Inter-laboratory collaborative trial of real-time PCR method for the relative quantitation of horse DNA and pork DNA in raw and processed beef DNA

Food Standards Agency Project Reference FS430818

Final Report for completion of Objective 5.4

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This report is the output of a Management System that is certified by LRQA to ISO 9001:2015 [1, 2].

Executive Summary

1. This project (FS430818) was initiated by the Food Standards Agency, UK, with support from the Department for Environment, Food and Rural Affairs, UK. The overall project describes the full international interlaboratory collaborative trial to define the performance characteristics of the real-time PCR method for horse and pork DNA in raw and processed beef matrix covering the range of concentrations 0.1-10% (w/w of raw meat).
2. The UK/EU Horse-meat issue of 2013, where a significant amount of horse DNA was found in a large number of beef meat products on sale at a supermarket store, prompted the development (Defra project FA0135) and interlaboratory validation (FSA project FS126001) of a real-time PCR approach for the quantitation of horse DNA in raw beef.
3. The real-time PCR approach was extended to develop three new methods for the quantitation of horse and pork DNA in raw and processed beef background (Defra project FA0171). A limited UK based ring-trial provided evidence of the fitness for purpose of the three new methods, applicable for DNA extracted from meat derived from horse and pork samples and demonstrated acceptable precision around the 1% (w/w) level for enforcement action. The methods can reliably distinguish between adventitious contamination at 0.1%, enforcement level at 1% and economically motivated adulteration at 10%.
4. This report is supplementary to the interim report against Objective 1.2 of the project which describes the validation of the method and associated verification of the test samples for the collaborative trial. Essential information is duplicated across both reports and this report, against Objective 5.4, remains the overall project report.
5. The interim report against Objective 1.2 of the project was initially reported in March 2022, to internally validate the previously-developed real-time PCR method for the quantification of horse and pork in beef (raw and processed). The interim report

additionally described the validation of the CTAB method for extraction of the DNA, quantification of extracts against standard curves, and the application of the method to verify the homogeneity of the test samples.

6. Test samples comprised DNA extracts from three types of analyte/matrix combinations: horse in processed beef, pork in processed beef, pork in raw beef. Each sample type was prepared at five nominal concentration levels: 0.1%, 0.5%, 1%, 3%, 10% (w/w of raw meats initially combined). The collaborative trial design was that of blind duplicates, comprising a total of 30 test samples.
7. Laboratories were recruited in the UK and internationally to take part in the collaborative trial. The 15 laboratories that were recruited were sent test samples, necessary consumables for undertaking the analysis and full instructions including SOP for the method in May 2022. All 15 laboratories had returned results by the time of the final July 2022 deadline, using a secure website facility.
8. Results were analysed according to established collaborative trial principles which initially involved the removal of non-compliant pairs (non-detects), Cochran's outlier removal and Grubbs' outlier removal. No more than three pairs of data needed to be removed per sample type.
9. The output of the data analysis was the measure of repeatability precision, reproducibility precision and critical differences. The pre-defined target precision was 25% relative standard deviation.
10. The ratio of observed precision to target precision was in the range 0.350 to 1.45 for repeatability and 0.371 to 1.43 for reproducibility. The collaborative trial has achieved acceptable precision for the method real-time PCR of DNA extracts for horse in processed beef, pork in processed beef and pork in raw beef.

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1. Introduction

Following the UK/EU Horse-meat issue of 2013, where a significant amount of horse DNA was found in a large number of beef meat products on sale at a supermarket store, a real-time PCR approach for the quantitation of horse DNA in raw beef was developed at LGC through Defra funding (project FA0135, Real-time PCR approach for quantitation of horse DNA and study into relevance of expression units (DNA/DNA and w/w tissue)) [3]. This method was validated through an international collaborative trial (project FS126001 - International collaborative trial of a real-time PCR method for the relative quantitation of horse DNA) by LGC and funded by the FSA [4]. The success of this validation study has enabled the method to be formally subjected to European Standardisation at CEN where it is currently undergoing standardisation through CEN Technical Committee CEN TC460 on Food Authenticity.

A requirement to expand the real-time PCR approach to include pork meat in both raw and processed beef has resulted in the development of three SOP methods for the quantitation of horse and pork DNA in raw and processed beef background. The study was funded by Defra under its Food Authenticity Programme (project FA0171, *Validation of Methods to Quantify Horse and Pork Meat Adulteration in Raw and Processed Beef*) [5]. The project looked at three new real-time-PCR methods for the relative quantitation of horse DNA in processed beef products, and the relative quantitation of pork DNA in raw and processed beef products. It included the development, the in-house validation and a limited UK based ring-trial (involving four UK laboratories). The study provided evidence of the fitness for purpose of the three new methods, as qualified by the limited (four laboratory) ring-trial. All three methods are applicable for DNA extracted from meat derived from horse and pork samples and demonstrate acceptable precision around the 1% (w/w) level for enforcement action. Furthermore, they can reliably distinguish between adventitious contamination at 0.1%, enforcement level at 1% and economically motivated adulteration at 10%.

Tenders were invited to plan a full-scale inter-laboratory collaborative trial on the methods that were developed under Defra project FA0171 [5] and additionally published [6] on the

quantitation of horse DNA in processed beef and pork DNA in raw and processed beef. This report is the culmination of that collaborative trial.

2. Materials and Methods

2.1 Raw material handling

Beef, pork and horse meats were purchased from local suppliers as whole joints. These were stored at -20 °C prior to preparation. Excess fat was trimmed from the joints before mincing (grinding) using an electric mincer. The beef was minced first to avoid any contamination from the pork and horse meat. For each meat species, the first ~200 g of minced meat was discarded so that any residue in the mincing equipment would be removed with that initial portion. The mincing equipment was cleaned before and after mincing each meat type using a combination of methods. After mechanical scrubbing of all parts of the mincer, the parts were soaked in a 10% bleach solution for 1 hour, sonicated in 10% bleach for 1 hour and then put through the dishwasher. Once out of the dishwasher all parts were sprayed with 1% Distel and wiped clean with paper towels. Swabs were taken from contact surfaces and analysed for the presence of each species DNA to ensure no cross-contamination occurred. The second ~200 g of minced meat for each species was retained as an environmental (negative) control for real-time PCR to ensure we had no contamination.

The meat mincing was located to a laboratory space that was several blocks removed from both the usual sample preparation facility at Fera and from the PCR laboratory. This was done to ensure the lowest possible risk of cross-contamination from the laboratory environment (where other meat sample handling is undertaken). An added benefit at the time of this project was to ensure sufficient physical working space when coronavirus social distance working mitigations were still in force at the Fera site.

Ingredients for the processed samples were tomato purée (Morrisons supermarket own-brand), cornflour (Sainsbury's supermarket own-brand) and gravy mix (Bisto gravy powder from ASDA supermarket). The gravy powder ingredients are listed as: potato starch, salt,

wheat starch, colour (ammonia caramel), onion powder, inactive yeast powder. The risk of an incurred cross-contamination with target meat DNA of these non-meat ingredients (including the gravy mix) was considered to be negligible. The same precautions were undertaken within the laboratory environment to minimise cross-contamination from the meat preparation.

2.2 DNA Extraction and Authenticity Testing

Prior to the verification of the sample types for the collaborative trial (homogeneity testing), the PCR method was validated in-house according to the principles of current standard methods [7, 8]. The full validation is the subject of a separate report and not detailed further here.

Following mincing, each meat species DNA was extracted from each species using the commercially available DNeasy® Mericon Food Kit (Qiagen) which is a cetyltrimethylammonium bromide (CTAB) method. Minced meat (2 g) was homogenised in 10 ml lysis buffer and proteinase K by mechanical disruption in a vortex. The homogenised mixture was then incubated at 60 °C, centrifuged and the cell lysis supernatant was phase-separated with chloroform. DNA was washed and precipitated in ethanol and eluted in 50 µl of elution buffer from the kit. The extraction method was also assessed in DNA fragmentation and a variety of modifications were trialled in the method. Modifications included differing lengths of incubation (30 or 60 minutes) and differing elution volumes (50 µl, 100 µl or 150 µl).

Because the input mass of each test portion of the meat was restricted for practical reasons (due to tube sizes) the eluates were individually measured on the spectrophotometer and the 260/280 nm and 260/230 nm ratios were noted. All individual extracts passed minimum performance criteria for quality and quantity and so were pooled after extractions to make one homogenous extract for each meat species.

Each meat species was also authenticated by Sanger sequencing using cytochrome B primers fwd: 5'-CCATCCAACATCTCAGCATGATGAA-3' and rev: 5'-CCCCTCAGAATGATATTTGTCCTCA-3'. Standard curves for calibration were made from

raw unprocessed 100% w/w horse and pork meat against the background myostatin mammalian gene target.

Meat combinations

The samples were prepared in the combinations described in Table 1, where the target percentage mixes of DNA relate to the w/w percentage of raw meats combined prior to extraction.

Sample: Horse	**Sample: Pork in raw beef**	**Sample Pork in processed beef**
Horse DNA in processed beef DNA (0.1%)	Pork DNA in raw beef DNA (0.1%)	Pork DNA in processed beef DNA (0.1%)
Horse DNA in processed beef DNA (0.5%)	Pork DNA in raw beef DNA (0.5%)	Pork DNA in processed beef DNA (0.5%)
Horse DNA in processed beef DNA (1%)	Pork DNA in raw beef DNA (1%)	Pork DNA in processed beef DNA (1%)
Horse DNA in processed beef DNA (3%)	Pork DNA in raw beef DNA (3%)	Pork DNA in processed beef DNA (3%)
Horse DNA in processed beef DNA (10%)	Pork DNA in raw beef DNA (10%)	Pork DNA in processed beef DNA (10%)

Table 1, combinations of target species DNA in matrix DNA with nominal w/w percentage of raw meats prior to extraction.

The final meat combinations of nominal targets 10% and 3% w/w were prepared first. Homogeneity on the raw meat combinations was assessed on the DNA extracted from these w/w combinations and was quantified slightly higher than the intended target (see results). The higher w/w combinations were then processed, and homogeneity was re-assessed. Lower final meat combinations of 1%, 0.5% and 0.1% w/w were made from the 3% w/w mixture. This was to ensure that no biases would be carried over from the 3%

combination to the lower % w/w combinations. The higher w/w combinations were then processed, and homogeneity was re-assessed.

Processed sample preparation

Processed samples were prepared according to the following recipe taken directly from the Defra report [5]:

65% meat, 27% water, 1.25% tomato puree, 3% cornflour and 3% gravy mix

The 65% meat was the total meat content of the processed sample, itself comprising the corresponding percentage combinations of beef and horse or pork, defined in Table 1 above. The meat was added to the other ingredients at ambient temperature, stirred and gradually heated in a lidded vessel on a hot plate to a temperature of 72 °C for 15 minutes. The mixture was stirred occasionally during heating and then left to cool at ambient temperature prior to subsampling.

The lower % combinations for processed matrices were made from 3% raw meat and then cooked to give processed % w/w combinations at 1, 0.5 and 0.1%, i.e. 3% processed meat was not used to make lower % combinations.

Consumables procurement

Protein swabs and Real-time PCR Universal Mastermix (reagents Taq polymerase, dNTPs, buffer) were purchased from Fisher Scientific. DNeasy® Mericon Food Kits were purchased from Qiagen. Skirted tubes (0.5 ml), tube caps, centrifuge tubes (50 ml and 1.5 ml) were purchased from Starlab. Labels for sample tubes were purchased from Barcode Warehouse (to be compatible with the LIMS). Myostatin horse and pig assays were purchased from Eurofins (assay details in Report Appendix 1, sub-Appendix 1, Table A1, page 22).

Sufficient PCR consumables were purchased for both the internal validation of the methods and for the collaborative trial itself (where this means all sample preparation and verification/homogeneity testing plus consumables for the participants). Consumables to be distributed to the participants in the interlaboratory trial were aliquoted out in preparation for eventual dispatch.

PCR equipment

All assays and standard curves were validated on QuantStudio 6 Flex Real-time PCR systems (Applied Biosystems).

2.3 Recruitment of participating laboratories

Laboratories were recruited for the collaborative trial by invitation. The criteria for selection of laboratories to invite were whether they were UK official control laboratories, commercial laboratories in the UK and overseas that had a known track record in meat authenticity analysis, and the four laboratories that had taken part in the previous interlaboratory validation [5, 6]. All communications were sent through the Fapas® general email account (info@fapas.com), rather than any personal email account, to reduce the potential for the invitation to be caught in any junk mail filter. The use of a central email account also ensured consistency of communications and avoidance of any unintended bias.

Positive responses to the invitation were received from five UK official control laboratories and ten commercial or official control testing laboratories from eight countries. The laboratories that took part in the collaborative trial are listed in alphabetic order in Table 2. The order in Table 2 does not correspond with the laboratory number in the results.

Table 2, laboratories that took part in the collaborative trial.

Laboratory name	Country
Campden BRI (Chipping Campden) Limited	UK
City of Edinburgh Council	UK
Eurofins Genomics Europe Applied Genomics GmbH	Germany
Fera Science Ltd (Fera)	UK
Glasgow Scientific Services	UK
Hampshire Scientific Service	UK

Laboratory name	Country
Laboratorio de Salud Publica - Madrid	Spain
Minton Treharne & Davies Ltd - Cardiff	UK
Premier Analytical Services	UK
Public Analyst's Laboratory - Cork	Ireland
Service de la consommation et des affaires veterinaires (SCAV)	Switzerland
SGS VIETNAM Ltd	Vietnam
Singapore Food Agency, National Centre for Food Science (Perahu Road)	Singapore
State General Laboratory	Cyprus
Tayside Scientific Services	UK

2.4 Collaborative trial design and logistics

The collaborative trial followed the randomised blind duplicate design [9], whereby each participating laboratory received 30 individual samples labelled PCR_01 to PCR_30 with no indication of what the sample number corresponded to. The 30 samples comprised 15 sample types with one replicate each. The sample numbers corresponded to the sample types as presented in Table 3.

Table 3, sample identifiers and the corresponding sample type and nominal concentration of raw meats (% w/w) prior to extraction.

Sample identifier	Sample type	Nominal level, %
PCR_01	Pork in raw beef	1
PCR_02	Horse in processed beef	1
PCR_03	Pork in processed beef	0.5
PCR_04	Pork in raw beef	0.5
PCR_05	Horse in processed beef	3

Sample identifier	Sample type	Nominal level, %
PCR_06	Horse in processed beef	1
PCR_07	Pork in processed beef	1
PCR_08	Horse in processed beef	3
PCR_09	Horse in processed beef	0.5
PCR_10	Pork in raw beef	3
PCR_11	Pork in processed beef	0.5
PCR_12	Horse in processed beef	0.5
PCR_13	Pork in processed beef	0.1
PCR_14	Pork in processed beef	0.1
PCR_15	Pork in raw beef	10
PCR_16	Pork in raw beef	3
PCR_17	Horse in processed beef	0.1
PCR_18	Horse in processed beef	0.1
PCR_19	Horse in processed beef	10
PCR_20	Pork in processed beef	10
PCR_21	Pork in processed beef	1
PCR_22	Pork in processed beef	3
PCR_23	Pork in raw beef	0.1
PCR_24	Pork in processed beef	10
PCR_25	Pork in processed beef	3
PCR_26	Horse in processed beef	10
PCR_27	Pork in raw beef	1
PCR_28	Pork in raw beef	0.1
PCR_29	Pork in raw beef	10
PCR_30	Pork in raw beef	0.5

In order to expedite results returns from the participating laboratories in the trial, the Fapas® database and secure website was set up to manage registrations, sample shipment and results entry. Use of the database negated any possibility of transcription errors from results returned by spreadsheet (for example). The Fapas® database is also

designed to handle sample shipments, including addressing and linking directly to couriers, which has clear logistical advantages. The internal project reference for the database was set up as round MA0201, with individual sample references of PCR_01 to PCR_30 for the 30 samples being sent, as per Table 3.

All samples, together with essential consumables, were dispatched on 9 May 2022 to all 15 laboratories, with a results return deadline of 1 July 2022. The samples and consumables were shipped in insulated boxes with dry ice. Detailed instructions on sample handling and experimental protocol to be used were provided with the samples, including the primer sequences and a suggested layout of the PCR plates to encompass the number of determinations required. These instructions are reproduced in Appendix 1. Raw data collected on spreadsheets were to be retained by the laboratories until requested.

3 Results

3.1 Results returns and exceptions

The deadline for results returns was extended to 15 July 2022, to allow some laboratories to submit results. One laboratory was sent a reminder two days before the deadline. All 15 laboratories returned results by the extended deadline.

One laboratory (006) reported difficulty with some of the analyses and requested additional reagents so that they could complete the work. A new set of reagents (but not test samples) was dispatched to this laboratory.

One laboratory (011) reported a delayed delivery of the original samples and that the samples eventually arrived at ambient temperature, having lost all the dry ice. Since it was anticipated that a re-send of samples would encounter a similar transit time, the laboratory was advised to continue with the analysis of the original samples.

Additional comments were received at the time of results submission from two laboratories. These were:

Lab 006; The 30 DNA samples and reagents/consumables were delivered with 2 ice packs on 20.06.2022. However, all items were at room temperature upon arrival.

Lab 014; Work carried out using MA0201 practical instructions. Pork Plate 3 was repeated using newly opened Mastermix as the first run failed on PCR Efficiency.

No additional modification to the data analysis was necessary as a result of these comments.

3.2 Results and Data Analysis

Results were downloaded from the database in csv format and imported into MS Excel. Data were filtered by sample type-replicate combinations against the known sample identifier code in Table 3. A template software tool (MS Excel) was used to analyse the data, one spreadsheet per each of the 15 sample types. The data analysis follows that of the IUPAC Protocol [9]. The following steps were used in sequence to identify non-valid data and their subsequent exclusion from the calculations:

- non-detects (both replicates excluded if only one replicate was a non-detect)
- Cochran's outlier (where the variance of the replicates is exceptionally large)
- Grubb's outlier (where both replicates are statistical outliers)
- secondary Cochran's outlier (if further identified following initial outlier removal)

The number n of valid paired data points for the statistical output was never less than 12. A majority of the data sets (9 of 15) had n = 14, one dataset had n = 15, and five data sets had n = 12.

Initial observations of the data showed two unusual occurrences. Lab 010 had submitted results that were almost exactly correspondent to the intended spike levels of the samples. Lab 012 results were mostly outliers (in fact, only two of the 15 sample types were not outliers). Both laboratories were contacted about their results with no indication of the nature of the problem, so as not to affect any bias. Lab 010 confirmed that their results were only available to a single significant figure, hence there was no reason to exclude their results from the data analysis. Lab 012 confirmed that they had followed the

instructions exactly. Since their results were mostly already excluded as statistical outliers, no further action was necessary.

All raw data are provided in Appendix 2. The associated Youden plots are presented in Appendix 3, graphically showing the outlier data. The associated mean and range plots are presented in Appendix 4.

Appendix 2 additionally provides the analysis performance data per laboratory for each plate assay (calibration coefficient of determination R^2 and PCR efficiency).

The principal outputs of the data analysis are the repeatability and reproducibility precisions, and the critical differences. In an analytical chemistry collaborative trial in the food sector, the acceptability of the observed precisions would ordinarily be measured against the precision predicted by the Horwitz equation. Thus, a Horwitz ratio (HorRat) of observed standard deviation to Horwitz-predicted standard deviation between 0.5 and 2 would be considered acceptable. In the case of this study, the use of the Horwitz equation is not appropriate, since the method in question is an amplification technique and the sample types are extracted DNA (so the whole method is not the subject of the trial). In the place of the HorRat, this study substitutes the 'target ratio' with the same indicator limits of 0.5 to 2, but the Horwitz-derived standard deviation is replaced with the relative standard deviation (RSD) of 25% (EURL-GMFF), which was verified as appropriate from the original method validation study [5, 6]. The critical difference value is the difference in result at 95% confidence at which a sample may be out of specification at a legal limit.

The summary data are presented in Table 4.

Table 4, principal outputs of the collaborative trial statistics. The intended level is % w/w of raw meats combined prior to extraction. n is the number of paired data points following outlier or non-compliant data removal. RSDr is relative standard deviation of repeatability r. RSDR is relative standard deviation of reproducibility R. CD95 is the critical difference at 95% confidence. Target ratio is observed RSD to target of 25%.

Horse in processed beef

Data type	Result	Result	Result	Result	Result
Intended level, %w/w	0.1	0.5	1	3	10
Mean, %w/w	0.175	0.823	1.73	3.30	10.5
n	15	12	14	12	14
RSDr, %	19.9	10.4	6.90	5.77	10.1
Target ratio r	1.21	0.630	0.418	0.350	0.611
RSDR, %	34.9	21.4	21.1	11.0	12.5
Target ratio R	1.39	0.857	0.844	0.441	0.501
CD95	0.11	0.33	0.70	0.67	2.14

Pork in processed beef

Data type	Result	Result	Result	Result	Result
Intended level, %w/w	0.1	0.5	1	3	10
Mean, %w/w	0.133	0.617	1.38	2.44	7.35
n	14	14	14	12	14
RSDr, %	16.2	11.9	16.3	6.96	10.8
Target ratio r	0.983	0.720	0.988	0.422	0.654
RSDR, %	32.6	19.9	28.4	17.5	17.3
Target ratio R	1.31	0.798	1.13	0.700	0.691
CD95	0.08	0.22	0.71	0.81	2.26

Pork in raw beef

Data type	Result	Result	Result	Result	Result
Intended level, %w/w	0.1	0.5	1	3	10
Mean, %w/w	0.105	0.619	1.26	3.21	10.2
n	14	14	14	12	12
RSDr, %	24.0	21.0	19.2	8.60	8.76
Target ratio r	1.45	1.28	1.16	0.521	0.531

RSDR, %	35.8	25.4	22.7	17.4	9.27
Target ratio R	1.43	1.01	0.908	0.696	0.371
CD95	0.07	0.25	0.45	1.04	1.39

The repeatability precision r target ratio varies from 0.350 to 1.45 and is less than 0.5 in only three instances (horse in processed beef at nominal 1% and 3% levels, and pork in processed beef at nominal 3% level). Clearly, no target ratio r exceeds the upper limit of 2. The repeatability precision is therefore generally compliant [9] with a target RSD_r of 25% and, in 11 of 15 sample types, the target ratio r is less than 1.0, indicating a high degree of repeatability precision. Furthermore, the observed RSD_r is never more than 24.0% at the lowest level of 0.1% w/w, which is in keeping with the minimum performance requirement of 25% as laid out by ENGL [10].

The reproducibility precision R target ratio varies from 0.371 to 1.43 and is less than 0.5 in only two instances (horse in processed beef at nominal 3% level, and pork in raw beef at nominal 10% level). No target ratio R exceeds the upper limit of 2. The reproducibility precision is therefore generally compliant [9] with a target RSD_R of 25% and, in 10 of 15 sample types, the target ratio R is less than 1.0, indicating a high degree of reproducibility precision. The observed RSD_R ranges between 32.6% and 35.8% at the 0.1%w/w level and is never more than 28.4% at the higher preparation levels. This is in keeping with the minimum performance requirement of 35% for reproducibility as laid out by ENGL [10] over the whole dynamic range and is less than the suggested 50% acceptable RSD_R at levels less than 0.2% w/w.

The 0.1%, 0.5% and 1% levels were prepared from the verified 3% preparations. Section 2.2 describes the mitigation actions taken to ensure correct preparation of the 3% levels prior to preparation of the lower levels and the additional verification steps taken. The data in Table 4 show the upper maximum bias of the 3% w/w levels to be 10% relative (horse in processed beef and pork in raw beef). This would not be sufficient to account for the potential bias observed in the lower prepared levels, i.e. the method bias is not due to propagation of inaccurate 3% w/w preparation. The bias observed in pork in processed beef at the 3% w/w level is -0.56% w/w, whereas the mean observed levels for the lower

preparations are apparently positively biased. The observed method bias for pork in processed beef at the lower levels is not attributable to propagation of inaccurate 3% w/w preparation.

As part of the serial preparation process, the 3% mixture underwent three times more mincing than the 10% mixture; the lower percentage mixture also underwent three times further mincing to ensure homogeneous mixtures. For the processed matrices, the pork was cooked first; initial trial and error of the cooking process for the 10% and 3% mixtures may have caused more DNA degradation than the subsequent processed samples (although the beef DNA would have similarly been affected, so the ratio should be unaltered to this degree). One further possibility is that the beef overall underwent more mincing than the contaminant meats, so the apparent positive bias might in fact be proportionally greater beef DNA degradation. There is also the potential for differences in extractability of DNA from different species which could be the subject of a different study in which beef is the contaminant in pork (for example).

Further analysis was undertaken (data not shown) of the trend between the mean results plotted against the intended target levels, for both the collaborative trial data and the homogeneity data (linear unweighted determination of slope and intercept). Across the full collaborative trial calibration range, the intercept is about 0.3% w/w for processed matrices and 0.1% w/w for the raw pork matrix. The intercept is negligible for the lower concentration range 0.1-1% w/w (but with a more positive slope), therefore the observed break point is between the 1% and 3% levels. The homogeneity data trend is similar to the processed horse and processed pork at the lower part of the range but more similar to the full range for raw pork in raw beef. The data don't quite support the premise that the sample production method derived from the 3% target level material may cause an over-estimation of contamination due to the extended processing, since the change in slope should be observed between the 3% and 10% levels, not the 1% and 3% levels. However, the slope of the pork in processed beef being <1 supports the possibility of over-processing of this initial material at the higher levels. This is particularly noticeable in the homogeneity data for this matrix where there is a clear distinction between the top two levels and lower three levels. In real-world samples, the propagation effects observed

here would not be expected to occur, hence the apparent positive bias may not be attributable to the PCR method nor to the ability of the laboratories in the trial. However, this is difficult to demonstrate at low levels unless the method of production of the test materials is radically altered which, in turn, may compromise their homogeneity.

The critical difference value (CD95) is the difference in result at 95% confidence at which a sample may be out of specification at a legal limit, i.e. whether the measurement uncertainty of a result may affect the interpretation of that result. At levels between 0.1% and 1%, the CD95 values are in keeping with these prepared levels for potential contamination detection. At the higher levels of 3% and 10%, the CD95 values are lower, as might be expected of adulteration levels. The principal discrepancy observed in the data is at the 1% target level in processed matrices, in which the CD95 values are 0.70 and 0.71 (horse and pork, respectively). For assumed regulatory levels between 0.5% and 1% there would be expected to be some potential for overlap of analytical results due to measurement uncertainty. However, since the CD95 values in the same processed matrices at the 3% levels are not too dissimilar (0.67 and 0.81, horse and pork, respectively) there remains the possibility that CD95 values are anomalies worth further investigation on a larger scale. Ultimately, further interpretation of the CD95 values would depend on decisions of regulatory limits in use.

The performance data of coefficient of determination R^2 and PCR efficiency are mostly within the acceptance criteria of the method SOP, i.e. $R^2 \geq 0.98$ and efficiency within the range 85 – 115%. One assay for each of two laboratories (9 and 14) had $R^2 < 0.98$ and laboratory 15 had three assays with $R^2 < 0.98$ (one pork and two horse). Laboratory 15 had a higher rate of outlier data than the other laboratories (with the exception of laboratory 12). Since the outliers were detected, any compromised calibration from laboratory 15 has not significantly affected the outcome of the study. The PCR efficiency for laboratory 2 was slightly <85% for two assays and laboratory 12 had low PCR efficiency for all six assays, five of which were <85%. The calibration performance for laboratory 12 was very good so the low PCR efficiency doesn't correlate with the outlier data from this laboratory, which are very high, often by more than a factor of two.

4. Conclusions

The analytical method for horse and pork DNA quantification using real-time PCR was internally validated and the CTAB method was found to be suitable to obtain DNA of sufficient quality and quantity. Using eight-fold standard curves covering the required dynamic range, all % w/w combinations of horse in processed beef and pork in raw and processed beef mixtures were quantified using the horse and pork standard curves respectively.

Processing of the meat and meat combinations in the laboratory was done so that there was minimal DNA degradation by dehydration of the meat. For the purpose of the collaborative trial, there was reliable detection of 0.1% w/w contamination by horse or pork meat in a beef background in laboratory processed samples.

Using the validated method, all the samples prepared for the full collaborative trial confirmed their fitness for purpose in terms of their homogeneity and expected concentration. The samples were then dispatched to participants in the collaborative trial, with results returned by all participants.

Repeatability and reproducibility precision was determined for all sample types at all preparation levels, including the lowest 0.1% w/w contamination level, and for all the processed samples. The acceptability of the precision was determined via the target ratio of observed precision to target precision of 25% relative. The target ratios never exceeded 1.45, i.e. there was no unacceptably large variance. The target ratios reduced at higher preparation levels (towards adulteration levels) so the method would always be suitable to detect economic adulteration of meats, either raw or processed.

5. References

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[4] FSA Report FS126001 (2016). International collaborative trial of a real-time PCR method for the relative quantitation of horse DNA.

[5] Defra report FA0171 (2018). Validation of Methods to Quantify Horse and Pork Meat Adulteration in Raw and Processed Beef

[6] Burns M., Nixon G., Cowen S., Wilkes T. International collaborative trial of a real-time PCR approach for the relative quantitation of horse DNA. Food and Nutrition Open Access, 2018, Dec;1(3):113. DOI: 10.31021/fnoa.20181113

[7] CEN/TS 17329-1. Foodstuffs – General guidelines for the validation of qualitative real-time PCR methods – Part 1: Single-laboratory validation. (Final draft at January 2021.)

[8] ISO/DIS 20813:2019. Molecular biomarker analysis – Methods of analysis for the detection and identification of animal species in foods and food products (nucleic acid-based methods) – General requirements and definitions

[9] IUPAC/AOAC Protocol for the design, conduct and interpretation of collaborative studies (Horwitz, 1993)

[10] European Network of GMO Laboratories (ENGL), Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing, 2015, European Commission, Joint Research Centre

Appendix 1; instructions to participants and SOP

(Reproduced exactly as provided originally to the participants, including the sub-Appendix 1. References in this entire Appendix are not numbered.)

Collaborative trial MA0201 practical instructions

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May 2022

1. Introduction

The UK/ EU Horse-meat scandal of 2013 highlighted the lack of traceability in the food supply chain and increased the risk of potentially harmful ingredients within the contaminating meats. As the first step to enforcing tighter measures on the sale of beef products within the UK, Defra commissioned through LGC, the development of a real-time PCR method for the detection and accurate quantification of horse DNA contamination in raw beef (FA0135). This method was successfully validated through an international collaborative trial (Burns M., et al. 2018) and is undergoing standardisation through the CEN Technical Committee on Food Authenticity (CEN TC460).

A requirement for the testing of pork meat in raw and processed beef and horse and pork DNA in raw and processed beef lead to the development of a second real-time PCR method funded by Defra (FA0171). These methods are now required to be validated by a full-scale inter-laboratory collaborative trial.

2. Scope

The method described in this SOP is to provide practical instructions on the set-up of the real-time PCR method and quantification of (i) horse DNA in raw beef (ii) horse DNA in processed beef (iii) pork DNA in raw beef and (iv) pork DNA in processed beef.

The purpose of this trial is for the quantification of DNA originating from contaminant meat (horse and/or pork) in raw and processed beef background, relative to mammalian DNA. We have provided 30 blind test samples that consist of a variety of percent contamination DNA in mammalian DNA (% w/w). The samples are labelled PCR_01 to PCR_30 and will give no indication on their respective percent contamination.

The method consists of building a set of calibration curves for the horse specific target and the mammalian (background) specific target using 100% horse DNA, as well as the pork specific target and the mammalian (background) specific target using 100% pork DNA. These calibration curves are based on estimates of the genome equivalent copy number for each horse and pork and will then be used to accurately quantify the 30 blind test samples. The final result is the quantification of the contaminant DNA relative to the mammalian DNA and is expressed as a ratio of the number of genome equivalents for the contaminant relative to the total number of mammalian genome equivalents in the sample.

3. Safety

Laboratory safety procedures must be followed at all times. Only trained and competent staff must undertake this procedure. Ensure all appropriate PPE is worn and the relevant laboratory COSHH and risk assessments are consulted prior to undertaking this method.

4. Method performance characteristics

4.1. DNA supplied

The standards of 100% horse and 100% pork DNA were extracted from authenticated horse and pork meat respectively, using the same methodology.

The test samples of contaminant (horse or pork) meat in raw and processed beef background were first mixed by weight to achieve a variety of percent contamination (% w/w). The test samples were then extracted using the same methodology as the standards.

The extracted DNA quality and quantity were assessed by a spectrophotometer (Nanodrop 1000) and all DNA passed quality control. A260/A230 ratio ≈ 1.8 and A260/A280 ratio ≈ 1.8 and 2.0.

4.2. Calibration Curves

This procedure was internally validated at Fera Science Ltd. following the methodology as developed by LGC. The validation was carried out on QuantStudio 6 Flex Real-time PCR system (Applied Biosystems).

Calibration curves for horse and pork are made from 100% horse DNA or pork DNA respectively. The horse and pork calibration curves cover a dynamic range of 40 ng/ µl to 9.8 pg/ µl. The estimated genome equivalent at 40 ng/ µl for both contaminant meats is 31,508 copies based on 1 haploid copy of the horse genome being 2474.9 Mb (Equus caballus NCBI genomes 2018) and the pig genome being 2458.2 Mb (Sus scrofa NCBI genomes 2018).

Within the dynamic range of the calibration curves the slope is between -3.6 and -3.1 and the coefficient of determination (R^2) is ≥0.98.

4.3. Limit of detection

The limit of detection (LOD) is defined as the lowest target concentration which is detectable 95% of the time. The LOD for the horse and pork genome was ≤9 pg/ µl (estimated genome equivalent of 7 copies), based on processed horse in beef and pork in beef samples.

4.4. Limit of quantification

The limit of quantification (LOQ) is defined as the lowest relative amount of contaminant meat (horse or pork) which is detectable 95% of the time. The LOQ for the horse and pork genome was ≤0.1% w/w of processed horse in processed beef and processed pork in processed beef.

5. Materials supplied

On receipt of the Collaborative trial MA0201 kit please ensure the integrity of the packaging as well as all the contents. Any tubes which are damaged, or where samples have leaked must be reported so a replacement sample can be dispatched. Please report any issues with the kit contents to info@fapas.com, include the exact name on the label of the tube as well as your laboratory identifying information.

5.1. DNA samples

30 samples are provided as DNA extracts prepared from contaminant meats in raw and processed beef in varying proportions.

Sample	Volume	Consumable
PCR_01 to PCR_30	150 µl	X1 Skirted tube (0.5 ml)

5.2. Reagents/Consumables

Reagent	Volume	Consumable
Horse forward and reverse primers	150 µl	X2 Skirted tube (0.5 ml)
Pork forward and reverse primers	150 µl	X2 Skirted tube (0.5 ml)
Myostatin forward and reverse primers	300 µl	X2 Skirted tube (0.5 ml)
Horse Probe	350 µl	X1 Skirted tube (0.5ml)
Pork Probe	350 µl	X1 Skirted tube (0.5ml)
Myostatin Probe	650 µl	X1 Skirted tube (1.5ml)
Master mix (Taq polymerase, dNTPs, buffer)	9 ml	X2 Skirted tube (10 ml)
DNA Standards (DNA in buffer)	350 µl	X2 Skirted tube (0.5 ml)

Primer and probes sequences can be found in Appendix 1.

6. Materials required

6.1. Equipment

Standard laboratory personal protective equipment (PPE)

Disinfectant

Vortex mixer

Benchtop centrifuge for centrifuging tubes

Benchtop centrifuge for centrifuging PCR plates

Calibrated micropipettes ranging from 1 µl to 1000 µl

Real-time PCR machine

6.2. Consumables

Optical 96 well clear PCR reaction plates

Plastic microtubes

Micropipette tips to fit the required micropipettes

7. Procedure

7.1. Laboratory organisation

Fera is an ISO17025 accredited laboratory and in accordance with our Quality policy we adhere to a unidirectional workflow plan that minimises cross contamination. The laboratory is organised into discrete spatially separated areas that contains dedicated equipment and PPE. All surfaces and equipment are decontaminated with appropriate disinfection agents and there are established waste disposal procedures. All equipment is regularly calibrated and serviced.

7.2. Experimental conditions

1. A suggested 96 well plate plan that includes as singleplex the horse or pork assay and the mammalian assay can be found in Appendix 2. The plan also incorporates samples in triplicate, Standards (horse or pork DNA) and non-template controls (NTC).

2. In order to quantify all test samples, they must be run for both the horse specific assay alongside the mammalian assay and the pork specific assay alongside the mammalian assay. I.e. you must run 3 plates for each assay.
3. In a clean lab briefly vortex all PCR reagent tubes in the kit and pulse spin to collect contents at the bottom of the tube.
4. Make up a working master mix solution for each assay as per **Table 1**.

Table 1: Real-time PCR reaction composition. Reaction composition for each assay (horse, pork and myostatin) for a total reaction volume of 25 µl. Volumes are provided for 1X and for 156X which corresponds to all PCR reactions required per assay. The template DNA volumes for the standards and for the test samples is 5 µl. MGW: Molecular grade water.

PCR reagent	**1X per assay**	**156X per assay**
2X Universal Master Mix	12.5	1950
Forward primer (20 µM)	0.375	58.5
Reverse primer (20 µM)	0.375	58.5
Probe (5 µM)	1	156
MGW	5.75	897
Template DNA (Standards and test samples)	5	5

5. Transfer 20 µl of the PCR reaction master mix solution into the appropriate wells according to the plate plan.
6. In the sample preparation laboratory prepare a dilution series (S1 to S5) for the horse and pork calibration curves.

6.1. The horse DNA standard is in the tube labelled Horse standard 1. This standard is 100% horse DNA at 40 ng/ µl and 350 µl volume. To prepare the 5 point and 8-fold calibration curve for horse refer to **Table 2**.

6.2. The pork DNA standard is in the tube labelled Pork standard 1. This standard is 100% pork DNA at 40 ng/ µl and 350 µl volume. To prepare the 5 point and 8-fold calibration curve for pork refer to **Table 2**.

Table 2: Dilution series for the horse and pork 5 point 8-fold calibration curve. This serial dilution is based on an initial 100% horse or pork DNA stock at a concentration of 40 ng/ µl at 350 µl. This dilution series will provide sufficient final volumes for each standard level for all required real-time PCR reactions. MGW: Molecular grade water.

Standard level	DNA (ng/ µl)	Genome equivalent (copies/ µl)	Volume stock (µl)	Volume MGW (µl)
S1	40	31508	350	0
S2	5	3938.5	15 (std 1)	105
S3	0.625	492.3	15 (std 2)	105
S4	0.078	61.5	15 (std 3)	105
S5	0.009	7.69	15 (std 4)	105

7. Briefly vortex all standards and test samples and pulse spin to collect contents at the bottom of the tube.
8. Place all the standards and test samples (PCR_01 to PCR_30) in a rack according to the plate plan. **Note: do not dilute test samples, they have already been diluted to 10 ng/ µl.**
9. Spike the test samples and the calibrant samples into the appropriate wells according to the plate plan. Seal the plate thoroughly with optical adhesive film, vortex the

sealed plate for 30 seconds and centrifuge the plate at 1000 g for 1 minute to collect contents to the bottom of the wells.

10. Place the plate into the real-time PCR machine.
11. Designate the wells with the FAM-NFQ detector per assay.
12. Ensure the correct cycling parameters are chosen (see **Table 3** for cycling parameter).

Table 3: PCR thermal cycling conditions.

Temperature (ºC)	Time (s)	Repeat cycles
50	120	1
95	600	1
95	15	45
60	60	45

7.3. Data analysis

13. View the graph in log mode and check that the threshold settings are adequate (sigmoidal curve and plateau level). If the data requires manual manipulation refer to the instrument manufacturer's recommendations.
14. If any of the water blank replicates give a positive result the plate should be assessed for probable contamination and a decision made on whether to reject and rerun.
15. Export the results into the Microsoft Excel **MA0201 Collaborative_Trial_Results 2022 spreadsheet** provided, into the appropriate tables for raw data (e.g. raw data from plate 1 should be in tab Raw_data_plate 1).
16. Repeat step 15 for the second contaminant assay relative to the mammalian assay. I.e. you must input the raw data from the horse ***and*** pork assays into the results spreadsheet.
17. The percentage of contaminant DNA in mammalian DNA is expressed as a ratio of the number of genome equivalents of the contaminant relative to the total mammalian genome equivalents present in the sample. The results spreadsheet will automatically generate a calibration curve to the contaminant assay and the mammalian (myostatin)

assay to the equation for a straight line ($y = mx + c$) and the coefficient of determination (R^2).

18. For each assay check the R^2 is above 0.98 and PCR efficiency is between 85 and 115%.
19. The spreadsheet will then automatically calculate the relative quantitative value of contaminant DNA compared to total mammalian DNA for each test sample using the following equation.

$$\%\ contaminant\ DNA = \frac{contaminant\ genome\ equivalent\ copy\ number}{mammalian\ genome\ equivalent\ copy\ number} \times 100$$

20. All % contaminant DNA values will be automatically copied in the Collated data tab.
21. Please report these results **for each assay** through the Fapas secure portal using your personal account as provided by following instructions available at fapas.com/technical documentation.

8. References

Defra project FA0135 (2013) “Real-time PCR approach for quantitation of horse DNA and study into relevance of expression units (DNA/DNA and w/w tissue).”

Defra project FA0171 (2018) “Validation of Methods to Quantify Horse and Pork Meat Adulteration in Raw and Processed Beef”.

Burns, M., Nixon, G., Cowen, S., Wilkes, T. International collaborative trial of a real-time PCR approach for the relative quantitation of horse DNA. Food and Nutrition Open Access, 2018, DOI: 10.31021/fnoa.20181113.

Collaborative trial MA0201 practical instructions

Appendix 1

Table A4:Sequences of the primers and probe for each of the horse, pork and mammalian (myostatin) assays used in the collaborative trial.

Target	Assay ref	Sequence (5' – 3')	Labelling
Horse (Köppel, R. et al. 2011)	Forward	CCAACTTCATCATGGACAACGC	-
Horse (Köppel, R. et al. 2011)	Reverse	GTTAAAGCTTGGCTCGACACG	-
Horse (Köppel, R. et al. 2011)	Probe	AAGTGCATCCCCGTGGCCCCTCA	6 FAM-NFQ
Pork (Köppel, R. et al. 2011)	Forward	GGAGTGTGTATCCCGTAGGTG	-
Pork (Köppel, R. et al. 2011)	Reverse	CTGGGGACATGCAGAGAGTG	-
Pork (Köppel, R. et al. 2011)	Probe	TCTGACGTGACTCCCCGACCTGG	6 FAM-NFQ
Mammalian Myostatin (Laube, I. et al. 2013)	Forward	TCTGACGTGACTCCCCGACCTGG	-
Mammalian Myostatin (Laube, I. et al. 2013)	Reverse	ATACCAGTGCCTGGGTTCAT	-
Mammalian Myostatin (Laube, I. et al. 2013)	Probe	CCCATGAAAGACGGTACAAGGTATACTG	6 FAM-NFQ

Köppel, R., Ruf, J., Rentsch, J. Multiplex real-time PCR for the detection and quantification of DNA from beef, pork, horse and sheep. Eur Food Res Technol. 2011;232(1):151-5. 10.1007/s00217-010-1371-y

Laube, I., Spiegelberg, A., Butschke, A., Zagon, J., Schauzu, M., Kroh, L., et al. Methods for the detection of beef and pork in foods using real-time polymerase chain reaction. International Journal Of Food Science & Technology. 2003;38(2):111-8. 10.1046/j.1365-2621.2003.00651.x

Collaborative trial MA0201 practical instructions Appendix 2

Suggested plate plans for the horse assay and the mammalian myostatin assay

Plate 1 - Horse

Letter	1	2	3	4	5	6	7	8	9	10	11	12	Note:
A	Std 1/H	Std 1/H	Std 1/H	Std 2/H	Std 2/H	Std 2/H	Std 3/H	Std 3/H	Std 3/H	Std 4/H	Std 4/H	Std 4/H	Horse assay
B	Std 5/H	Std 5/H	Std 5/H	NTC	NTC	NTC	1	1	1	2	2	2	-
C	3	3	3	4	4	4	5	5	5	6	6	6	-
D	7	7	7	8	8	8	9	9	9	10	10	10	-
E	Std 1/H	Std 1/H	Std 1/H	Std 2/H	Std 2/H	Std 2/H	Std 3/H	Std 3/H	Std 3/H	Std 4/H	Std 4/H	Std 4/H	Myostatin
F	Std 5/H	Std 5/H	Std 5/H	NTC	NTC	NTC	1	1	1	2	2	2	-
G	3	3	3	4	4	4	5	5	5	6	6	6	-
H	7	7	7	8	8	8	9	9	9	10	10	10	-

Plate 2 - Horse

Letter	1	2	3	4	5	6	7	8	9	10	11	12	Note:
A	Std 1/H	Std 1/H	Std 1/H	Std 2/H	Std 2/H	Std 2/H	Std 3/H	Std 3/H	Std 3/H	Std 4/H	Std 4/H	Std 4/H	Horse assay
B	Std 5/H	Std 5/H	Std 5/H	NTC	NTC	NTC	11	11	11	12	12	12	-
C	13	13	13	14	14	14	15	15	15	16	16	16	-
D	17	17	17	18	18	18	19	19	19	20	20	20	-
E	Std 1/H	Std 1/H	Std 1/H	Std 2/H	Std 2/H	Std 2/H	Std 3/H	Std 3/H	Std 3/H	Std 4/H	Std 4/H	Std 4/H	Myostatin
F	Std 5/H	Std 5/H	Std 5/H	NTC	NTC	NTC	11	11	11	12	12	12	-
G	13	13	13	14	14	14	15	15	15	16	16	16	-
H	17	17	17	18	18	18	19	19	19	20	20	20	-

Plate 3 - Horse

Letter	1	2	3	4	5	6	7	8	9	10	11	12	Note:
A	Std 1/H	Std 1/H	Std 1/H	Std 2/H	Std 2/H	Std 2/H	Std 3/H	Std 3/H	Std 3/H	Std 4/H	Std 4/H	Std 4/H	Horse assay
B	Std 5/H	Std 5/H	Std 5/H	NTC	NTC	NTC	21	21	21	22	22	22	-
C	23	23	23	24	24	24	25	25	25	26	26	26	-
D	27	27	27	28	28	28	29	29	29	30	30	30	-
E	Std 1/H	Std 1/H	Std 1/H	Std 2/H	Std 2/H	Std 2/H	Std 3/H	Std 3/H	Std 3/H	Std 4/H	Std 4/H	Std 4/H	Myostatin
F	Std 5/H	Std 5/H	Std 5/H	NTC	NTC	NTC	21	21	21	22	22	22	-
G	23	23	23	24	24	24	25	25	25	26	26	26	-
H	27	27	27	28	28	28	29	29	29	30	30	30	-

Suggested plate plans for the pork assay and the mammalian myostatin assay

Plate 1 - Pork

Letter	1	2	3	4	5	6	7	8	9	10	11	12	Note:
A	Std 1/P	Std 1/P	Std 1/P	Std 2/P	Std 2/P	Std 2/P	Std 3/P	Std 3/P	Std 3/P	Std 4/P	Std 4/P	Std 4/P	Pork assay
B	Std 5/P	Std 5/P	Std 5/P	NTC	NTC	NTC	1	1	1	2	2	2	-
C	3	3	3	4	4	4	5	5	5	6	6	6	-
D	7	7	7	8	8	8	9	9	9	10	10	10	-
E	Std 1/P	Std 1/P	Std 1/P	Std 2/P	Std 2/P	Std 2/P	Std 3/P	Std 3/P	Std 3/P	Std 4/P	Std 4/P	Std 4/P	Myostatin
F	Std 5/P	Std 5/P	Std 5/P	NTC	NTC	NTC	1	1	1	2	2	2	-
G	3	3	3	4	4	4	5	5	5	6	6	6	-
H	7	7	7	8	8	8	9	9	9	10	10	10	-

Plate 2 - Pork

Letter	1	2	3	4	5	6	7	8	9	10	11	12	Note:
A	Std 1/P	Std 1/P	Std 1/P	Std 2/P	Std 2/P	Std 2/P	Std 3/P	Std 3/P	Std 3/P	Std 4/P	Std 4/P	Std 4/P	Pork assay
B	Std 5/P	Std 5/P	Std 5/P	NTC	NTC	NTC	11	11	11	12	12	12	-
C	13	13	13	14	14	14	15	15	15	16	16	16	-
D	17	17	17	18	18	18	19	19	19	20	20	20	-
E	Std 1/P	Std 1/P	Std 1/P	Std 2/P	Std 2/P	Std 2/P	Std 3/P	Std 3/P	Std 3/P	Std 4/P	Std 4/P	Std 4/P	Myostatin
F	Std 5/P	Std 5/P	Std 5/P	NTC	NTC	NTC	11	11	11	12	12	12	-
G	13	13	13	14	14	14	15	15	15	16	16	16	-
H	17	17	17	18	18	18	19	19	19	20	20	20	-

Plate 3 - Pork

Letter	1	2	3	4	5	6	7	8	9	10	11	12	Note:
A	Std 1/P	Std 1/P	Std 1/P	Std 2/P	Std 2/P	Std 2/P	Std 3/P	Std 3/P	Std 3/P	Std 4/P	Std 4/P	Std 4/P	Pork assay
B	Std 5/P	Std 5/P	Std 5/P	NTC	NTC	NTC	21	21	21	22	22	22	-
C	23	23	23	24	24	24	25	25	25	26	26	26	-
D	27	27	27	28	28	28	29	29	29	30	30	30	-
E	Std 1/P	Std 1/P	Std 1/P	Std 2/P	Std 2/P	Std 2/P	Std 3/P	Std 3/P	Std 3/P	Std 4/P	Std 4/P	Std 4/P	Myostatin
F	Std 5/P	Std 5/P	Std 5/P	NTC	NTC	NTC	21	21	21	22	22	22	-
G	23	23	23	24	24	24	25	25	25	26	26	26	-
H	27	27	27	28	28	28	29	29	29	30	30	30	-

Appendix 2; collaborative trial raw data

Horse in processed beef

Nominal level, %	0.1	0.1	0.5	0.5	1	1	3	3	10	10
Sample ID	PCR_17	PCR_18	PCR_09	PCR_12	PCR_06	PCR_02	PCR_08	PCR_05	PCR_19	PCR_26
Lab no. 1	0.26	0.2	1.04	0.83	2.34	2.58	4.55 c	5.11 c	13.44	10.65
Lab no. 2	0.23	0.25	1.17	1.15	2.2	2.22	3.92	3.9	12.83	10.15
Lab no. 3	0.19	0.17	1.01	0.8	2.05	1.85	3.72	3.47	10.96	11.26
Lab no. 4	0.24	0.33	0.89	1.04	1.76	1.81	3.65	3.67	13.18	11.63
Lab no. 5	0.15	0.18	0.74	0.79	1.62	1.57	3.07	3.19	11.18	10.26
Lab no. 6	0.16	0.16	0.9	0.81	1.76	1.72	3.5	3.55	10.06	9.74
Lab no. 7	0.13	0.16	0.8	0.68	1.63	1.47	3.2	3.35	9.57	10.98

Lab no. 8	0.16	0.18	0.72	0.88	1.59	1.4	3.05	2.99	9.16	10.73
Lab no. 9	0.14	0.12	0.77	0.78	1.52	1.61	2.84	3.1	9.18	10.62
Lab no. 10	0.1	0.1	0.5	0.5	1	1	3	3	10	10
Lab no. 11	0.1	0.18	0.87	0.77	1.83	1.9	3.47	3.42	10.95	10.12
Lab no. 12	0.17	0.23	1.66 c	1.14 c	3.38 g	3.5 g	5.45 g	5.29 g	9.98	12.08
Lab no. 13	0.09	0.14	ND	0.69	1.67	1.85	3.47	3.02	8.99	9.08
Lab no. 14	0.1	0.13	0.62	0.68	1.17	1.58	2.42	3.12	9.08	7.94
Lab no. 15	0.21	0.3	0.98	ND	1.86	1.73	5.07 c	3.86 c	18.68 g	20 g

Pork in processed beef

Nominal level, %	0.1	0.1	0.5	0.5	1	1	3	3	10	10
Sample ID	PCR_13	PCR_14	PCR_03	PCR_11	PCR_07	PCR_21	PCR_25	PCR_22	PCR_20	PCR_24
Lab no. 1	0.11	0.09	0.6	0.6	1.28	1.66	3.31	2.78	5.85	7.48
Lab no. 2	0.18	0.13	0.84	0.76	1.74	1.58	2.67	2.6	7.28	7.04
Lab no. 3	0.18	0.17	0.57	0.64	1.21	1.14	2.85 c	1.86 c	9.24	7.42
Lab no. 4	0.19	0.22	0.72	0.88	1.89	1.49	2.37	2.42	9.16	6.82
Lab no. 5	0.09	0.12	0.5	0.45	1.36	1.37	2.41	2.34	7.63	7.24
Lab no. 6	0.1	0.11	0.58	0.55	1.19	1.57	2.16	2.45	6.58	6.56
Lab no. 7	0.09	0.16	0.63	0.61	1.3	1.36	2.29	1.99	8	5.8
Lab no. 8	0.19	0.2	0.71	0.7	1.36	1.42	2.54	2.55	7.24	7.29
Lab no. 9	0.18	0.15	0.61	0.75	1.31	1.47	2.69	2.41	6.62	6.78
Lab no. 10	0.1	0.1	0.5	0.5	0.5	1	3	3	10	10

Lab no. 11	0.13	0.17	0.6	0.77	1.33	1.56	2.48	2.53	6.82	7.09
Lab no. 12	0.44 g	0.48 g	1.38 g	1.55 g	2.7 g	3.89 g	7.39 g	5.38 g	12.14 g	12.49 g
Lab no. 13	0.11	0.1	0.54	0.42	1	1.3	2.17	2.33	6.41	6.13
Lab no. 14	0.09	0.07	0.41	0.53	0.93	0.9	1.3	1.64	6.19	5.23
Lab no. 15	0.1	0.09	0.74	0.56	2.62	1.9	6.26 c	2.24 c	8.94	9.07

Pork in raw beef

Nominal level, %	0.1	0.1	0.5	0.5	1	1	3	3	10	10
Sample ID	PCR_23	PCR_28	PCR_30	PCR_04	PCR_27	PCR_01	PCR_16	PCR_10	PCR_29	PCR_15
Lab no. 1	0.13	0.12	0.57	0.48	1.59	1.26	3.17	3.08	10.72	9.02
Lab no. 2	0.18	0.14	0.74	0.87	1.59	1.75	3.53	3.94	10.94	10.28
Lab no .3	0.07	0.05	0.63	0.43	1.63	0.99	3.83	2.69	10.58	11.67
Lab no. 4	0.07	0.16	0.64	0.82	1.22	1.72	4.27	4.67	10.94	11.68
Lab no. 5	0.13	0.1	0.64	0.42	1.29	1.09	2.78	3.06	10.74	9.62
Lab no. 6	0.12	0.08	0.57	0.55	1.18	1.03	2.91	2.78	9.86	9.36
Lab no. 7	0.07	0.11	0.59	0.62	0.99	1.48	3.88 c	1.89 c	8.52	11.52
Lab no. 8	0.14	0.13	0.74	0.55	1.37	1.42	3.49	3.5	10.49	10.74
Lab no. 9	0.18	0.13	0.61	0.57	1.32	1.15	3.34	3.04	10.48	10.49
Lab no. 10	0.1	0.1	1	0.5	1	1	3	3	10	10

Lab no. 11	0.1	0.12	0.65	0.57	1.19	1.18	3.31	3.37	10.09	9.69
Lab no. 12	0.66 g	0.48 g	1.77 g	1.29 g	3.08 g	2.77 g	6.15 g	4.83 g	16.3 g	16.42 g
Lab no. 13	0.08	0.09	0.49	0.5	1.1	1.11	2.56	2.63	9.6	7.76
Lab no. 14	0.03	0.05	0.33	0.51	0.58	1.01	2.57	2.57	6.12 g	7.39 g
Lab no. 15	0.09	0.08	0.88	0.85	1.83	1.26	3.35 c	7.83 c	40.18 c	11.3 c

Calibration performance reported by laboratories

	Coefficient of determination R^2					
Lab number	**Pork 1**	**Pork 2**	**Pork 3**	**Horse 1**	**Horse 2**	**Horse 3**
1	0.999	0.999	0.994	0.998	0.997	0.999
2	0.993	0.995	0.994	0.996	0.997	0.998
3	0.997	0.985	0.998	0.994	0.996	0.994
4	0.994	0.996	0.997	0.990	0.997	0.999
5	0.998	0.998	0.999	0.992	0.998	0.995
6	0.998	0.998	0.995	0.998	0.998	0.992
7	0.998	0.997	0.998	0.999	0.996	0.999
8	0.996	0.997	0.999	0.995	0.991	0.998
9	0.999	0.999	0.999	0.995	0.993	0.953#
10	0.991	0.993	0.997	0.990	0.996	0.994
11	0.999	0.998	0.998	0.999	0.998	0.991
12	0.996	0.999	0.997	0.999	1.00	0.995
13	0.999	0.995	0.999	0.995	0.993	0.998
14	0.998	0.999	0.995	0.977#	0.996	0.988
15	0.991	0.997	0.963#	0.977#	0.958#	0.993

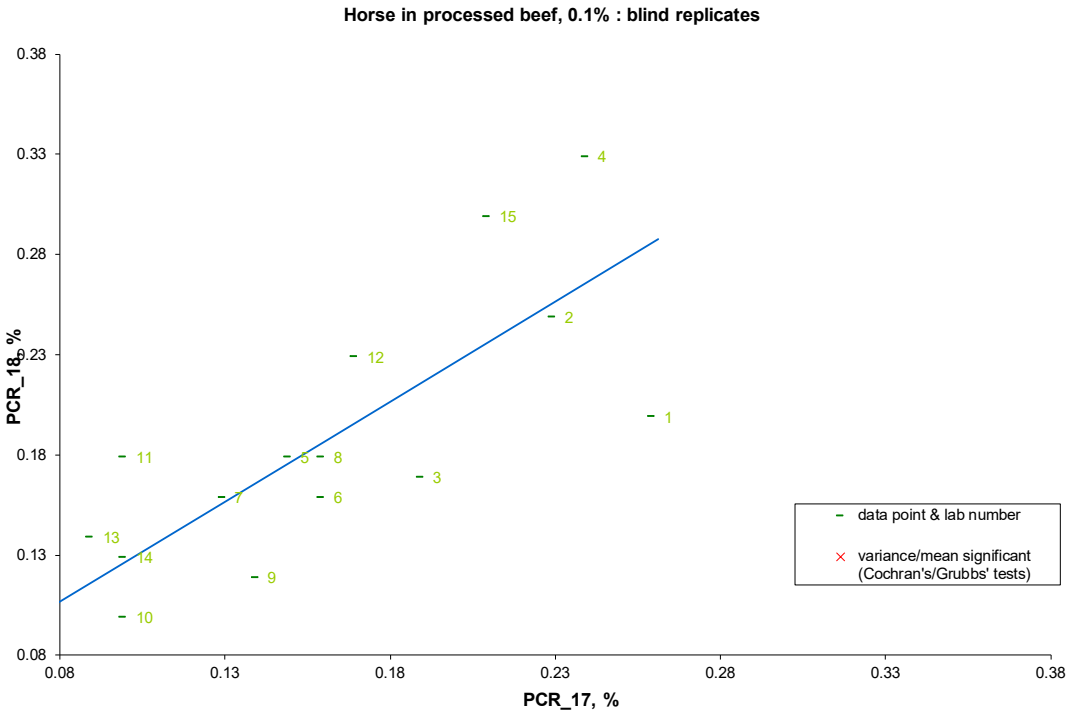
Acceptance criterion ≥0.98, # indicates <0.98. Assay plate number indicated by 1, 2 or 3 following the target matrix of pork or horse.

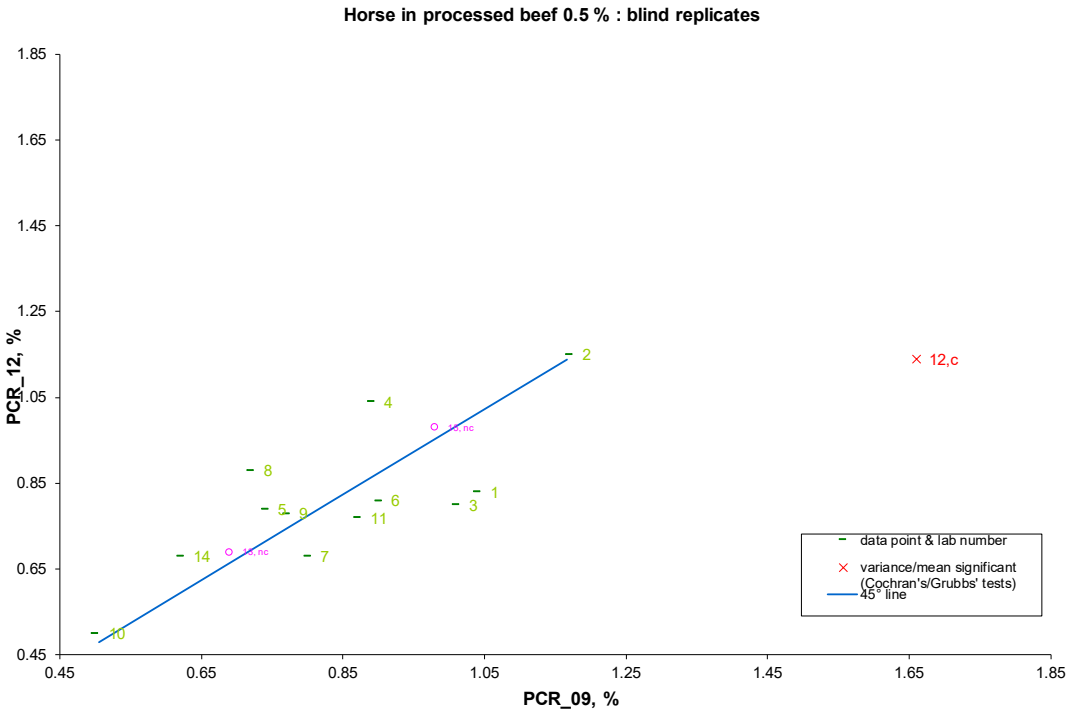
PCR efficiency reported by laboratories

	PCR efficiency, %					
Lab number	**Pork 1**	**Pork 2**	**Pork 3**	**Horse 1**	**Horse 2**	**Horse 3**
1	96.6	92.5	91.5	101	103	96.4
2	83.2#	89.3	84.9#	87.7	86.6	88.9
3	99.1	91.0	97.2	89.7	94.5	87.6
4	91.6	88.4	94.7	95.0	85.4	88.1
5	98.5	100	93.3	97.0	93.0	95.2
6	100	98.8	93.2	91.6	96.8	91.3
7	90.2	91.6	96.2	94.6	94.4	96.9
8	95.8	92.1	93.5	98.8	92.6	94.3
9	94.8	89.8	90.6	93.7	93.7	98.3
10	94.8	90.5	90.7	104	93.3	89.8
11	93.8	95.6	90.9	94.2	97.3	91.0
12	80.5#	79.5#	71.8#	72.1#	82.8#	89.1
13	95.6	98.9	96.1	92.9	102	98.0
14	95.4	99.1	104	92.4	95.9	88.8
15	106	105	105	104	109	108

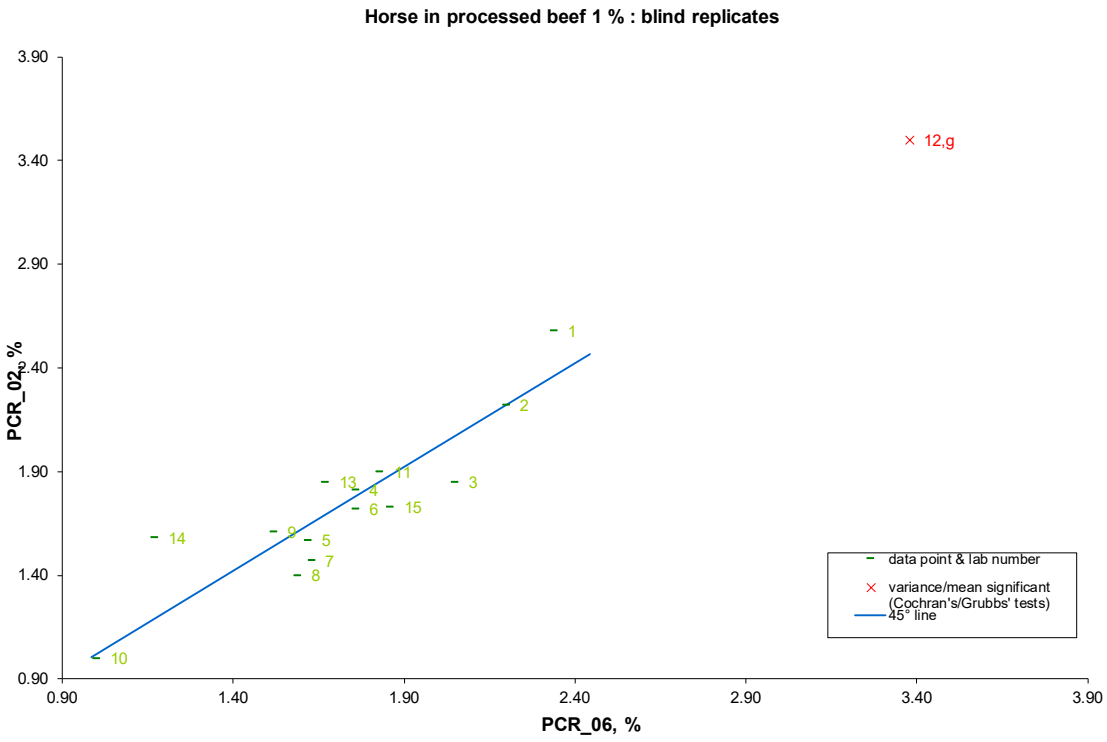
Acceptance criterion 85 – 115%, # indicates value outside acceptance criterion. Assay plate number indicated by 1, 2 or 3 following the target matrix of pork or horse.

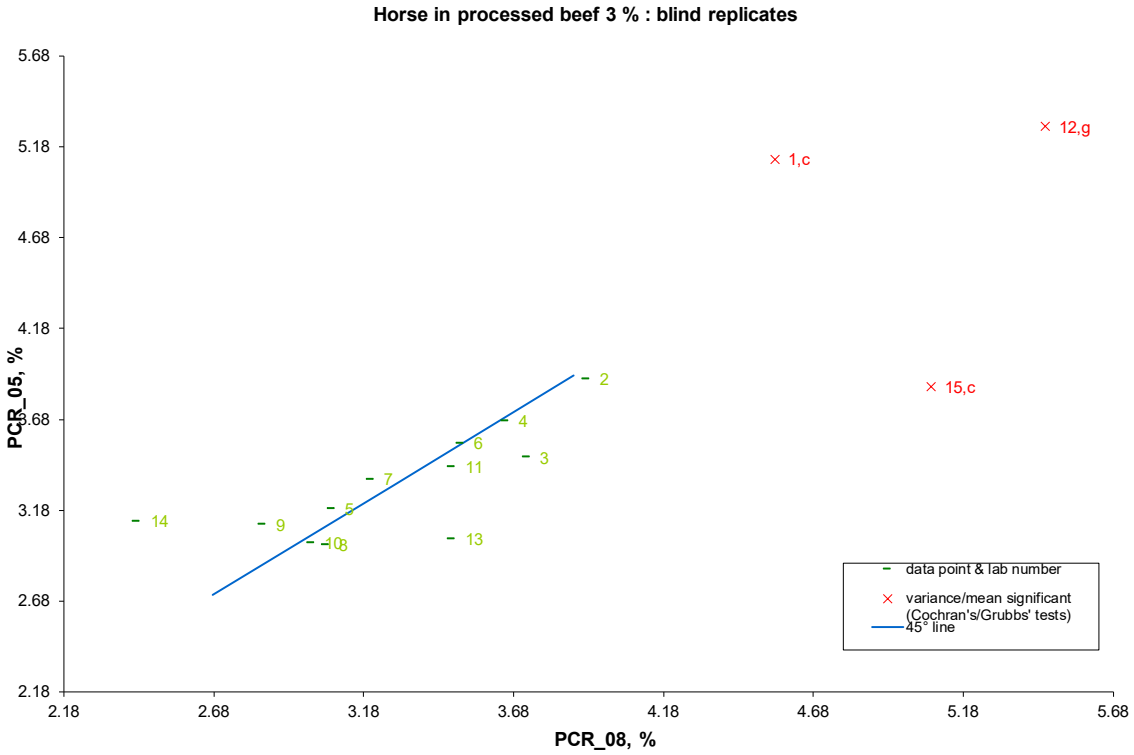
Appendix 3; Youden plots of raw data from the collaborative trial

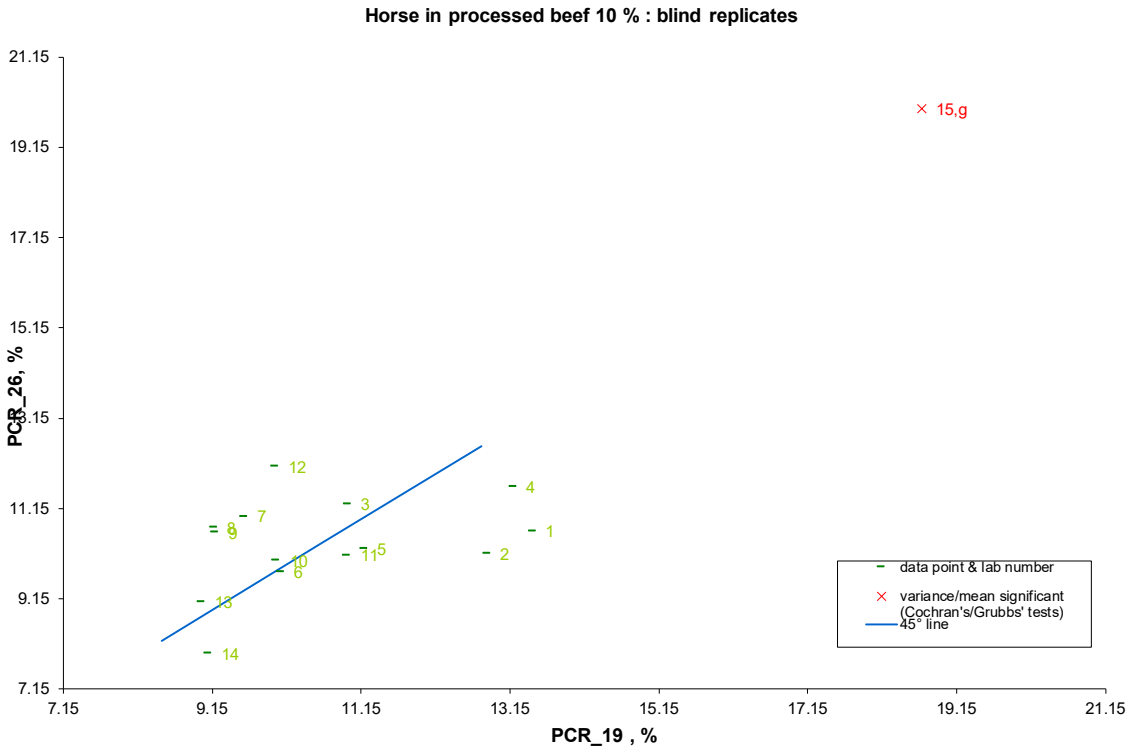


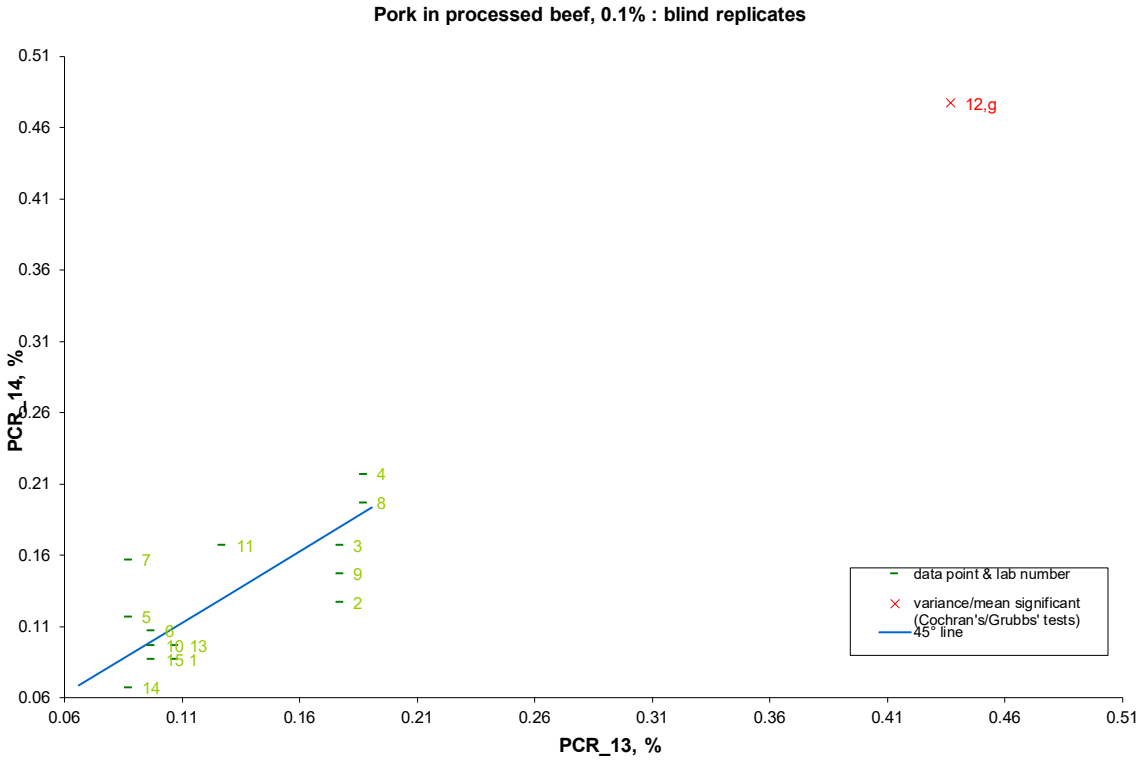


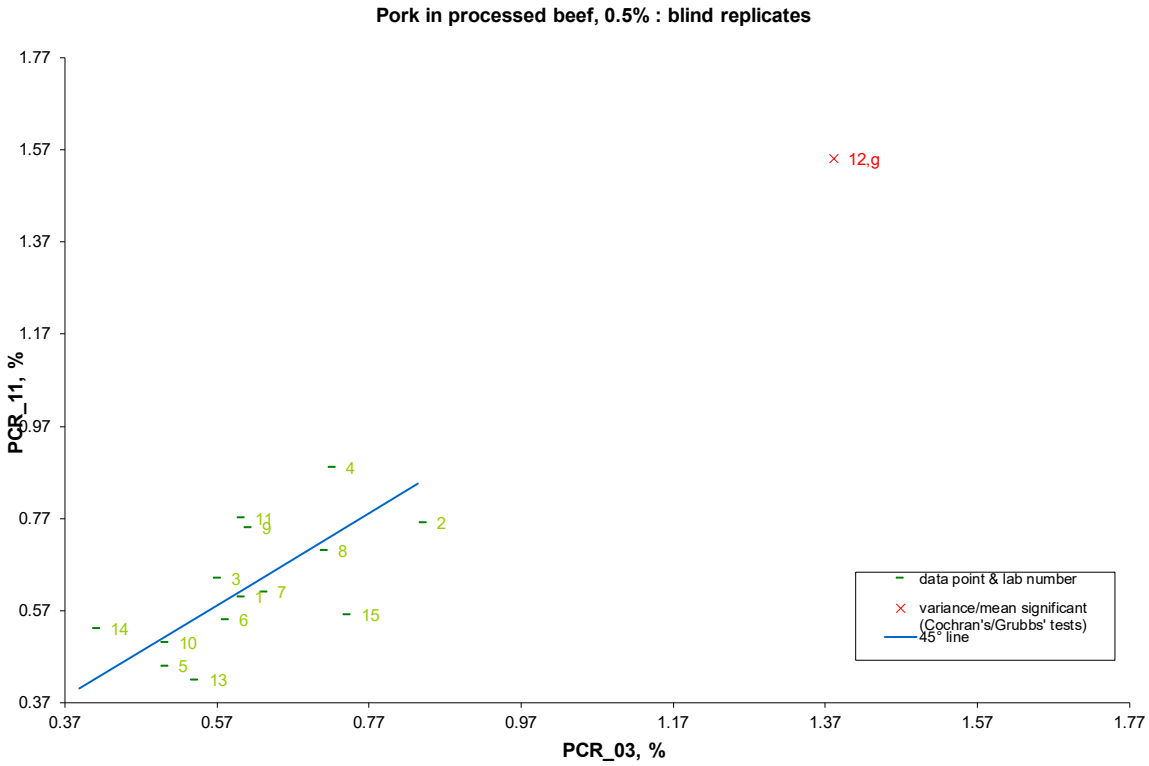
Laboratories 13 and 15 were non-compliant due to single data points only.

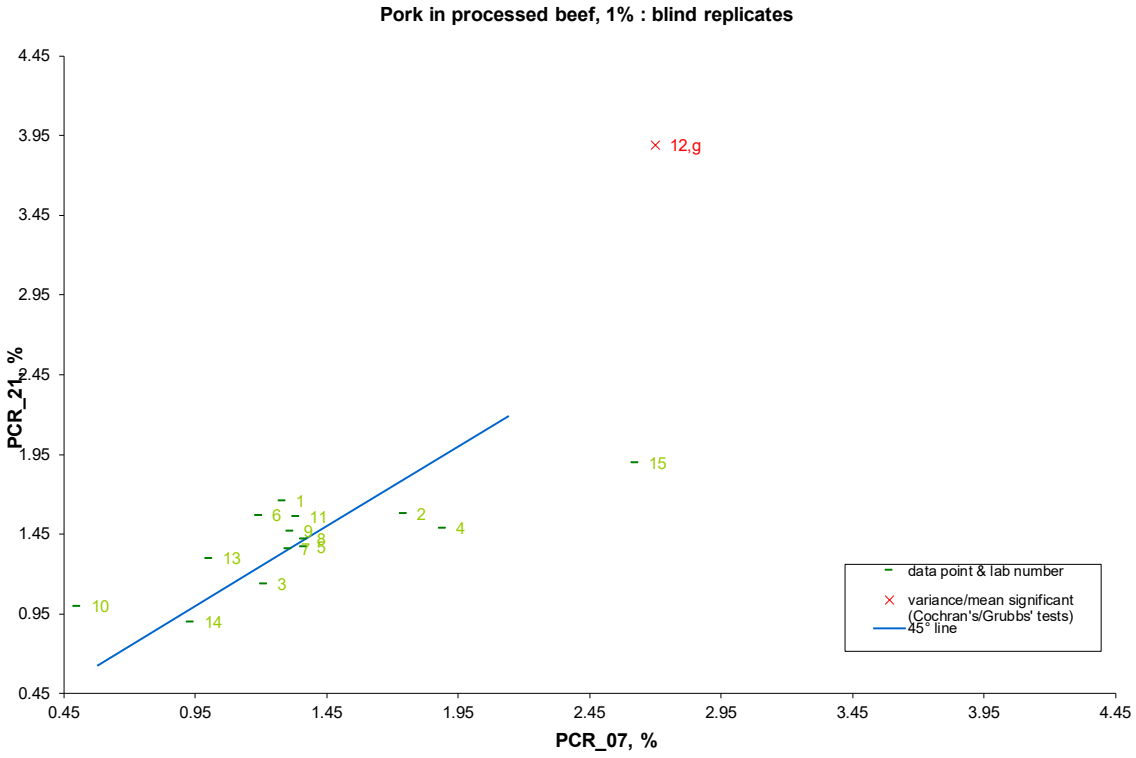


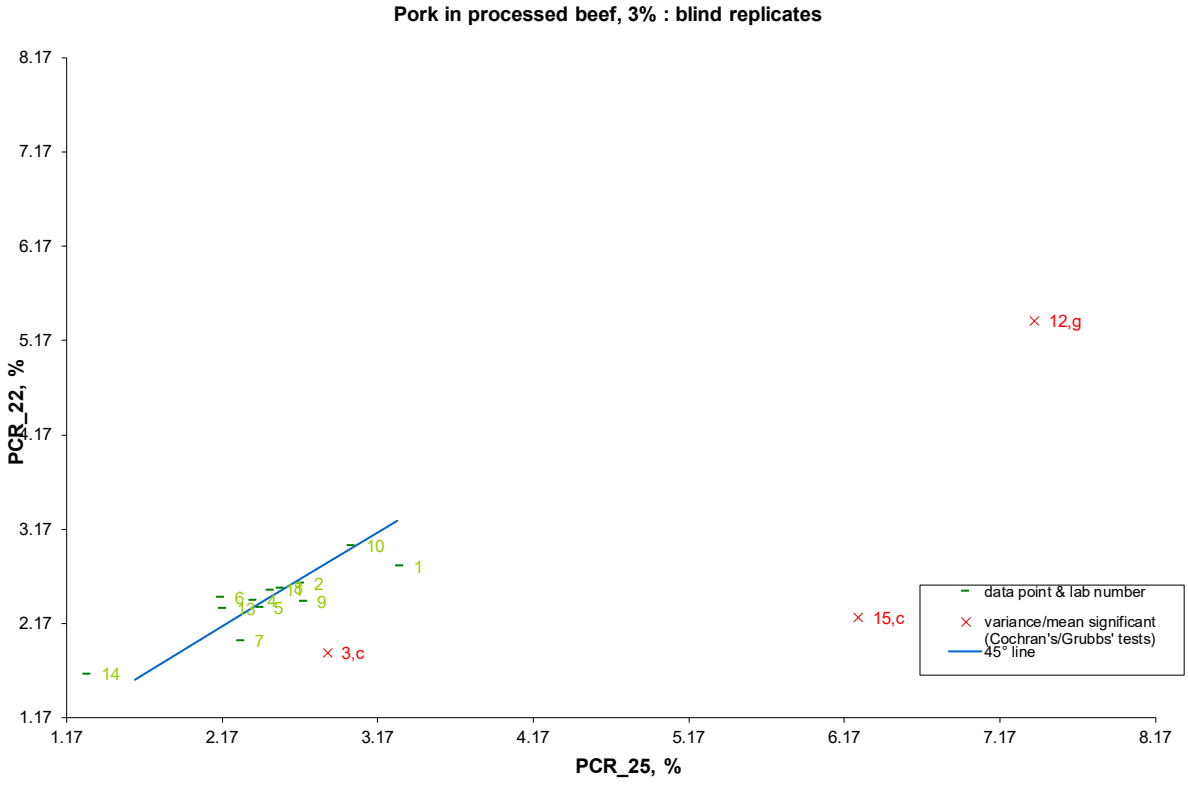

Horse in processed beef 3 % : blind replicates
PCR_05, %
PCR_08, %
5.68
5.18
4.68
4.18
3.68
3.18
2.68
2.18
12,g
1,c
15,c
data point & lab number
variance/mean significant (Cochran's/Grubbs' tests)
45° line

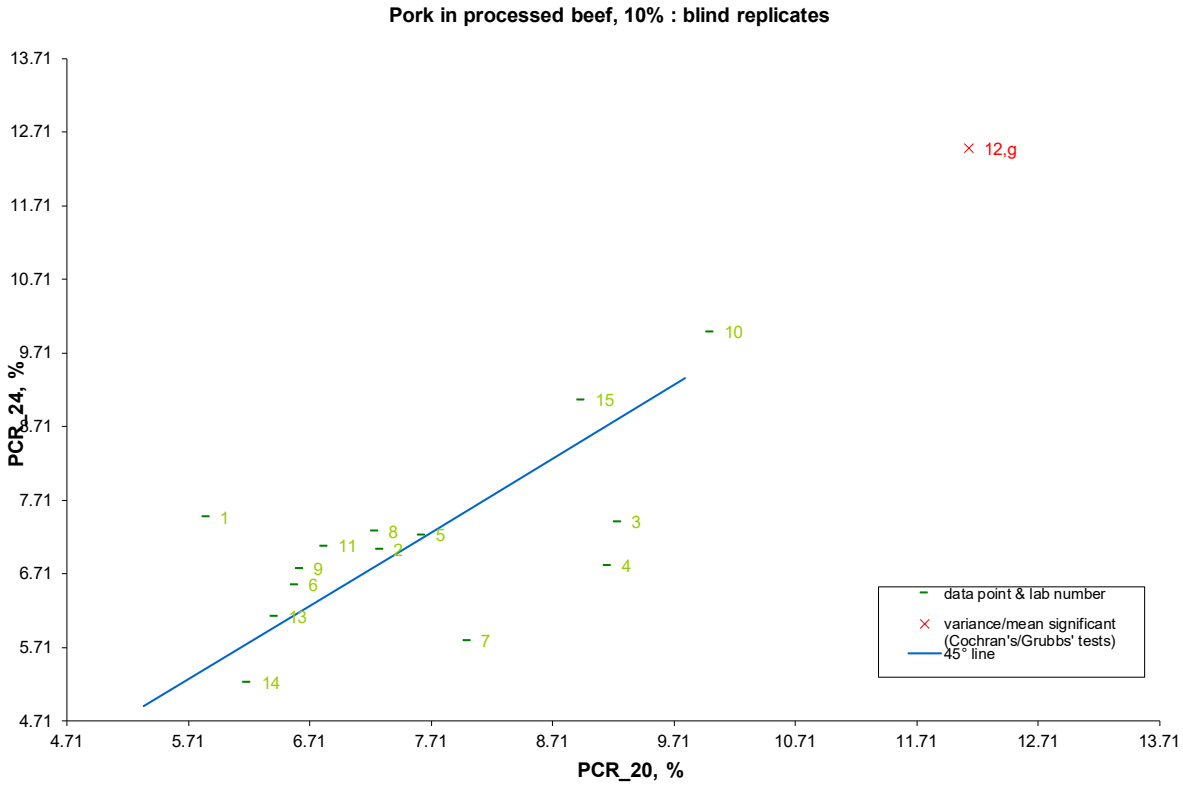

Horse in processed beef 10 % : blind replicates
PCR_26, %
PCR_19 , %
21.15
19.15
17.15
15.15
11.15
9.15
7.15
15,g
data point & lab number
variance/mean significant (Cochran's/Grubbs' tests)
45° line

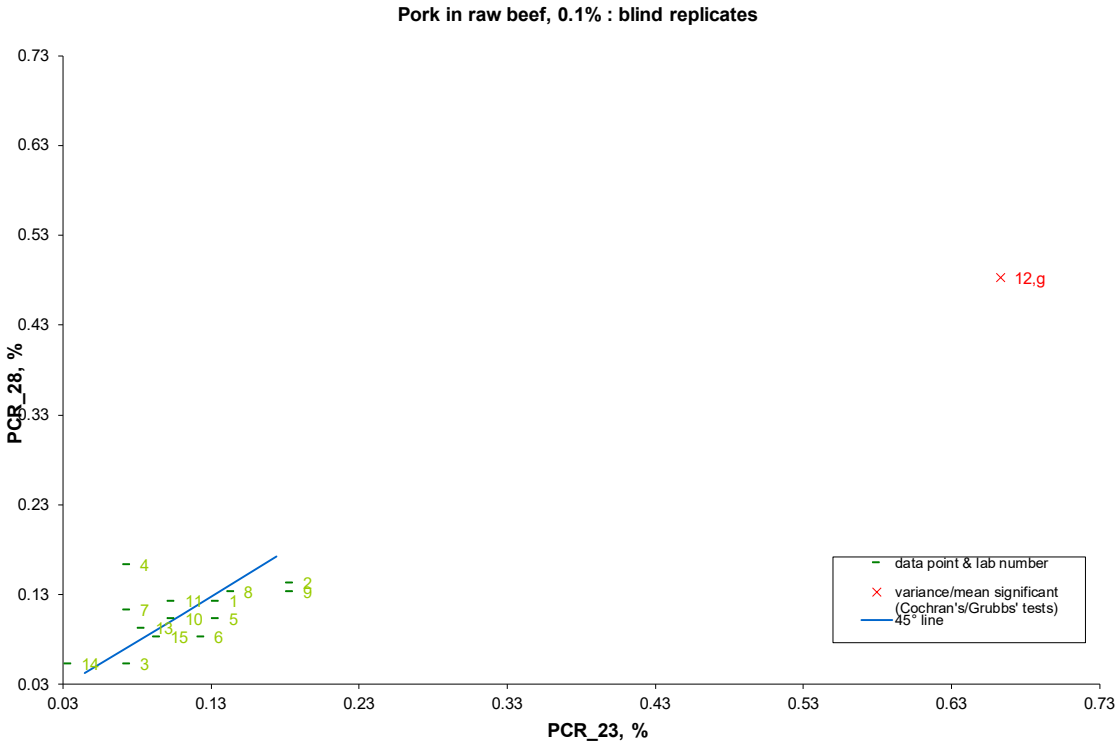

Pork in processed beef, 0.1% : blind replicates
PCR_14, %
PCR_13, %
0.06
0.11
0.16
0.21
0.26
0.31
0.36
0.41
0.46
0.51
12,g
4
8
11
3
7
9
2
5
6
10
13
15
1
14
data point & lab number
variance/mean significant (Cochran's/Grubbs' tests)
45° line

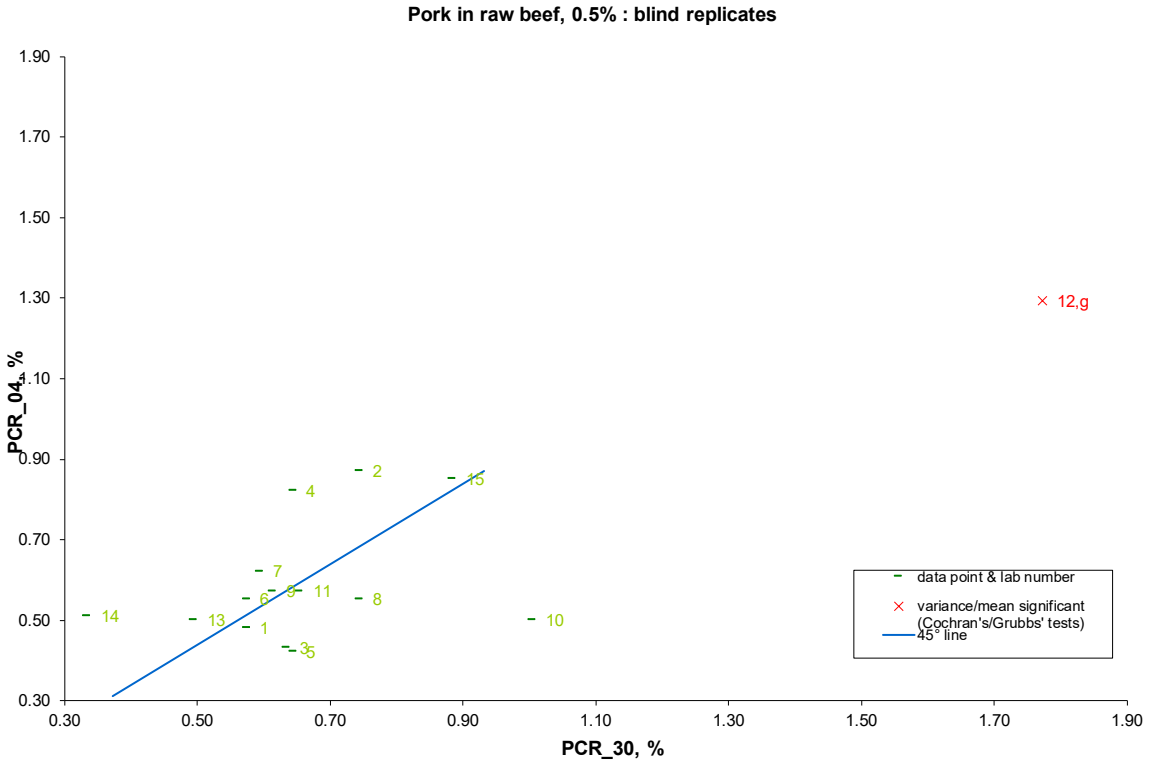

Pork in processed beef, 0.5% : blind replicates
PCR_11, %
PCR_03, %
0.37
0.57
0.77
0.97
1.17
1.37
1.57
1.77
12,g
4
11
9
2
8
3
1
7
6
15
14
10
5
13
data point & lab number
variance/mean significant (Cochran's/Grubbs' tests)
45° line

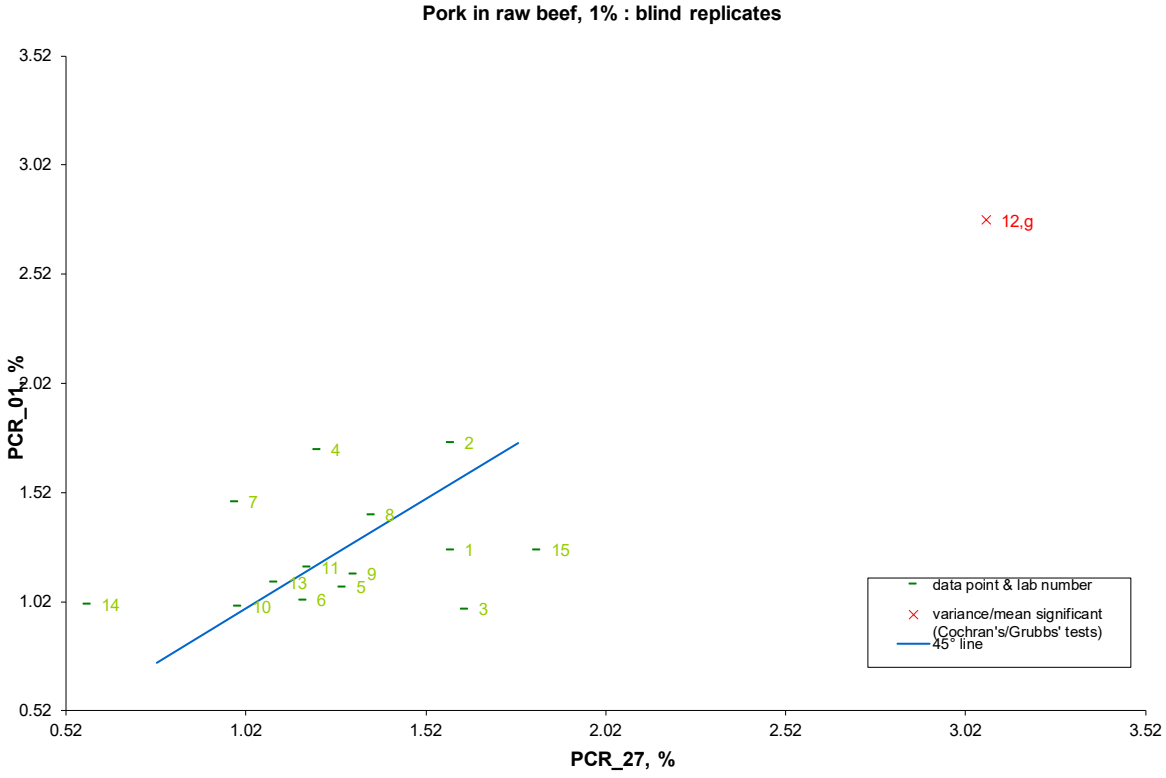

Pork in processed beef, 1% : blind replicates
PCR_21, %
PCR_07, %
12,g
15
1
6
11
9
8
2
4
7
5
13
3
10
14
data point & lab number
variance/mean significant (Cochran's/Grubbs' tests)
45° line
0.45
0.95
1.45
1.95
2.45
2.95
3.45
3.95
4.45

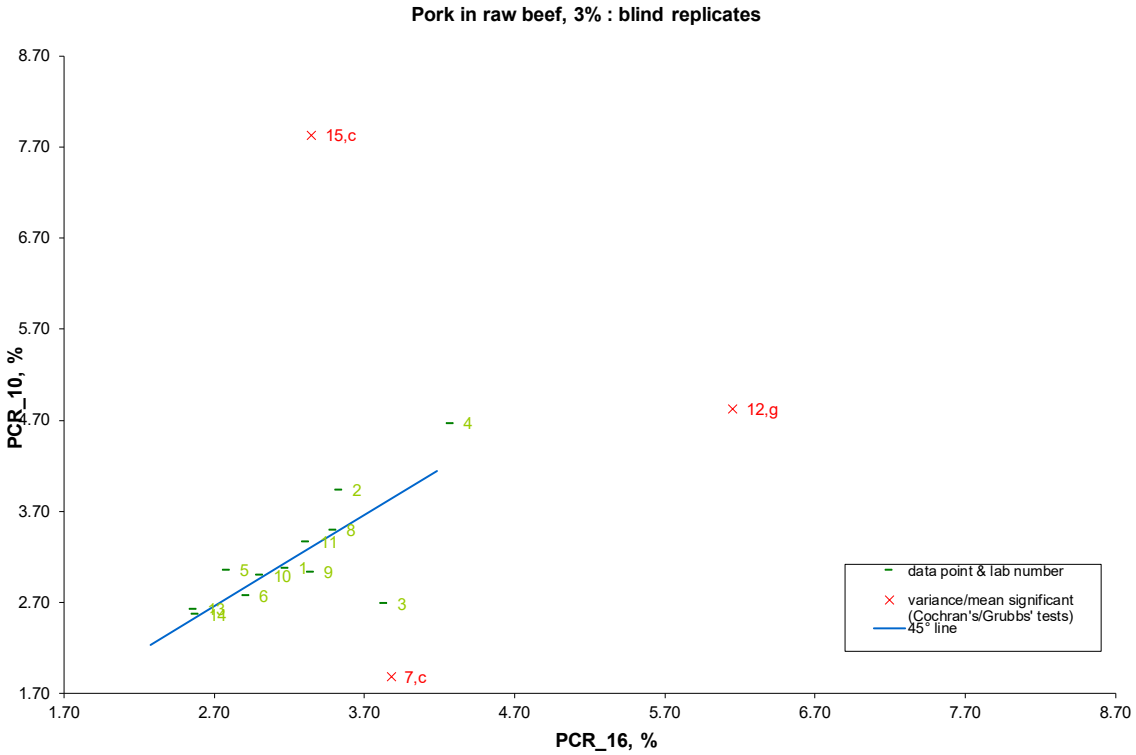

Pork in processed beef, 3% : blind replicates
PCR_22, %
PCR_25, %
12,g
15,c
3,c
10
1
2
6
4
9
5
7
14
data point & lab number
variance/mean significant (Cochran's/Grubbs' tests)
45° line
1.17
2.17
3.17
4.17
5.17
6.17
7.17
8.17

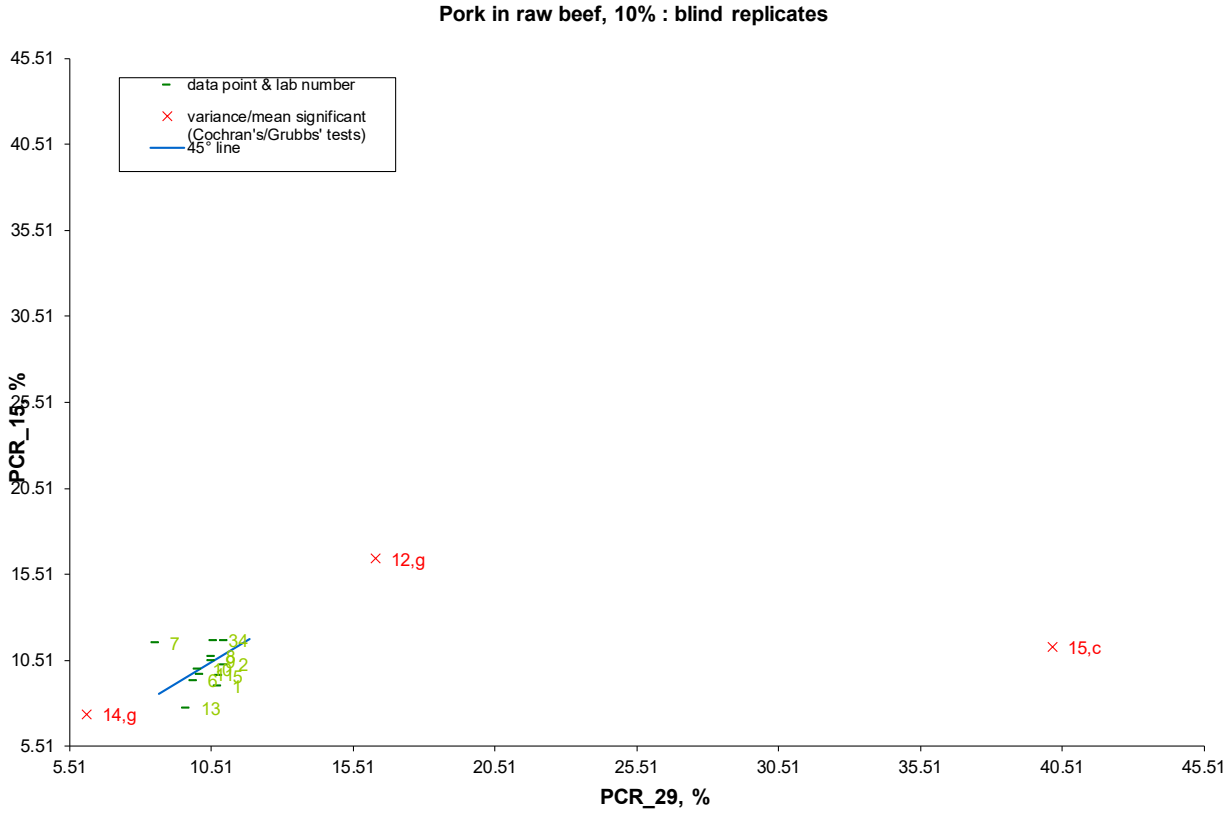

Pork in processed beef, 10% : blind replicates
PCR_24, %
PCR_20, %
13.71
12.71
11.71
10.71
9.71
8.71
7.71
6.71
5.71
4.71
12,g
10
15
1
3
8
5
11
2
9
4
6
13
7
14
data point & lab number
variance/mean significant (Cochran's/Grubbs' tests)
45° line


Pork in raw beef, 0.1% : blind replicates
PCR_28, %
PCR_23, %
0.73
0.63
0.53
0.43
0.33
0.23
0.13
0.03
12,g
4
7
11
8
1
2
9
10
5
13
15
6
14
3
data point & lab number
variance/mean significant (Cochran's/Grubbs' tests)
45° line

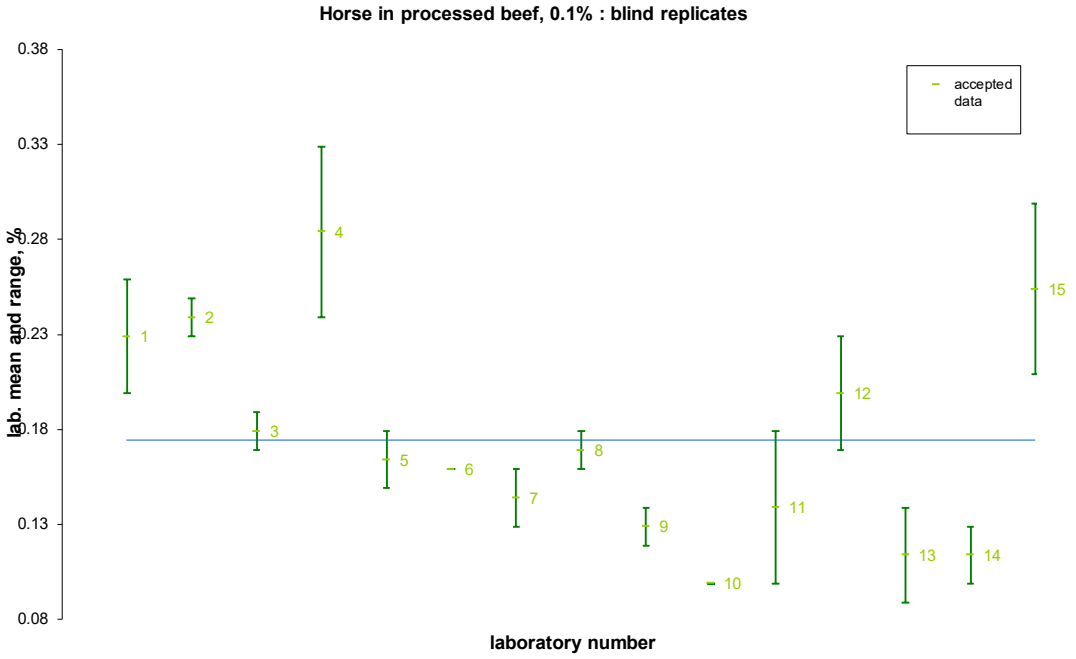

Pork in raw beef, 0.5% : blind replicates
PCR_04, %
PCR_30, %
1.90
1.70
1.50
1.30
1.10
0.90
0.70
0.50
0.30
0.30
0.50
0.70
0.90
1.10
1.30
1.50
1.70
1.90
12,g
2
4
15
7
6
9
11
8
14
13
1
3
5
10
data point & lab number
variance/mean significant (Cochran's/Grubbs' tests)
45° line

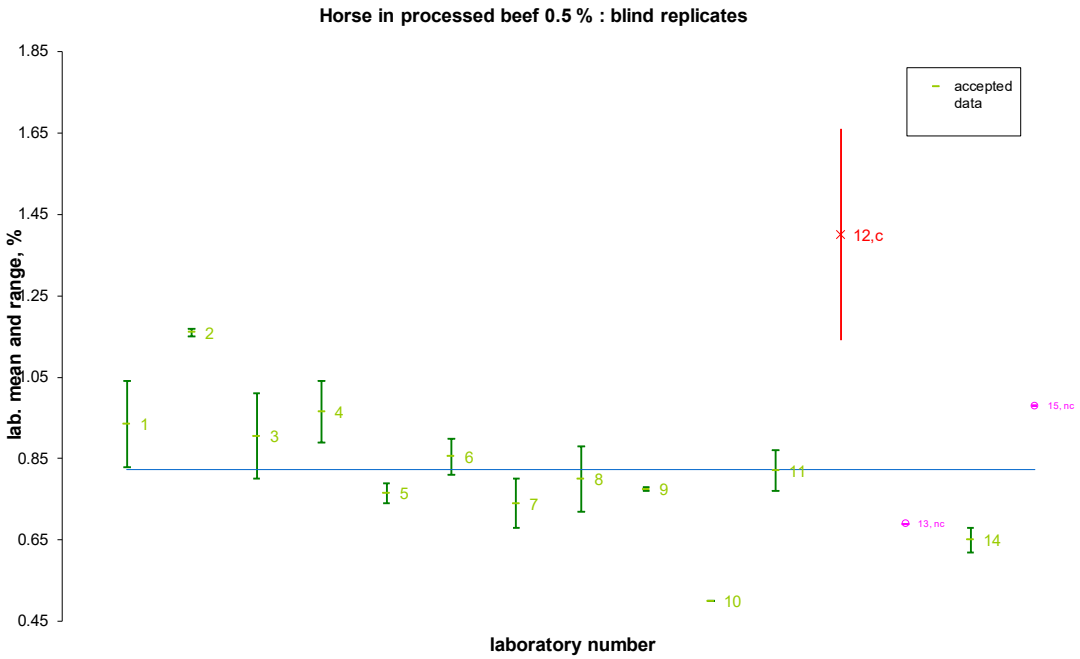

Pork in raw beef, 1% : blind replicates
PCR_01, %
PCR_27, %
3.52
3.02
2.52
2.02
1.52
1.02
0.52
0.52
1.02
1.52
2.02
2.52
3.02
3.52
12,g
4
2
7
8
1
15
11
9
13
5
14
10
6
3
data point & lab number
variance/mean significant (Cochran's/Grubbs' tests)
45° line

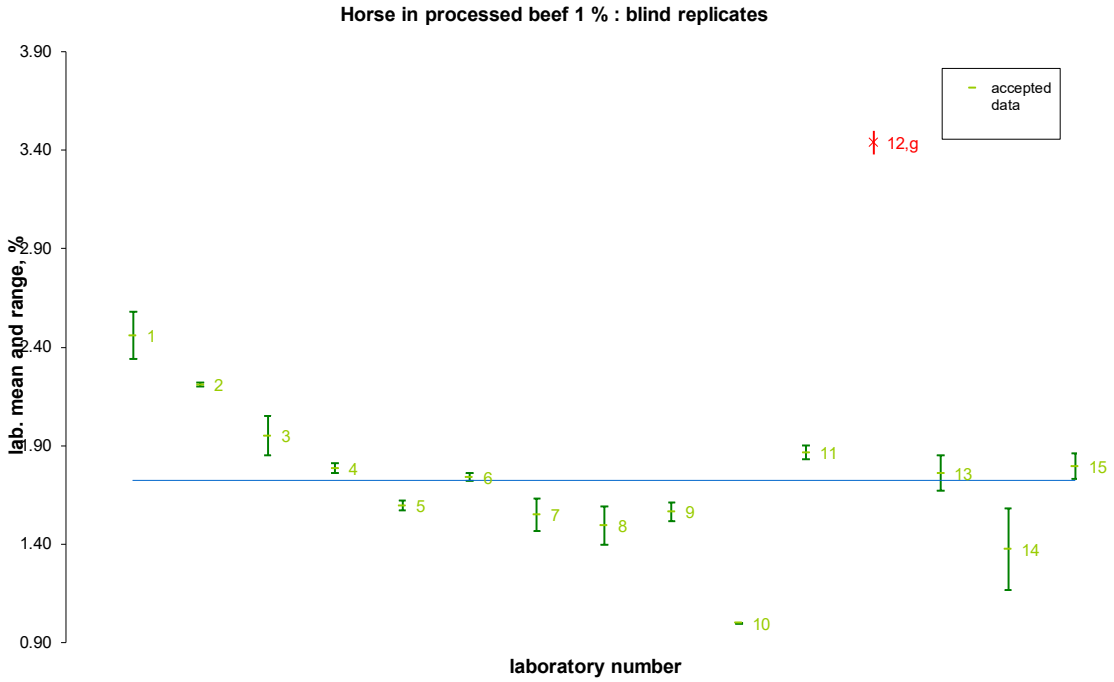

Pork in raw beef, 3% : blind replicates
PCR_10, %
PCR_16, %
8.70
7.70
6.70
5.70
4.70
3.70
2.70
1.70
15,c
12,g
7,c
data point & lab number
variance/mean significant (Cochran's/Grubbs' tests)
45° line

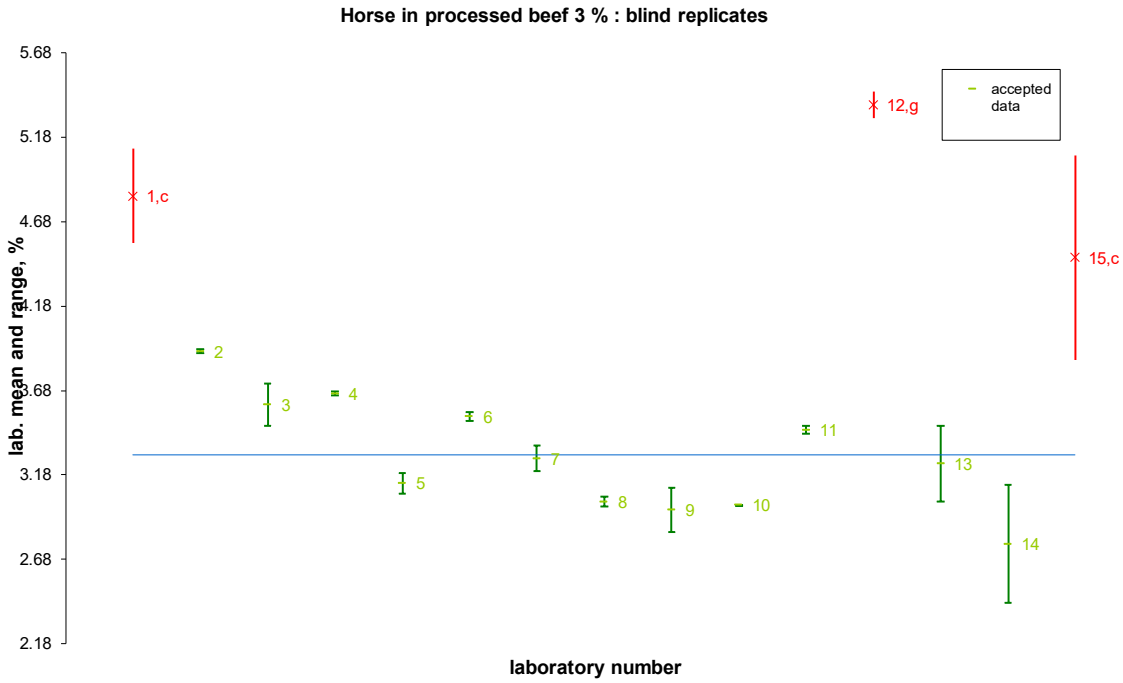

Pork in raw beef, 10% : blind replicates
PCR_15, %
PCR_29, %
45.51
40.51
35.51
30.51
25.51
20.51
15.51
10.51
5.51
12,g
15,c
14,g
data point & lab number
variance/mean significant (Cochran's/Grubbs' tests)
45° line

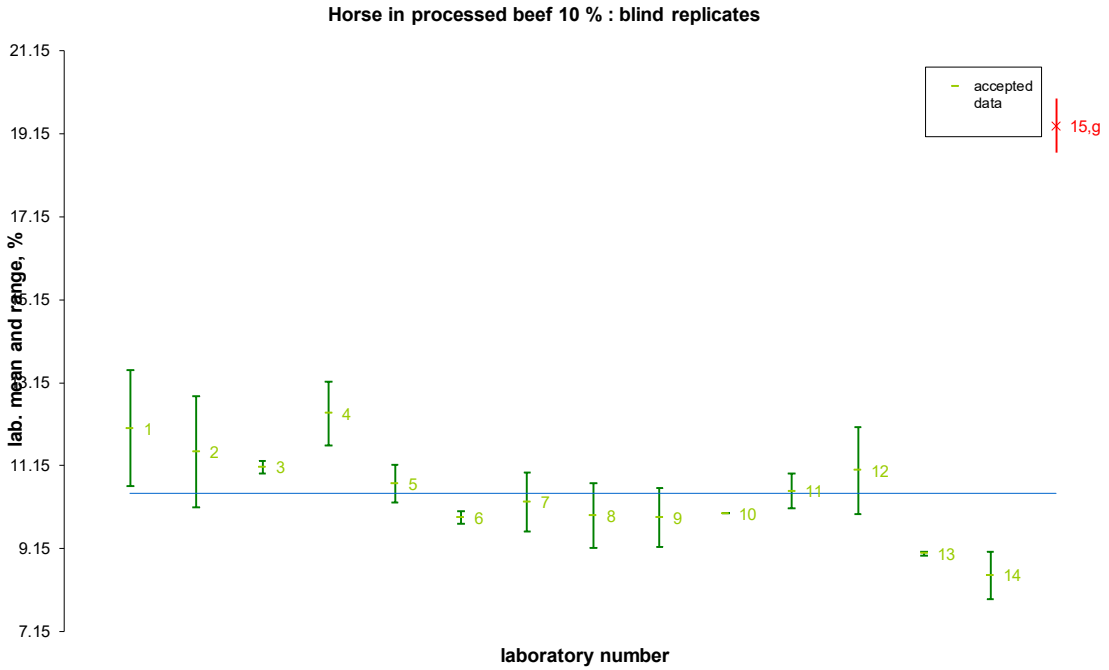
Appendix 4; mean and range plots of raw data from the collaborative trial

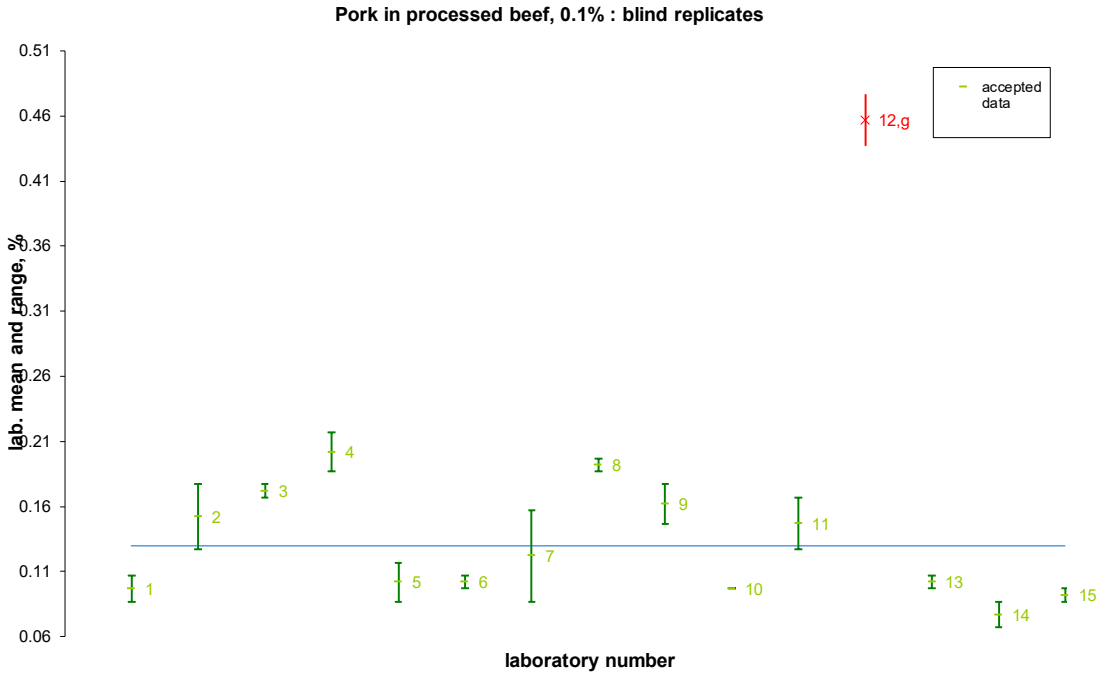


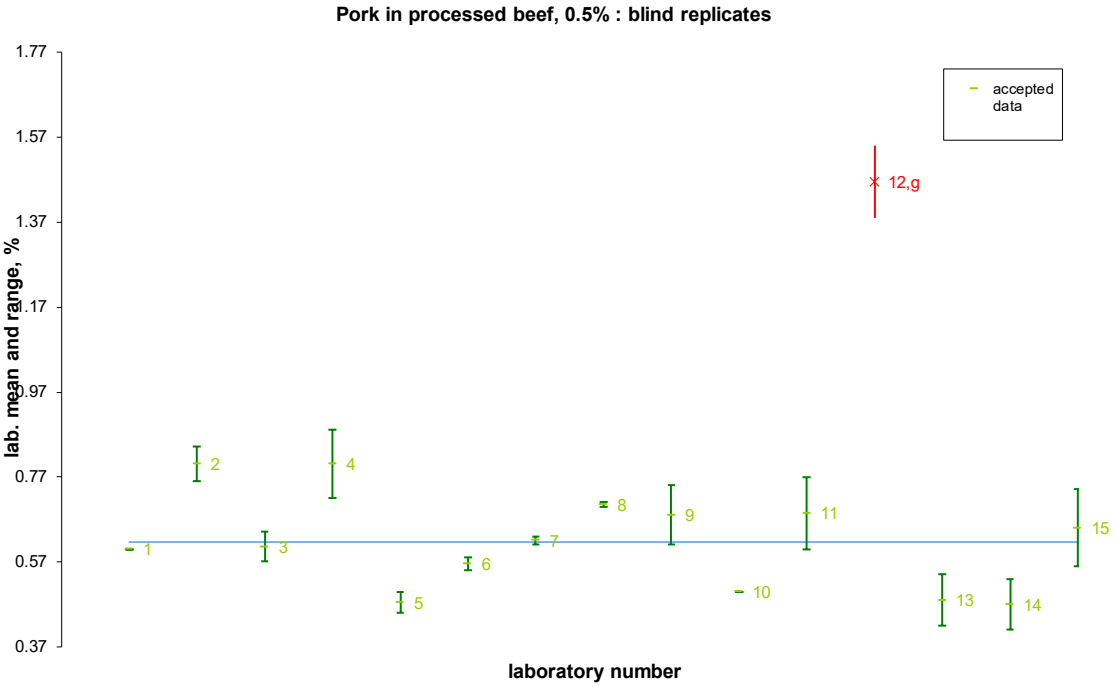


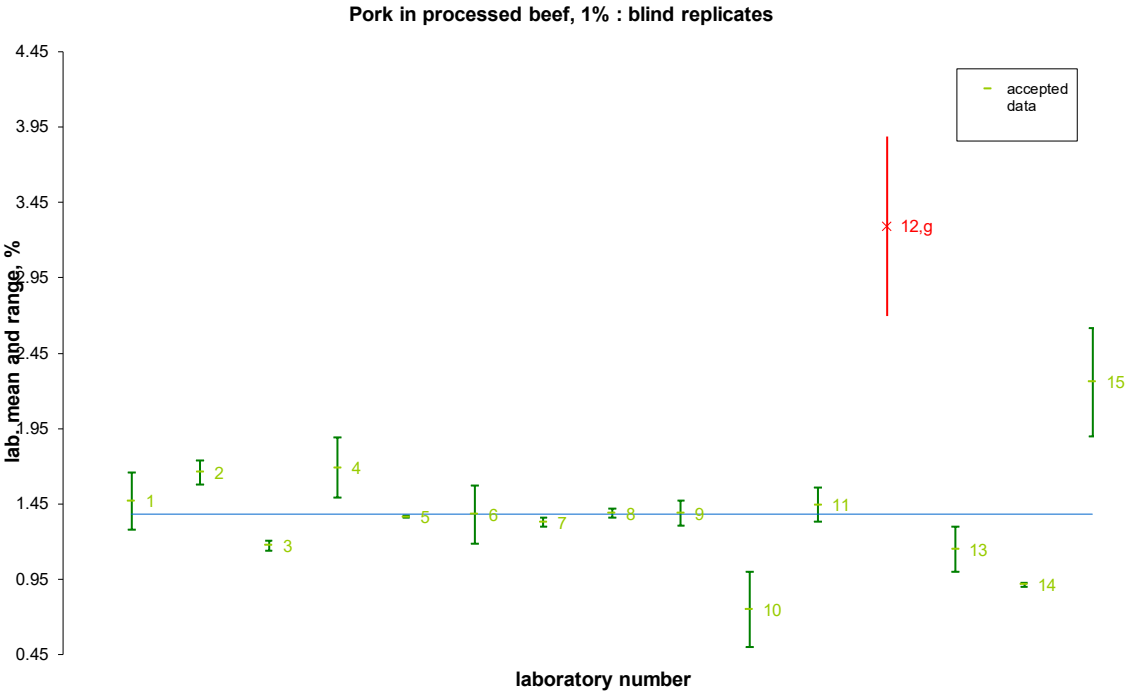

Horse in processed beef 1 % : blind replicates
3.90
3.40
2.90
2.40
1.90
1.40
0.90
lab. mean and range, %
accepted data
12,g
1
2
3
4
5
6
7
8
9
10
11
13
14
15
laboratory number

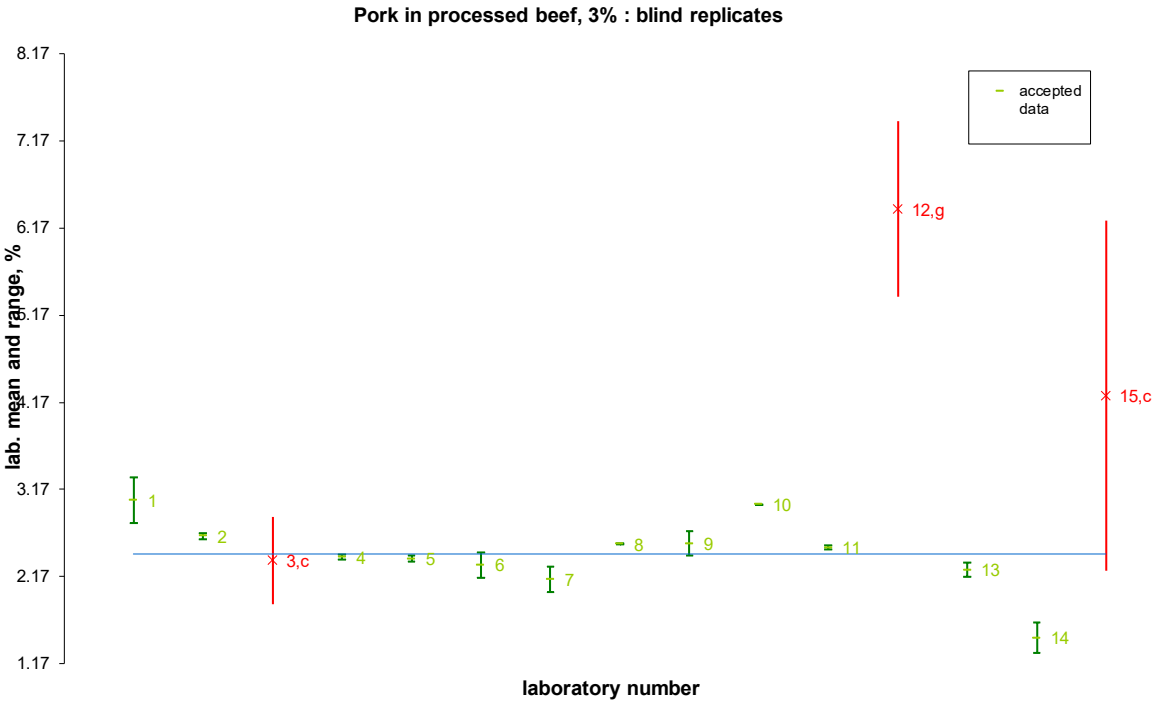

Horse in processed beef 3 % : blind replicates
5.68
5.18
4.68
4.18
3.68
3.18
2.68
2.18
lab. mean and range, %
accepted data
12,g
1,c
15,c
2
3
4
5
6
7
8
9
10
11
13
14
laboratory number

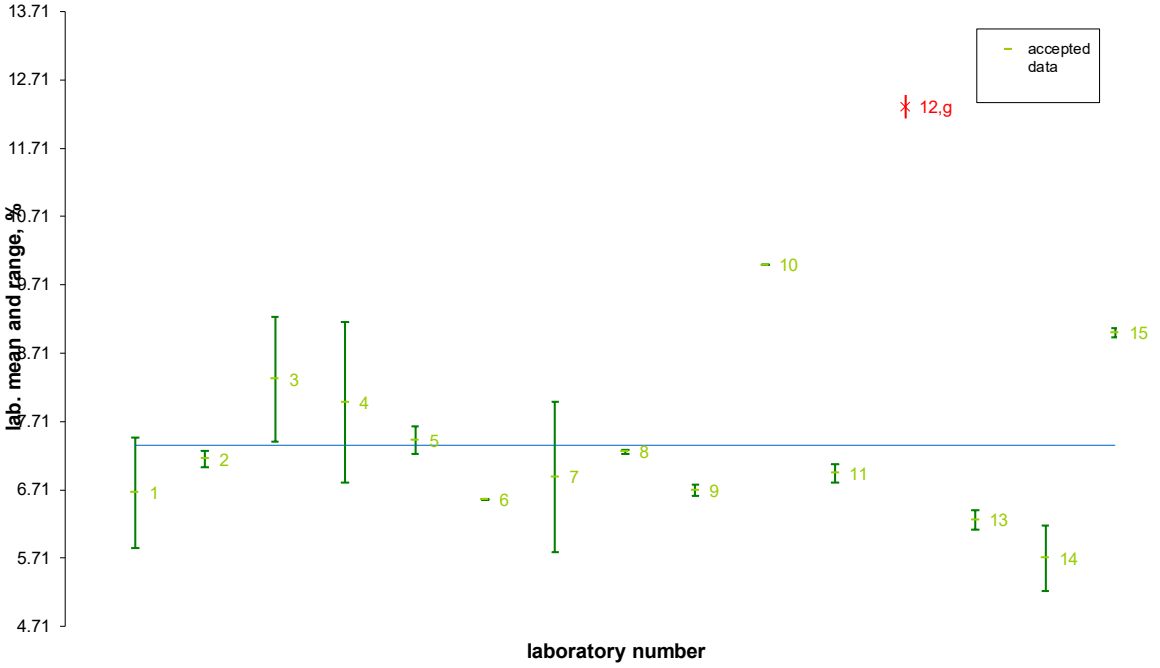

Horse in processed beef 10 % : blind replicates
21.15
19.15
17.15
15.15
13.15
11.15
9.15
7.15
lab. mean and range, %
accepted data
15,g
1
2
3
4
5
6
7
8
9
10
11
12
13
14
laboratory number

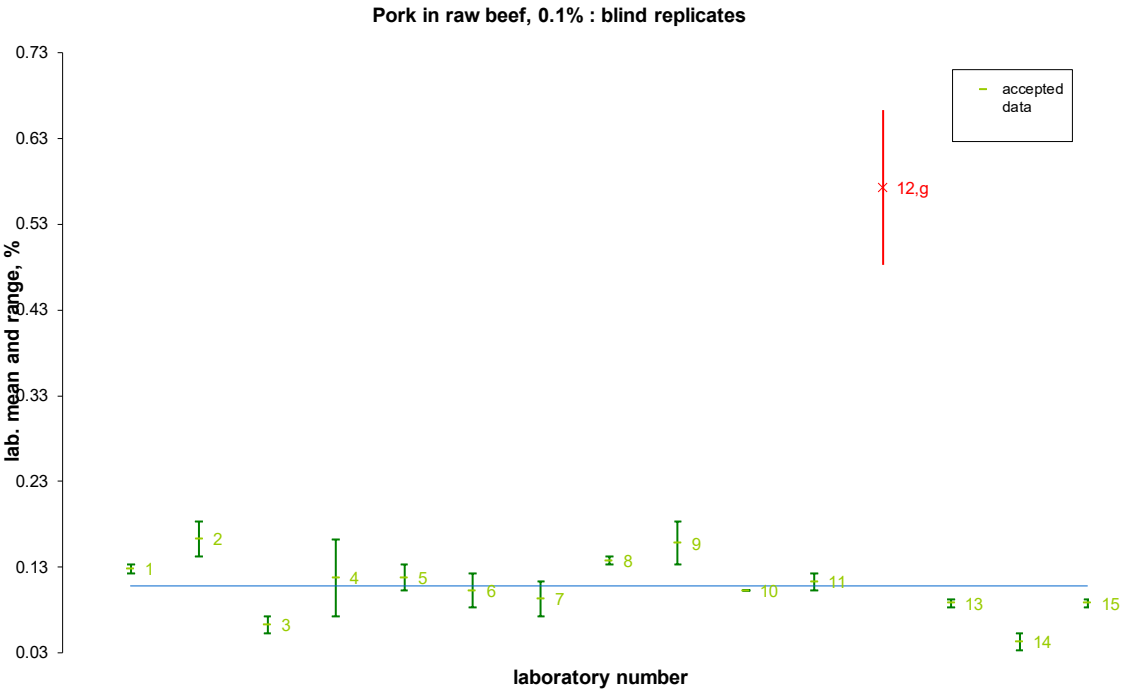

Pork in processed beef, 0.1% : blind replicates
0.51
0.46
0.41
0.36
0.31
0.26
0.21
0.16
0.11
0.06
lab. mean and range, %
accepted data
12,g
1
2
3
4
5
6
7
8
9
10
11
13
14
15
laboratory number

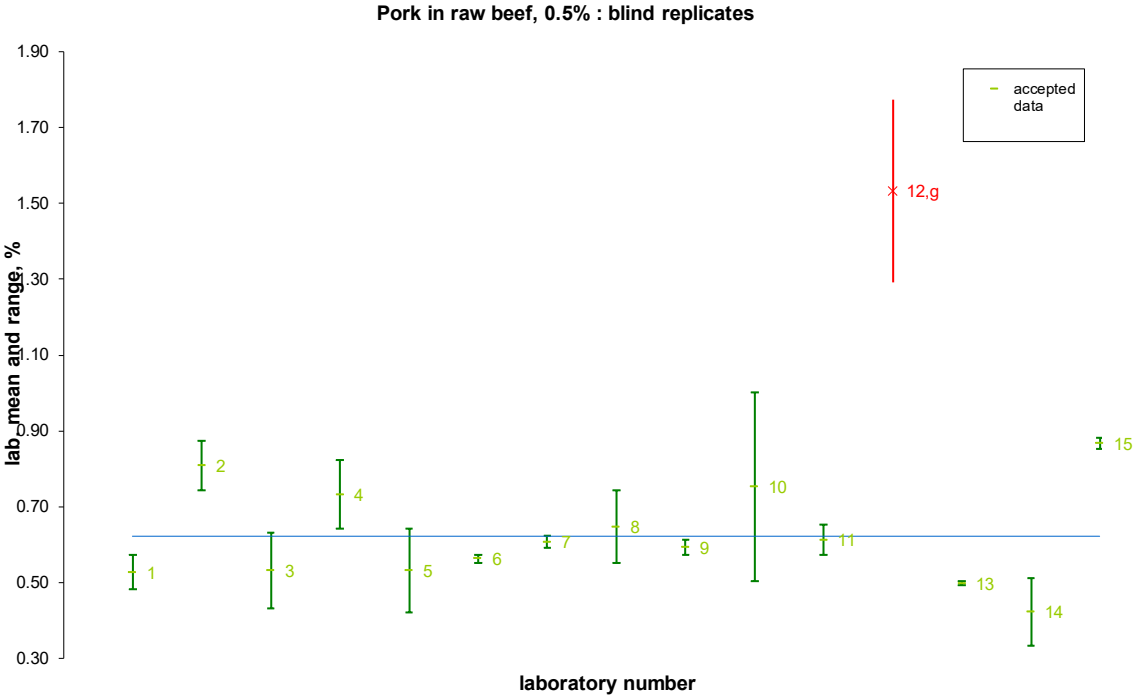

Pork in processed beef, 0.5% : blind replicates
lab. mean and range, %
1.77
1.57
1.37
1.17
0.97
0.77
0.57
0.37
accepted data
12,g
1
2
3
4
5
6
7
8
9
10
11
13
14
15
laboratory number

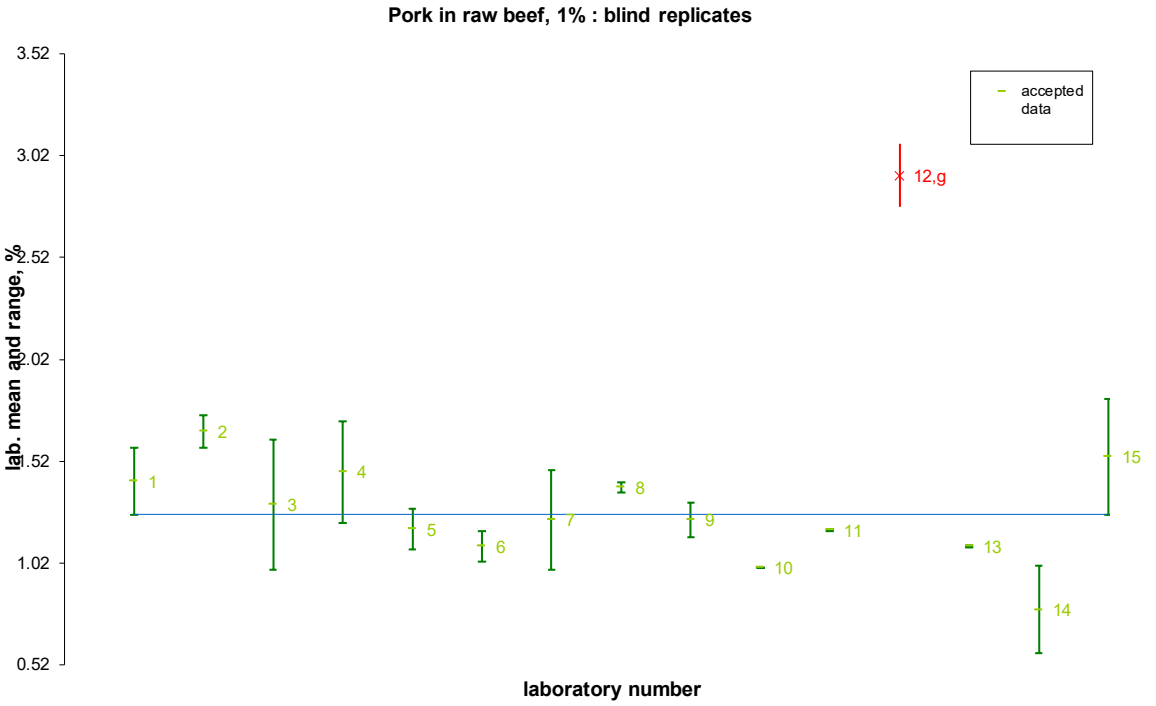

Pork in processed beef, 1% : blind replicates
lab. mean and range, %
4.45
3.95
3.45
2.95
2.45
1.95
1.45
0.95
0.45
accepted data
12,g
1
2
3
4
5
6
7
8
9
10
11
13
14
15
laboratory number

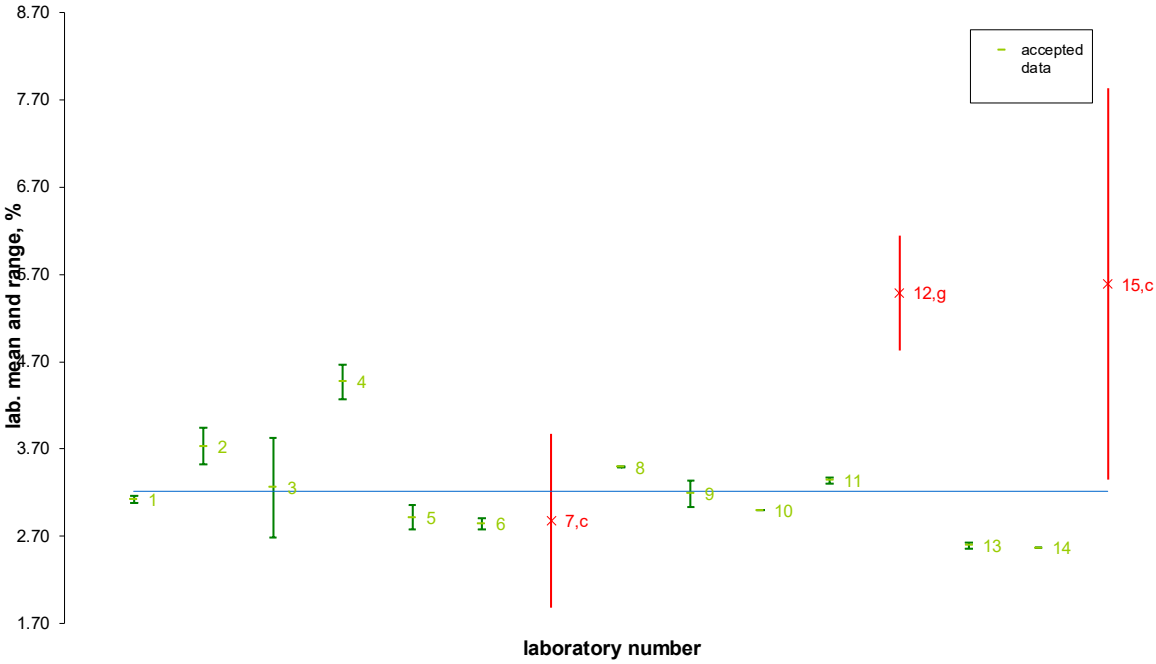

Pork in processed beef, 3% : blind replicates
lab. mean and range, %
8.17
7.17
6.17
5.17
4.17
3.17
2.17
1.17
accepted data
12,g
15,c
3,c
1
2
4
5
6
7
8
9
10
11
13
14
laboratory number

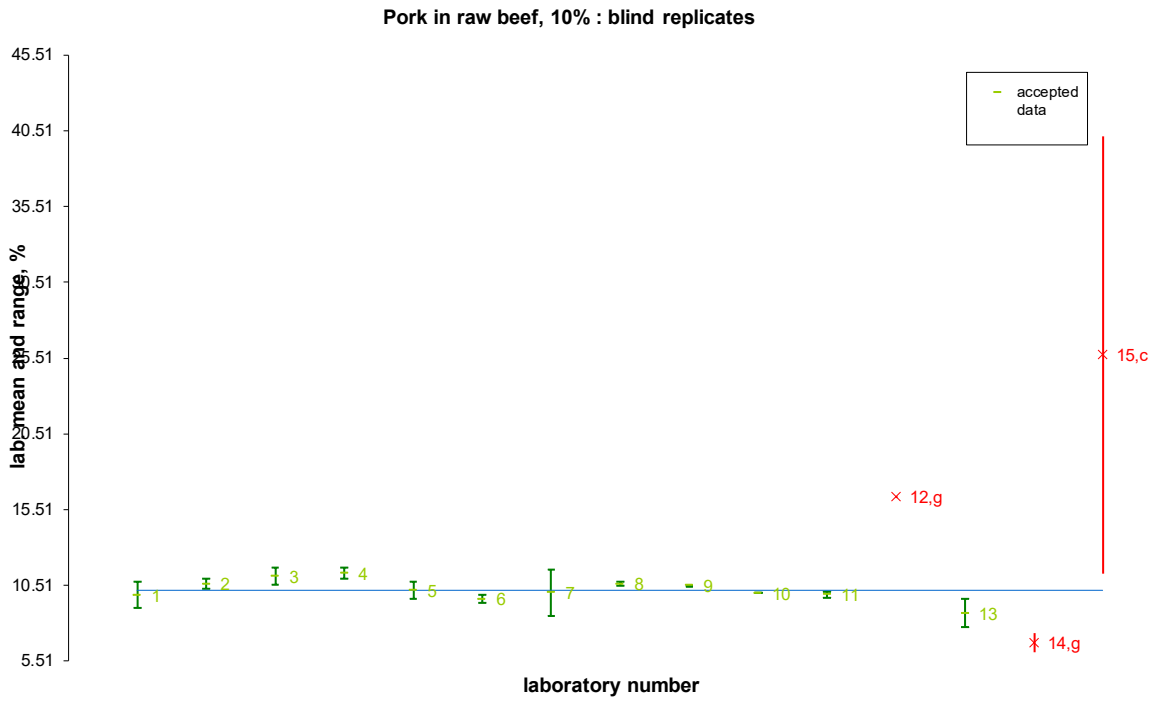

Pork in processed beef, 10% : blind replicates
lab. mean and range, %
13.71
12.71
11.71
10.71
9.71
8.71
7.71
6.71
5.71
4.71
accepted data
12,g
1
2
3
4
5
6
7
8
9
10
11
13
14
15
laboratory number


Pork in raw beef, 0.1% : blind replicates
lab. mean and range, %
0.73
0.63
0.53
0.43
0.33
0.23
0.13
0.03
accepted data
12,g
1
2
3
4
5
6
7
8
9
10
11
13
14
15
laboratory number


Pork in raw beef, 0.5% : blind replicates
lab. mean and range, %
1.90
1.70
1.50
1.30
1.10
0.90
0.70
0.50
0.30
accepted data
12,g
1
2
3
4
5
6
7
8
9
10
11
13
14
15
laboratory number


Pork in raw beef, 1% : blind replicates
lab. mean and range, %
3.52
3.02
2.52
2.02
1.52
1.02
0.52
accepted data
12,g
1
2
3
4
5
6
7
8
9
10
11
13
14
15
laboratory number


Pork in raw beef, 3% : blind replicates
lab. mean and range, %
8.70
7.70
6.70
5.70
4.70
3.70
2.70
1.70
accepted data
12,g
15,c
1
2
3
4
5
6
7,c
8
9
10
11
13
14
laboratory number


Pork in raw beef, 10% : blind replicates
45.51
40.51
35.51
15.51
10.51
5.51
lab mean and range, %
accepted data
1
2
3
4
5
6
7
8
9
10
11
12,g
13
14,g
15,c
laboratory number