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Validation of Two Real-Time PCR Approaches for the Relative Quantitation of Pork and Horse DNA in Food Samples

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Abstract

Two real-time PCR methods for the relative quantitation of DNA from meat species in food samples are described: these methods are applicable for horse in processed beef meat products, and pork in raw/processed beef meat products. Test samples were prepared using raw meat admixtures or processed horse/pork in beef food products made to an industry-standard recipe. The methods were subjected to single laboratory method validation, evaluating the performance characteristics of specificity, PCR efficiency and r-squared (r^2), Limit of Detection (LOD), Limit of Quantitation (LOQ), and precision and trueness. A limited UK-based inter-laboratory trial of the two methods was completed involving four participating laboratories. Full statistical analysis of the data qualified the applicability of the methods for accurate and sensitive trace-level analysis. The methods were deemed fit for purpose for reproducibly distinguishing between adventitious contamination at 0.1% (w/w), the level for further enforcement action at 1% (w/w), and a level representative of deliberate economically motivated adulteration (10% (w/w)). The data provided evidence that the precision of the two methods was applicable for qualitative and quantitative detection at topically important levels of adulteration. This work has added significant value to the current state of the art in quantitative determination of topical meat species adulteration, allowing analysts to distinguish between adventitious contamination and deliberate adulteration. The resulting methods described in this paper can easily be deployed and used by analytical laboratories for controls and due-diligence testing based on standard laboratory equipment.

Keywords

Food Authenticity, Food Adulteration, Meat Speciation, Meat Quantitation, Real-Time PCR

1. Introduction

In 2013 a significant amount of horse meat was found in some beef products which were on sale to the general public within the EU and in the UK [1]. The UK Government commissioned an independent review into the food supply network and in 2014, HM Government Elliott Review into the Integrity and Assurance of Food Supply Networks was published [2]. This report included recommendations for improving systems to deter, identify and prosecute food adulteration, as well as recommendations on improving laboratory testing capability to ensure a harmonised approach for food authenticity testing.

EU Regulation 1169/2011 on the provision of food information to consumers, stipulates the requirement for origin labelling for most fresh and frozen meat products, as well as the provision of information on the quantity of certain ingredients [3]. As highlighted by the 2013 horse-meat issue and associated HM Government Elliott Review, there is a need to improve traceability and trackability of meat produce in the food supply chain with particular emphasis on quantitative estimation of key food ingredients at trace levels, in order to help increase consumer confidence and ensure fair trade.

In line with and reinforcing the recommendations from HM Government Elliott Review, the Department for Environment, Food & Rural Affairs (Defra) commissioned a project to develop a real-time PCR method for the relative quantitation of horse DNA [4]. This method was subject to validation as part of subsequent Defra projects [5] [6], resulting in the production of a standard operating procedure (SOP) and a peer-reviewed publication on the method [7]. An international collaborative trial of the method was then organised, funded by the UK Food Standards Agency (FSA) [8], which established the fitness for purpose of the method at an international level. This method is now undergoing international standardisation via Technical Committee CEN/TC 460 "Food Authenticity" [9].

Proof of principle of applying the method to processed food samples was further described in another publication [10], alongside a real-time PCR method in development for the relative quantitation of pork DNA.

A previous publication described the application of a real-time PCR method for the relative quantitation of horse DNA in raw meat samples [7]. This current paper describes the development of additional real-time PCR methods, for the relative quantitation of pork DNA in raw/processed food products, and horse DNA in processed food products, as a result of a further Defra project [11]. The development and validation of these methods are presented, alongside results from an intra- and a small inter-laboratory-based study, in order to provide objective evidence of the fitness for purpose of these methods for the quantitative estimation of topical levels of food adulteration.

2. Experimental

2.1. Raw Meat Admixture Preparation

Raw pork, horse, and beef meat materials from multiple animals were sourced

from reputable suppliers, species identity verified by real-time PCR, and used as the basis for the development of the horse and pork qPCR methods.

Raw beef, pork, and horse meat materials were trimmed of fat/connective tissue and passed through a meat grinder using a 7 mm grinder plate. Single adulterated admixtures were gravimetrically prepared by mixing and passing the component materials three times through a meat grinder to generate a range of admixtures comprising 100% (w/w) (pork, horse, and beef), 10% w/w (pork or horse in beef), 1% (w/w) (pork or horse in beef) and 0.1% (w/w) (pork or horse in beef). Prepared materials were stored at −20˚C until required for analyses and associated freeze-thaw cycling minimised.

2.2. Processed Meat Admixture Preparation

Additional processed meat materials required for developing/validating the processed meat methodologies were sourced as a cooked cottage pie type processed food material prepared by Campden BRI Limited (UK) prepared using food industry-standard processes and methodologies [10]. "Cottage pie" was selected on the basis of it representing a typical ready meal-type food product found within the marketplace with good matrix complexity and was anticipated to exhibit limited processing-based template degradation.

The meat content of the samples representing the processed food material was as follows:

- 0.1% (w/w) pork/10% (w/w) horse (89.9% (w/w) beef, 0.1% (w/w) pork and 10% (w/w) horse mince meat);
- 1% (w/w) pork and horse (98% (w/w) beef, 1% (w/w) pork and 1% (w/w) horse mince meat);
- 10% (w/w) pork/0.1% (w/w) horse (89.9% (w/w) beef, 10% (w/w) pork and 0.1% (w/w) horse mince meat).

Prepared materials were stored at −20˚C until required for analyses and associated freeze-thaw cycling minimised.

2.3. DNA Extraction

DNA extraction was performed on 100 mg sub-samples using the Maxwell RSC® (Promega, UK) automated nucleic acid system with the Maxwell® RSC PureFood GMO and Authentication Kit (Promega, UK). Extracted DNA materials were characterised by Nanodrop™ 2000 spectrophotometer (Thermo Fisher Scientific, UK) to determine DNA yield (A_{260}) and material quality metrics (A_{260}:A_{230} and A_{260}:A_{280}).

Relevant sampling guidance (e.g. from the National Competent Authority) should be followed in order to ensure analytical samples are representative of the original sample or batch.

2.4. Real-Time PCR

Singleplex relative quantitative-based methods were developed to determine

both the horse and pork DNA content through the comparative analyses of raw horse and pork meat in raw and processed beef samples.

The methods utilised published hydrolysis probe-based real-time PCR assays (**Table 1**) developed by Köppel *et al.* (2011) which target the porcine beta actin-gene and the equine growth hormone receptor gene (GHR) [12], and Laube *et al.* (2003) which targets the mammalian and poultry myostatin gene [13].

The methods were developed for a total volume of 25 µL for the Laube (mammalian) reaction and 25 µL for the Köppel (pork or horse) reaction. 12.5 µL 2 × TaqMan™ Universal PCR Master Mix with oligonucleotides (forward primer, reverse primer, and probe to appropriate final reaction mixture concentration) were made up to 19 µL with DNase/DNA-free water (**Table 2(a)** and **Table 2(b)**). Reagent volumes were scaled to prepare a sufficient reaction mixture for 1.2 times the number of reaction replicates. 19 µL of the reaction mixture was transferred into the appropriate wells using a manual or automated micropipette.

Genomic copy number estimations were based on the assumption that a single haploid copy of the horse (*Equus caballus*) genome equates to 2474.93 Mb and a single copy of the pig (*Sus scrofa*) genome equates to 2457.91 Mb (NCBI Genomes, reference genome sizes correct in 2019).

A 5-point (8-fold) serial dilution series (S1 - S5) was prepared, ranging from approximately 24,576 to 6 domestic pig (pork) or horse (equine) genome equivalent copies, using quantitated 100% (w/w) domestic pig (pork) or horse (equine) genomic DNA as the calibrant, diluted in DNase/DNA-free water.

Real-time PCR assays were performed under standard 2-step real-time PCR cycling conditions (10 min/95˚C; 15 s/95˚C, 1 min/60˚C, 45 cycles) on a QuantStudio™ 7 Flex Real-Time PCR System (Thermo Fisher Scientific, UK) utilising a 96-well block format with TaqMan™ Universal PCR Master Mix (Thermo Fisher Scientific, UK) at a 25 µL final PCR reaction volume. 50 ng DNA test samples and appropriate controls per target assay were represented by a triplicate PCR technical replicate level.

The following describes the analysis of results associated with the pork real-time PCR method. Data analysis for the horse method follows the same description

Table 1. Primer and probe sequence information for the pork and horse methods.

Target	Assay details	Sequence Names	Sequence (5’ - 3’)	Labelling
Pig beta-actin gene	[12]	Sus_ACTB-F	GGAGTGTGTATCCCGTAGGTG	
		Sus_ACTB-R	CTGGGGACATGCAGAGAGTG	
		Sus1 TMP	TCTGACGTGACTCCCCGACCTGG	6-FAM/NFQ
Horse	[12]	EC-GHR1-F	CCAACTTCATCATGGACAACGC	
		EC-GHR1-R	GTTAAAGCTTGGCTCGACACG	
		EC-GHR1_(P)	AAGTGCATCCCCGTGGCCCCTCA	6-FAM/NFQ
Mammalian & poultry myostatin gene	[13]	MY-f	TTGTGCAAATCCTGAGACTCAT	
		MY-r	ATACCAGTGCCTGGGTTCAT	
		MY-Probe	CCCATGAAAGACGGTACAAGGTATACTG	6-FAM/NFQ

Table 2. (a) qPCR reaction composition and final concentrations (25 µL total reaction volume) for the pork method; (b) qPCR reaction composition and final concentrations (25 µL total reaction volume) for the pork method.

(a)

Reagent Component	Laube Mammalian	Köppel Pork
2 × TaqMan Universal PCR Master Mix	1 ×	1 ×
Forward Primer	0.3 µM	0.3 µM
Reverse Primer	0.3 µM	0.3 µM
Probe	0.2 µM	0.2 µM
Water	Make up to 19 µL	
Template DNA	6 µL volume	
	Test samples: 50 ng DNA Standards: 129.36 ng to 0.05 ng DNA	

(b)

Reagent Component	Laube Mammalian	Köppel Horse
2 × TaqMan Universal PCR Master Mix	1 ×	1 ×
Forward Primer	0.9 µM	0.3 µM
Reverse Primer	0.9 µM	0.3 µM
Probe	0.2 µM	0.2 µM
Water	Make up to 19 µL	
Template DNA	6 µL volume	
	Test samples: 50 ng DNA Standards: 130.25 ng to 0.05 ng DNA	

below, except all instances of the pork-specific (Köppel) assay, should be replaced with the horse-specific (Köppel) assay.

Separate pork-specific (Köppel) and mammalian universal (Laube) calibration curves were generated (**Figure 1**). The $\log_{10}$ transformed estimated copy number (x-axis) was plotted against the mean C_q (y-axis) value for each of the five dilutions used for the calibration curve.

Independent simple linear regression curves were applied to the Köppel and Laube assay calibrant data sets to determine the equation of the straight line ($y = mx + c$) and coefficient of determination (r^2). Percentage PCR efficiencies for both qPCR assays were calculated. In terms of method acceptance criteria, the r^2 should be ≥ 0.98 and PCR efficiency 100% ± 10%, otherwise, the experiment was repeated.

The mean C_q values for the pork specific (Köppel) and mammalian (Laube) assays for the test sample were tabulated. The equation of the straight line for both assays was rearranged to calculate estimated genomic DNA copy numbers for the pork and mammalian targets.

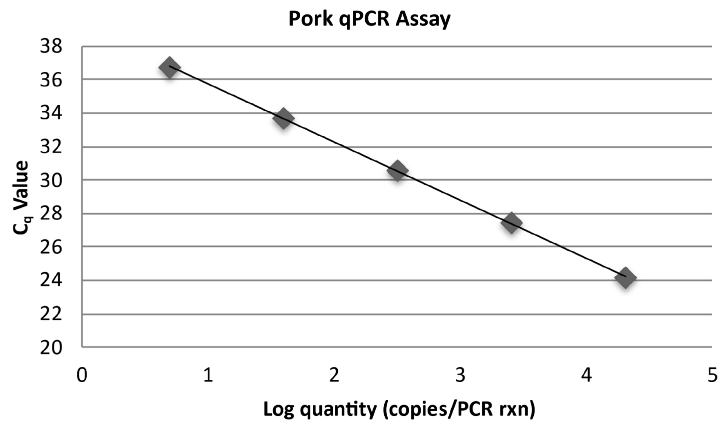

Figure 1. Example calibration curve for the pork specific (Köppel) assay. The $\log_{10}$ of the estimated copy number is plotted against the mean C_q value for the five serial dilutions used in the calibration curve.

The estimated relative quantitative value of pork DNA (%) compared to total mammalian DNA for each test sample was calculated based on the estimated pig genome equivalent copy numbers, divided by the estimated total mammalian genome equivalent copy numbers, multiplied by 100. This value provided an estimate of the amplifiable pork DNA content relative to the total mammalian DNA present in raw or processed beef meat products.

2.5. Single Lab Validation Exercise

2.5.1. Assay Specificity Evaluation

A panel of DNA control materials sourced from Zyagen (San Diego, US) and BioChain Institute, Inc. (Newark, US), comprising beef, chicken, donkey, horse, human, mouse, pig, salmon, sheep, and turkey DNA was used to assess the specificity of the component qPCR assays. 50 ng DNA test samples and appropriate controls per target assay were represented by a triplicate PCR technical replicate level. PCR amplification responses (positive and negative) to the DNA control materials were used to determine specificity and non-specificity characteristics.

2.5.2. Method Validation

Initial method validation of the two methods was performed using a standard curve-based approach in order to determine performance metrics including PCR efficiency and r^2, Limit of Detection (LOD), Limit of Quantitation (LOQ), and precision and trueness. Matrix matched test samples comprising 0.1%, 1%, and 10% (w/w) pork or horse in beef (raw and processed) were quantified using the appropriate method.

2.6. Inter-Laboratory Validation Exercise

2.6.1. Trial Design and Material Preparation

A limited UK-based inter-laboratory trial was conducted to provide actionable evidence for taking the methods forward as part of a full international collaborative trial. Four UK-based laboratories participated in this limited study. The in-

ter-laboratory trial conformed with IUPAC and published ENGL guidelines for the design, conduct, and interpretation of collaborative studies [14] [15].

The four participating laboratories were provided with 27 coded DNA samples to analyse using the two real-time PCR methods as applied to horse in processed beef products, or pork in raw/processed beef products. These samples represented the 0.1%, 1%, and 10% (w/w) mixtures of raw pork in beef, or processed horse or pork in beef. To minimise inter-laboratory variability (not attributable to the method), all of the required reagents were provided.

Following consultation with a statistical advisor, a robust trial design was developed that built upon expertise gained as part of a previous international collaborative trial for the relative quantitation of horse DNA from raw meat products [8].

Participants used a range of qPCR instruments including an Applied Biosystems™ 7500 Real-Time PCR System (Thermo Fisher Scientific, UK), an Applied Biosystems™ QuantStudio™ 12 K Flex Real-Time PCR System (Thermo Fisher Scientific, UK), an Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System (Thermo Fisher Scientific, UK) and an Applied Biosystems™ QuantStudio™ 7 Flex Real-Time PCR System (Thermo Fisher Scientific, UK).

DNA samples were provided to the participating laboratories as the samples for analysis. The nature of the samples provided (DNA) was chosen on the basis of minimising problems with interference from sample extraction, mitigating stability issues of meat samples, and administrative problems with sending meat via a courier. The real-time PCR methods measure the relative DNA content of a sample, and the purpose of the inter-lab trial was to evaluate the method itself and not the performance of the laboratories in terms of their ability to extract DNA.

The trial comprised duplicate experimental plates per method application (processed horse in processed beef products, raw pork in raw beef meat, processed pork in processed beef products) for 6 plates in total, based on a 96-well plate format. Each duplicate plate was partitioned into two equal and identical sections in order to accommodate the species-specific and mammalian/poultry assays. The sectional plate design included the five-point calibration curve described above, negative/positive controls, and 9 test samples (0.1%, 1%, and 10% (w/w) adulteration, 3 replicates per level) per method.

Participants submitted datasets to demonstrate that the method conformed with specified quality metrics ($r^2 \geq 0.98$ and PCR efficiency = 100% ± 10%) as outlined in published acceptance criteria for real-time PCR methods [14]. Collated results were filtered to identify and discard significant data outliers. Study performance characteristics, inclusive of repeatability and reproducibility estimates of RSD_r and RSD_R, were evaluated.

2.6.2. Trial Design and Material Preparation

Both the raw data and the real-time PCR analyses submitted from participating laboratories were evaluated. Data were converted into tab-delimited text format

in preparation for statistical analysis using the R statistical computing package (version 3.01). The statistical status of putative outlying data points was confirmed with the use of Grubb's/Cochran's tests [16] [17], and any significant outlying values were removed.

Values for the repeatability and reproducibility of the method were calculated for each test sample level within a method with the use of a mixed-effects model based on maximum likelihood. The model specified three random effects which were: 1) unit-to-unit variation; 2) between-plate effect nested within the laboratory; 3) between-laboratory variation. The three sample levels were represented by three identical test units assigned to units U1 to U9 (processed horse), U10 to U18 (raw pork), and U19 to U27 (processed pork). 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 (%). Performance characteristics associated with real-time PCR assays (PCR efficiency and r^2 values) were also estimated

3. Results and Discussion

3.1. DNA Extractions

DNA extractions derived from the sample admixtures showed good DNA yields (144 - 344 ng/μL) and quality as determined by spectrophotometry (A_{260}:A_{280} ≤ 1.9 and A_{260}:A_{230} ≤ 2.4, indicating a lack of carryover from RNA, proteins, and inorganic salts).

3.2. Single Lab Validation Exercise

A full single-lab validation exercise was undertaken to provide objective evidence of the fitness for purpose of the horse and pork real-time PCR methods. Performance characteristics were assessed according to Codex document CAC/GL 74-2010 [18].

3.2.1. Assay Specificity Evaluation

A short *in vivo* specificity evaluation was conducted to confirm the published specificity characteristics determined by the assay developers. A panel of control materials comprising beef, chicken, donkey, horse, human, mouse, pig, salmon, sheep, and turkey DNAs was used to assess the specificity of the component qPCR assays.

The pork and horse assays were specific to their reported target species, and the Köppel horse assay also amplified the presence of donkey DNA as previously reported [12] [19]. The Laube mammalian/poultry assay successfully amplified all the expected test DNAs and demonstrated very late/sporadic amplification with salmon fish DNA. This observation was not reported by the developers [13] and suggests that the primer/probe sequences may share some limited sequence identity with fish myostatin or related genes resulting in non-specific amplification under the experimental conditions employed. Whilst trace level cross-reactivity is observed for the Laube assay, this should not compromise analytical perfor-

mance due to the defined scopes of the methods and the negligible impact of any fish-related materials on baseline quantitative measurements.

3.2.2. Method Validation

Method validation work was undertaken to establish the initial performance characteristics associated with the methods. The methods utilised raw meat-derived DNA calibrants to quantify a range of assay appropriate raw/processed meat admixtures (0.1%, 1%, and 10% (w/w)) across three replicate experiments.

The core assay quality metrics derived from the dual calibration curves per test plate demonstrated that all species-specific assays (horse and pork) fell within expected performance parameters with similar r^2 (coefficient of determination) values ≥ 0.996 and PCR efficiencies ≥ 90%. The mammalian/poultry assay showed comparable performance across both methods (r^2 ≥ 0.995, and PCR efficiencies ≥ 94%).

The limit of detection (LOD) for each method was calculated from the lowest calibrant concentration which was repeatedly detected (where ≥ 95% of replicates were detected) and, except for the processed pork in beef application, all qPCR assays could detect at least 5 genomic equivalent copies. The higher LOD (≤ 40 genomic copies) associated with the processed pork in beef application was due to the failure of a single replicate PCR reaction.

The limit of quantitation (LOQ) for the processed horse and raw pork in beef samples was ≤ 0.1% (w/w). A higher LOQ (≤ 1% (w/w)) was associated with the processed pork in beef application, due to only 4 out of 6 replicates generating a quantitative value at the 0.1% (w/w) level which may be due to processivity/sampling issues associated with the processed meat materials.

Intra-assay precision was determined based on two replicate extractions with a technical level of PCR replication of three, for both methods at the three levels of adulterant (0.1%, 1%, and 10% (w/w)). For both methods, the coefficient of variation (CV) was 16% or less for the 10% and 1% (w/w) samples. At the 0.1% (w/w) level, the CV was 22% when applied to the processed horse and raw pork ad-mixtures, and 30% or less when applied to the processed pork ad-mixtures.

The 1% (w/w) adulteration level is often adopted as a practical measure within the UK and the EU to represent a threshold level for further enforcement action. At this level, the CV of samples varied between 3% and 16%. These results highlighted good precision levels associated with both methods.

Trueness estimates (expressed as percentage bias) associated with both methods ranged from −33% and 41% bias at the important 1% (w/w) adulteration threshold level for further enforcement action. The results showed limited positive and negative bias trends associated with the test methodologies.

It should be noted that the 1% (w/w) assigned value is the best estimate of the contributing w/w measurements at this trace level and the true value may be different from this. Of more direct relevance is the precision associated with this estimate which, for example, had a CV of 9% or less for the raw pork admixture at this level. The higher level of biases observed with some of the processed meat

admixtures are likely due to the impact of processivity/long-term storage on the test material.

These initial method validation results based on a single-laboratory study indicated that the methods were fit for purpose and suitable for further validation as part of the inter-laboratory trial.

3.3. Inter-Laboratory Validation Exercise

3.3.1. Trial Design and Material Preparation

A limited UK-based inter-laboratory trial was conducted to provide actionable evidence for taking the methods forward prior to a full international collaborative trial. Four UK-based laboratories participated in this limited study. As far as possible given the scope of the study, the inter-laboratory trial conformed with IUPAC and published ENGL guidelines for the design, conduct, and interpretation of collaborative studies [14] [15].

Following consultation with a statistical advisor, a robust trial design was developed that built upon expertise gained as part of the previous international collaborative trial for the relative quantitation of horse DNA from raw meat products [8].

3.3.2. Real-Time PCR Assay Performance

Based on the participant's data, the mean r^2 (coefficient of determination) and PCR efficiencies for the species-specific and mammalian targets for both methods were calculated. The r^2 was ≥ 0.997 and ≥ 0.998 for the species-specific and mammalian targets respectively, across both methods. The PCR efficiencies varied from 91% to 93% for both the species-specific and mammalian targets.

Published guidance for minimum performance requirements for real-time PCR methods for GMO analysis [14], which is used as a model system for the validation of many qPCR assays, stipulates acceptance criteria of r-squared ≥ 0.98 and PCR efficiency of 100% ± 10%. The r^2 and PCR efficiencies for both methods evaluated in the current study were therefore considered fit for the purpose of general quantitative PCR methods.

3.3.3. Preliminary Inspection of Data

Four laboratories participated in the initial inter-lab trial. A Grubb's test [17] was applied to each sample level within each of the three applications (processed horse in processed beef products, raw pork in raw beef meat, processed pork in processed beef products) method in order to identify any outliers. For each sample level within each application six units (across duplicate plates) were measured by each of the four laboratories, making a total of 24 values per sample level/application combination.

For the processed horse and the raw pork applications, no individual outliers were identified using the Grubbs test. These entire data sets were used in subsequent analysis.

For the processed pork application, one outlier was identified by the Grubbs

test. This constituted one estimate for the 10% (w/w) processed sample. This value was removed from the processed pork data set, leaving 23 values for the 10% (w/w) sample overall.

The between and within plate variance for Laboratory 2 for the 0.1% (w/w) sample for the processed pork application was noted as being relatively large and was not consistent with the rest of the sample data sets within this application. A closer examination of the data showed that three estimates of the 0.1% (w/w) test sample, localised on a single plate for Laboratory 2 could be considered as outliers. These three data points were further confirmed as outlying values through the application of a Cochran's outlier test [16] with a P-value of 0.0015. On this basis, the 0.1% (w/w) estimates on this plate were removed from the remaining data set for the processed pork application.

3.3.4. Statistical Analysis of Measurement Data

Repeatability and reproducibility estimates were calculated for each sample level within a method application, compliant with the IUPAC guidance for collaborative trials [15], and CEN Technical Specification CEN/TS 17329-2 (2019-06) on collaborative trials using real-time PCR methods [20]. A mixed-effects model based on maximum likelihood was used to more effectively partition the variance based on laboratory, plate, and sample effects within each of the three applications.

For the purposes of the current report, 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 3**.

Table 3 reveals a general trend where the repeatability and reproducibility improve (decrease) with increasing levels of adulteration. Excluding the 0.1% (w/w) level, the reproducibility is below 20% and the repeatability is below 10% for the horse and pork methods, providing evidence for the applicability of these methods for precise quantitation at the level for further enforcement action (1% (w/w)) and for quantitation at economically motivated adulteration levels (e.g., 10% (w/w)). This trend is frequently observed due to the impact of stochiometric and Poisson processes on low-level PCR-based detection.

For full international collaborative trials, the IUPAC and ENGL guidelines [14] [15] provide guidance on measurement criteria that are required to be satisfied in order that an experimental method be considered fit for purpose. Collectively, these require that: 1) 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; 2) the relative repeatability standard deviation (RSD_r) should be below 25% across the levels of analyte tested.

Table 3. Results from the interlaboratory trial of the two methods. (a) Horse qPCR method: processed horse application; (b) Pork qPCR method: raw pork application; (c) Pork qPCR method: processed pork application.

(a)

Horse qPCR method	Sample level (w/w)		
	0.1%	1.0%	10%
Number of data points	24	24	24
Mean estimated relative horse DNA content (%)	0.12	1.30	10.78
Relative repeatability standard deviation RSD_r (%)	22.06	6.18	3.22
Relative reproducibility standard deviation RSD_R (%)	46.04	19.43	10.90
Bias (absolute)	0.02	0.30	0.76
Bias (%)	15.27	30.32	7.75

(b)

Pork qPCR method	Sample level (w/w)		
	0.1%	1.0%	10%
Number of data points	24	24	24
Mean estimated relative pork DNA content (%)	0.13	1.17	12.48
Relative repeatability standard deviation RSD_r (%)	18.14	9.27	3.31
Relative reproducibility standard deviation RSD_R (%)	22.94	18.45	11.51
Bias (absolute)	0.03	0.17	2.48
Bias (%)	27.44	16.71	24.84

(c)

Pork qPCR method	Sample level (w/w)		
	0.1%	1.0%	10%
Number of data points	21	24	23
Mean estimated relative pork DNA content (%)	0.11	1.62	14.89
Relative repeatability standard deviation RSD_r (%)	19.53	8.00	4.09
Relative reproducibility standard deviation RSD_R (%)	24.59	18.21	9.61
Bias (absolute)	0.01	0.62	4.89
Bias (%)	10.31	61.79	48.92

Both qPCR methods fulfil this criterion for the reproducibility relative standard deviation (RSD_R), even at the 0.1% (w/w) adulteration levels whose highest RSD_R is 46%. For the relative repeatability standard deviation (RSD_r), both qPCR methods also fulfil the acceptance criteria. There is therefore evidence of the good precision associated with the methods.

At the 0.1% (w/w) level irrespective of method, all replicates provided an in-

strument measurement response and were detected, showing the excellent reproducibility of the methods for trace level detection. Equally well, the 95% confidence intervals associated with the quantitative estimates of the samples at the 0.1% (w/w) level show that this estimate (deliberately chosen to model adventitious contamination) never overlapped with the confidence interval associated with the 1% (w/w) level for further enforcement action. The methods are therefore applicable for easily and reliably distinguishing between samples that may have trace level adventitious contamination (0.1% (w/w)) and those that should be subject to further enforcement action (1% (w/w)).

Across the range of sample levels examined as part of this limited inter-lab trial, the relative bias ranged from 8% to 62% compared to the assigned w/w adulteration levels. This may not be unexpected as the assigned values are based on best estimates from the gravimetric preparations and therefore may not be true values. Equally well, genome size differences between the background bovine genome and the target species of either horse or pork can produce such a positive bias, typically in the region of around 15% of the w/w value as previously reported [10]. Any observable bias can be accounted for and a correction factor introduced, as previously described [10]. The main focus of the current study was on the precision with which these estimates were made and thus how reliable and reproducible the methods were within and between laboratories, with a particular focus around the 1% (w/w) level for further enforcement action. It is important that further assessment of any differences between the sizes of the target species genome (horse and pork) compared to the background (beef) be further elaborated upon in future studies, should the methods proceed to a full collaborative trial and be required to give full quantitative estimates on a w/w basis as opposed to the current relative DNA: DNA measurement.

3.4. Discussion

Two methods have been validated describing the use of quantitative real-time PCR for the purposes of accurate detection and quantitation of adulterant meat species in food samples. These methods are applicable for the relative quantitation of horse DNA in processed beef meat products, and the relative quantitation of pork DNA in raw/processed beef meat products. Both methods complement previously published approaches including the method for the relative quantitation of horse DNA in raw meat products [7] [8].

A limited inter-laboratory trial using four UK-based laboratories provided evidence of the applicability of the methods, which needs to be further qualified as fit for purpose through any additional optimisation studies prior to deployment as a full inter-laboratory or international collaborative trial. Optimisation studies and inter-laboratory trials can be used to further qualify the applicability of the methods across different meat samples, as well as elaborate on performance characteristics (e.g., Limit of Quantitation) using a larger replicate size.

The pork real-time PCR method for the relative quantitation of raw pork in raw beef was shown to be fit for purpose for full quantitation at levels represent-

ative of adventitious contamination (0.1% (w/w)), the level for further enforcement action (1% (w/w)) and at an economically motivated adulteration level (10% (w/w)).

Equally well, the pork and horse real-time PCR methods for the relative quantitation of processed pork and processed horse in beef products were shown to be fit for purpose for detection of adventitious contamination (e.g. at 0.1% (w/w)) and fully quantitative at levels representative of further enforcement action (1% (w/w)) and economically motivated adulteration levels (10% (w/w)). The methods can reliably distinguish between adventitious contamination (0.1% (w/w)) and the level for further enforcement action (1% (w/w)).

Both methods use regular quantitative real-time PCR methodologies and should therefore be able to be routinely applied by relevant analytical laboratories, for testing, and for enforcement purposes.

The results of the current study clearly demonstrate the fitness for purpose of the real-time PCR methods for the relative quantitation of DNA from horse and pork in raw and processed food products. However, this was demonstrated in a limited UK-based inter-lab trial involving only four laboratories. In order to qualify the fitness for purpose of these methods further, additional optimisation of the methods should be considered, alongside a larger inter-laboratory or international collaborative trial, using IUPAC guidance to provide evidence for consideration of the methods for international standardisation by CEN/ISO following qualification of the reproducibility of the methods.

Quantitative real-time PCR methods assess instrument measurement responses generated from individual DNA targets. Any difference in the relative abundance of these DNA targets means that a DNA copy number to copy number measurement does not necessarily translate directly into a weight per weight measurement. An estimate of the relative equine and bovine genome sizes was used when calculating the assigned value of the test samples, as part of the previous international collaborative trial of the real-time PCR approach for the relative quantitation of horse DNA in raw meat products [8]. Further work needs to be conducted for the methods described in the current paper to test the efficacy of implementing conversion factors if results need to be expressed on a w/w basis as opposed to relative DNA contents. In particular, modern technologies such as digital PCR, which can afford absolute single-molecule quantitation without reference to a calibration curve, should be used to help accurately value assign copy number estimates to reference materials and standards for these purposes.

4. Conclusions

Two real-time PCR methods for the relative quantitation of DNA from meat species in food samples were successfully developed as part of this work. These methods are applicable for processed horse in beef meat products, raw pork in raw beef meat products, and processed pork in beef meat products.

Using test samples prepared using raw meat admixtures or dual spiked

processed horse and pork in beef food products made to industry standard recipes, in house assessment of important performance characteristics (inclusive of specificity, PCR efficiency and r^2, Limit of Detection (LOD), Limit of Quantitation (LOQ) and precision and trueness), as part of the method validation process, provided evidence that the methods were fit for purpose.

Following on from this, a limited inter-laboratory trial involving four UK-based laboratories was conducted. Full statistical analysis of the data was conducted following IUPAC guidance for inter-laboratory trials, providing evidence of the reproducibility of the two methods. The methods were deemed fit for the purpose of reliably distinguishing between adventitious contamination at 0.1% (w/w), the level for further enforcement action at 1% (w/w), and a level representative of deliberate economically motivated adulteration (10% (w/w)). The results of this project provided evidence that the precision of the two methods was satisfactory for quantitative and qualitative detection at topically important levels of adulteration.

This work has added significant value to the current state of the art in quantitative determination of topical meat species adulteration, allowing analysts to easily distinguish between adventitious contamination and deliberate adulteration. The two methods can readily be deployed by analytical laboratories for routine testing (e.g. by enforcement or testing labs) based on standard laboratory equipment. These methods support quantitative estimation of key food ingredients at trace levels, in order to help increase consumer confidence and ensure fair trade. The methods will further help ensure honesty in labelling, consumer confidence in the food chain's integrity, and traceability in the origin of food and associated ingredients.

The methods currently provide estimates for the relative quantitation of pork and horse DNA, as applied to raw and processed (beef) food products. Should the methods be required to be used for expression of results in terms of w/w meat materials, then further validation is required to investigate copy number differences for the relevant meat species used.

In order to further qualify the applicability of these methods, they need to be subjected to a full international collaborative trial to demonstrate the reproducibility of the methods. This will provide objective evidence for considering the methods for international standardisation via the relevant CEN or ISO committees.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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