. Performance was evaluated based on the resulting PCR efficiencies and the Pearson product-moment correlation coefficient (R^2).

DNA Calibration Standards

DNA extracted from 100% w/w raw horse meat was used as the calibrant for the generation of standard curves for the universal mammalian and equine specific real-time PCR assays as specified in the published method [8]. The initial DNA concentration was estimated with use of spectrophotometry (A_{260}). Dilution to a suitable working concentration was performed with the addition of an appropriate volume of EB buffer (Qiagen). Participants were instructed to prepare a five point, seven fold dilution series (encompassing a genome equivalent copy number range from 24,010 copies/ μ L, to 10 copies/ μ L) based on the calibrant that was provided.

Test Units

Twenty blinded samples (labelled from U1 to U20), representing the five different levels of horse DNA (extracted from gravimetrically prepared samples of 0.1%, 0.5%, 1.0%, 5.0% and 20.0% of raw horse meat in a raw beef background), were used in the collaborative trial. Each level of horse DNA was replicated four times: two of the technical replicates were randomly assigned to test samples between U1 to U10, and the remaining two technical replicates were randomly assigned to test samples between U11 and U20. All test samples were stored at -80°C until they were shipped on dry ice by courier to participants.

Real-time PCR

Real-time PCR was performed as described in the published method [8]. Two Taqman™ real-time PCR assays were run, one targeting a region within the equine growth hormone receptor gene (*EC-GHR1*) [12], and the second targeting a region within the mammalian myostatin gene (*MSTN*) [13]. For the relative quantitation of the horse DNA in a test sample, the seven-fold, five point serial dilution of the calibrant (DNA extracted from 100% w/w raw horse meat) was performed, which was then used as the template for the two real-time PCR assays in order to generate two standard curves for a fixed mass of input DNA. Each of these calibration curves represented the approximate genomic equivalent copy number of either the total mammalian or total horse DNA present. The mass,

and hence the approximate relative copy number of both horse and mammalian DNA, was determined for the test sample by comparing the C_q obtained for each assay with the calibration curves that had been generated with use of the calibrant. The contents of the calibration samples are provided in Table 2.

Limited Stability Study

A limited stability study was undertaken to check how consistently the values of the test samples were estimated across three consecutive time points (T1, T2, T3) corresponding to 1, 14 and 62 days following test unit generation and freezing for storage. At each time point, four replicates of each sample level were analysed, with each sample level represented by a triplicate level of PCR replication within a time point.

Collaborative Trial Design

For the international collaborative trial thirteen laboratories participated. The collaborative trial was designed in accordance with IUPAC and ENGL guidelines for the design, conduct and interpretation of collaborative studies [10,11]. Participating laboratories were provided with the 20 coded (blind) DNA test samples to analyse using the real-time PCR method, representing the five levels of raw horse meat in a raw beef meat background. The use of five levels, as recommended in the IUPAC guide [10], enabled an assessment of the usefulness of the test method at various levels of adulteration, and provided data for the estimation of levels of repeatability precision (S_r ; RSD_r) and reproducibility precision (S_R ; RSD_R). To control for inter-laboratory variability not attributable to the method, all of the required reagents were provided, with the exception of nuclease free water.

All participants received electronic copies of detailed working instructions for performing the method, plus an electronic copy of an Excel (Microsoft®) pro/forma reporting sheet to be returned for statistical analysis. Participants were also required to report, in detail, any additional information that could have influenced their results, including: (i) make and model of real-time PCR instrument used, (ii) date of instrument calibration, (iii) level of accreditation held by laboratory (e.g., ISO 17025), and (iv) the number of analysts involved in the trial. In addition, participants were required to return a file containing the raw data generated from each of the PCR experiments performed.

PCR was performed in triplicate for each of the test samples U1 to U20. Test samples were assigned to two, ninety-six well PCR plates, such that each level of horse DNA was replicated twice on each plate and in total four replicates for each level of horse DNA were analysed. Details of the plate layouts used are illustrated in Figures 1.1 and 1.2.

Data Analysis

Both the raw data and the real-time PCR analyses from

participating laboratories were evaluated. Data was converted into tab-delimited text format in preparation for statistical analysis using the R statistical computing package (version 3.01). Box plots were generated for the data and a preliminary inspection of the data performed in order to determine the presence of outlying data points. The statistical status of putative outlying data points was confirmed using a Grubb's test [18], and any significant outlying values removed.

Values for the repeatability and reproducibility of the method were calculated for each level with use of a mixed effects model, based on maximum likelihood. The model specified three random effects as follows: (a) unit-to-unit variation (where each sample level was represented by four identical test units randomly assigned from U1 to U20); (b) between-plate effect nested within laboratory; (c) between-laboratory variation. Output from the model was used to derive precision estimates of the relative repeatability standard deviation RSD_r (%) and the relative reproducibility standard deviation RSD_R (%). In addition to these estimates, the performance characteristics of PCR efficiency and the R^2 value of the calibration curve for both real-time PCR assays were also determined.

Results and Discussion

Sample Authenticity

Authenticity of the meat samples sourced from Kezie Ltd (Duns, UK), was confirmed in-house with use of DNA sequencing and real-time PCR.

DNA Sequencing Analysis

DNA was analysed by Sanger DNA sequencing. Two mitochondrial genes (12S rRNA and cytochrome b) were used for species specific identification of each joint of meat. All samples were identified as the correct species by alignment of candidate DNA sequence to reference sequence databases using BLASTN [19]. Species identity of all samples were confirmed with beef samples demonstrating a 100% match level (max score 708, E value 0.0) and 98% for the horse (max score 2069, E value 0.0).

qPCR analysis

Samples were further validated based on the application of the horse specific (*EC-GHR1*) and universal mammalian (*MSTN*) real-time PCR assays (data not shown).

DNA extraction

DNA was extracted and purified from all of the samples for each level of horse meat (w/w) used. DNA yield was at least 240 ng/ul in a final elution volume of 500 µl. Values for the $A_{260}:A_{280}$ ranged from 1.8 to 2.0 indicating a general lack of proteins, phenol and other inhibitors, and the $A_{260}:A_{230}$ measurement ranged from 2.0 to 2.2 indicating the general absence of other contaminants which absorb at 230 nm.

	1	2	3	4	5	6	7	8	9	10	11	12
A	STD1	STD1	STD1	STD2	STD2	STD2	STD3	STD3	STD3	STD4	STD4	STD4
B	STD5	STD5	STD5	NTC	NTC	NTC	U1	U1	U1	U2	U2	U2
C	U3	U3	U3	U4	U4	U4	U5	U5	U5	U6	U6	U6
D	U7	U7	U7	U8	U8	U8	U9	U9	U9	U10	U10	U10
E	STD1	STD1	STD1	STD2	STD2	STD2	STD3	STD3	STD3	STD4	STD4	STD4
F	STD5	STD5	STD5	NTC	NTC	NTC	U1	U1	U1	U2	U2	U2
G	U3	U3	U3	U4	U4	U4	U5	U5	U5	6	U6	U6
H	U7	U7	U7	U8	U8	U8	U9	U9	U9	U10	U10	U10

Figure 1.1: Plate set-up and loading order for Plate A: samples U1 - U10; shaded cells: Horse specific (*EC-GHR1*) assay; clear cells: universal mammalian (*MSTN*) assay

	1	2	3	4	5	6	7	8	9	10	11	12
A	STD1	STD1	STD1	STD2	STD2	STD2	STD3	STD3	STD3	STD4	STD4	STD4
B	STD5	STD5	STD5	NTC	NTC	NTC	U11	U11	U11	U12	U12	U12
C	U13	U13	U13	U14	U14	U14	U15	U15	U15	U16	U16	U16
D	U17	U17	U17	U18	U18	U18	U19	U19	U19	U20	U20	U20
E	STD1	STD1	STD1	STD2	STD2	STD2	STD3	STD3	STD3	STD4	STD4	STD4
F	STD5	STD5	STD5	NTC	NTC	NTC	U11	U11	U11	U12	U12	U12
G	U13	U13	U13	U14	U14	U14	U15	U15	U15	U16	U16	U16
H	U17	U17	U17	U18	U18	U18	U19	U19	U19	U20	U20	U20

Figure 1.2: Plate set-up and loading order for Plate B: samples U11 - U20; shaded cells: Horse specific (EC-GHR1) assay; clear cells: universal mammalian (MSTN) assay

	Sample				
Factor	S1	S2	S3	S4	S5
Genome equivalents (copies)	24,010	3,430	490	70	10
Mass of horse DNA in each reaction (g)	1.30 E-07	1.86 E-08	2.66 E-09	3.80 E-10	5.42 E-11

Table 2: Genome equivalents and mass of DNA present in each of the experimental test samples

Laboratory	Nominal level (% horse DNA)	Plate	Unit	Measured level (% horse DNA)	Grubbs' test (<i>p</i> -value)
F	5%	1	U7	16.92	0.0072
K	1%	1	U9	1.72	0.053

Table 3: Data points initially identified from the plot as requiring further investigation

Limited stability study: Statistical analysis was performed for the quantitative real-time PCR data generated for the pre-shipment quality control of samples, across three consecutive time points (corresponding to 1, 14 and 62 days following test unit generation and freezing for storage). The pre-shipment testing was undertaken in order to determine the existence of any significant difference in the mean values obtained for each of the five sample levels (20%, 5%, 1%, 0.5% and 0.1% horse DNA) between sampling time points. At each time point, four replicates of each sample level were analysed, with each sample level represented by a triplicate level of PCR replication within a time point. A one-way ANOVA was conducted for each of the five sample levels across the three time points, the results showing that there were no significant differences between the time points for each of the five sample levels ($p > 0.05$ in all cases).

Real-time PCR assay performance: Across all laboratories the mean PCR efficiencies for the horse specific (*EC-GHR1*) and universal mammalian (*MSTN*) assays were 94.1% and 96.4% respectively, corresponding to calibration curve gradients of -3.476 and -3.418 respectively. Mean values for R^2 for the horse specific (*EC-GHR1*) and universal mammalian (*MSTN*) assays were 0.998 and 0.997 respectively.

Statistical Analysis of Quantitative Data

Preliminary inspection of data: Thirteen laboratories participated in the collaborative trial. Initial quality inspection of the data was performed on a laboratory by laboratory basis. All 13 laboratories returned full datasets and met the quality acceptance criteria for PCR performance as detailed in the minimum performance requirements for analytical methods of GMO testing [11] with the average value of the slope of the standard curve being in the range of -3.1 to -3.6, and

the R^2 coefficient being greater than or equal to 0.98.

The data sets from the 13 laboratories were combined. Box plots for all five levels of adulteration for the combined data set are shown in Figure 2. Initial visual inspection of these plots suggest that: (a) plate and lab variation appear consistent across all levels; (b) test unit-to-test unit variation appears small compared with plate-to-plate and lab-to-lab variation; (c) a general small but positive bias exists with respect to the nominal level. Notable outlying data points were detected for laboratory F at the 5% level for horse DNA, and for laboratory K at the 1% level for horse DNA (Table 3). Examination of the underlying C_q triplicate data revealed that the results for the test unit U7 (5% w/w) discrepancy observed with laboratory F were dispersed to an unacceptable extent, making this data point unreliable. In addition, applying Grubbs' test to the four lab F data points was strongly significant ($p = 0.0072$). This, together with the poor triplicate variability, was considered sufficient reasons to exclude the test unit U7 value from the laboratory F data set. The test unit U9 data point discrepancy associated with the Laboratory K data set was not identified as a statistical outlier and was retained ($p = 0.053$, Grubbs' test).

Quantification of horse DNA: Table 4 represents the summarised results returned by each laboratory as part of the international collaborative trial. Values in Table 4 represent the quantitative estimates associated with the five levels of test samples (20%, 5%, 1%, 0.5% and 0.1% (w/w) horse meat).

Figure 2 represents the summarised results pictorially, with the estimated concentration shown by laboratory and plate. Box plots for all five levels of adulteration for the combined data set are shown.

Table 5 presents a summary of the quantitative results including the overall mean values estimated with each test sample as part of

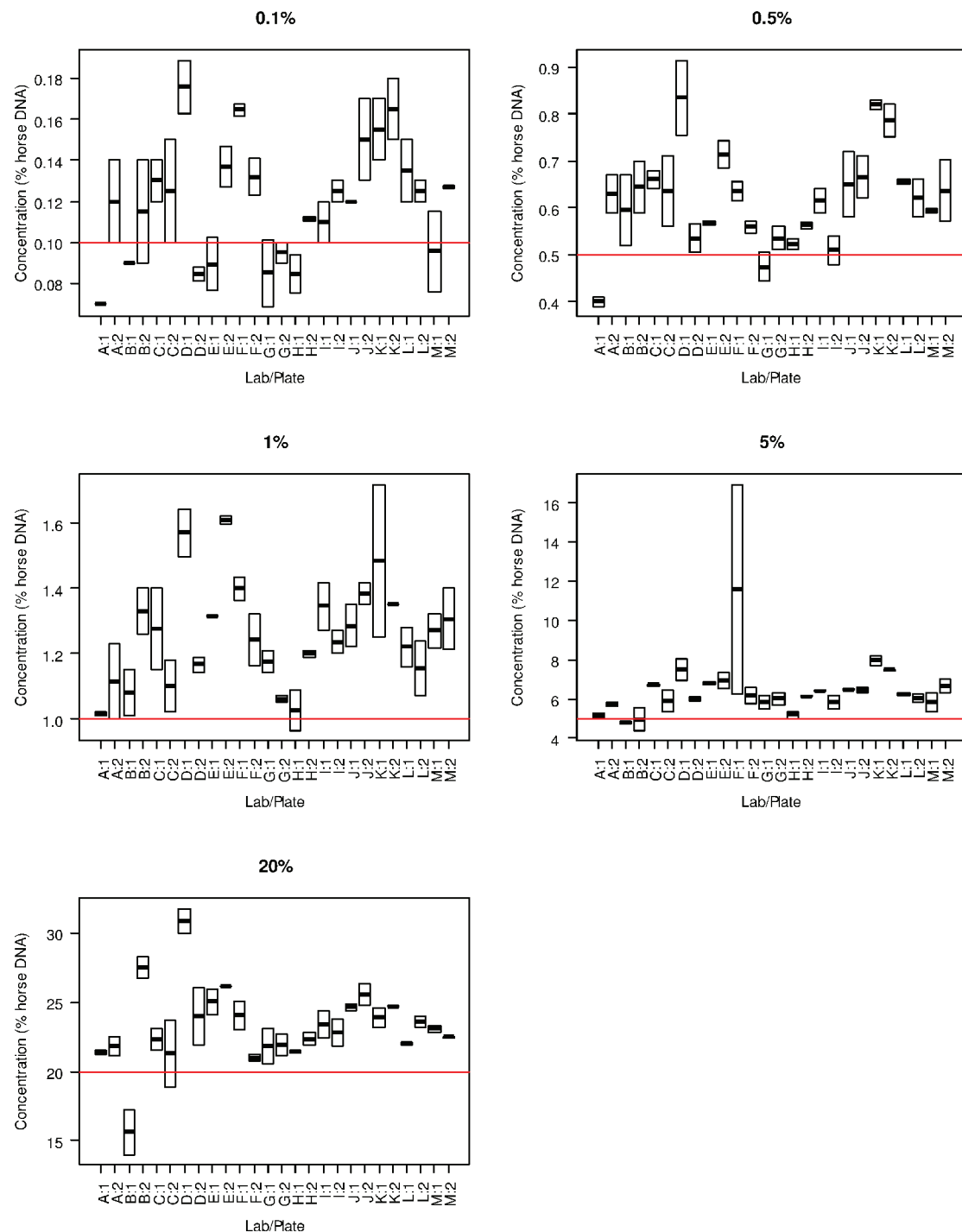


Figure 2: Measured concentration (% horse DNA) shown by lab (A to M) and plate (1 or 2). Two units were each measured once on each plate. Nominal concentration (% w/w) is indicated by the red line

the collaborative trial, alongside the standard deviations associated with the laboratory, plate, test unit and residual effects.

Statistical analysis of measurement data: Repeatability and reproducibility estimates were calculated for each sample level with use of a mixed effects model, based on maximum likelihood. The model specified three random effects which were: (a) unit-to-unit variation; (b) between-plate effect nested within laboratory; (c) between-laboratory variation. For the purposes of this paper, repeatability was defined as the standard deviation between repeat measurements taken by the same analyst in the same laboratory using the same instrument and corresponds to the residual standard

deviation in the specified model. Reproducibility was defined as the standard deviation between different laboratories performing the same experiment, and contains additional sources of variation over the repeatability. A summary of the results obtained for the precision and trueness estimates is presented in Table 6.

Since the original method for the relative quantitation of horse DNA was published in 2013 [5], advances in the sequencing of mammalian genomes have resulted in estimates of the equine and bovine genomes becoming available. According to the NCBI database [20] the size of the equine genome can be estimated as 2474.94 M base pairs [21] and the bovine (cattle) genome as 2724.98 million base pairs [22]. These estimates suggest that the horse genome is

Laboratory	Level % (w/w)																			
	0.10				0.50				1.00				5.00				20.00			
	U3	U8	U13	U17	U5	U10	U11	U15	U1	U9	U16	U19	U4	U7	U14	U18	U2	U6	U12	U20
A	0.07	0.07	0.14	0.10	0.41	0.39	0.67	0.59	1.02	1.01	1.23	1.00	5.24	5.00	5.80	5.64	21.27	21.49	22.52	21.13
B	0.09	0.09	0.14	0.09	0.52	0.67	0.70	0.59	1.15	1.01	1.40	1.26	4.70	4.83	4.40	5.52	17.23	13.99	28.29	26.71
C	0.14	0.12	0.10	0.15	0.64	0.68	0.71	0.56	1.40	1.15	1.02	1.18	6.69	6.73	5.33	6.44	21.50	23.10	18.84	23.74
D	0.19	0.16	0.08	0.09	0.91	0.75	0.56	0.50	1.50	1.64	1.14	1.19	8.06	6.93	5.92	6.07	31.80	30.04	26.04	21.92
E	0.10	0.08	0.13	0.15	0.57	0.56	0.74	0.68	1.32	1.31	1.60	1.62	6.71	6.90	6.55	7.34	24.14	26.00	26.16	26.13
F	0.17	0.16	0.14	0.12	0.61	0.65	0.57	0.55	1.44	1.36	1.16	1.32	6.27	16.92*	6.57	5.78	25.10	22.98	21.19	20.71
G	0.10	0.07	0.10	0.09	0.44	0.50	0.56	0.51	1.14	1.21	1.05	1.07	5.49	6.20	5.70	6.33	23.10	20.58	22.71	21.13
H	0.08	0.09	0.11	0.11	0.53	0.51	0.57	0.56	0.96	1.09	1.21	1.19	5.37	4.98	6.18	6.09	21.30	21.52	22.80	21.92
I	0.12	0.10	0.13	0.12	0.59	0.64	0.48	0.54	1.27	1.42	1.27	1.20	6.44	6.35	5.50	6.14	24.36	22.43	23.77	21.78
J	0.12	0.12	0.13	0.17	0.72	0.58	0.71	0.62	1.22	1.35	1.42	1.35	6.45	6.52	6.33	6.56	24.38	24.91	24.82	26.33
K	0.14	0.17	0.18	0.15	0.81	0.83	0.75	0.82	1.25	1.72	1.35	1.35	7.73	8.15	7.51	7.42	23.17	24.58	24.67	24.76
L	0.12	0.15	0.12	0.13	0.65	0.66	0.66	0.58	1.16	1.28	1.07	1.24	6.16	6.34	6.23	5.84	21.96	22.13	23.19	24.00
M	0.11	0.08	0.13	0.13	0.59	0.60	0.70	0.57	1.22	1.32	1.40	1.21	5.32	6.35	6.98	6.31	22.86	23.35	22.44	22.50

Table 4: Summary of the collated quantitative data for the International Collaborative Trial of the real-time PCR method for the quantitation of horse DNA (A single data point of 16.92 (Lab F, Test unit U7) was identified as a statistical outlier and removed from subsequent analysis of the raw data. All remaining data from Lab F was retained)

	Sample nominal value (% horse meat w/w)				
Metric	0.10	0.50	1.00	5.00	20.00
Mean estimated quantitative value of sample	0.12	0.62	1.26	6.20	23.26
Laboratory standard deviation	0.01	0.05	0.08	0.62	<0.01
Plate standard deviation	0.02	0.08	0.11	0.36	2.54
Test unit standard deviation	<0.01	0.02	<0.01	<0.01	<0.01
Residual standard deviation	0.02	0.05	0.11	0.41	1.34

Table 5: Summary of the quantitative results returned by participating laboratories. The values represent the results derived from the data set with the statistical outlier removed. Values for laboratory, plate, test unit and residual standard deviation were derived with use of the maximum likelihood model used for statistical analysis

around 17% smaller than the bovine genome. The assigned values of the test samples for the percentage horse DNA relative to total mammalian DNA can therefore also be estimated based on the relative genome sizes of the equine and bovine genomes (Table 6). Based on the difference between the assigned and estimated values on the percentage horse DNA content, there is a small but consistent positive bias in this data set, with estimated values varying between 8 and 15% depending upon the sample level.

Table 6 reveals a general trend in the data where the repeatability and reproducibility improve (decrease) with increasing concentration. The reproducibility is below 26% across the range of DNA levels examined, while the repeatability is consistently 15% or below.

The measurement criteria which are required to be satisfied in order that an experimental method be considered fit for purpose have been outlined in the relevant IUPAC guidance [10]. Additionally, as the method used during the international collaborative trial was a real-time PCR approach, further acceptance criteria were adhered to as outlined by published ENGL guidance, which is regarded as one of the leading fields associated with real-time PCR analysis for food and feed [11]. Collectively, the published guidance require that:

(a) the relative reproducibility standard deviation (RSD_R) should be below 35% over the majority of the dynamic range, and below 50% at the lower end of this range; (b) the relative repeatability standard deviation (RSD_r) should be below 25% across the levels of analyte tested. Table 7 shows that the real-time PCR method satisfies both of these requirements at all of the levels of horse DNA tested. Specifically, the highest value obtained for the RSD_R (%) was 25.83% at the 0.1% w/w level, which is below the critical threshold of 50% set for this lower level. With respect to the RSD_r , a maximum value of 15% was recorded for this metric at the 0.1% w/w level. This is below the critical threshold of 25% stipulated by the collective IUPAC and ENGL guidance (Table 7).

In terms of the real-time PCR assays used with the method, both satisfy the required performance criteria specifically stipulated in the ENGL guidance [11]. Specifically, mean PCR efficiencies of 94.1% and 96.4% were achieved by the horse specific (*EC-GHR1*) and universal mammalian (*MSTN*) assays respectively. These values correspond to a gradient of the calibration curve of -3.5 and -3.4 respectively, which fall within the $-3.1 \geq \text{slope} \geq -3.6$ range stipulated in the ENGL guidance [11]. In addition, the mean values for the correlation coefficient of a standard curve obtained by linear regression (R^2)

	Assigned value (% horse DNA) based on equine and bovine genome size estimates				
Metric	0.11	0.55	1.10	5.48	21.58
Assigned value (% horse watt w/w)	0.10	0.50	1.00	5.00	20.00
Number of participating laboratories	13	13	13	13	13
Number of significant outliers determined with use of Grubbs outlier test	0	1*	0	0	0
Relative repeatability standard deviation RSD_r (%)	15.00	8.39	8.65	6.66	5.74
Repeatability standard deviation S_r	0.02	0.05	0.11	0.41	1.34
Relative reproducibility standard deviation RSD_R (%)	25.83	17.58	13.73	13.35	12.35
Reproducibility standard deviation S_R	0.03	0.11	0.17	0.83	2.87
Mean value based on collaborative trial (% horse cp/cp)	0.12	0.62	1.26	6.20	23.26
Bias (%) relative to assigned value based on % horse DNA	9.00	12.68	14.55	13.19	7.76

Table 6: Summary of quantitative measurement data (*A single test unit (U7) was removed from the 0.5% (w/w) data set for laboratory F as it was identified as a significant statistical outlier. All of the remaining data for this laboratory was retained).

Measurement	Estimate from Collaborative Trial	Acceptance criteria (IUPAC/ENGL)
Relative repeatability standard deviation(RSD_r)	9% (15%)	<25%
Relative reproducibility standard deviation(RSD_R)	18% (26%)	<35% (<50% at lowest concentration)

Table 7: Estimated relative standard deviations of repeatability and reproducibility associated with the results from the collaborative trial, as well as the levels to comply with to show that the method is fit for purpose as outlined in IUPAC and ENGL published guidance. Estimated values in parenthesis indicate the relative standard deviations for the lowest gravimetric test sample (0.1% w/w) whilst the other value indicates the largest relative standard deviation associated with the remaining test samples (0.5, 1, 5 and 20% w/w)

were 0.998 and 0.997 for the horse specific (*EC-GHR1*) and universal mammalian (*MSTN*) assays respectively, which are above the threshold of acceptance stipulated in the ENGL guidelines [11].

Values for the trueness of the method can be estimated from the levels of bias observed at each of the levels of analyte used. According to IUPAC and ENGL guidelines, trueness should be $\pm 25\%$ across the measured range. Based on the relative sizes of the equine and bovine genome based on DNA sequencing projects (the equine genome being, on average, 9% smaller than the bovine genome), the bias compared to the assigned value of the samples (on a copy number by copy number basis) varied between 7.76 and 14.55%, well within the $\pm 25\%$ criteria for acceptance. Figure 3 shows the combined repeatability and mean estimated associated with the five test samples evaluated as part of the collaborative trial, taking into account the average difference between the equine and bovine genomes.

The published IUPAC and ENGL guidance is also intended for use with well characterised reference materials and not necessarily for in-house materials which were prepared for use in a collaborative trial. The focus of this trial was to assess the performance of the method, and not to assign a value to the samples used. A relatively consistent and positive bias is shown throughout all sample levels, which is likely to have contributions from the production of the gravimetric samples. This is particularly relevant for the 0.1%, 0.5% and 1% w/w sample levels which were derived from an appropriate gravimetric amount of the 5% w/w material, and were thus not completely independent. However, the estimates for repeatability, reproducibility and bias still satisfy the combined requirements specified in the published IUPAC and the ENGL guidance, providing evidence of the fitness for purpose of the method.

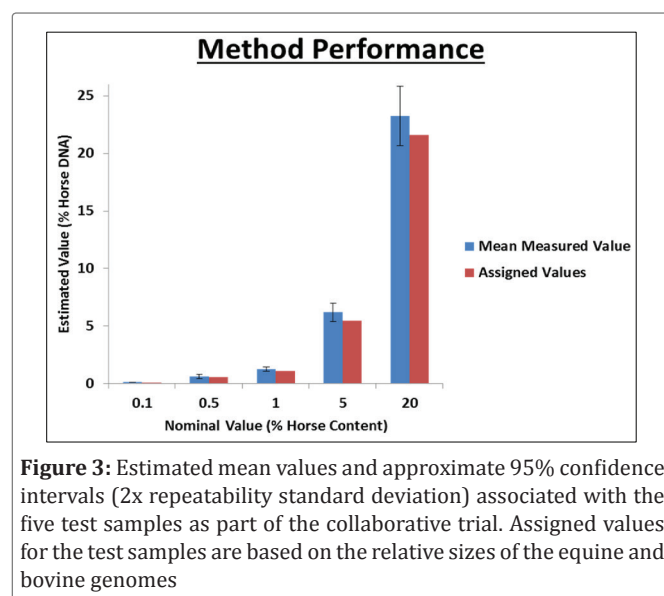
The focus of the current study was to characterise the repeatability and reproducibility of the method as part of a collaborative trial. The debate whether to express results in terms of w/w or cp/cp, the advantages and disadvantages of the two, and the conversion between them, continues to be a topic of discussion at an international level between scientists, with no immediate agreement on a solution. From a metrological and traceability scientific point of view, estimates made in copy numbers are often preferable, but from a practical, enforcement and legislative view point, estimates based on w/w are

often the preference. Copy number values of a sample can be affected by tissue type, DNA recovery, matrix background, DNA degradation, sample preparation etc., and it was not within the remit of the reported study to resolve this situation, where discussions on the area continue to be the subject of many international working groups with no clear resolution to the issue. However, as estimates of mammalian genome sizes become increasing more accurate and available, for examples as a result of Next Generation Sequencing projects, it should become possible to take into account these calculations to provide more accurate estimates of sample levels on a copy number by copy number basis in controlled situations. Alternatively, digital PCR can be used to assign values to test samples based on absolute single molecule quantitation of the various mammalian genomes. The true value of a test sample will be dependent upon what approach and what measurement units were used to assign the value. One approach to calculate the assigned value of a test sample is to use the robust mean taken from the current collaborative trial. For each test sample, the robust mean was based on four biological replicates measured three times each across thirteen different laboratories (Table 6). The very tight repeatability and reproducibility estimates associated with each of the five samples provides testament to the fact that there was reasonable confidence in the value that was being estimated.

Template provided: The nature of the samples provided (DNA) was chosen on the basis of minimising problems with interference from sample extraction, mitigating against stability issues of meat samples, and administrative problems with sending meat as a sample for an international collaborative trial. The real-time PCR method measures the relative DNA content of a sample, and the purpose of the international collaborative trial was to evaluate the method itself and not the performance of the laboratories in terms of their ability to extract DNA. A number of international validation trials which are currently in effect also use this premise where DNA is provided to participating laboratories as the template [23].

Conclusion

The tight repeatability and reproducibility estimates associated with this international collaborative trial of the real-time PCR approach for the relative quantitation of horse DNA provides evidence for the good precision of the method within and between laboratories.



Additionally, only low dispersion is observed around the expected value of the test samples with some consistent but small positive bias. Coupled with the good performance characteristics associated with real-time PCR assays, these values fulfil the acceptance criteria for the precision and trueness associated with a method considered fit for purpose as part of a collaborative trial. The method is now being considered for international standardisations through the relevant CEN sub-committee.

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