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Standardisation and application of a semi-quantitative SDS-PAGE method for measurement of myofibrillar protein fragments in bovine *longissimus* muscle

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Abstract

A semi-quantitative SDS-PAGE method was standardised for the routine analysis of myofibrillar protein fragments, 30 and 110 kDa, associated with the meat tenderisation process. Bovine serum albumin was used as an internal standard. Standard curves for purified proteins demonstrated a linear response using this method. Mean analytical recoveries were 95.5 and 102.8% for separation on 7.5 and 15% acrylamide gels, respectively. The ability of the test to measure 30 kDa and 110 kDa fragments independently of sample volumes was demonstrated. Quality control studies showed that levels of 110 kDa protein fragment quantified on 7.5% gels varied by 23.1% within gels and 36.4% between gels. Quantification of 30 kDa protein fragment on 15% gels varied by 7.3% within gels and 7.0% between gels. This thoroughly standardised SDS-PAGE method may be used to measure 30 and 110 kDa bands in extracts of bovine *M. longissimus dorsi* over the post-mortem ageing period and to evaluate other quantitative methods developed in the future for measurement of the same protein fragments.

1. Introduction

The production of meat of consistently good eating quality is of major economic importance to the meat industry. The use of different feeding regimes, pre-slaughter treatments and meat processing mechanisms can result in large variations in quality of the same cut of meat from different animals. With existing knowledge of the biochemical mechanisms involved in the meat tenderisation process, potential markers of meat quality could be targeted and tested for their ability to predict variations in meat quality.

In previous reports the ageing process in meat has been associated with the degradation of myofibrillar proteins such as troponin T and the appearance of protein fragments such as the 30 and 110 kDa fragments (Casserly, Stoeva, Voelter, Healy & Troy, 1998; Ho, Stromer & Robson, 1994; Negishi, Yamamoto & Kuwata, 1996; O'Halloran, Troy & Buckley, 1997). These observations suggest that the 30 and 110 kDa fragments may act as potential markers for the process that results in tenderness. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) has been used extensively for qualitative visual analysis of these myofibrillar proteins and fragments (Huff-Lonergan, Parrish & Robson, 1995; McBride & Parrish, 1977; Pommier, Poste & Butler, 1987). However, the development of a quantitative test is necessary for detecting the variation in post-mortem changes between meat samples. With the advent of new imaging technology and software for image analysis there is a growing trend in the use of SDS-PAGE for quantitative analysis. This introduces the need for standardisation of procedures being developed in different laboratories prior to their use in routine analyses.

This study involved the standardisation of a semi-quantitative SDS-PAGE method for routine analysis of myofibrillar proteins in meat and testing the potential of the method for quantifying changes in myofibrillar proteins and fragments over the ageing process.

2. Materials and methods

2.1. Meat samples

2.1.1. Isolation of myofibrils

Myofibrillar proteins were extracted from 30 g freeze-dried bovine *M. longissimus dorsi* samples using the method of Etlinger, Zak and Fischman (1976) as

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modified by Wang (1982). Samples were homogenised in 100 ml of pyrophosphate relaxing buffer [100 mM KCl (BDH), 2 mM $MgCl_2$ $6H_2O$ (BDH), 2 mM EGTA (Sigma), 10 mM Tris-maleate (Sigma), 0.5 mM Dithiothreitol (BDH), 2 mM $Na_4P_2O_7$ (BDH) and 0.1 mM PMSF (Sigma)]. The homogenate was centrifuged (10 min at ~3000 *g*) and the pellet was resuspended and homogenised in wash buffer (100 mM KCl, 2 mM $MgCL_2.6H_2O$, 2 mM EGTA, 10 mM Tris-maleate, 0.5 mM DTT and 0.1 mM PMSF, pH 6.8) and re-centrifuged. The pellet was resuspended in wash buffer, filtered and centrifuged. A detergent extraction step using Triton X-100 buffer [wash buffer plus 0.5% (w/v) Triton X-100] was carried out on the pellet followed by three further washes with wash buffer. Each time the pellet was resuspended, mixed and centrifuged as previously described. The final pellet was freeze dried and stored in a dessicator.

2.1.2. Electrophoresis

SDS-PAGE was performed according to the procedure of Greaser, Yates, Krzywicki and Roelke (1983). Samples of myofibrillar extracts were applied to 2 mm vertical slab gels (Atto, Mason Technology). Resolving gels containing 15 and 7.5% acrylamide were prepared with a cross-linking ratio for bis to monomer of 1:200. Proteins of 20–66 and >66 kDa were resolved by SDS-PAGE on 15 and 7.5% gels, respectively. The gels contained a mixture of Tris (3M), glycerol (50%), distilled water, 0.1% SDS, 0.02% ammonium persulphate and 0.1% *N,N,N′,N′*,-tetramethyl-ethylene-diamine (TEMED). Gels were poured 16–24 h before use to allow the generation of free radicals to cease. All samples were heated at 100°C for 2 min. Samples were loaded into the wells of a 3% stacking gel at a concentration of 200 μg myofibrillar protein per well. Electrophoresis was performed at a constant 160 volts (E871 Consort power supply). Gels were stained in a mixture of 50% v/v methanol (BDH, Analar), 9.2% v/v acetic acid (BDH, Analar) and 0.5% w/v coomassie Brilliant Blue R-250 (BDH, Electran). Destaining was carried out using a mixture of 25% v/v ethanol (BDH, Analar) and 5% v/v acetic acid. Gels were shrunk in 12% polyethylene glycol (PEG) solution for approximately 24 h before scanning.

2.2. Internal standard

A standard solution of BSA (Sigma Chemicals) was prepared at 1 mg/ml in de-ionised water ($\varepsilon_{0.1\%} = 0.666$). A one in two dilution of this standard in sample buffer gave a final stock concentration of 0.5 mg/ml. Appropriate volumes of this stock were added to protein standards and myofibrillar extract samples to give a final concentration of 5 μg BSA/50 μl sample. Sample volumes of 50 μl were loaded routinely.

2.3. Standards

Protein standards of phosphorylase b (97,400 M.Wt.), ovalbumin (45,000 M.Wt.), glyceraldehyde-3-phosphatase (36,000 M.Wt.) and carbonic anhydrase (29,000 M.Wt.) were obtained from Sigma Chemicals Co. Ltd. Stock solutions were dissolved at a final concentration of 0.5 mg/ml in sample buffer containing 8 M urea (BDH, Analar), 2 M thiourea (BDH, GPR), 0.05 M tris base (Sigma chemicals), 7 mM DTT (BDH, Biochemical), 3% SDS (BDH, Biochemical) and 0.005% bromophenol blue (BDH, Electran).

2.4. Sample preparation

After protein concentration was determined by the Kjeldahl method, the freeze-dried myofibrillar extracts were solubilized overnight at room temperature in sample buffer at a concentration of 5 mg/ml. BSA solution was added as internal standard to give a final concentration of 4 mg/ml myofibrillar protein and 0.1 mg/ml BSA. Solutions were mixed by inversion, aliquoted and frozen at −20°C.

2.4.1. Semi-quantitative determination of proteins

After de-staining and shrinking the gels were quantitatively analysed. Gels were scanned using a colour image scanner (Sharp, JX-330) and then analysed using ImageMaster VDS 3.0 software (Pharmacia Biotech). All computer imaging parameters (i.e. 256 grey scale, 150 Dpi, drop-out colour red and filtered profile baseline) were the same for each captured gel image. The use of ImageMaster VDS 3.0 software enabled lane and band analysis, gel annotation, printing and documentation.

The limits of protein bands of interest in each lane were defined. A filtered profile analysis was used for baseline determination. The optical density (OD) for each protein band was calculated as a ratio of that for the standard BSA protein band in the same lane [relative optical density, (ROD)]. Each well contained 200 μg myofibrillar protein and 5 μg BSA per 50 μl volume loaded. The amount of protein was expressed in BSA equivalents (μg) using the following calculation:

$$\mu g \text{ protein/mg myofibrillar crude protein} = \frac{\text{OD protein band}}{\text{OD BSA band}} \times 5 \times 5$$

2.5. Standardisation experiments

Standardisation involved the use of semi-quantitative imaging analysis in the preparation of standard curves, determination of analytical recovery for added standards

and independence of volume for the assay. Finally the variation for analysis of the same myofibrillar extract sample within and between gels was determined.

2.5.1. Quantification of myofibril extracts from aged meat samples

Meat samples were collected from bovine *M. Longissimus dorsi* after ageing at 4°C for 2, 7 and 14 days. A total of 66 myofibrillar extracts from 22 *M. Longissimus dorsi* at these three different time points post-mortem were prepared as described above and semi-quantitatively analysed for 30 kDa protein fragment bands on 15% SDS-PAGE gels. Myofibrillar extracts from the same *M. Longissimus dorsi* at different times post-mortem were always analysed in duplicate on the same gel.

3. Results

3.1. SDS-PAGE

The results for this standardisation study show the importance of developing a reproducible procedure if SDS-PAGE is to be employed for routine quantitative purposes. Also a consistent approach to sample preparation and analysis is important for comparison of myofibrillar extracts at different age times post-mortem from the same muscle and also for comparison of myofibrillar extracts at the one age time post-mortem from muscles of different animals.

Fig. 1 illustrates the band separation achieved on both 7.5 and 15% acrylamide gels using this method. Apart from titin which is not heat stable (Locker & Wild, 1984) the other myofibrillar bands are indicated and have been previously described by numerous authors (Claeys, Uytterhaegen, Buts & Demeyer, 1995; Negishi et al., 1996; O'Halloran et al., 1997; Pommier et al., 1987).

3.2. Standard curves

Standard curves prepared on 7.5 and 15% acrylamide gels are shown in Fig. 2(A) and (B) respectively. Duplicate standards at five different concentrations were used for each standard curve. An internal standard of purified bovine serum albumin (BSA) was added to give the same final concentration (0.1 mg/ml) in each standard concentration prepared. The standard curve was plotted as the concentration of the protein in μg versus the corresponding μg of BSA equivalents. All standard curves were found to be linear within a range of 0.025 to 5 mg for each standard tested with an average R^2 value of 0.985 and 0.987 for 7.5 and 15% gels, respectively.

3.3. Analytical recovery

This was determined by preparing tubes with four different concentrations (12.5, 25, 50 and 100 μg/ml) of each protein standard and a constant amount of BSA internal standard (0.1 mg/ml) in a solution of sample buffer. Volumes of 50 μl for each concentration of added protein were run in duplicate. The amount in each protein band was calculated in μg of BSA equivalents. Using the standard curve the corresponding concentration of protein recovered was recorded. The latter results were calculated as a percentage value of the original amount

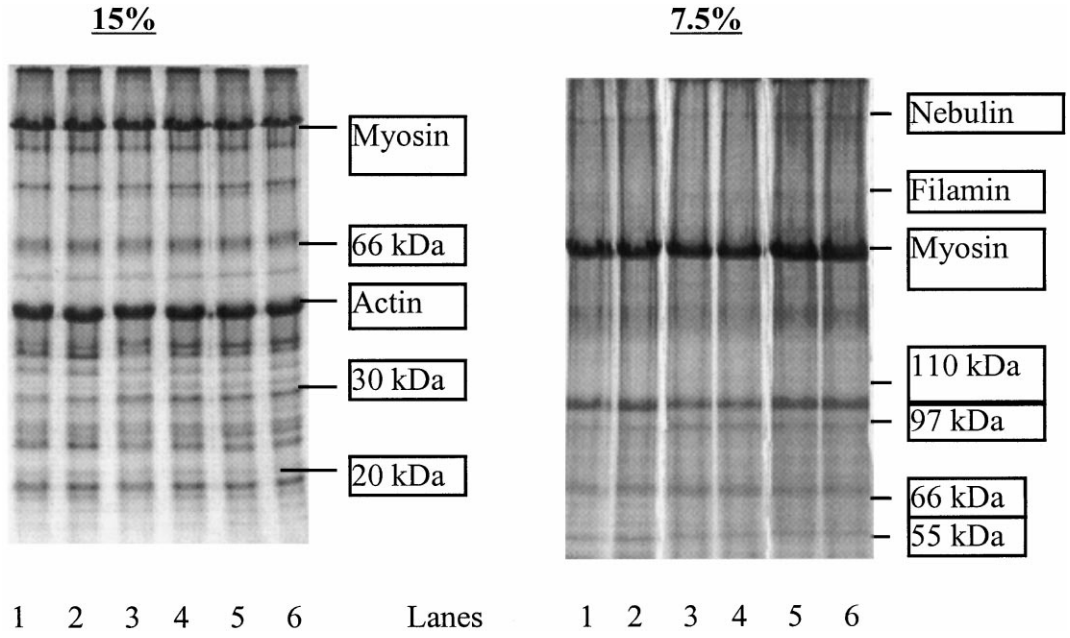

Fig. 1. Typical separation profiles for myofibrillar extracts from bovine *M. longissimus dorsi* using 7.5 and 15% polyacrylamide gels.

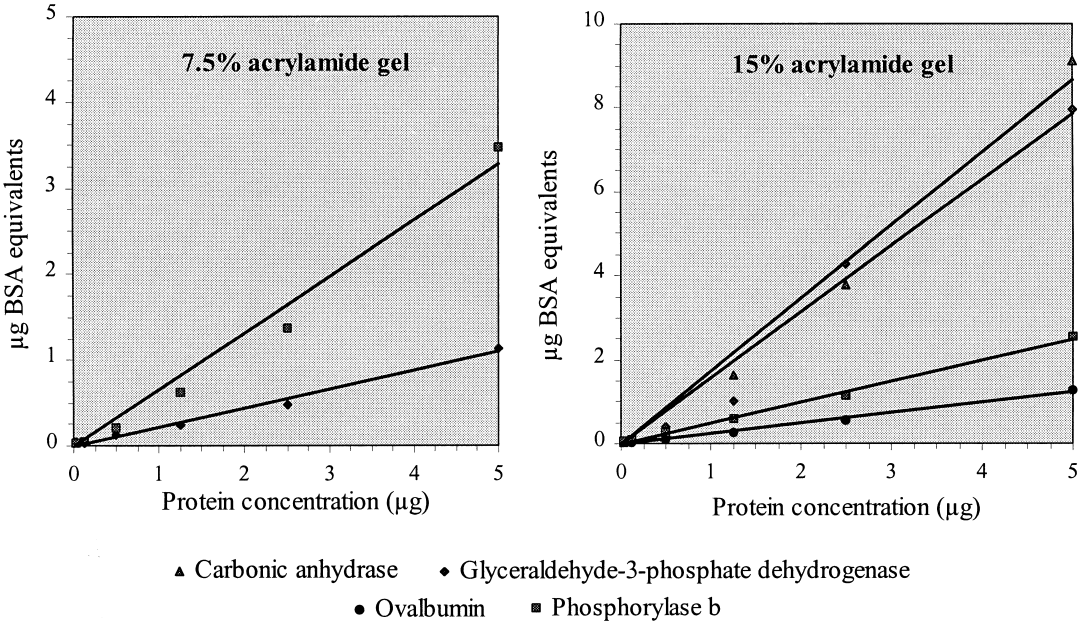

Fig. 2. Standard curves for phosphorylase b and ovalbumin on 7.5% SDS-PAGE and phosphorylase b, ovalbumin, carbonic anhydrase and glyceraldehyde-3-phosphate dehydrogenase on 15% SDS-PAGE using BSA as internal standard.

of protein added (Table 1). Mean analytical recoveries for the individual standard proteins shown range from 77.2 to 119.9%. The results compare well with a previous publication which demonstrated overall analytical recovery of 92–110% (Claeys et al., 1995).

3.4. Independence of volume

The ability of the assay to measure myofibrillar proteins as distinct bands independently of the sample volume was determined by dilution of a single myofibrillar extract in sample buffer with addition of internal standard. Samples were analysed in duplicate on two separate gels. The concentration of myofibrillar protein in each band was calculated as µg of BSA equivalents and corrected for the dilution factor. Results are plotted for protein bands of interest against the corresponding dilution factor (Fig. 3).

Graphic presentation of independence of volume in this way should yield straight lines parallel to the x-axis. Abnormalities in the data can be seen clearly using this method (Power & Fottrell, 1991). For our separation protocol on 7.5% gels the filamin, 110, 97 and 55 kDa bands demonstrated independence of volume. On 15% gels measurements for 55, 20, 32 and 30 kDa bands were also independent of sample volume.

Table 1
Recovery of added standard for 7.5 and 15% resolving gels

	Protein added (µg/50 µl)				
	0.625	1.25	2.5	5.0	
	% Analytical recovery				Mean (±S.D.)
7.5% acrylamide gel					
Phosphorylase b	125.7	106.7	78.5	160.7	117.9 (±34.5)
Ovalbumin	70.0	75.1	94.7	111.1	87.73 (±18.9)
% Recovery, mean of means	102.8% (SEM ± 21.3)				
15% acrylamide gel					
Phosphorylase b	140.6	101	85.9	151.9	119.9 (±31.4)
Ovalbumin	94.8	105.1	115	113.6	107.1 (±9.3)
Glyceraldehyde-3-phosphate dehydrogense	65.7	55.8	70.1	117.2	77.2 (±27.3)
Carbonic anhydrase	65.3	54.3	92.4	99.2	77.8 (±21.4)
% Recovery, mean of means	95.5% (SEM ±21.4%)				

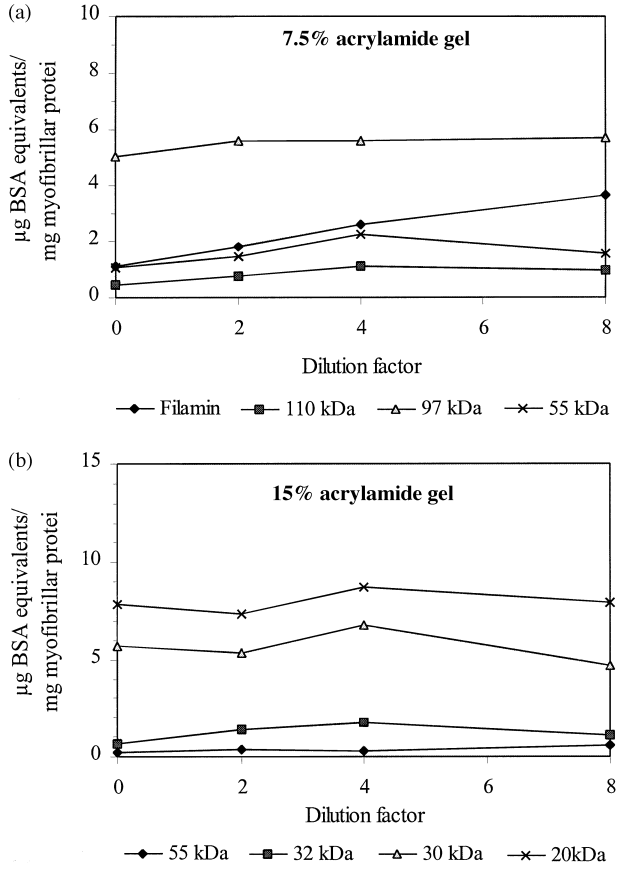

Fig. 3. Determination of the influence of sample volume assayed on the different myofibrillar proteins quantified using (A) 7.5% and (B) 15% resolving gels.

Table 2
Quality control results for variation in quantification of myofibrillar proteins on the same (within) and on different (between) 7.5% SDS-PAGE gels

	Variation					
	Within-assay ($n=10$)			Between-assay ($n=8$)		
Protein band	Mean[a]	±S.D.	%CV	Mean[a]	±S.D.	%CV
Nebulin	6.1	0.55	9.0	5.75	0.7	2.2
Filamin	1.45	0.25	17.2	1.4	0.35	25.0
Myosin	62.05	2.9	4.70	52.6	5.45	10.4
110 kDa	0.65	0.15	23.1	0.55	0.2	36.4
104 kDa	10.9	0.65	6.00	9.4	1.0	10.6
97 kDa	4.95	0.35	7.1	4.7	0.50	10.6
55 kDa	1.3	0.50	38.5	1.85	0.7	37.8

[a] Values in µg BSA equivalents/mg myoffibbrillar protein.

Table 3
Quality control results for variation in quantification of myofibrillar proteins on the same (within) and on different (between) 15% SDS-PAGE gels

	Variation					
	Within-assay ($n=10$)			Between-assay ($n=8$)		
Protein band	Mean[a]	±S.D.	%CV	Mean[a]	±S.D.	%CV
Actin	88.5	2.95	3.3	84.35	8.7	10.3
36 kDa	17.7	1.4	7.9	21.65	5.1	23.6
34 kDa	0.8	0.15	18.8	0.95	0.2	21.1
32 kDa	1.0	0.1	10.0	1.1	0.1	9.1
30 kDa	5.5	0.4	7.3	6.4	0.45	7.0
20 kDa	0.85	0.1	11.8	0.9	0.1	11.1
16 kDa	0.195	0.05	25.6	0.135	0.05	37.0

[a] Values in µg BSA equivalents/mg myofibrillar protein.

3.5. Assay variation

3.5.1. Within assay variation

A single 14 day sample of myofibrillar protein was prepared. BSA internal standard was added to the final concentration of 0.1 mg/ml BSA and 4 mg/ml of myofibrillar sample. The same volume (50 µl) and concentration (200 µg) of this sample was run repeatedly on a single gel. The concentration of myofibrillar protein in each band was calculated as µg of BSA equivalents. The mean values for resolved protein bands of interest are represented in Tables 2 and 3 for 7.5 and 15% resolving gels, respectively. The overall variation (%CV) for protein bands quantified on 7.5% SDS-PAGE ranged from 6 to 38.5% with a variation of 23.1% for levels of 110 kDa protein fragment. On 15% resolving gels the variation for quantified protein bands ranged from 3.3 to 25.6% with a variation of 7.3% for the quantification of the 30 kDa band.

3.5.2. Between assay variation

For determination of between assay variation a single myofibrillar protein sample loaded at the same concentration (200 µg/50 µl) was run in duplicate on four separate gels. The concentration of myofibrillar protein in each band was calculated as µg of BSA equivalents. The values for each myofibrillar protein were averaged over the four gels analysed and the results represented in Tables 2 and 3 for 7.5 and 15% resolving gels respectively. Values for percentage coefficient of variation ranged from 10.6 to 37.8% for protein bands quantified on 7.5% gels with a variation of 36.4% for the levels of 110 kDa band quantified. On 15% resolving gels the variation ranged from 7 to 37% with a variation of 7.0% for quantification of the 30 kDa band between gels.

It has been observed that the extent of variation for analyses both within and between gels changed with the concentration of the protein band being quantified, i.e. variation tends to be higher for protein bands at the lower concentrations. Analytical procedures such as enzyme immunoassays have a long history of use for quantification purposes and the acceptable limit for variation within and between assays is below 15% (Price

& Newman, 1991). However, unlike enzyme immunoassays this SDS-PAGE method is only semi-quantitative and therefore a greater variation in precision and accuracy of results would be acceptable. If this is the case, then our results show that the precision of this method is acceptable for measurement of 30 kDa bands. The 110 kDa band on 7.5% gels is present in smaller quantities (10-fold) than the 30 kDa band on 15% gels. This is reflected in the results for variation within and between gels. Taking these considerations into account the precision of this method is probably within the upper acceptable limits for measurement of the 110 kDa band.

3.6. Myofibrillar proteins as indicators of meat ageing

The ability of the standardised semi-quantitative SDS-PAGE method to detect quantifiable levels of the 30 kDa protein fragment was investigated in order to assess its usefulness in studies for prediction of meat quality. Bovine *M. longissiumus dorsi* samples were aged and the myofibrils extracted as described. (Table 4).

The results (Table 4) demonstrate the ability of the test to detect changes in the appearance of the 30 kDa protein fragment over the meat ageing period. The rate of appearance of this band may vary between animals depending on the rate of glycolysis post-mortem (O'Halloran et al., 1997).

Protein concentrations for the 30 kDa band in extractions from aged meat samples showed a 2–3-fold increase from 2 to 7 days ageing post-mortem. A further 1–2-fold increase was observed from 7 to 14 days post-mortem. The rate at which this increase occurred was different between animals. The variation for the levels of 30 kDa protein fragment between animals in extracts from meat aged for 2 days was 72%. This variation decreased to 40 and 34% in extracts from meat aged for 7 d and 14 days, respectively. The high variation for 30 kDa fragment levels between animals after 2 days ageing can be partly explained by the fact that the precision of this method decreases (%CV increases) for quantification of bands with protein concentrations below 1.0 μg BSA equivalents/mg protein. Quality control results indicate that the variation in results obtained in this region will be greater. The development of a more sensitive antibody based detection system, which is part of the proposed objectives of this research, would alleviate this problem.

Future studies will involve using protein concentration levels for the 30 kDa protein fragment at specific time points post-mortem to evaluate this test for prediction of meat tenderness. Alternatively it may be possible to use the value for the rate of appearance of the 30 kDa fragment in individual animals to evaluate the test for its potential to predict meat quality.

Table 4
Quantification of 30 kDa and 110 kDa protein bands in aged myofibrillar samples on 15% gels

	30 kDa protein band quantified (γ BSA equivalents/mg myofibrillar protein)
Age of meat sample	Range (mean ± S.D., *n*)
2 days	0.62–9.78 (3.61 ± 2.6, *n* = 22)
7 days	1.46–19.0 (11.87 ± 4.81, *n* = 23)
14 days	6.1–26.81 (15.28 ± 5.2, *n* = 22)

4. Discussion

The main difficulties with standardisation of a semi-quantitative SDS-PAGE method for analysis of myofibrillar protein extracts are the complexity of the material being analysed and the lack of available purified standard myofibrillar proteins. Only one other author has shown the use of purified standards to demonstrate the linearity of an SDS-PAGE semi-quantitative method (Claeys et al., 1995). Other studies (Fritz, Mitchell, Marsh & Greaser, 1993; Negishi et al., 1996) have employed SDS-PAGE for quantitative purposes and determined protein band intensities using computer image analysis as in this study. However, few of these methods make use of an internal standard such as bovine serum albumin (BSA). BSA is useful as an internal standard because the SDS-PAGE separation profile for resolved myofibrillar extracts shows a convenient lack of bands in this molecular weight region (i.e. 66 kDa). The use of BSA improves the precision of the assay both within and between gels. It is a vital component of any standardised SDS-PAGE system used for quantitative purposes because it minimises the differences that occur between replicate analyses within gels. Also it reduces the inconsistencies between gels that may be caused by staining and destaining procedures. Coomassie blue is the most commonly used staining system for SDS-PAGE. For reasons that are both practical and economical it is necessary to recycle gel staining and destaining solutions. The consistency of stain and destain times cannot overcome the inevitable differences that will occur between gels because of the reduced efficiency of recycled solutions. Also, variation between different protein bands is introduced through differential binding of the Coomassie stain. Therefore, use of BSA as an internal standard is vital for routine analysis.

5. Conclusion

Currently, quantitative SDS-PAGE is the only method available to measure levels of the 30 kDa fragment produced by proteolytic degradation of troponin T.

There is tremendous potential for the development of immunoassay based tests for this type of analyses in the future. These tests are more rapid, require less sample preparation time, are relatively simple and can be automated for high throughput. The performance of an ELISA test for analysis of soya protein in meat products has been investigated previously by comparison with an SDS-PAGE method (Olsman, Dobbelaere & Hitchcock, 1985). In the same way it will be necessary to use a recognised standardised semi-quantitative SDS-PAGE method for analysis of myofibrillar protein to evaluate the precision, accuracy and sensitivity of newly developed enzyme immunoassay type tests.

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