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Development of a real-time PCR approach for the relative quantitation of horse DNA

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Abstract

The 2013 European-wide issue involving the undeclared presence of horse meat in beef products emphasised the need for the development of accurate analytical approaches for the quantitative detection of meat adulteration. As part of a UK Government, Department for Environment, Food & Rural Affairs (Defra) project, a real-time PCR method was developed for the quantitation of horse DNA relative to the total amount of mammalian DNA present in raw meat samples. Single copy nuclear DNA targets were chosen and assays selected that targeted an equine growth hormone receptor and a mammalian/poultry myostatin gene. The method was challenged against a range of gravimetrically prepared raw horse meat in raw beef ad-mixtures, and demonstrated good performance characteristics, including high mean r-squared values (> 0.995) and PCR efficiencies (> 90 %). The limit of detection was estimated at less than five horse genome equivalents, and the limit of quantitation to be ≤ 0.1 % w/w gravimetric preparation of raw horse meat in a raw beef meat background. Assessment of multiple 1 % w/w raw gravimetric samples estimated the mean and analytical measurement uncertainty (based on a 95 % confidence interval) to be 1.58 ± 0.54 % w/w, thereby demonstrating good trueness and precision.

The method was validated for DNA extracted from samples that consisted of raw horse meat in a raw beef meat background. The development and publication of this non-proprietary novel real-time PCR method for the quantitation of horse DNA will inform and strengthen the decision making processes of food companies and regulators.

1) Introduction

Food authenticity and food fraud are becoming increasingly problematic owing to pressures on food production and the current climate of financial constraint. In January 2013, the Food Safety Authority of Ireland (FSAI) published a report which stated that a significant amount of horse DNA had been detected in beef burger products which were on sale to the public [1]. As part of the response to the 2013 European Union (EU) horse-meat issue, an EU survey of beef products was commissioned by the European Commission [2] which also coincided with a UK wide survey initiated by the Food Standards Agency (FSA). Following advice from regulators, enforcement agencies and industry, a 1 % weight for weight (w/w) threshold to distinguish between adventitious contamination and deliberate adulteration of meats was adopted [3, 4]. The adoption of such a threshold highlighted the requirement for a harmonised and freely accessible quantitative approach to be developed in order to accurately measure the amount of horse DNA present in food samples.

Official food controls can only detect food adulteration with the aid of suitable methods of analysis. Methods already in use include isoelectric focusing in polyacrylamide gels (PAGIF) [5], polyacrylamide gel electrophoresis (PAGE) [6], and enzyme-linked immunosorbent assays (ELISA) [7, 8]. All of these methods are based on the detection of species-specific proteins. Identification of animal species has proved to be difficult, in particular in samples which have been exposed to high temperatures, because of the denaturation of proteins. However, methods of DNA analysis based on the polymerase chain reaction (PCR) offer enhanced characteristics such as specificity and sensitivity when applied to the identification of

animal species components, even in products which have been subject to intensive processing [9]. A number of publications exist detailing the detection of commercially important species using real-time PCR [10-12]. In general, it is agreed amongst experts that the use of mitochondrial DNA allow highly sensitive assays to be developed due to the abundance of the mitochondrial genome in cells [13, 14]. However, because of the variability in the number of mitochondrial copies amongst species, and even between tissue types within the same species [15, 16], mitochondrial DNA should generally not be used for quantitative purposes and recent scientific literature favour the use of single copy nuclear DNA targets [15, 17].

The work described here details the development of a real-time PCR approach for the relative quantitation of horse DNA, based on single copy nuclear DNA targets, valid for samples consisting of raw horse meat in a raw beef background. Published hydrolysis probe-based assays with the appropriate performance characteristics were identified and evaluated. The selected assays comprised of a Koppel *et al*., (2011) assay targeting the equine growth hormone receptor [18] and a Laube *et al*., (2003) assay targeting the mammalian and poultry myostatin gene [11]. The assays were optimised and then evaluated to determine key performance characteristics. The fitness for purpose of the quantitative approach was qualified through single laboratory method validation and application to a range of w/w gravimetrically prepared raw horse meat in raw beef materials.

The method described uses real-time PCR to quantitate the amount of horse DNA relative to the amount of total mammalian DNA extracted from a raw meat sample. The method has been applied to and is validated for DNA extracted from samples that consist of raw horse meat in a raw beef meat background. The results have provided evidence that a relative quantitation based approach can accurately

measure the amount of horse DNA present in a sample relative to the amount of beef DNA. In addition, when there is similarity in species, tissue type and ingredients between test samples and calibrants, this quantitation approach can be extended to w/w gravimetric materials with acceptable levels of precision and bias.

2) Results and discussion

Development of a real-time PCR method for the relative quantitation of horse DNA

A review of current molecular approaches for the detection of horse DNA identified a panel of potential DNA target sequences for use in the quantitation of horse meat in food samples using real-time PCR methodologies. These biomarkers included single copy targets (e.g. the equine growth hormone gene [18]) which are suited to quantitative analysis, and multiple copy targets (e.g. mitochondrial cytochrome b [19] and 16s rRNA [20] genes) that enhance detection sensitivity at the cost of quantitative potential. However, owing to the fact that the number of copies of mitochondrial DNA can vary between species, and between tissue types within a species, it was decided to focus on single copy nuclear DNA targets in order to afford the greatest potential for quantitation, in line with other expert views in the field [17].

Real time PCR assays were selected that targeted the nuclear DNA single copy horse growth hormone receptor gene (GHR) [18] and mammalian and poultry myostatin gene [11]. Koppel *et al* (2011) have justified the use of the GHR gene as an equine specific target on the basis of the high levels of assay specificity predicted for the target *in silico.* Laube *et al* (2003) proposed the use of myostatin as a cross species target due to the high degree of sequence conservation for myostatin existing between mammals which also extends to poultry, thereby ensuring broad meat tissue coverage. The selected assays were not used in a multiplex format due to potential issues such as reduced assay flexibility, requirement for multiplex optimisation and the interference of competing qPCR assays on quantitative measurements.

Single-laboratory method validation

Initial evaluation work was performed to establish general real-time PCR assay performance characteristics including sensitivity and specificity. Specificity testing (data not shown) demonstrated that the equine GHR assay exhibited some cross-reactivity with the closely related domesticated donkey species (*Equus asinus asinus*), as described in the original paper (Koppel *et al*., 2011), whilst the mammalian/poultry myostatin assay correctly detected mammalian and poultry chromosomal DNA target from genomic DNA templates. The published specificity data [18] shows that the equine GHR assay does not cross react with 50 other animal and plant species often found in food, and whilst the assay does cross react with DNA from mule/donkey, neither of these species are common meats used within the UK and their use as labelled ingredients is very unlikely. The undeclared presence of any meat species in a sample is considered non-compliant with EU labelling legislation [21]. The equine GHR assay gave no detectable response when applied to the 100 % w/w beef control.

Single-laboratory method validation following best-practice guidelines in this area [22-24] was conducted using a range of w/w gravimetric preparations of raw horse meat in a raw beef background as test samples. Figure 1 shows typical amplification profiles and calibration curves associated with the methodology and highlights good quantitative measurement responses and linearity. Both assays share similar performance characteristics, which is desirable for a relative quantitative method, as shown by $R^2 > 0.995$ and PCR efficiencies calculated to be 90.4 % and 94.6 % (Table 1). No amplification was detected when applying the equine GHR assay to the 100 % w/w beef control. These performance characteristics support the use of the two selected assays in the development of a relative quantitation method.

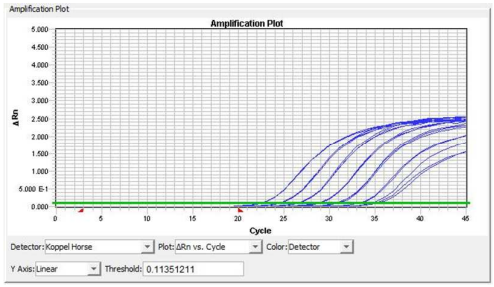
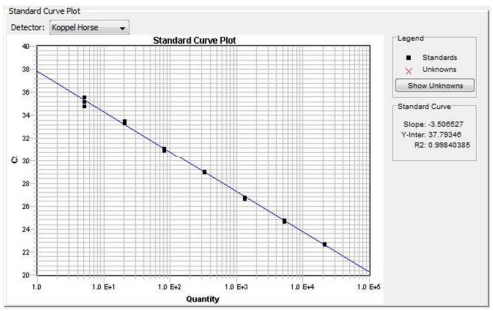
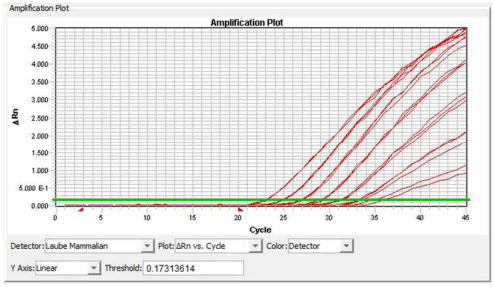
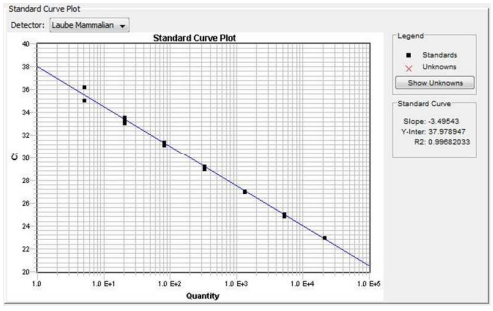
Assay	Calibrant Amplification Plots	Calibration Curve
Equine GHR		
Mammalian/Poultry Myostatin		

Figure 1. Typical amplification plots and associated calibration curves for the equine GHR and total mammalian/poultry myostatin assays derived from a single experimental plate. The plots were based on a 7 point equine genomic DNA calibration curve (20,480 to ~ 5 horse genome equivalents) prepared from 100% raw horse meat at a triplicate technical replicate level.

Table 1. Equine GHR and mammalian/poultry myostatin real time PCR assay performance data. Performance metrics generated from three replicate plates (n=3) comprising 7 point equine genomic DNA calibration curve (20,480 to ~ 5 horse genome equivalents) derived from 100% w/w raw horse meat, per target assay at a triplicate technical replicate level (all replicates detected).

Assay	Mean R^2	Mean Intercept	Mean Slope	Mean PCR Efficiency
Equine GHR	0.995	38.496	-3.577	90.4 %
Mammalian/Poultry Myostatin	0.997	37.924	-3.458	94.6 %

The Limit Of Detection (LOD) was defined as the lowest target analyte concentration (estimated nominal copy numbers) that could still be detected on 95 % of occasions. This was determined experimentally to be at least 5 genomic equivalent copies (~10 target gene copies) for both the horse genome and mammalian genome (raw meat samples) based on the lowest dilution on the respective calibration curves through single-laboratory validation (data not shown). The Limit of Quantitation (LOQ) was defined as the lowest relative amount of horse content of a sample that could still be reliably quantified (95 % confidence interval incorporates assigned value). This was determined experimentally to be ≤ 0.1 % w/w gravimetric materials of raw horse meat in a raw beef (meat) background, based on the successful detection and quantitation of all nominal 0.1 % w/w test samples. The minimal quantitative performance equates to approximately 20 equine GHR gene copies (~50 pg horse DNA in ~50 ng total mammalian DNA) and compares favourably with the LOD which was estimated to be 5 genomic equivalent copies that equates to approximately 10 gene copies.

Relative quantitative analyses of the w/w gravimetric materials showed good comparability between the estimated and expected percentage horse DNA levels across the range of the gravimetric materials, as demonstrated by the trueness estimates (Table 2). The trueness estimates showed that the most bias was associated with the nominal 0.1 % w/w gravimetric sample, which was to be expected given the low concentration of the target analyte present in the sample and the inherent variability associated with gravimetrically prepared mixed meat materials. The trueness estimates are broadly comparable with those typically observed with complex food matrices quantified using qPCR [25].

Table 2. Relative quantitation data and % bias and % coefficient of variation associated with the real-time PCR method. The mean estimated relative horse DNA content, based on real-time PCR, for six w/w gravimetrically prepared ad-mixtures is shown. The results represent three replicate real-time PCR plates based on a 7 point genomic DNA calibration curve (20,480 to ~ 5 genome equivalents) derived from 100% w/w raw horse meat, with w/w horse in beef ad-mixture test samples (100 %, 30 %, 5 %, 1 %, 0.5 % and 0.1 %) per target assay at a triplicate technical replicate level (n=3). The percentage ratio of horse genome equivalents relative to the total mammalian genome equivalents present in each of the test samples was used to calculate the mean estimated % relative Horse DNA content of each the test samples, based on real-time PCR.

w/w Gravimetric Materials	Mean Estimated % Relative Horse DNA Content	% Bias (Trueness)	% CV (Precision)
0.1 % Horse	0.2 %	+69.2 %	25.8 %
0.5 % Horse	0.5 %	+6.3 %	7.8 %
1 % Horse	0.8 %	-24.0 %	15.7 %
5 % Horse	2.2 %	-57.1 %	6.4 %
30 % Horse	33.9 %	+13.0 %	7.7 %
100 % Horse	106.7 %	+6.7 %	1.5 %

Coefficients of variation (CV) varied between 1.5 and 25.8 % with poorer levels of precision generally exhibited with the lower level w/w gravimetric materials. The trueness was much improved at the 0.5 and 1 % w/w levels, and the precision associated with the method, as captured by the coefficient of variation, was never greater than 26 %. The focus of the study was not to validate a set of internally prepared ad-mixtures, but to characterise the performance of the methodology.

Further work will be required to develop/source a set of appropriate w/w gravimetric materials for follow-on inter-laboratory method validation activities.

To ensure results were reproducible across different horse samples, tissue samples from different horse specimens were sourced and used to provide calibration curves and test samples (w/w gravimetric materials). Pairwise comparisons using calibration curves derived from horse specimens different from the test samples were prepared. There was statistically no significant difference ($P > 0.05$) when using tissue samples derived from different horse specimens for the calibrants and the test samples on the quantitative capability or the performance characteristics of the method (data not shown). This result demonstrates that the methodology can be applied to the quantitation of unknown and independent horse meat material within a test sample consisting of raw meat.

Due to the importance of the 1 % w/w level for food labelling purposes, a study was conducted to estimate the mean horse content and associated measurement uncertainty at this level. The study was based on four independently prepared 1% w/w samples with a PCR technical level of replication of six across three replicate PCR plates (each sample therefore being represented by 18 PCR replicates). Figure 2 shows the narrow range of observed mean % relative horse DNA content values (1.32 ± 0.08 to 1.93 ± 0.19) and good associated precision levels. The pooled associated analytical measurement uncertainty (based on a 95 % confidence interval) was determined to be 1.58 ± 0.54 % w/w raw horse meat in a raw beef background. This equates to a coefficient of variation of around 17 %, comparable to the data in Table 2 for the 1 % w/w sample. Given the low level of target analyte, the trueness and precision estimates compare well with other estimates based on real-

time PCR approaches for food authenticity testing estimates in the published literature [18, 26].

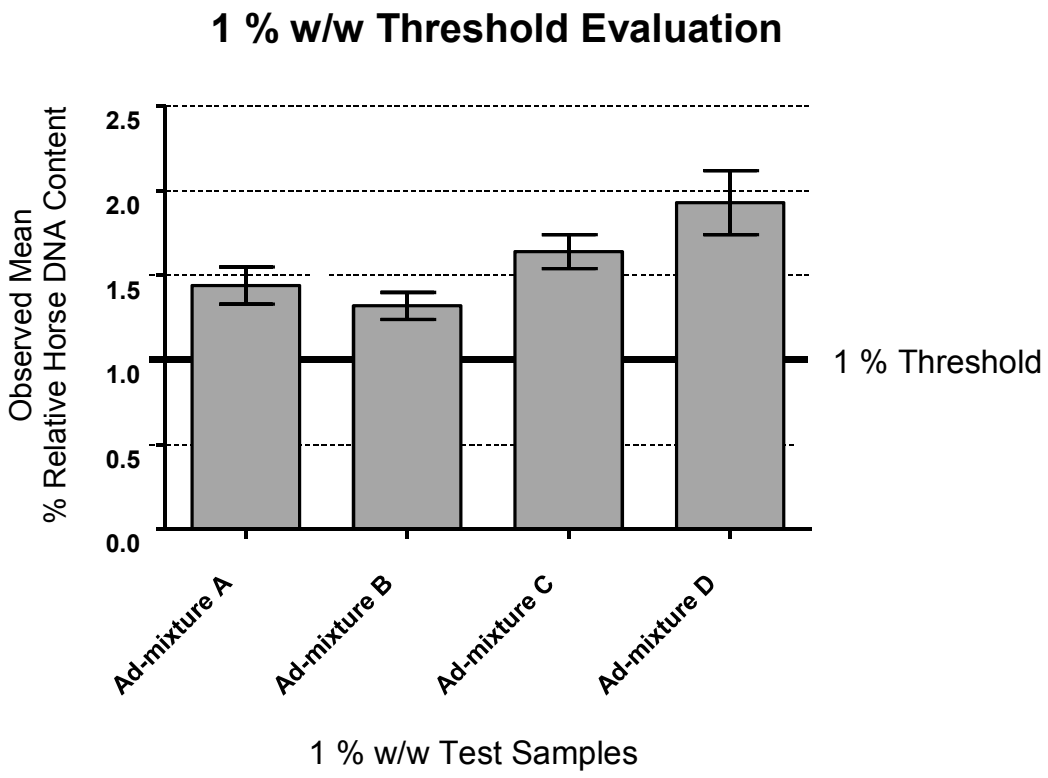


Figure 2. An evaluation of individual 1 % w/w horse in a background of beef test samples (ad-mixtures A – D). Mean % relative DNA content values were calculated from triplicate data sets (n=3). Error bars represent ± Standard Deviation

The 2013 horse meat issue highlighted that there was a lack of harmonisation and guidance on how to quantitate meat species in food products. There is lack of agreement on how to express the results of meat quantitation studies (e.g. on a weight per weight basis of gravimetric meat preparations, or on a DNA to DNA copy

number basis), as well as lack of agreement on what DNA targets should be used for quantitation (inclusive of debates on the appropriateness of mitochondrial and nuclear DNA targets). The term “quantitation” is often used as a relative expression, but once again there is lack of agreement on what the species-specific meat content should be expressed relative to. For example, should this be in relation to the total sample, the total meat content of the sample, a particular species of meat in the sample, the total amount of DNA, or the total amount of mammalian DNA present in the sample? The relationship between DNA copy numbers and actual meat content is often poorly understood and dependent upon a number of factors including the level of processing of the food sample, the matrix background, etc.

The results described here provide evidence that relative quantitation based real-time PCR can be employed to accurately determine the amount of horse DNA present in a raw meat sample relative to the amount of beef DNA. In addition, it was also found that where there was an exact match in species, tissue type and ingredients between test samples and calibrants, this quantitation could be extended to w/w gravimetric materials. The performance characteristics associated with the equine GHR and mammalian/poultry myostatin real-time PCR assays were found to be suited to quantitative measurements as demonstrated by good PCR efficiencies, LODs of around 5 genome equivalents, good dynamic range and good trueness and precision estimates.

3) Experimental

Sourcing and authentication of materials

Raw horse and beef muscle tissue samples were sourced from Kezie Ltd (Duns, UK). The meat samples were surface trimmed and then prepared by removing any separable fat, gristle, etc. retaining the lean meat. The lean meat was cubed, thoroughly homogenised in a food processor, combined and then mixed, and the resultant paste stored as separate horse and beef species. Meat species were authenticated by subjecting the samples to species typing through a mixture of ELISA, DNA sequencing and qPCR-based approaches.

Preparation of weight for weight ad-mixtures

Weight for weight ad-mixtures compromising 100 %, 30 %, 5 %, 1 %, 0.5 % and 0.1 % w/w of raw horse-meat in a raw beef meat background were gravimetrically prepared by weighing the required amounts of the authenticated raw horse-meat into the authenticated raw beef (meat) background. Ad-mixture samples pre pared for the initial method validation activities contained 2 to 10 g of total material and the 1% w/w threshold evaluation studies utilised larger ad-mixture preparations comprising 100 % w/w horse (500 g), 100 % w/w beef (3 Kg) and 1 % w/w horse in beef (100 g) to ensure effective homogenisation.

DNA extraction

DNA extraction was performed on 1 or 2 g samples (whole or homogenised sub-sample) using the silica-based Kleargene™ DNA extraction method (LGC Genomics, Hoddesdon, UK). The extraction process comprised sample homogenisation followed by incubation, lysis of cellular components in an SDS buffer with proteinase K and binding of the isolated DNA to positively charged silica beads.

Multiple washing stages were used to clean the DNA which was eluted in 0.5/1 ml of elution buffer. DNA yield (A260) and quality characteristics (A260:230 and A260:280) were determined using a Nanodrop™ ND-1000 Spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, USA).

Calibration curves

The source of the calibrant was the 100% w/w raw horse-meat sample, treated as described as in the 'Preparation of weight for weight ad-mixtures' section. A 7-point (4 fold) calibration series ranging from approximately 20,480 horse to 5 genome equivalent copies (111.10 to 0.03 ng equivalent) was prepared in DNase/DNA-free water (Ambion brand, Life Technologies, Paisley, UK) using spectrophotometrically quantitated 100% w/w horse genomic DNA derived from raw horse meat. Two separate calibration curves were produced based on the above description and assessed using the Koppel *et al*., (2011) [18] and Laube *et al*., (2003) [11] real-time PCR assays.

Relative quantitative qPCR method development

A singleplex relative quantitative-based method was developed to determine horse DNA content through the comparative analyses of raw horse meat in raw beef ad-mixture samples. The method utilised published hydrolysis probe-based real-time PCR assays developed by Koppel *et al*., (2011) which targets the equine growth hormone receptor gene (GHR) [18], and Laube *et al*., (2003) which targets the mammalian and poultry myostatin gene [11]. Both assays were optimised to run under the same thermal cycling conditions and then evaluated to determine basic performance characteristics.

Twenty-five µl singleplex reactions were prepared comprising 12 µl 2x TaqMan Universal PCR Master Mix (Life Technologies, Paisley, UK), assay specific primers at 0.3 µM (mammalian/poultry myostatin) or 0.2 µM (equine GHR) and dual-labelled hydrolysis format probes (labelled with 6-FAM/BHQ®-1) at 0.2 µM (mammalian/poultry myostatin) or 0.08/0.2 µM (equine GHR). HPLC purified primers and probes (see Table 3) were sourced from Eurofins Genomics (Ebersberg, Germany). The reactions were made up to 20 µl with DNase/DNA-free water (Ambion brand, Life Technologies, Paisley, UK) and 5 µl of the template DNA/water control was added to each reaction as appropriate, to bring the total volume to 25 µl. Template DNA input was normalised to 50 ng for each test sample.

Real time PCR was performed under standard 2-step thermal cycling conditions (10 min/ 95^{o}C; 15 s/ 95^{o}C, 1 min/ 60^{o}C, 45 cycles) using a 7900HT Fast Real-Time PCR System (Life Technologies, Paisley, UK) set to monitor FAM-based fluorescence. The data was analysed using SDS 2.4.1 software (Life Technologies, Paisley, UK) using automated baseline and threshold settings.

Table 3. Primer and probe sequence information. Oligonucleotide primers were HPLC purified and hydrolysis probes labelled with 6-FAM (reporter) and BHQ®-1 (non-fluorescent quencher).

Target	Assay Details	Sequence Names	Sequence (5' – 3')
Equine growth hormone receptor gene	Koppel *et al.* (2011)	EC-GHR1-F	CCAACTTCATCATGGACAACGC
		EC-GHR1-R	GTTAAAGCTTGGCTCGACACG
		EC-GHR1-P	AAGTGCATCCCCGTGGCCCCTCA
Mammalian & poultry myostatin gene	Laube *et al.* (2003)	MY-f	TTGTGCAAATCCTGAGACTCAT
		MY-r	ATACCAGTGCCTGGGTTCAT
		MY-Probe	CCCATGAAAGACGGTACAAGGTATACTG

Method validation

The developers of the mammalian/poultry myostatin gene assay and equine GHR gene assay provided data qualifying the specificity associated with each of the tests [11, 18]. Assay specificity was confirmed by challenging the assay across a range of genomic DNAs (horse, donkey, beef, pork, lamb, duck, mouse, human and chicken) sourced from BioChain Institute, Inc. (Newark, USA) and Zyagen Laboratories (San Diego, USA).

Validation of the relative quantitation-based method was performed using triplicate real-time PCR plates. A 7-point (4 fold) calibration series ranging from approximately 20,480 horse to 5 genome equivalent copies (111.10 to 0.03 ng equivalent) was prepared in DNase/DNA-free water (Ambion brand, Life Technologies, Paisley, UK) using spectrophotometrically quantitated 100% w/w horse genomic DNA. Genomic copy number estimations assumed that 1 haploid copy equates to 2474.93 MB [27]. The calibrants, 100 %, 30 %, 5 %, 1 %, 0.5 % and 0.1 % w/w horse in beef tissue ad-mixture test samples and appropriate controls per target assay were represented

by a minimum triplicate technical level of replication per plate. Assay performance-based metrics including PCR efficiency, trueness, precision and LOD were derived from the validation work.

Evaluating fitness for purpose for labelling enforcement at the 1 % threshold

Calibration curves were produced based on serial dilutions of 100 % w/w horse genomic DNA (single horse specimen). Triplicate qPCR plates were undertaken comprising a 20,480 to 5 horse genome equivalents (assuming a haploid genome size of 2474.93 MB) seven point calibration curve. Test samples consisting of 100 %, and 1 % (A – D) w/w horse in beef ad-mixtures were evaluated as well as appropriate controls per target assay. All samples and controls were represented by a triplicate PCR technical replicate level.

Data analyses

Mammalian/poultry myostatin assay and equine GHR assay calibration curves were generated separately by plotting the $\log_{10}$ transformed estimated copy number (x-axis) versus mean C_q (y-axis) value for each of the seven standards used in the calibrant set, based on DNA extracted from 100% w/w raw horse meat. Independent simple linear regression curves were fitted to the mammalian/poultry myostatin and equine GHR assay calibrant data sets to determine the equation of the straight line ($y = mx + c$) and coefficient of determination (R^2). Minimum performance criteria were applied to each calibration curve ($R^2 > 0.98$ and 100 % +/- 10 % PCR efficiency), otherwise the experiment was repeated.

The percentage ratio of horse genome equivalents relative to the total mammalian genome equivalents present in each of the test samples was calculated. This was achieved by tabulating the mean C_q values for the mammalian/poultry myostatin and

equine GHR assays for each test sample, and using the previously derived equation of the straight line for both assays, to calculate the estimated genomic DNA copy numbers for the horse and mammalian targets. The calculated value of the horse DNA copy number divided by the total mammalian DNA copy number of the sample provided an estimate of the horse content of each sample consisting of raw meat, relative to the amount of raw horse meat in a raw beef (meat) background on a gravimetric (w/w) basis.

4) Conclusions

The results have provided evidence that a relative quantitation based approach can accurately measure the amount of horse DNA present in a sample relative to the amount of beef DNA. The method described in this paper quantifies the amount of horse DNA relative to total mammalian DNA in raw meat samples, using real-time PCR. The method has been applied to and is validated for DNA extracted from samples that consist of raw horse meat in a raw beef (meat) background only. The results can be expressed in relation to a gravimetric w/w meat basis but only in terms of the relative amount of raw horse meat in a raw beef (meat) background. The authors anticipate that the described methodology will be further subjected to an international Collaborative Trial in order to fully demonstrate fitness for purpose across multiple laboratories and benchmark core performance characteristics.

The development and validation of a real-time PCR approach for the quantitation of horse DNA, as described in this report, will allow food companies to make decisions on their supply chain based on accurate results, which will help to identify the source of the adulterant. Regulators will also be able to confidently enforce labelling laws in cases where this method identifies a non-compliant result. Having a fully quantitative method for the determination of horse DNA in beef based meat products will help regulators to enforce this UK/EU legislation and enable honest traders to robustly defend their food supply chain. Greater standardisation and guidance at an EU level with respect to the expression of the amount of meat adulteration in a sample will help provide a framework for more meaningful results and discussions to be had.

The provision of a real-time PCR approach for the relative quantitation of horse DNA, as described in this work, provides a solid foundation to help underpin and

accurately measure the amount of horse DNA present relative to total mammalian DNA in a test sample.

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Graphical Abstract

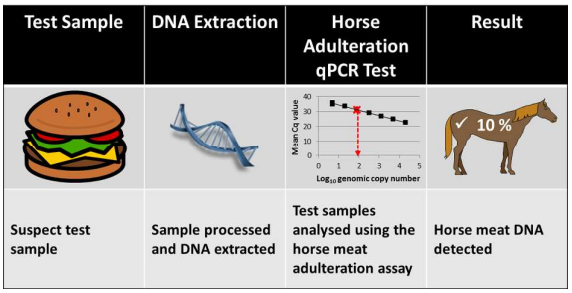

Figure illustrating the basic processing steps required to identify and quantify potential horse meat adulteration