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Inter-laboratory validation of a method for detecting previously frozen poultrymeat by determination of HADH activity

9th July 2013

LGC Report Number LGC/R/2013/279

Inter-laboratory validation of a method for detecting previously frozen poultrymeat by determination of HADH activity.

A report of a collaborative trial organised by the Food and Consumer Safety team at LGC.

The study was jointly funded by DG-AGRI for the European Commission [AGRI-2012-0648] and by the Department for Environment, Food and Rural Affairs (DEFRA) [FA0114] for the UK.

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Date: 09th July 2013.

Report Number: LGC/R/2013/279

This study was co-funded by the UK Department of Food and Rural Affairs and the European Commission.

Executive Summary

Poultry is a perishable product which requires careful control of temperature during production and retail processes. The EU poultry marketing legislation[1] requires that poultry be marketed either as fresh poultry or as frozen (or quick-frozen) poultry. It is not permitted to market poultry which has been frozen and thawed as fresh poultry. A robust analytical method that is capable of distinguishing between fresh and previously frozen poultry is therefore required.

A method to detect whether poultry and other meats had been previously frozen was developed in earlier work and was validated by collaborative study in the UK in 1997. The method relied on measuring the β-hydroxyacyl-CoA-dehydrogenase (HADH) activity of intracellular juice obtained from the test samples. The ratio of the HADH activity of sub-samples tested before and after laboratory freezing is compared to a reference cut-off limit to determine whether the sample has previously been frozen.

The cut-off limit calculated for chicken (0.9) was found to be too high to effectively distinguish between chilled and previously frozen poultry. Additional work was funded by the UK Foods Standards Agency with the aim of improving the method to achieve a more effective cut-off limit for chicken. LGC undertook this work and improvements were made to the method which was then applied to additional poultry samples. The method was validated at LGC and a cut-off limit of 0.5 for chicken was recommended.

The main aim of the current study was to validate the revised cut-off value for chicken via a collaborative trial. The study was conducted with two groups of participants. The first group consisted of twelve laboratories selected from the National Reference Laboratories (NRLs) for added water in poultry from EU Member States. This part of the study was funded by DG-AGRI on behalf of the European Commission. The second group consisted of twelve laboratories from Official Control (Public Analyst) Laboratories in the UK. This part of the study was funded by the UK Department for the Environment, Food and Rural Affairs (Defra). The study was planned and executed so that all laboratories performed the same tasks within the same time period, enabling the data to be combined to maximise the dataset available.

Each laboratory was asked to analyse 24 chicken breast samples and to use the results to determine the thermal history (chilled or previously frozen) according to the Standard Operating Procedure (SOP) provided. All samples were supplied as chilled blind samples.

The results of the study showed inter-laboratory variation between the results obtained for chilled and for previously frozen chicken however, despite this, almost all of the samples were correctly identified and reported in terms of their thermal history. Although some reduction in the analytical variability of the method would be beneficial, the results confirmed the suitability of the revised cut-off limit of 0.5 for the official control of chicken.

The collaborative trial was successful in validating an analytical method that is suitable for the detection of previously frozen chicken which can be used to enforce legislation relating to the marketing of chicken within the European Union. The study will be published in a peer-reviewed journal in due course but the report including the SOP is freely and publically available on the European Commission website http://ec.europa.eu/agriculture/external-studies/index_en.htm.

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Introduction

Poultrymeat is a perishable product which requires careful control of temperature during all stages of the production and retail process.

The EC Poultrymeat Marketing Standards Regulation (EEC) No 1906/90 which is now repealed and incorporated into Annex XIV Chapter B, to Council Regulation (EC) No. 1234/2007[1], so known as "Single CMO", requires that poultrymeat (whole birds and portions) is only marketed within certain conditions – fresh, frozen or quick frozen . It gives definitions for each product type and defines 'fresh poultrymeat' as that "which has not been stiffened at any time by the cooling process prior to being kept at a temperature not below – 2 °C and not higher than + 4 °C."

Fresh poultry is perceived by many consumers as a superior product and therefore can attract a price premium. However, at the point of sale, previously frozen and thawed poultry is visually indistinguishable from fresh poultry, and this could result in the customer being mislead. Regulators have reported that they cannot effectively enforce the requirements of the regulations due to the lack of a validated method.

A method which measures the activity of a mitochondrial enzyme (β-*hydroxyacyl-CoA-dehydrogenase (HADH)*) in poultry has been used in the UK to distinguish between fresh and previously frozen poultry and other meats[2]. The procedure cannot however be applied to poultry preparations where mechanical processing (mincing etc) has been used. Moreover, it cannot distinguish between chilled poultry and that which has been deep chilled rather than conventionally frozen (-12 ºC or below).

HADH is a muscle mitochondrial enzyme, which is released when the mitochondrial membranes are damaged during freezing and thawing. The HADH activity of the intracellular fluid pressed from "as received" and "laboratory frozen" sub-samples of a meat sample is determined using absorbance spectroscopy and the ratio between them can be used as an indicator of the thermal history of the sample. Data obtained by collaborative study in the UK[2] was used to develop cut-off limits for this ratio, above which the sample is deemed to have been previously frozen. The cut-off limits at 99 % confidence interval for chicken were 0.90 for chicken and 0.62 for turkey.

During a subsequent study[3], the HADH method was applied to chilled and frozen chicken and turkey which had been processed using new refrigeration technologies, to assess the extent to which the method could distinguish between these and poultry which had been conventionally chilled or frozen. As part of this work, improvements were made to the method in respect of the pressing technique to be used and to the sample dilution required before assay. Data obtained from a single-laboratory study at LGC showed that the cut-off limit specified for chicken in the previous study was too high and a new limit for the R_1 ratio of 0.5 was proposed for chicken.

The aim of the current study was to validate the improved procedure and in particular, to confirm the suitability of the revised cut-off limit ($R_1 < 0.5$) to distinguish between chilled and previously-frozen poultry via a collaborative trial.

Method Principle and Experimental Approach

HADH is a muscle mitochondrial enzyme, which is released when the mitochondrial membranes are damaged during freezing and thawing. Therefore, the HADH activity will be higher in juice obtained from samples which have been frozen compared with those that have not. The intracellular juice is extracted from the samples using a pressing technique. Determination of the HADH activity is based on the following reaction:

$$\text{Acetoacetyl-coenzyme A} + \text{NADH} + H^+ \xrightarrow{\textbf{HADH}} \beta\text{-hydroxybutryl-coenzyme A} + NAD^+$$

The HADH activity is determined by measuring the rate of decrease of NADH which is proportional to the decrease in the absorption of a sample extract at 340nm. This is monitored using a UV spectrophotometer.

In order to determine the thermal history of a single sample received by a laboratory, two sub-samples should be prepared from the meat portion. Juice is expressed from the first sub-sample immediately while the second sub-sample is stored frozen for at least two days at -18 ± 2 °C. After thawing the meat juice is expressed from the second sub-sample. The ratio (R_1) of the HADH activity for the first (original) and second (laboratory-frozen) sub-sample is then calculated. Where a sample has been stored at chilled temperatures only, the R_1 ratio will be low. A sample with an R_1 value approaching 1 is likely to have been pre-frozen. The actual R_1 value obtained should be compared with the cut-off value (threshold limit) for that particular meat type. The procedure is summarised in Diagram 1.

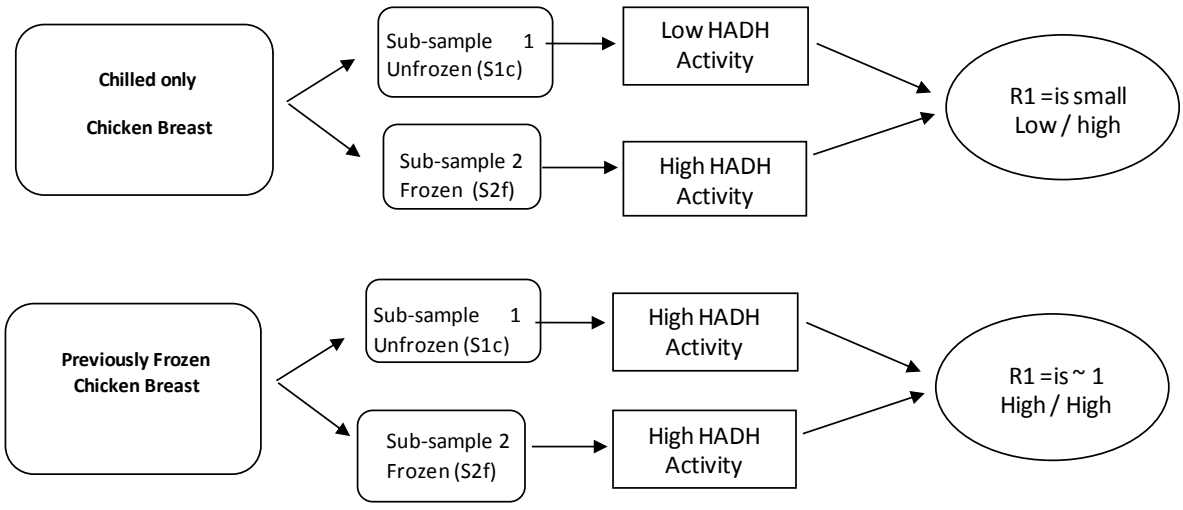

Diagram 1. Schematic of HADH method for the determination of previously frozen chicken.

Collaborative Trial Study Design

A total of twenty four laboratories were included in the collaborative trial. Originally a UK-wide study was planned using twelve UK Official Control (Public Analyst) laboratories. However, as this is an issue that affects many countries both within and outside of the European Union and there is currently no official EU method for determining whether a sample has been previously frozen, the collaborative trial was expanded to include twelve NRL laboratories from EU Member States.

Whole chicken breast fillets, because of their importance in the poultry market, were used as the test samples for the study.

A pre-trial round was conducted to enable participants to familiarise themselves with the improved analytical procedure where necessary and to demonstrate that they could obtain appropriate data. Participants purchased samples locally to use for the pre-trial stage.

For the main trial, each laboratory was provided with twelve samples of chilled chicken breasts and twelve samples of previously frozen chicken breasts which were all supplied in a chilled condition. The samples were packaged in a similar manner and the sample numbers were randomised so that it was not possible to distinguish between the samples visually. Each participant was asked to analyse the samples using the analytical protocol (SOP) provided and to report the results obtained. The laboratories were instructed to conduct the initial stages (sub-sampling) within ten days of receipt of samples to avoid any problems due to the perishability of the chicken samples.

Note: It was originally intended to analyse each chicken breast in duplicate, however it proved impossible to obtain sufficient sub-samples from commercial chicken breast portions; individual chicken breasts were therefore provided to be analysed once only (2 sub-samples were taken from each breast per assay).

Press manufacture

The press used to extract the intracellular fluid from the chicken breast is specified in the SOP**.** Although other designs may be suitable, the press design was standardised in the interests of reducing variability. The presses were manufactured by the UK National Physical Laboratory Workshops. One press was sent to each participating laboratory. The press is shown in Figure 1.

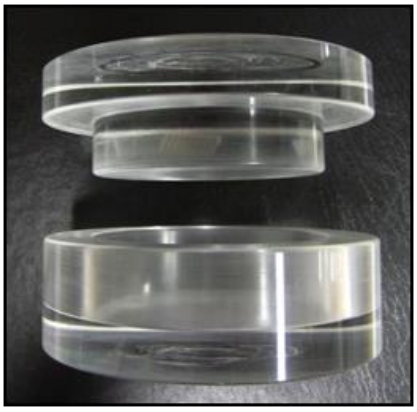

Figure 1: Press for extracting intracellular juice from chicken meat samples

Preparation of Quality Control Materials

Purified HADH enzyme is not commercially available in a suitable quantity to be used for reference purposes. Control solutions were therefore prepared from chilled and frozen chicken breast portions at LGC.

Chilled chicken breasts were purchased from a reputable retailer and were tested to check that the HADH activity was appropriate for a chilled product. Half of the samples were then frozen at approximately -18 °C for 48 h before thawing for 24 h in a refrigerator. Intracellular juice was extracted from the chilled and frozen samples and the juices for each group were pooled.

The pooled samples were partially diluted (x10) with phosphate buffer, filtered through a 0.45 µm filter under vacuum and aliquoted into eppendorf tubes. The two control solutions (one chilled & one frozen) were then stored at -80 °C until required.

The HADH activities of these solutions were determined at LGC.

Homogeneity

The HADH activity of each solution was determined using the procedure specified in the SOP and the prepared aliquots were tested for homogeneity using the procedure for "sufficient homogeneity" developed by Thomson & Fearn [5]; ten units of each solution, taken at random were thawed and analysed in duplicate using the assay procedure. The data obtained was analysed statistically and is shown graphically in Figure 2. The full data is given in Annex 1.

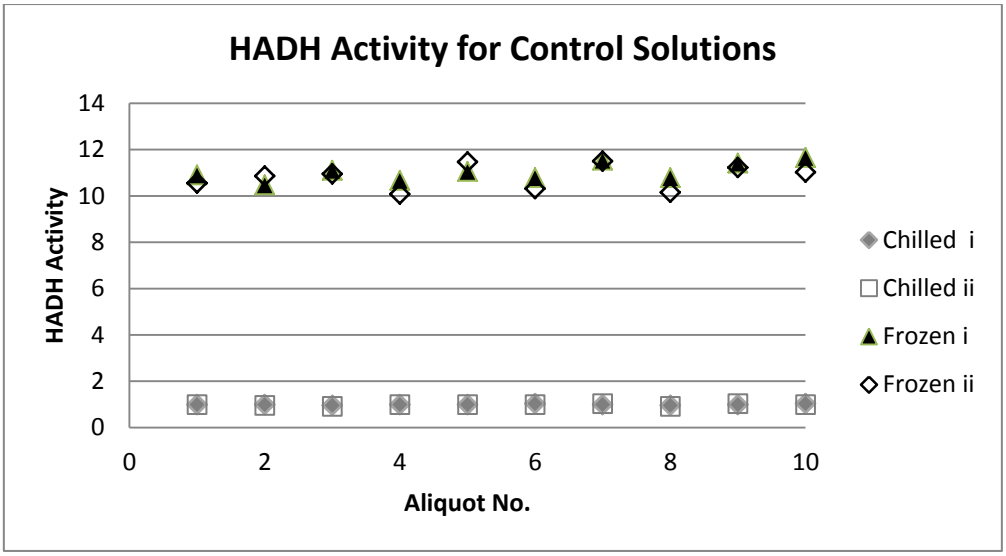

Figure 2: Homogeneity data for Control solutions.

A total of 10 aliquots were selected for each QC solution and the HADH Activity in each measured in duplicate. The duplicate results are shown (i and ii) on the graph.

The mean HADH activity for the chilled samples was 0.99 (σ = 0.036) and for the frozen sample it was 10.93 (σ = 0.468). If it is assumed that these have arisen from the same sample, the R_1 ratio produced would be 0.09 proving that the source chicken breasts were chilled.

A plot of the reaction kinetics for each sample type is shown in Figure 3; this demonstrates the small change in absorbance expected for the chilled control solution compared with the greater change in absorbance for the frozen control solution. The HADH activity is measured as the rate of decrease in the absorption of the sample extract.

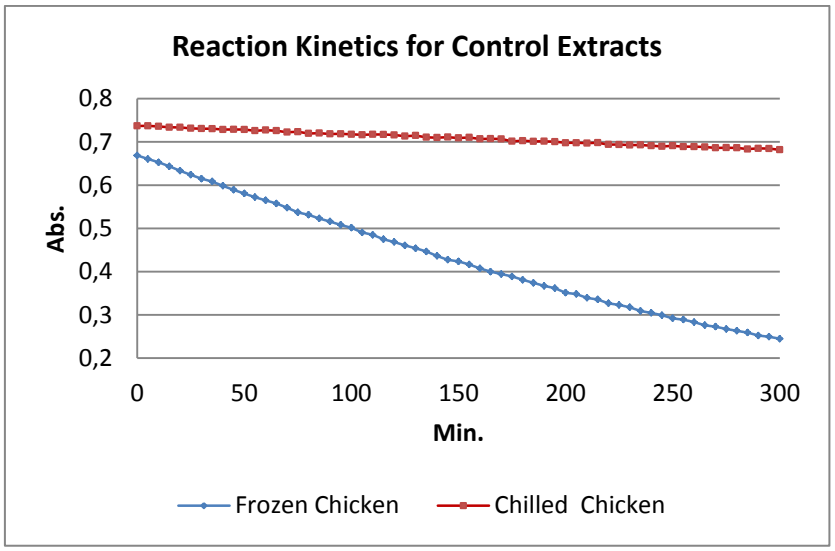

Figure 3: Reaction kinetics for the control solutions

Two aliquots of each juice were supplied to each participant for use as control solutions. Acceptability criteria were not provided but the typical values obtained at LGC for the HADH activity (and combined R_1 value) for these materials were given to the participants. The intention was to test the ability of each laboratory to determine significantly different HADH activities for the two solutions; low for the chilled solution and high for the frozen solution.

Participants

There were two groups of participants:

1. Twelve National Reference Laboratories for Poultrymeat from the EU Member States shown in Table 1. The participating Institutes are shown in Annex 2.
2. Twelve Official Control (Public Analyst) laboratories from the regions of England, Wales & Scotland shown in Table 2. Participating laboratories are shown in Annex 2.

Table 1: EU Participants

Country
Austria
Belgium
Denmark
Italy
Ireland
Lithuania
The Netherlands
Poland
Slovenia
Spain
Sweden
United Kingdom

Table 2: UK Participants

Region
Cardiff
Cardiff
Edinburgh
Glasgow
Hampshire
Kent
Lancashire
Norwich
Staffordshire
Swansea
West Yorkshire
Worcester

Pre-Trial

A pre-trial was held to ensure that all participants were familiar with the analytical procedure and to demonstrate that they could obtain the expected values for chilled and frozen chicken and for the control solutions.

Participants were asked to obtain samples of chilled and frozen chicken from local sources and to carry out the procedure, in duplicate on these samples. Control solutions were provided for reference.

Each laboratory was provided with a press, a copy of the SOP, instructions for the pre-trial and a results sheet. Discussions were held with individual laboratories where required, to resolve any analytical issues or queries. The study documents are shown in Annex 3.

The pre-trial results are shown graphically in Figures 4 to 9 and are presented in Annex 4. For each sample there are two results for HADH activity; the value obtained from the first sub-sample and the value obtained from the second sub-sample after a laboratory freezing step. The R_1 ratio is calculated by dividing the activity measured in the first sub-sample by the activity measured in the second sub-sample.

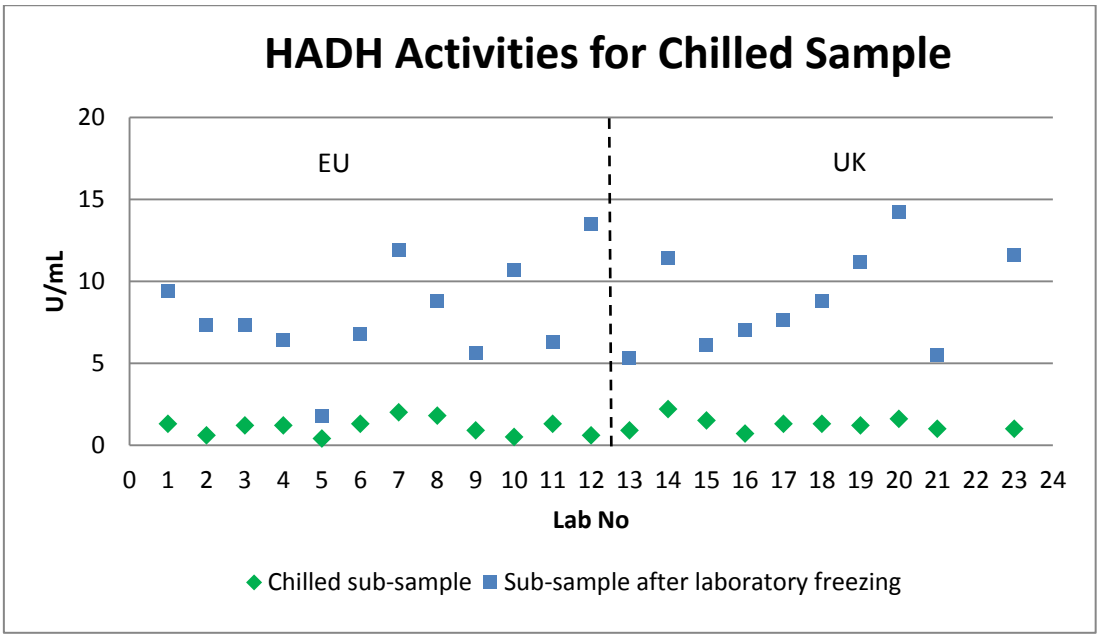

Figure 4: Reported HADH activity for chilled sample

The green symbol shows activity measured in juice from the first sub-sample and the blue symbol shows activity measured in the second, laboratory frozen, sub-sample.

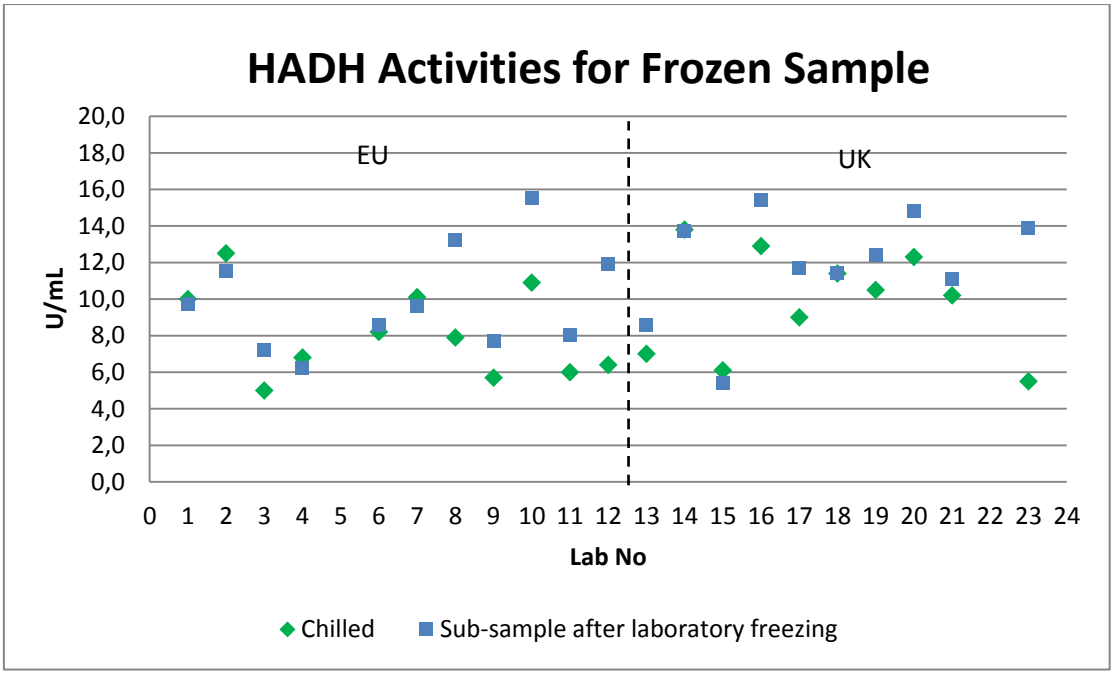

Figure 5: Reported HADH activity for frozen sample.

The green symbol shows activity measured in juice from the first sub-sample and the blue symbol shows activity measured in the second, laboratory frozen, sub-sample.

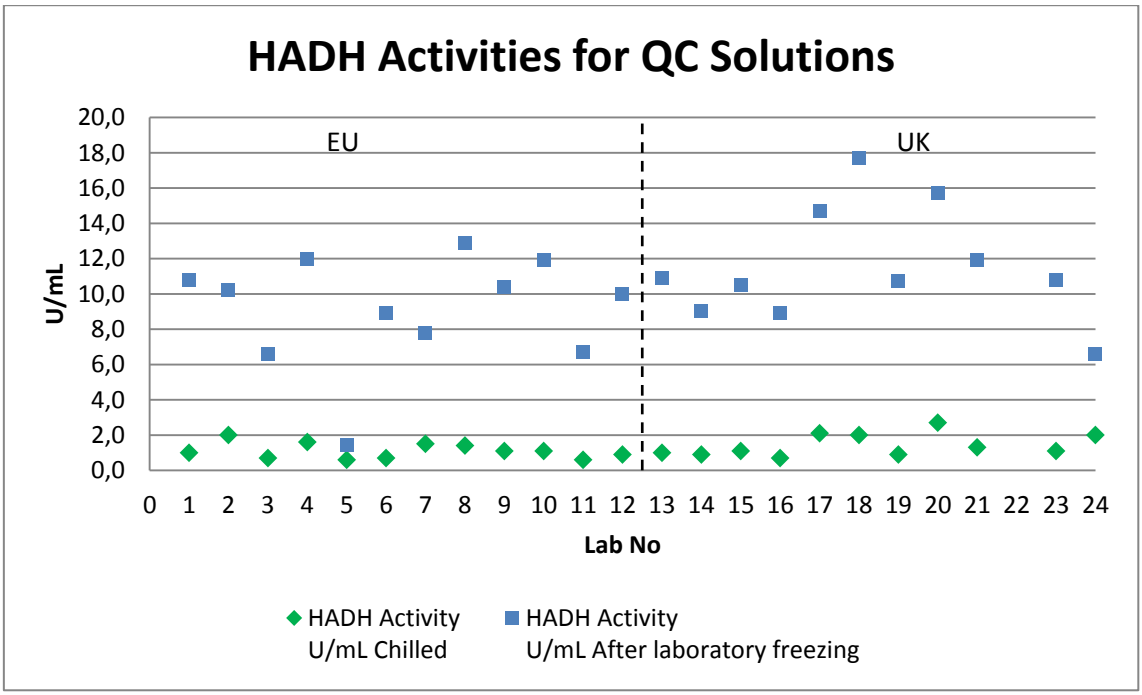

Figure 6: Reported HADH activity for QC solutions

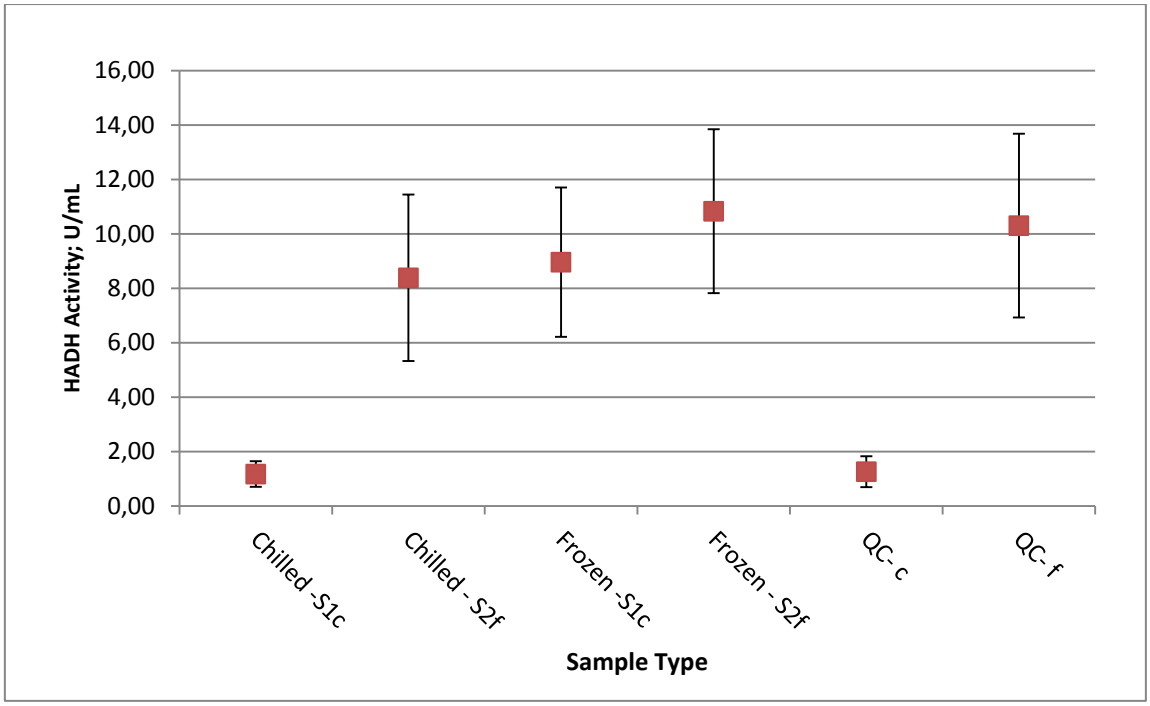

Figure 7: Inter-laboratory mean and standard deviation (±1SD) for the pre-trial HADH Activity data.

Sample S1c is the first sub-sample and S2f the second sub-sample from each chicken breast.

For the chilled chicken samples (Figure 4), there was some variation in the HADH activities obtained from pressed solutions however, with the exception of Lab 5, there was a clear separation between the first and second (laboratory frozen) sub-samples.

For the frozen (when purchased) samples (Figure 5), there was again a similar variation in the data but as expected, the data for the two sub-samples were interspersed. In just under half of the laboratories, the activity for the laboratory-frozen sample was higher than the activity for the samples analysed when first thawed (i.e. the sub-sample analysed "as received"). This indicates some additional cell damage occurred during the second freezing step which has resulted in a reduction in the R_1 ratio obtained for these laboratories. In other laboratories, a similar value was obtained for both sub-samples as is expected for frozen samples. The cause of this is unknown but it is thought that perhaps the freezing conditions or temperatures used in some laboratories are more vigorous than those used commercially leading to greater cell damage and correspondingly higher HADH activity in the laboratory frozen sub-sample. This issue has contributed to the variability seen in this study. Lab 23 had the greatest difference between the first and second sub-sample from the frozen chicken which resulted in an artificially low R_1 value and misclassification of the sample as fresh.

For the two QC solutions (Figure 6), there was a similar pattern of variation and the two groups (chilled and frozen) were again separated as expected with the exception of Laboratory 5 who reported a low value for both QC solutions.

The inter-laboratory mean and standard deviation for the whole dataset is shown in Figure 7. These data reflect the within laboratory data and show higher variability for the frozen

samples or sub-samples. However, the HADH activities for chilled and frozen samples (as purchased) and QC solutions are all clearly separated, with the exception of Lab 5, demonstrating the laboratories ability to correctly execute the method. This is further illustrated in Figures 8 to 9.

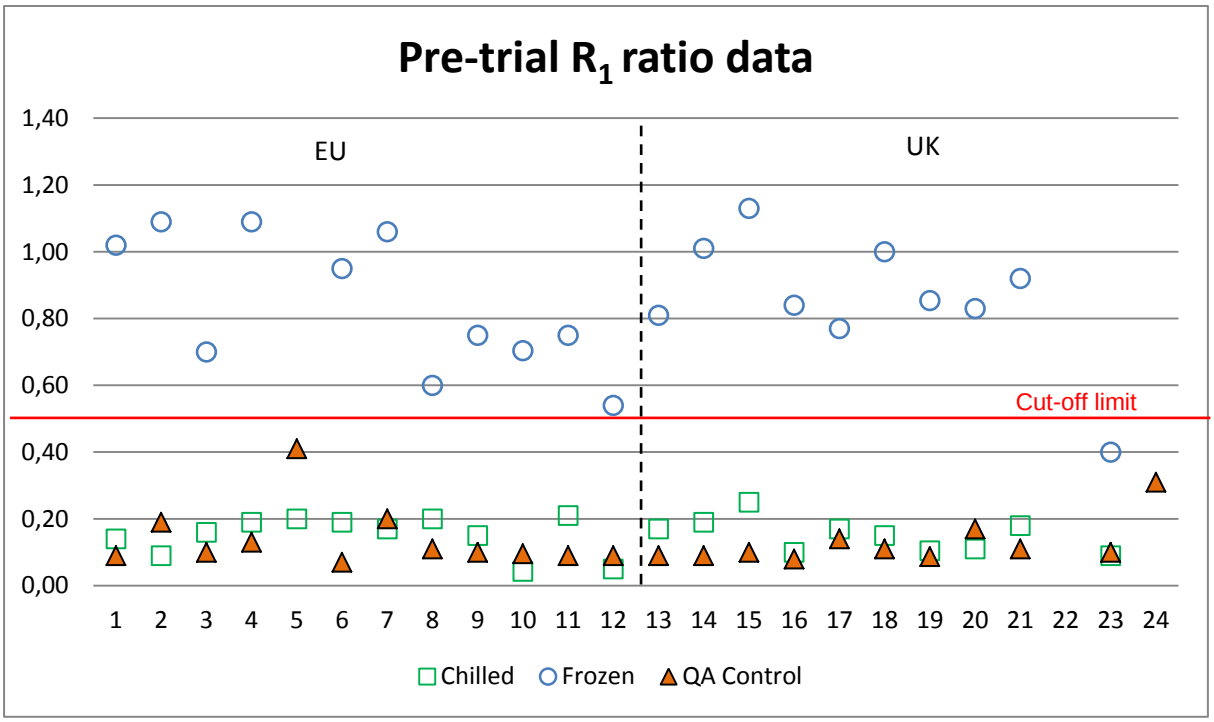

Figure 8: R_1 ratio data for chilled & frozen/thawed samples and QC solutions.

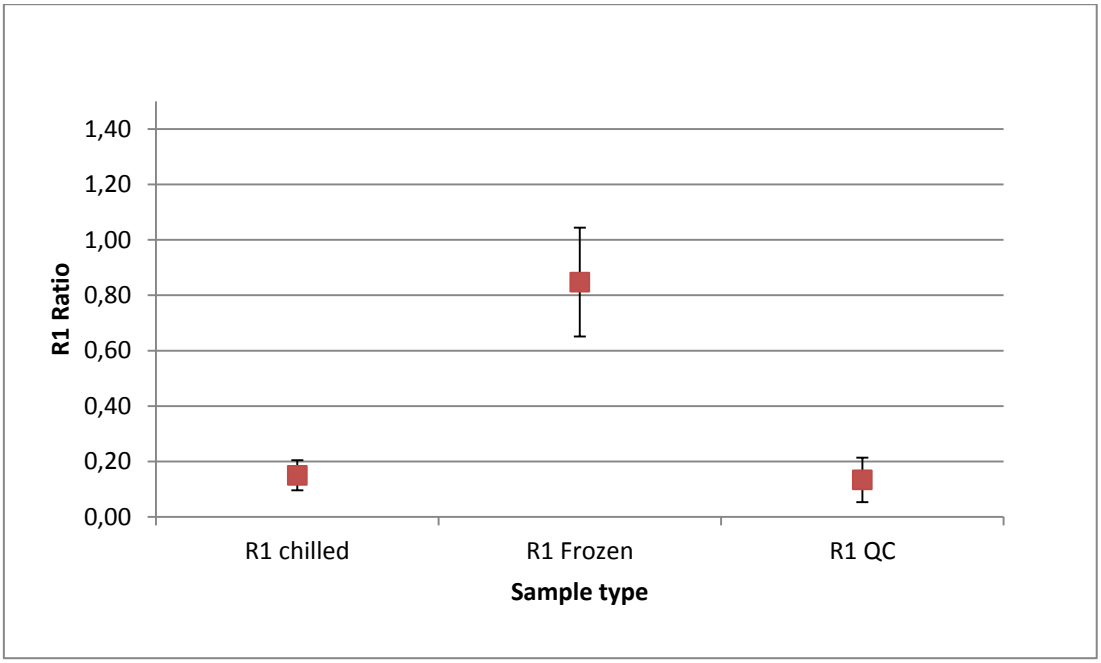

Figure 9: *Inter-laboratory mean and standard deviation (±1SD) for the pre-trial R1 ratio data.*

R_1 ratios

The R_1 ratios reported are shown in Figures 8 & 9.

Despite the low HADH activities reported by Lab 5 for the chilled chicken sample, the R_1 ratio for the chilled chicken sample was acceptable correctly identifying the sample as chilled chicken. The R_1 value reported by laboratory 5 for the QC solution was high compared to the majority of laboratories but once again it correctly identified that the QC solutions had been prepared from chilled chicken breasts. Laboratory 5 did not report data for a frozen sample. Laboratory 24 also reported a high QC value but again it correctly identified that the QC solutions had been prepared from chilled chicken breasts.

There was a reasonably high inter-laboratory variability, but with the exception of one laboratory (23), the thermal history of the samples was correctly identified using the revised cut-off limit of 0.5 for chicken.

Two laboratories had technical issues with the analysis and only returned partial data. One laboratory failed to return any data in the allotted timeframe but had previous experience of the procedure and therefore was retained for the main trial.

Analytical problems and outlying results were discussed with the laboratories concerned and resolved where possible although in most cases, it was not possible to identify any specific problems. In view of this and that the QC results returned by laboratories were as expected, all of the laboratories were retained for the main trial.

Main Trial

Sample Collection

Samples of chicken breast were supplied by a major UK poultry producer (Bernard Matthews Limited) whose assistance is gratefully acknowledged. All critical steps in preparing the samples were witnessed by LGC staff.

The previously frozen samples were prepared as follows: 288 samples of chicken breasts were taken from a commercial poultry slaughterhouse on the 9th April 2013. The samples were placed into individual, heat-sealed polythene bags together with a moisture absorbing mat. The samples were then frozen using a commercial blast freezing process and stored in a freezer at -18 °C for six days. The samples were removed from the freezer on the morning of the 15th April 2013 and stored overnight in a chilled room at approximately 10 °C.

A further 288 samples of chicken breasts were taken from the same slaughterhouse on the 15th April 2013. These samples were placed into individual, heat-sealed bags together with a moisture absorbing mat identically to the previously frozen samples. The samples were stored overnight in a chilled room at approximately 10 °C.

On the following day, the two groups of samples were divided into 24 sets each containing 12 chilled and 12 frozen & thawed samples. These were randomly labelled so that the participants would be unable to distinguish between the samples, either by visual assessment or by number.

The samples were packed into insulated boxes for distribution under the supervision of LGC staff.

Sample Distribution

The samples for UK destinations were distributed to all of the participants using the UK poultry producer's chilled distribution vehicles. Samples were delivered within 24 h at a temperature of approximately 4 °C.

The samples for the non-UK laboratories were distributed using a chilled distribution carrier specialising in logistics for the life science and pharmaceutical industries. Samples were packed into cooled, insulated boxes containing a phase-gel coolant (this was not in direct contact with the samples). A temperature monitor was included in each box. The samples were distributed by air & road within 24-48 h of packing. Temperatures were monitored to ensure that they remained chilled without freezing. The laboratories confirmed that the temperature monitors typically showed a temperature around 5 °C on receipt at the destination.

Participants were instructed to place the samples in a refrigerator on receipt and to carry out the initial sub-sampling within 10 days of receipt.

Study Instructions

The participants were provided with study instructions and a results sheet for the main trial. The press, SOP and the QC control solutions were provided during the pre-trial stage. A copy of the main trial documentation is shown in Annex 5.

Results and Discussion

All of the participating laboratories returned data for the main trial. (The closing date for submission of data was the 10th May; most of the data was returned before this but the final set of data was received on the 15th May). All of the data was accepted. The full data are shown in Annex 6**.**

Each laboratory had calculated the R_1 ratio data for each sample. The R_1 value for the chilled chicken, frozen chicken and the QC solutions are shown graphically in Figures 10, 11 & 12. The graphs show all of the data reported before any removal of outliers.

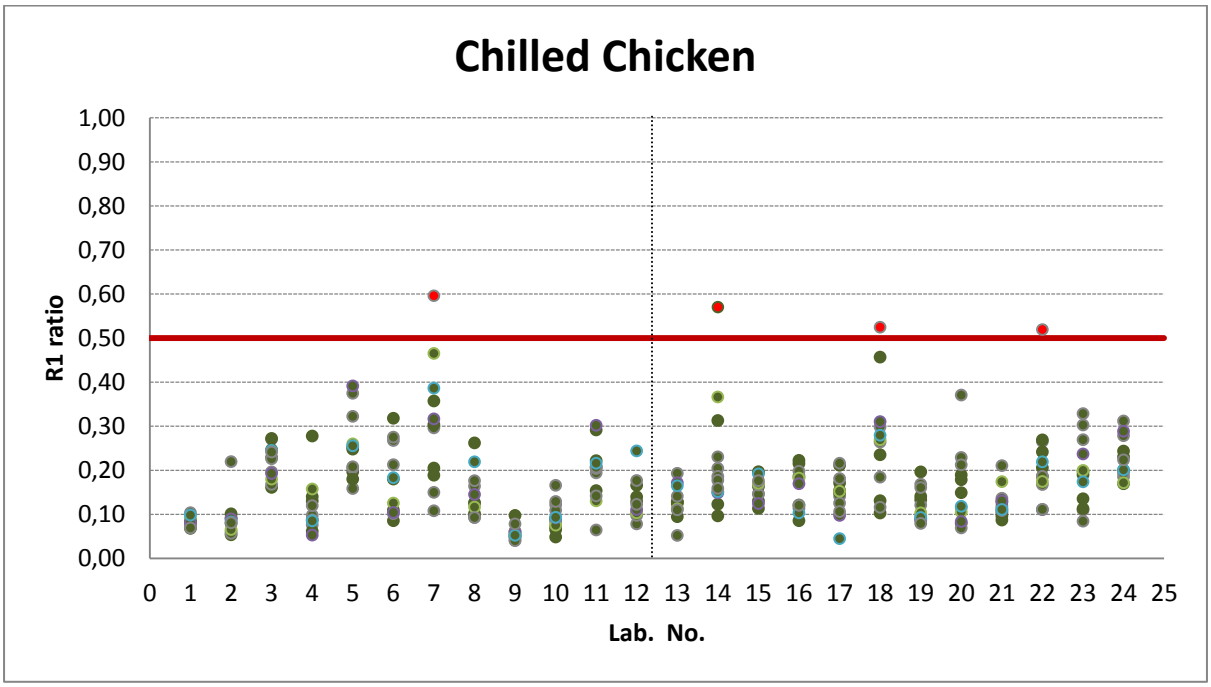

Figure 10: Study data (R1 ratio) for Chilled chicken.

Laboratories 1-12 are EU laboratories and 13-24 are UK laboratories.

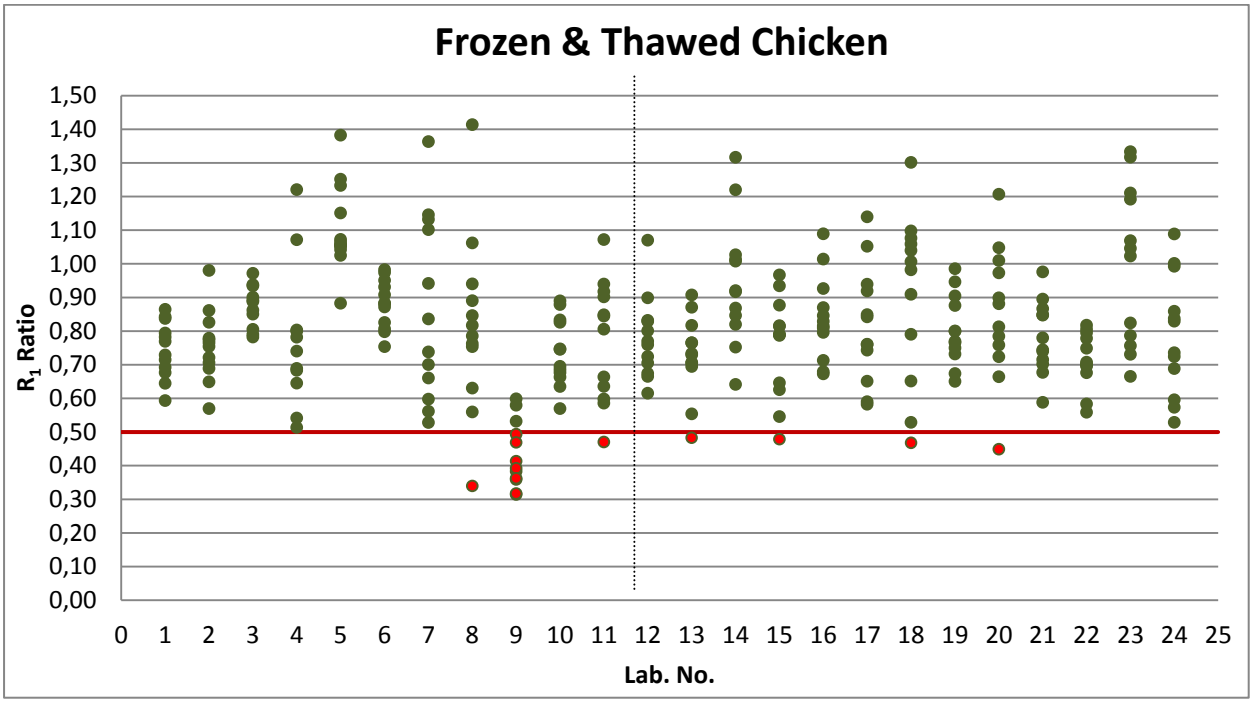

Figure 11 Study data (R1 ratio) for frozen & then thawed chicken

Laboratories 1-12 are EU laboratories and 13-24 are UK laboratories.

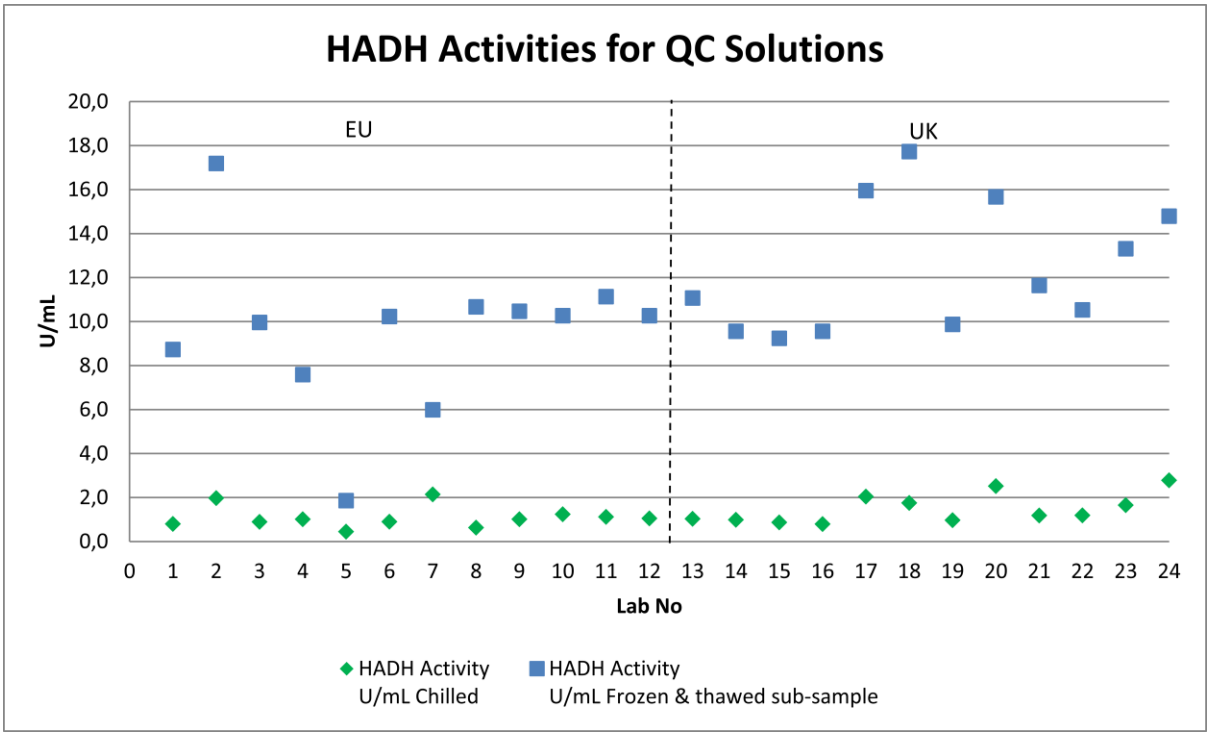

Figure 12 Study data for chilled and frozen QC solutions

The majority of the laboratories reported QC data that was around the expected value. The mean and standard deviation of these data were 1.3 ± 0.6 for the 'chilled' solution and 11.0 ± 3.5 for the 'frozen' solution. This demonstrated the laboratories' ability to execute the HADH assay procedure correctly.

As shown in Figure 10, the data for the chilled chicken shows some variation (both within and between laboratory), the extent of which differed between laboratories (see Figure 13). However with the exception of four results, all of the data was below the proposed cut-off limit of 0.5 for chicken. The failure rate i.e. the percentage of chilled chicken which were mis-identified as previously frozen using all of the data was 1.4 % (4 out of 288 samples). However, the four laboratories concerned were all removed as statistical outliers by application of the Cochran's test. (The full statistical evaluation of the data is shown in Annex 7). When these values are excluded, all of the chilled data were below the cut-off limit.

The data for the frozen chicken (Figures 11 & 13) showed higher variability which again varied between laboratories. Excluding the data from three laboratories whose results were identified as outliers using Grubb's tests, the failure rate (frozen samples incorrectly identified as fresh) using the proposed cut-off limit of 0.5 for chicken was 2.1 % (6 out of 288).

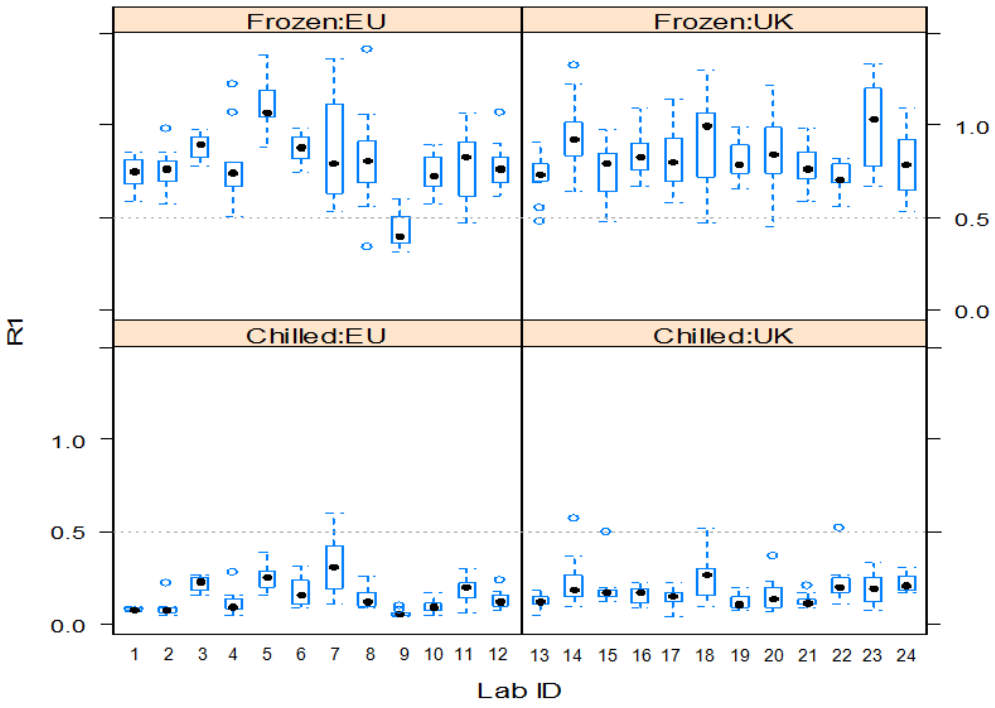

Figure 13: Boxplots of Study data (R1 ratio) grouped by Study group and sample type.

The means and standard deviations for all laboratories are shown in Table 3 below.

Table 3: Within Laboratory Mean, Standard deviation for study data (R1 ratio)

Lab	Group	Type	Mean	SD	Type	Mean	SD
1	EU	**Chilled**	0.09	0.011	**Frozen**	0.74	0.084
2	EU	**Chilled**	0.09	0.044	**Frozen**	0.76	0.105
3	EU	**Chilled**	0.22	0.039	**Frozen**	0.88	0.064
4	EU	**Chilled**	0.12	0.061	**Frozen**	0.77	0.211
5	EU	**Chilled**	0.26	0.072	**Frozen*g**	1.11	0.130
6	EU	**Chilled**	0.18	0.079	**Frozen**	0.88	0.072
7	EU	**Chilled *c**	0.33	0.150	**Frozen**	0.86	0.273
8	EU	**Chilled**	0.14	0.054	**Frozen**	0.82	0.264
9	EU	**Chilled**	0.06	0.017	**Frozen*g**	0.43	0.099
10	EU	**Chilled**	0.10	0.032	**Frozen**	0.74	0.101
11	EU	**Chilled**	0.19	0.067	**Frozen**	0.77	0.179
12	EU	**Chilled**	0.13	0.047	**Frozen**	0.78	0.122
13	UK	**Chilled**	0.13	0.037	**Frozen**	0.73	0.121
14	UK	**Chilled *c**	0.23	0.133	**Frozen**	0.95	0.189
15	UK	**Chilled *c**	0.19	0.100	**Frozen**	0.76	0.150
16	UK	**Chilled**	0.16	0.047	**Frozen**	0.84	0.126
17	UK	**Chilled**	0.15	0.049	**Frozen**	0.82	0.174
18	UK	**Chilled *c**	0.26	0.128	**Frozen**	0.91	0.251
19	UK	**Chilled**	0.12	0.038	**Frozen**	0.81	0.106
20	UK	**Chilled**	0.16	0.085	**Frozen**	0.85	0.200
21	UK	**Chilled**	0.13	0.034	**Frozen**	0.78	0.109
22	UK	**Chilled *c**	0.22	0.107	**Frozen**	0.72	0.084
23	UK	**Chilled**	0.20	0.079	**Frozen*g**	1.00	0.236
24	UK	**Chilled**	0.22	0.048	**Frozen**	0.79	0.179

*c Laboratory identified as outlier by Cochran's test and removed from the "chilled" dataset.
*g Laboratory identified as outlier by Grubbs tests and removed from the "frozen" dataset

The data was evaluated statistically according to ISO 5725-3: 1994[6], which require the removal of statistical outliers (identified as such with 99% confidence). To prevent excessive removal of outliers the number of outliers removed was restricted in accordance with IUPAC guidelines[7]. The parameters calculated for the analytical method are shown in Table 4. The mean of the data for fresh chicken was 0.148 ± 0.073 and 0.807 ± 0.168 for frozen chicken was 0.807 ± 0.168. The full statistical analysis is available in Annex 7.

Table 4: Analytical validation parameters after statistical evaluation.

Type	Estimate of mean	s_{Lab}	s_r	S_{R1}	S_{R12}	RSD_r	RSD_1	RSD_{12}
Chilled	0.148	0.050	0.053	0.073	0.052	0.359	0.491	0.350
Frozen	0.807	0.042	0.163	0.168	0.063	0.202	0.208	0.078

Where S_{lab} *= between-laboratory*

S_r *= within-run repeatability for single observations*

S_{R1}*= reproducibility SD for single observation*

S_{R12}*= reproducibility SD for mean of 12 observations*

RSD_r *=Repeatability relative standard deviation*

RSD_1 *=Relative standard deviation for single observation*

RSD_{12} *= Relative standard deviation for mean of 12 observations*

The results from the UK laboratories, many of whom had previous experience with the method, were compared with the results from the EU laboratories. There was no significant difference between the data produced from the two sets of laboratories. The data is shown in Figure 14 below.

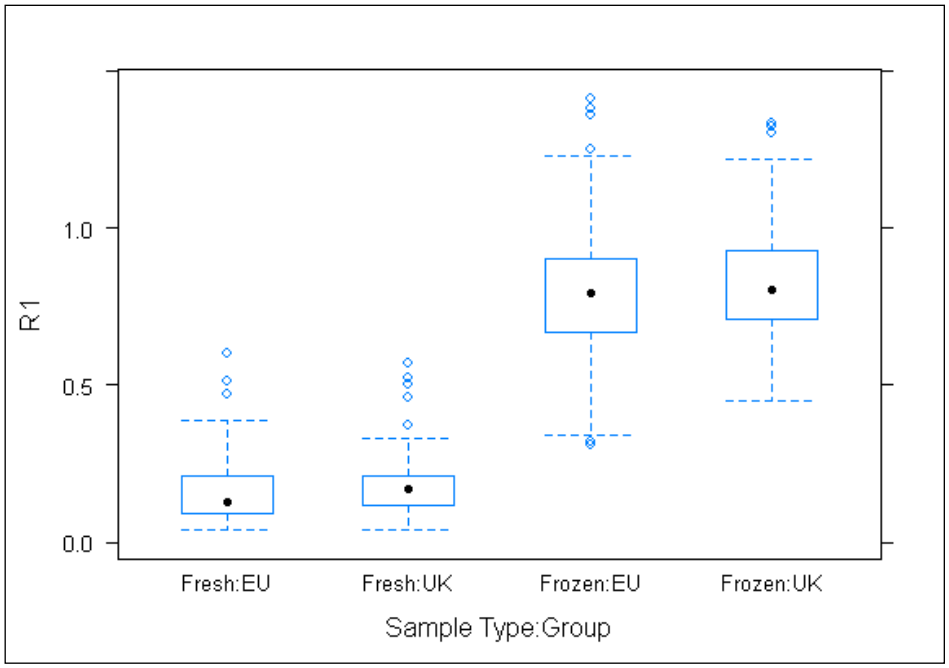

Figure 14: Comparison between UK and EU data, grouped by sample type.

Method variability

The % relative standard deviation (RSD) obtained by LGC for chilled chicken was 12.9 % and that for frozen chicken was 11.3%. For all remaining laboratories (including outliers), the within-lab % RSD's for chilled chicken ranged from 17.7 to 58.3 % and those for frozen & thawed chicken ranged from 7.3 to 32.3 %. This variability could arise from a number of sources as shown in Figure 15.

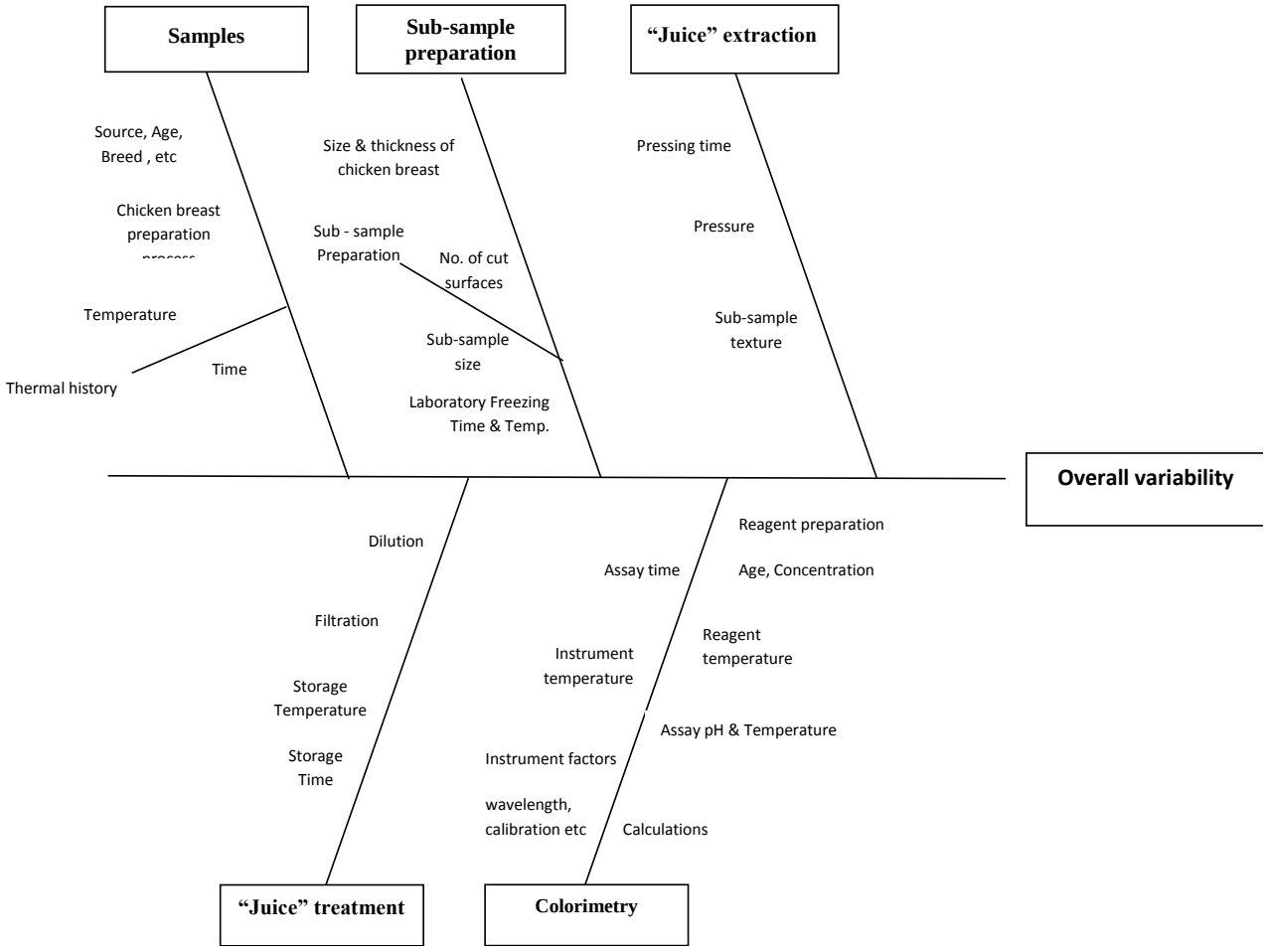

Figure 15: Potential sources of variation in the HADH procedure

As far as possible, these factors were standardised in the SOP but it is clear that significant variability remains. Although the method is fit for purpose in its current state, a reduction in the variation obtained would be beneficial. It is difficult to determine which of these parameters are most important but two crucial areas are the laboratory freezing step and the ease with which juice can be extracted from the sub-samples. Some laboratories reported quite large differences between the HADH activities of samples frozen only once (i.e. previously frozen samples) and those frozen twice (i.e. sub-sample 2 for previously frozen samples). This would reduce the R_1 ratio. Some participants also reported difficulty in

extracting enough juice from a small number of samples. These issues can be addressed by:

1) Specifying a temperature range for laboratory freezing e.g. -18 °C to - 22 ºC
2) Preparing a training video covering key aspects of the method including the pressing stage.

Laboratories also varied in the temperature and time of storage of diluted, filtered juice before analysis. This may also be a source of variation although this has not been found to be so in previous studies at LGC. These areas would warrant further investigation however, despite this, the method is still fit for purpose as demonstrated by the fact that almost all of the chilled and frozen chicken samples were correctly identified by participating laboratories.

Cut-off value

An aim of this collaborative study was to validate the use of a lower cut off limit for differentiating between fresh chicken and chicken which had been frozen. The original cut-off value (0.9) was considered too high.

The dataset (once outliers had been removed) was analysed to compare the number of samples correctly identified using the current cut-off value compared with the new limit. The fraction of samples expected to exceed the current limit (0.5) found in this study, was estimated from the percentages of the normal distribution with the mean and standard deviation. The number of samples that would be correctly identified as frozen using the new limit was 96.6 % compared with only 29 % using the previous limit (0.9). This is based on a single observation. If the mean of three observations were used, 99.9 % of frozen samples would be correctly identified using the new limit. This is illustrated in Figures 16 and 17. Using this analysis all of the chilled chicken samples would be correctly identified as fresh using either limit.

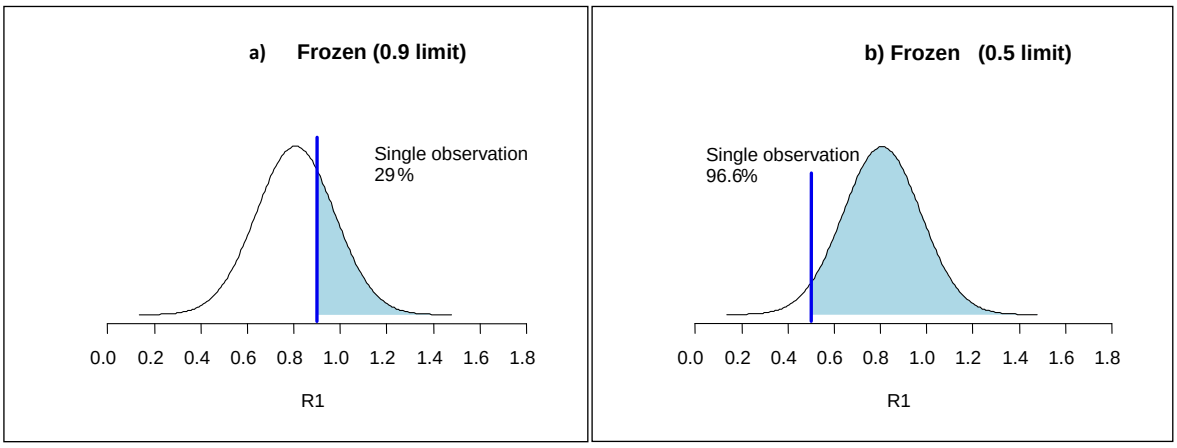

Figure 16: The fraction of test samples above a) the previous (0.9) and b) the proposed (0.5) cut-off limit for identifying previously frozen samples based on a single observation.

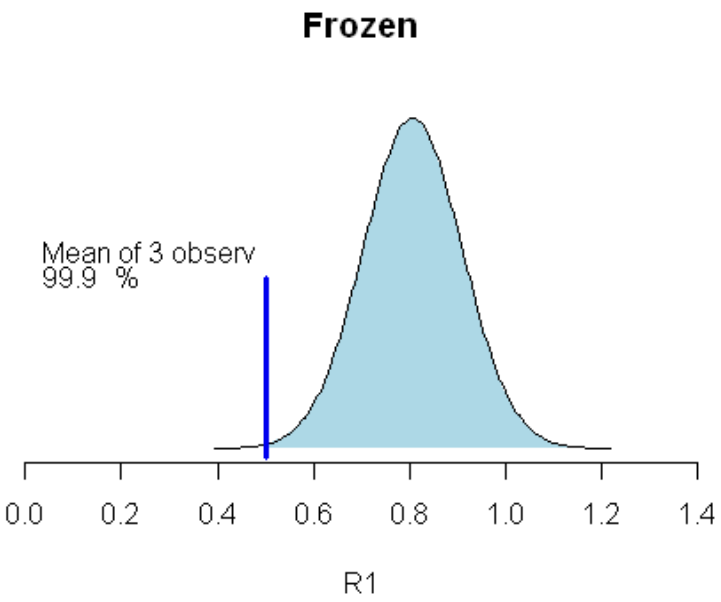

Figure 17: The fraction of test samples above the proposed cut-off limit (0.5) for identifying previously frozen samples based on the mean of 3 observations.

Study conclusions

The method was successfully transferred to all 24 laboratories, which included some laboratories which had no previous experience of the method.

The majority of samples were correctly classified as either chilled or previously frozen despite there being analytical variability in results reported by different laboratories. Some improvement in the analytical variability would be beneficial but is not essential as the performance achieved in the collaborative trial has shown that the method is fit for the purpose of identifying previously frozen chicken to a high degree on confidence.

The results of the study show that the procedure is suitable to distinguish between chilled and pre-frozen chicken breast meat using the proposed cut-off limit of $R_1 = 0.5$. If the mean of 3 observations are used then 99.9 % of frozen samples will be correctly identified using this limit. On a practical level, this means analysing three individual chicken breasts from a batch.

Recommendations

1. The R_1 ratio cut-off limit is used to determine whether samples of chicken breast meat have been previously frozen. The proposed limit of 0.5 for chicken breast meat has been shown to be fit for purpose and should be officially adopted.

2. The method is suitable to enforce the provisions of the EU poultry marketing legislation. A European Union inter-laboratory validated method offers a very powerful tool to regulators and allows effective enforcement of the requirements in Annex XIV, Chapter B of Council Regulation (EC) No 1234/2007 by detecting possible fraud in thawed poultry meat.

   The consistent application of the improved HADH method across Europe will offer protection to consumers and legitimate traders in the EU against the illegal practise of selling previously frozen poultry as ‘fresh’.

   We recommend that method described in this report and as presented in Appendix 1 is adopted as an approved procedure for the official control of poultry breast meat.

Acknowledgements

This study was jointly funded by the European Commission and by the UK Department for the Environment, Food and Rural Affairs (defra). The Steering Committee of DG-AGRI, headed by Mrs Stefania Marrone and Mrs Theresa Ekong of Defra are thanked for their support and assistance.

Additional thanks are due to:

- Mr K Burgon of the manufacturing facility, UK National Physical Laboratory, Teddington for the production of the sample presses.
- Mr Jeremy Hall and Mr Gary Smith of Bernard Matthews Limited for their help and assistance with the procurement and distribution of the chicken breast samples for this study.
- Biocair Ltd for their assistance with the European distribution.
- Colleagues in the Food and Consumer Safety team at LGC, particularly Magdalena Mazur and Stephen Taylor for technical assistance
- Jesus Minguez and Steve Ellison from the LGC statistics team for advice regarding the trial design and for performing the statistical analysis of the data.
- Staff and analysts of all participating laboratories for their efforts in this study.

References:

1. Annex XIV, Chapter B of COUNCIL REGULATION (EC) No 1234/2007 of 22 October 2007, establishing a common organisation of agricultural markets and on specific provisions for certain agricultural products (Single CMO Regulation). (OJ L 299, 16.11.2007, p. 1)

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3. Lawrance P, Woolfe M, Tsampazi C; *"The Effect of Superchilling and Rapid Freezing on the HADH Assay for Chicken and Turkey"*; Journal of the Association of Public Analysts (Online), 2010, 38, 13-23.

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5. Fearn T, Thompson M; *"A new test for 'sufficient homogeneity'*, Analyst, 2001, 126, 1414–1417.

6. ISO 5725:-3: 1994 Part 3 Intermediate measures of the precision of a standard measurement method ISO, Geneva, (1994)

7. Horwitz, W. "Protocol for the design, conduct and interpretation of method performance studies." Pure & Appl. Chem. 1995, 67, 331-343

Appendix 1

Method for the detection of previously frozen poultrymeat by determination of HADH activity

1 Scope and field of application

This procedure can be used to determine whether poultry breast meat has been previously frozen and can be used to verify the labelling of poultry breast meat sold as chilled poultry.

When meat is frozen and thawed, the muscle mitochondria are damaged and the HADH enzyme is released into the intracellular fluid. The relative increase in the amount of HADH found in fluid pressed from a sample before and after laboratory freezing can be used to indicate whether it has been previously frozen. The HADH activity is determined using a spectrophotometric procedure.

This protocol is specific for chicken and turkey breast meat but may be used for other cuts and/or species with appropriate limit values. Additional validation may be required. The method is not applicable to minced meat or to poultry preparations.

2 Definitions

HADH activity is expressed in International Units per mL of meat press juice (U/mL). 1 U represents 1 micromole of substrate converted per minute at pH 6.0 and 25 °C.

The ratio R_1 is the ratio of the HADH activities obtained for samples analysed before (X_0) and after (X_1) a laboratory freezing step. $R_1 = X_0/X_1$.

3 Principle

Two sub-samples are prepared for each poultry breast sample using the procedure described. The first sub-sample is analysed directly and the second sub-sample is analysed after freezing for at least 2 days at -18 °C ± 2 °C. After thawing, where required, the meat juice is expressed from the sample and diluted with a phosphate buffer.

Determination of the HADH activity is based on the following reaction:

$$\text{Acetoacetyl-coenzyme A} + \text{NADH} + H^+ \xrightarrow{\textbf{HADH}} \beta\text{-hydroxybutryl-coenzyme A} + NAD^+$$

The HADH activity is measured by the rate of decrease in the absorption of a treated sample extract at 340 nm using a UV spectrophotometer. The ratio (R_1) of the HADH activity for the original and laboratory-frozen sub-samples is then used to determine whether the meat sample has previously been frozen by comparison against threshold limits.

4 Health and Safety

There are no particular hazards associated with this procedure however general laboratory precautions such as the use of eye protection and other appropriate protective work wear should be applied. Care should be exercised in handling knives or scalpels and other equipment and chemicals. Suitable procedures should be in place for the disposal of poultry after use.

5 Equipment required.

5.1 Meat press: The meat press consists of a perspex container comprising a circular base unit and lid, and a G clamp. Details are shown in Appendix 1A. These presses are not commercially available but can be manufactured to the specifications shown by an appropriate, engineering facility.

5.2 Knife or scalpel: A sharp kitchen knife is required to cut sub-samples from the poultry breasts. Although a laboratory scalpel can be used, a longer blade enables the samples to be cut more easily.

5.3 Ruler: *A plastic or metal ruler is used to measure the sub-samples.* (*If a lot of samples are being analysed, a 30 mm template can be constructed from stainless steel or similar material*)

5.4 Petri dishes: Plastic, petri dishes or similar containers are required to catch the drip from the pressed samples.

5.5 Volumetric flasks, 25 mL.

5.6 Pasteur pipettes, *plastic or glass.*

5.7 Syringe filters, *PVDF or nylon, 25 mm, 0.45 µm.*

5.8 Plastic syringes.*5 mL*

5.9 Automatic pipettors, (50 µL – 5 mL)

5.10 Timer. *Capable of timing 3 minutes*

5.11 Spectrophotometer: Capable of measurement at 340 nm. (If possible, the spectrophotometer cell should be temperature controlled at 25 °C, however this is not vital in normal laboratory operating temperatures as solutions are attemperated before use and the measurement time is short.

5.12 Plastic or glass cuvettes, 1 cm x 1 cm

5.13 Analytical balance. *Capable of weighing to at least 2 decimal places.*

5.14 Water bath. Capable of maintaining 25 °C ± 0.5 °C.

6 Reagents

Laboratory reagent grade (GPR) or analytical reagent grade (AR) reagents are suitable unless otherwise stated. Water should be de-ionised or of similar quality.

6.1 Potassium dihydrogen orthophosphate (KH_2PO_4), 0.1 M solution.
Weigh 13.6 ± 0.1 g into a volumetric flask and make to 1 L with water.

6.2 Dipotassium hydrogen orthophosphate trihydrate ($K_2HPO_4.3H_20$), 0.1 M solution.
Weigh 22.8 ± 0.1 g into a volumetric flask and make to 1 L with water.

6.3 Potassium phosphate buffer solution, pH 6.0 ± 0.05

To 1 L of potassium di-hydrogen phosphate solution, (6.1.1), slowly add the dipotassium hydrogen phosphate solution (6.1.2) until a pH of 6.0 ± 0.05 is obtained using a pH meter. The solution can be stored under refrigeration (approximately 4 °C) for at least one month. The pH should be checked before use.

6.4 Ethylenediamine tetraacetic acid, disodium salt. (EDTA)

6.5 EDTA solution (26.9 mM)

Weigh out 0.500 ± 0.005 g EDTA, disodium salt into a 50 ml beaker, and add approximately 40 mL of water. Warm on a hotplate, with stirring, until dissolved. Transfer quantitatively to a 50 mL volumetric flask with water, make up to the mark and mix. This solution can be stored under refrigeration for at least one month.

6.6 β-nicotinamide adenine dinucleotide, reduced-form, disodium salt hydrate (anhydrous molecular weight 709.4) [NADH] (e.g. Sigma N8129*)

6.7 NADH solution (7.05 mM)

Weigh 25 ± 2 mg of NADH [Note] into a 5 mL volumetric flask. Make up to the mark with water [Note] and mix. The solution can be stored for up to a week a refrigerator.

Note: The weight taken should be corrected for the % purity, and the water content if necessary, as described on the supplier's information sheet for the product used.

Note: Purified water is often slightly acid which can adversely affect the stability of the NADH in solution. The pH of the water used should be checked using a pH meter and if less than 7.0, a drop of 0.01 M sodium hydroxide should be added to raise the pH to between 7.0 and 8.0.

6.8 Acetoacetyl - Coenzyme A, sodium salt (e.g. Sigma A1625; 5 mg vials*)

6.9 Acetoacetyl – Coenzyme A solution (5.9 mM)

Add 1 mL of water into a vial containing 5 mg of acetoacetyl – Coenzyme A as purchased and mix. The solution can be stored in a refrigerator for up to two days.

*The products shown are for information only and no endorsement is intended. Alternative products may be used if equivalent.

7 Procedure

7.1 Sub-sampling

Two sub-samples are required from each poultry breast. Each sub-sample should be a block of approximate dimensions: 30 mm x 30 mm x 25 mm (height). A suitable procedure is as follows:

7.1.1 Remove any skin and extraneous fat from the poultry breast.

7.1.2 Using a sharp, kitchen knife or scalpel, trim the breast so that a 30 mm strip is taken from the thickest part of the breast. (See Fig 1)

7.1.3 Cut two 30 mm blocks from the strip.

7.1.4 Carefully cut the top and bottom surfaces from each block so it is approximately of the size specified and each block has six cut surfaces.

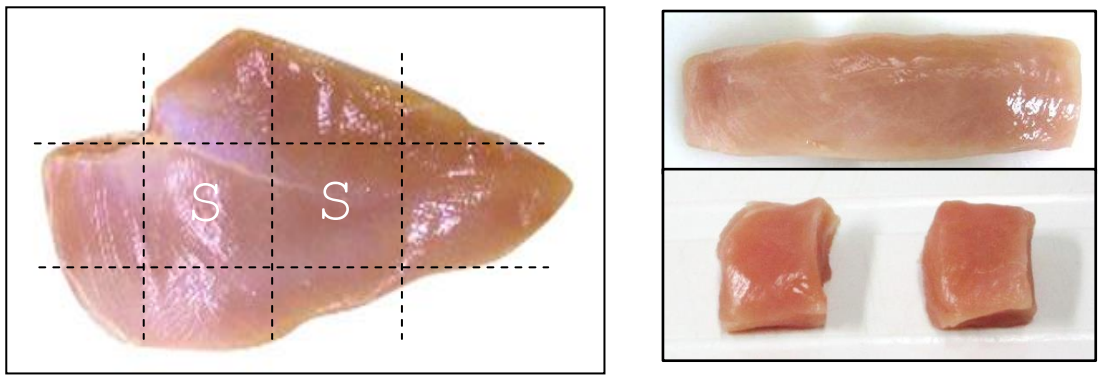


Figure A1: Preparation of sub-samples

The sub-sampling procedure should be standardised as much as possible. The blocks should ideally be single pieces of meat but if necessary, two thinner pieces, each with six cut surfaces can be combined to produce the necessary height. *If the block dimensions are very different from those described, there may be difficulty in operating the press.*

7.1.5 Place each block into separate plastic bags, seal and label. The blocks should be placed into the bags immediately after cutting. Do not allow the blocks to dry out before placing into the bags.

7.1.6 One of the sub-samples is set aside for pressing on the day of cutting. The other should be placed into a freezer at ~ -18 °C and allowed to freeze. Do not fast-freeze as slow freezing is important to ensure maximum disruption of the mitochondria for the release of HADH. Store the sub-sample in the freezer for at least 48 h.

Note that freezing for longer periods should not affect the subsequent determination however samples should be analysed as soon as practicable.

7.1.7 After freezing, the sub-sample should be slowly thawed for 24 h in a refrigerator at ~ 2 to 5 °C before pressing.

7.2 Pressing

7.2.1 Place the test portion into the cavity of the base unit of the press.

Place the lid onto the press and press together (Fig 2).

Insert the press into the G-Clamp and tighten gently to hold it in place.

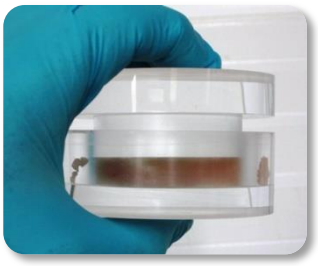


Figure A2

7.2.2 Hang the clamp and press onto two horizontal rods or similar so that the press is suspended vertically. Place a petri dish under the press. Tighten the G-clamp screw until the press is almost closed and juice begins to drip from the press. (Fig 3)

Figure A3: Sample pressing

7.2.3 Allow the juice to drip into the petri dish for ~10 minutes or until at least 1 mL of juice is obtained.

7.2.4 Swirl the dish gently, to mix the juice obtained and transfer 1 mL of juice to a 25 mL volumetric flask. Dilute the juice to 25 mL with phosphate buffer (6.3) and filter an aliquot of ~ 5 mL through a 0.45 µm syringe filter (Figs 4 & 5).

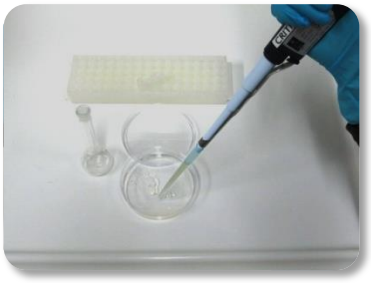


Figure A4

Figure A5

7.2.5 Analysis of the pressed juice, after dilution and filtration, may be carried out on the day of pressing or if necessary the extracts may be stored overnight in a fridge or may be frozen at -18 °C for at least one week.

Note: Do not store unfiltered juice as it may contain small pieces of meat residue.

7.3 Spectrophotometry

7.3.1 Place a bottle containing the phosphate buffer solution (6:3) into a water bath at 25 °C for 30 min before use.

7.3.2 Add 200 µL of EDTA solution (6.5) to 2.6 mL of buffer (7.3.1) in a 1 cm cuvette and then add 50 µL of NADH solution (6.7) and 100 µL of the diluted and filtered press juice. Cap and mix by inversion.

7.3.3 To start the reaction, add 50 µL of Acetoacetyl-Coenzyme A (6.9). Immediately, mix by inversion, wipe the cuvette and place into the spectrophotometer. Read the absorbance at 340 nm and start the timer.

Reagent	**Volume**
Phosphate buffer (25 °C)	2.6 mL
EDTA Soln.	0.2 mL
NADH	50 µL
Sample solution	100 µL
Acetoacetyl CoA	50 µL

Table A1: Addition of sample and reagents

7.3.4 After 3 min, read the absorbance at 340 nm again.

7.3.5 Repeat the measurements with a second, 100 µL portion of the sample press juice.

Note: Ideally, all operations should be carried out at 25 °C, however providing that the buffer solution is at 25 °C and that the ambient temperature is relatively constant, no additional temperature control is normally required.

8 Calculations

The HADH activity of each sample extract obtained from the frozen and unfrozen samples is determined and is used to calculate a factor R_1,

8.1 Calculate the rate of decrease in absorbance ΔE/min as:

$$\frac{\boldsymbol{E_0 - E_{3\,min}}}{\mathbf{3}}$$

Note 1: If the three minute period is slightly exceeded, the rate of decrease should be obtained by dividing by the actual time between the start and end measurements.

Note 2: The starting absorbance for a filtered sample solution, diluted as described should be around 0.6. The decrease in absorbance (ΔE) is typically 0.3 – 0.4 for frozen poultry samples and less than 0.1 for unfrozen samples. (See Appendix 1B for details)

8.2 Calculate the HADH activity (U/mL) using the following equation

$$\frac{V}{\varepsilon \text{ x d x a}} \text{ x } \Delta E/min \cdot D$$

Where:

V	= Total volume in cuvette (3 mL)
ε	= Millimolar extinction coefficient for NADH at 340 nm {mmol/L} (= 6.3)
d	= light path of the cuvette (1cm)
a	= volume of diluted meat juice (0.1 mL)
ΔE/min	= rate of decrease in absorbance
D	= dilution factor of the press juice (25)

Therefore, using the default volumes and dilutions described above, the equation becomes:

HADH activity = ΔE/min * 119 U/mL

8.3 Calculate the R1 value for each sample as follows:

$$R_1 = \frac{X_0}{X_1}$$

Where:

X_0 = HADH activity of the unfrozen sub-sample.
X_1 = HADH activity of the frozen sub-sample.

9 Interpretation of results

The R_1 value is used to indicate whether the sample has previously been frozen by comparing it to threshold values. The threshold values for chicken and turkey breast meat are as follows:

Chicken: 0.50
Turkey: 0.62

If the R_1 value obtained is greater than the threshold values given above, then the sample is considered to have previously been frozen and thawed.

Further information about these threshold values is given in Appendix 1C.

10 Analytical quality assurance

There are no certified materials available, and it is difficult and prohibitively expensive, to obtain pure HADH enzyme for this purpose. Juices from fresh and frozen samples should be prepared in-house and used for quality assurance purposes. This can be done either by in-laboratory freezing of chilled chicken breast samples or by purchasing chilled and frozen chicken breasts from a reputable source. The HADH activity of each juice should be confirmed by analysis before storing at -18 °C (or -80 °C if available).

These solutions can be used for control purposes with every batch of samples and to confirm that the absorbance readings are optimised. In the author's laboratory and with the conditions described in this protocol, typical HADH activities are ~ 10 – 15 U/mL for frozen chicken and 1U/mL or below for unfrozen chicken. The long-term stability of such solutions must be established by the user.

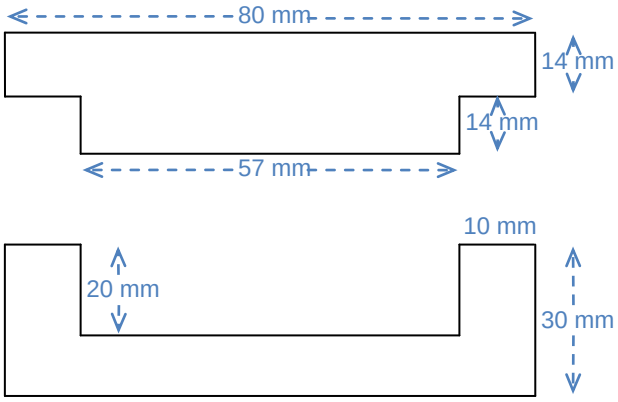
Record the HADH activities of each sample and analyse a vial of each solution with every batch of samples to demonstrate consistent results.

Note: If the activities of the two solutions are not as expected, check that the spectrophotometric procedure has been carried out correctly and if so, repeat with new reagents. If the results are still unusual, check that the remaining procedures have been carried out correctly and repeat using new samples purchased from a different source.

Appendix 1A: Design Specifications for "Juice" extraction Press

The meat press consists of a perspex container comprising a circular base unit and lid, and a G-clamp. The dimensions of each are given below in Figure 1A-1.

Figure 1A-1 Dimensions of meat press



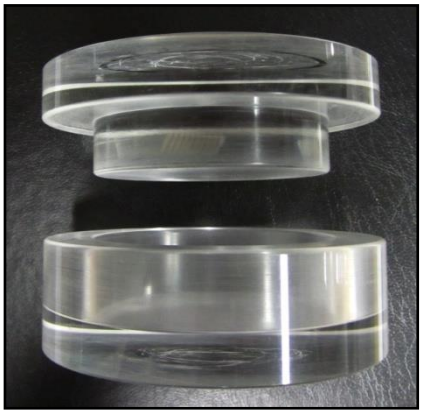


Figure 1A-2 G-Clamp

A G-clamp similar to that shown below is used to operate the press.

A clamp with jaw-size of approx.100-150 mm is required. The precise dimensions of the clamp are not important provided that it fits around the press. Suitable clamps can be obtained from DIY or hardware stores.

Appendix 1B For information only

Reaction kinetics vs sample extract concentration

The HADH activity is measured using a spectrophotometric technique by determining the rate of conversion in the following reaction:

$$\text{Acetoacetyl –coenzyme A + NADH + H}^{+} \xleftrightarrow{\text{HADH}} \beta\text{-hydroxybutyryl-coenzyme A + NAD+}$$

The HADH activity is measured by the decrease in the absorption of the reaction solution at 340 nm. The reaction rate is dependant on the HADH activity of the sample extract as shown in Figure 1B-1 below

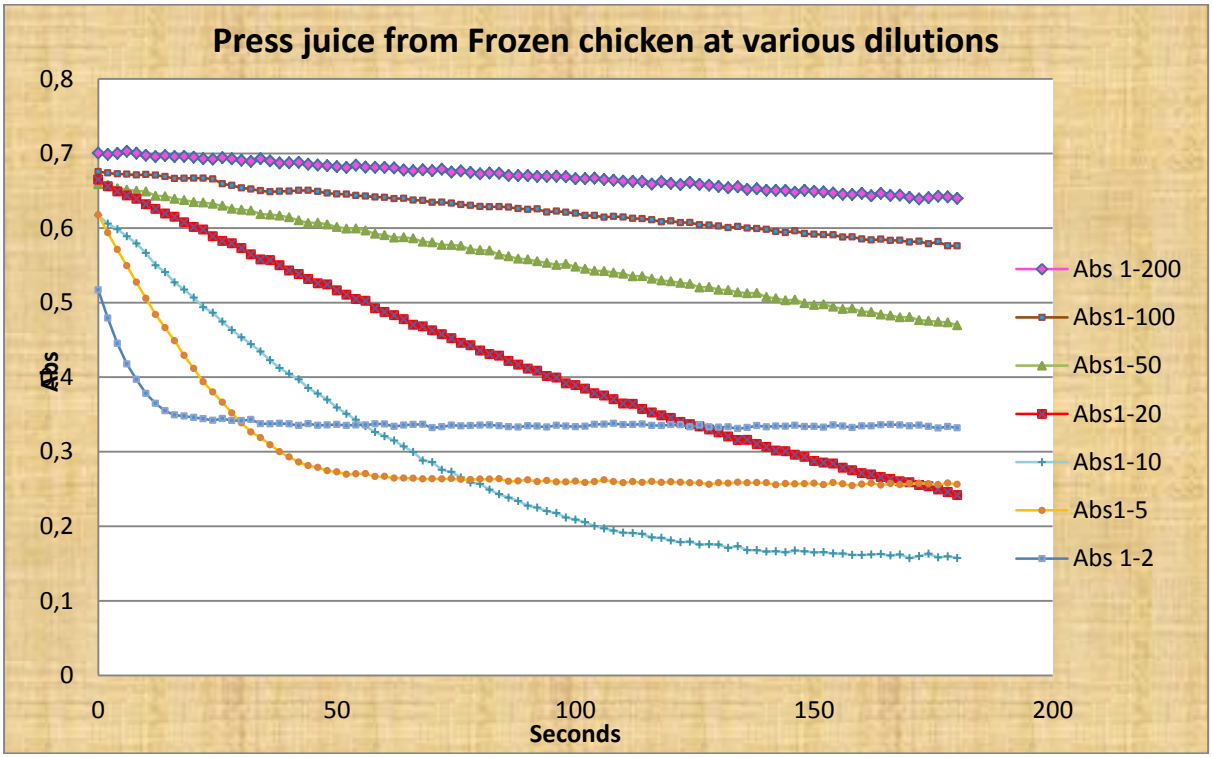


Figure 1B-1 Reaction kinetics vs. sample dilution (x2 to x200)

Figure 1B-1 shows the reaction kinetics for a frozen chicken extract diluted with decreasing amounts of phosphate buffer before analysis.

If the dilution is too large, the decrease in absorption is small which makes differentiation between chilled and frozen samples difficult.

If the dilution is too small, the reaction kinetics become non-linear and whilst this can be used to differentiate between fresh and frozen samples directly, the reaction rates are heavily dependant on the sample extract concentration and would be subject to variations in HADH activity between different samples and to variations arising from the analytical procedure. It is also not possible to determine ΔE /min, if the kinetics are non-linear.

For these reasons, a comparative assay (before and after freezing) is recommended and the

sample extract is diluted so that the maximum decrease in absorption is obtained for frozen samples whilst the reaction kinetics remain linear. This is achieved by diluting the press juice 1 mL to 25 mL and using 100 µl of diluted extract for the spectrophotometric reaction as described in the protocol.

If a spectrophotometer capable of kinetic measurement (continuous measurement over fixed time periods) is available, the optimum dilution rate may be confirmed in the user's laboratory.

Using the conditions described typical reaction curves for chlled and frozen samples were as shown in Figure 1B-2.

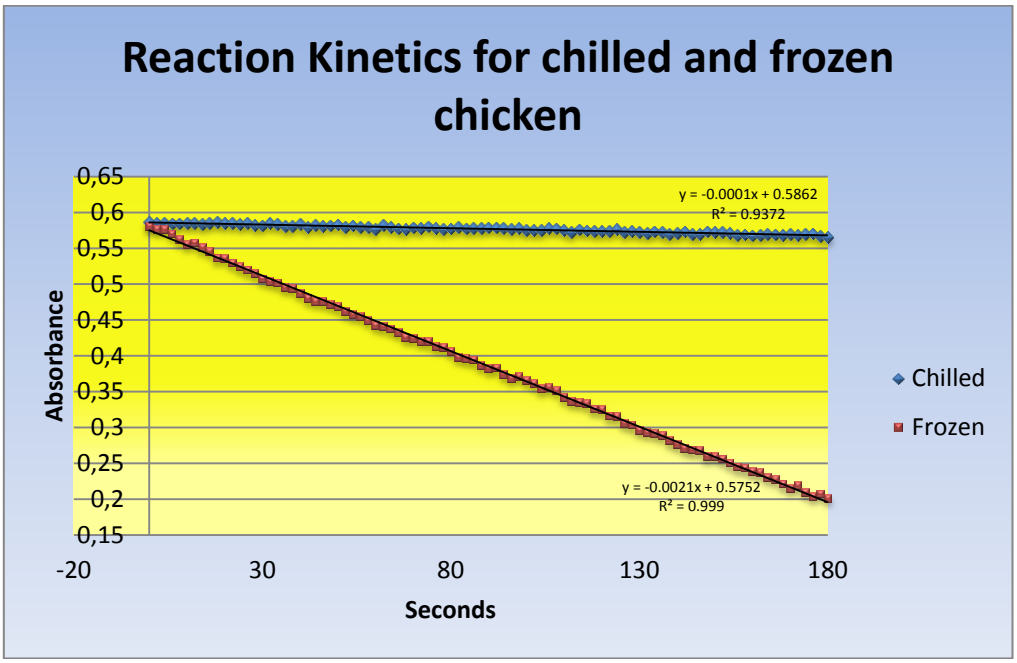


Figure 1B-2

Appendix 1C For Information Only

Threshold values

The threshold values for chicken and turkey were originally determined by Hargin et al[1]. In his paper, Hargin quotes R1 cut-off values for a range of meats including chicken and turkey. The threshold values quoted were:

Chicken	0.90
Turkey	0.62

The HADH method was re-evaluated in 2008 at LGC with funding from the Food Standards Agency[2]. This work was undertaken to address concerns about some aspects of the analytical procedure and to assess the effect of new freezing and chilling techniques on the results obtained. Modifications were made to the original procedure in respect of the pressing procedure and the working range for the spectrophotometry.

As part of this work, the threshold value for chicken was reassessed and found to be too high. A new value was calculated using a confidence interval of 99 %. Although this limit was obtained in a single, laboratory, it is believed to be more appropriate to the modified procedure and will prevent false negatives (i.e. the non-detection of previously frozen samples). The limit for turkey is much lower and remains unchanged. The revised threshold limits are therefore as follows:

Chicken	0.50
Turkey	0.62

Where these limits are exceeded, the samples may be considered to have been previously frozen and thawed.

References

1. *Hargin, K., J.Assoc.Publ.Analysts, 1997, 33, 1-46*
2. *Lawrance, P., Wolfe, M., Tsampazi, C., J.Assoc.Publ.Analysts(Online), 2010, 38, 13-23.*