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Development of a rapid and accurate method for separation and quantification of myofibrillar proteins in meat

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The adulteration of meat and meat products with organ meats or alternate species is a problem both for meat producers and consumers. Therefore, a rapid method was developed and verified for the separation of proteins in fresh meat samples with the goal of identifying contaminating proteins. An extraction buffer system was optimized for protein recovery from meat samples and muscle proteins were separated on a size exclusion high performance liquid chromatography column. The optimal protein extraction was obtained with a buffer of 0.4 M NaCl at pH 6.0. The effects of storage on the extract were carefully examined and it was determined that the muscle protein extract could be stored for 10–12 h prior to analysis. The relationship between peak area and injection concentration was found to be linear for myosin, myosin light chains and troponin. Mass recovery studies found that recovery was not significantly different from 100% for beef or pork protein samples. Muscle samples from beef, veal, lamb, pork and turkey were compared and identifying differences were found in all chromatograms. This method should provide a rapid method for detection of meat adulteration or for separation and purification of muscle proteins.

Keywords: meat, muscle, protein, separation.

INTRODUCTION

Quality control in the meat industry is usually associated with prevention of meat contamination from chemicals, antibiotics or debris and micro-organisms. However, there are other aspects of quality control that are not as widely publicized but are equally important. Two examples are the substitution of one meat species for another or the addition of organ meats to ground meat, both of which come under the heading of meat adulteration. These occurrences not only represent customer fraud but may have serious health implications (allergic reactions) or cause cultural and religious conflicts (Swatland, 1985). The meat producer's income and reputation can be negatively affected if either unintended contaminations or deliberate fraudulent activities are exposed. However, rapid and accurate procedures for the detection of meat substitution or adulteration are not widely available.

Previous research in this laboratory had suggested that a high performance liquid chromatography (HPLC) method had the potential to fulfill this need (Murch *et al.*, 1992). This original method separated muscle proteins by size exclusion chromatography and was initially developed to assess differences in myofibrillar and sarcoplasmic proteins extracted by classification buffers (Helander, 1957; Lobley and Lovie, 1979). The adaptation of this analysis system for potential use by commercial laboratories required further methods development, verification and an expansion of the methods to specifically address those issues involved in meat adulteration.

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The current research was undertaken with the goal of providing the meat industry with a valuable and useful tool for maintenance of high standards in meat quality. The use of these techniques for the comparison of different muscles of the same species and different meat species facilitates the most common commercial applications. Therefore, a variety of meat samples from lamb, turkey, veal, beef and pork was compared and examples of possible meat adulterations were assessed. The results of this research clearly show that this method can be used efficiently to address issues of quality and consumer confidence in the meat industry.

MATERIALS AND METHODS

Many factors were tested for their effects on the accuracy, precision, specificity and ruggedness of this method. For purposes of clarification, a summary of the conditions of these experiments is found in Table 1.

Muscle sampling

To compare the extraction buffers, beef longissimus muscle (LM) samples were obtained from three steers (14 to 15 months old) from the Elora Beef and Kapuskasing Research Stations, ON. Pork longissmus muscle (LM) samples were also used and were obtained from three pigs (5 to 6 months old) from Arkell Research Station and slaughtered at the University of Guelph abattoir.

For determination of mass recovery, pork inside round (pectineus, adductor and semimembranosus muscles) samples from four pigs (5 to 6 months old, gilts, Yorkshire) were obtained from the Arkell Research Station, University of Guelph and slaughtered at the University of Guelph abattoir. The carcasses showed no evidence of being pale, soft, exudative (PSE) or dark, firm, dry (DFD). Veal inside rounds from four different animals were obtained from Delft Blue Inc., Cambridge, ON. All the veal samples were from Holstein bull calves 17 to 18 weeks old. Approximately 200 g of each of four inside round muscles (pectineus, adductor and semimembranosus muscles) were used in this experiment. In addition, a mixture of 90% beef semitendinosus (ST) and 10% beef heart tissue was prepared. The beef ST and whole heart samples were obtained from Better Beef, Guelph, ON (Canadian yield class A1, grade A).

For studies of protein extract stability, pork inside round samples from four pigs (as described earlier) were used. Stability tests were performed on fresh, raw sample extracts after 0, 4, 8, 12, 24 and 48 h of storage at 4°C. These times reflect the possible delays between completion of protein extraction and injection into the HPLC under normal laboratory conditions.

Recovery of sample mass from different species were also compared. Lamb (female, 10 months old) LM from one animal was obtained from the University of Guelph and slaughtered at the University of Guelph abattoir. Twelve turkey breasts (Nicholas toms approximately 18 weeks old) were obtained from Cold Spring Farms, Thamesford, ON. Extracts of turkey were prepared with a buffer composed of 0.5 M KCl, pH 6.0.

All carcasses were stored post mortem for 24 h prior to sampling. Samples of 100–200 g were vacuum packed and stored at 2–4°C for 3 days for pork and veal samples and 5 days for beef samples. These time periods were chosen to correspond to the usual length of time it takes for meat to arrive at the retail level.

Muscle sample preparation

The preparation of all muscle samples began with the removal of any visible fat, connective tissue and covering muscles. Samples were then comminuted with a hand grinder for uniform subsampling. For comparison of extraction buffers, pork samples were passed through the grinder once (using a 3/8″ (9.5 mm) grinding plate), and beef samples were passed through the grinder twice (3/8″ then 1/8″ (3.2 mm) plate). The comminuted sample was stored at 3–4°C overnight, prior to further preparation. The samples in all of the remaining experiments were cut into blocks and comminuted with a food processor (Braun Canada Ltd, Mississauga, ON) immediately prior to preparation. A subsample of approximately 1 g was taken from each comminuted sample and kept on ice for the remainder of the procedure. To each subsample, 15 ml of the extraction buffer (see later) was added and the combination was homogenized for 15 s at 60% of maximum output (12 000 rpm) (IKA-WERK, Ultra-turrax, Janke & Kunkel GmbH & Co., Staufen, Germany). The resulting slurry was centrifuged for 10 min at $6480 \times g$ at 3–4°C (Beckman J2-21 centrifuge, rotor J14, Beckman Instruments Inc., Mississauga, ON). The supernatant was filtered with a nylon 0.45 micron syringe filter (Mandel Scientific Company Ltd, Guelph, ON) into 30 ml polypropylene bottles (Nalgene, Fisher Scientific Co., Napean, ON). A 100 μl subsample was pipetted into a microtest-tube (BioRad Laboratories Inc., Mississauga, ON) for injection. To eliminate any bubbles that may have been introduced into the sample during pipetting, the extracts were centrifuged (Medifuge/Biofuge 13, Baxter Corp., Canlab Division, Mississauga, ON) at 5000 rpm ($5741 \times g$) for 3 min. The final extract for each subsample was injected into the HPLC within approximately 4 h following preparation unless otherwise indicated.

Comparisons of extraction buffers

Fifteen buffer combinations were prepared using five different NaCl concentrations and three pH levels to

Table 1. Summary of the experimental conditions for testing of the analytical method

Experimental parameter	Species	Muscle	Column	Mobile phase	Flow rate (ml/min)
Extraction buffer	Beef	Longissmus dorsi	40XL	0.5 M KCl, pH varied with buffer	0.6
Extraction buffer	Pork	Longissmus dorsi	40XL	0.5 M KCl, pH varied with buffer	0.6
Extract stability	Pork	Pectineus, adductor, semimembranosus	3000PWXL	0.4 M KCl pH 6.0	0.6
Mass recovery	Pork and veal	Pectineus, adductor, semimembranosus	40XL and 3000PWXL	0.5 M KCl pH 6.0	0.6
				0.4 M KCl pH 6.0	0.6
Mass recovery	Beef	Semitendinosus, heart	40XL and 3000PWXL	0.5 M KCl pH 6.0	0.6
				0.4 M KCl pH 6.0	0.6
Flow rate	Pork	Longissmus dorsi	40XL	0.5 M KCl pH 6.0	Varied
Flow rate	Beef	Longissmus dorsi	40XL	0.5 M KCl pH 6.0	Varied
Flow rate	Standards	Muscle protein standards	40XL	0.5 M KCl pH 6.0	Varied
Elution buffer	Pork	Longissmus dorsi	40XL	Varied KCl and pH	0.6
Elution buffer	Beef	Longissmus dorsi	40XL	Varied KCl and pH	0.6
Elution buffer	Standards	Muscle protein standards	40XL	Varied KCl and pH	0.6
Limit of detection	Standards	Muscle protein standards	40XL	0.5 M KCl pH 6.0	0.6
Linearity of response	Standards	Muscle protein standards	40XL	0.5 M KCl pH 6.0	0.6
Limit of quantification	Standards	Muscle protein standards	40XL	0.5 M KCl pH 6.0	0.6
Column comparison	Standards	Muscle protein standards	40XL and 3000PWXL	0.5 M KCl pH 6.0	0.6
				0.4 M KCl pH 6.0	0.6
Column comparison	Beef	Longissmus dorsi, semitendinosus	40XL and 3000PWXL	0.5 M KCl pH 6.0	
	Pork			0.4 M KCl pH 6.0	0.6
Species comparison	Veal	Pectineus, adductor, semimembranosus, longissmus dorsi	40XL	0.5 M KCl pH 6.0	0.6
	Pork				
	Lamb				
	Beef				
Species comparison	Turkey	Breast, longissmus dorsi	40XL	0.5 M KCl pH 6.0	0.6
	Pork				
	Beef				
Muscle comparison	Beef	Semitendinosis, heart	40XL	0.5 M KCl pH 6.0	0.6

determine the optimal extraction buffer. NaCl (biological grade, Fisher Scientific Co., Napean, ON) concentrations used were 0.1, 0.154, 0.4, 0.5, 0.6 M with 10 mM 2-(N-morpholino)ethanesulfonic acid (MES) (Calbiochem Corporation, Windsor, ON). The pH was adjusted to 5.5, 6.0 and 7.0 with either 1 N HCl or 1 N NaOH (Fisher Scientific Co., Napean, ON). The physiological concentration of NaCl in muscle (0.154 M) was always included in the calculated salt concentrations. Extraction buffers were filtered using 0.45 micron nylon filters (Fisher Scientific Co., Napean, ON), stored at 3–4°C and warmed to room temperature prior to use. Fifteen subsamples, each approximately 1 g (1.01 to 1.17 g) were taken from each pork and beef LM sample.

Liquid chromatography

A Waters 625 LC protein system (Waters Canada Ltd, Mississauga, ON) was used in conjunction with a Waters Intelligent Sample Processor (WISP 712®) for the injection and separation of the myofibrillar proteins. The proteins were detected with a Waters 484 variable absorbance detector set at 280 nm, together with a Waters 840 data station. For comparisons of the extraction buffers, four elution buffers were used in combination, blended through an Autoblend® system (Waters), to maintain a molarity of 0.5 KCl and pH similar to the extraction buffer with 100 mM Tris HCl (Fisher Scientific Co., Nepean, ON), 100 mM Tris Base (Fisher Scientific Co., Nepean, ON), milli-Q® water

(Milli-Q®; Waters, Millipore Ltd, Milford, MA) and 1 M KCl (Fisher Scientific Co., Nepean, ON). The proportions of each buffer were adjusted to achieve the desired pH level and salt concentration of the eluent. Once the optimal extraction buffer was chosen, the elution buffers were blended to maintain a constant ionic concentration of 0.4 M KCl at pH 6.0. Two columns, a Bio-Gel 40XL large molecular weight size exclusion column (Bio-Rad Laboratories Ltd, Mississauga, ON) and a 3000PW×l mid-range molecular weight size exclusion column (Supelco Canada Ltd, Oakville, ON) were compared at room temperature (23°C). All comparisons of extraction buffers were made with a flow rate of 0.6 ml min^{-1}.

Comparisons of chromatographic conditions

Molecular weight standards, comprised of thyroglobulin (bovine), gamma globulin (bovine), ovalbumin (chicken), myoglobin (horse) and vitamin B-12, were prepared as per manufacturer's (Bio-Rad Laboratories Ltd, Mississauga, ON) instructions and injected onto the 3000PWxL column at flow rates of 0.6, 0.7 and 0.8 ml min^{-1} (0.5 M KCl, pH 6.8) for determination of optimal flow rates. A standard injection of 200 μg of myosin light chains (bovine: Sigma Chemical Co., St Louis, MO) was separated at 0.3, 0.4 and 0.5 M KCl at pH 6.0 and a flow rate of 0.7 ml min^{-1} for comparisons of the salt composition of the elution buffer.

Linearity of sample response

Myosin (bovine), myosin light chains (bovine) and troponin (bovine) standards (Sigma Chemical Co., St Louis, MO) were injected to test for linearity in their UV absorbance. Standards were injected three times each at three concentrations (myosin: 60, 150, and 300 μg; myosin light chains and troponin: 80, 200 and 400 μg).

Mass recovery

Duplicate samples from fresh pork and veal inside round and a mixture of 90% beef ST and 10% beef heart tissue were used to calculate mass recovery. A micro BCA protein assay reagent kit (Pierce, Rockford, IL distributed by Chromatographic Specialties Inc., Brockville, ON) was used to measure protein concentration in the sample and in the pooled fraction eluted from the column. The assay was performed using the standard manufacturers protocol. The concentration of protein in the muscle extract and eluted from the column were compared to determine mass recovery.

Determination of molecular weight

A series of protein molecular weight standards (Sigma Chemical Co., St Louis, MO) were injected every two weeks to generate molecular weight calibration curves and verify system stability. Each standard was prepared to a concentration of 1 mg ml^{-1} using the same extraction buffer as used for the meat samples, and injected. The standards were prepared in advance and stored at −80°C for a maximum of 3 weeks.

Statistical analyses

All statistical analyses were performed by Statistical Analysis Software (SAS 1989), version 6.09, SAS Institute Inc., Cary, NC, USA). The raw areas (absorbance units full scale (aufs)) under the individual peaks were used for statistical analysis for the comparison of extraction buffers. The optimum combination of pH and NaCl was evaluated using a 3×5 factorial, randomized complete block design with three pH levels (5.5, 6.0, 7.0) and five NaCl concentrations (0.1 M, 0.154 M, 0.4 M, 0.5 M, 0.6 M) that was applied to the three replicates for each species. Analysis of variance was used to test for significant changes in peak area caused by animal, pH and NaCl effects with the General Linear Model procedure. The protein peaks that were significantly different, were analyzed further by using multiple regression to test for linear, quadratic and interaction effects of treatment. Response surfaces were generated to estimate the optimum combination of salt and pH.

To determine the linearity of sample response, regression analysis was used to compare the area under individual peaks. The experiment for characterization of extract stability was designed as six repeated measures over time on four animals. The area under individual peaks was converted to a percentage of the total area under all peaks as a measure of total protein. Regression over natural logarithm of time was used to analyze these percentage areas of individual peaks. With this analysis, the overall means, linear and quadratic coefficients were calculated for each animal and compared to the null hypothesis with a t-test.

RESULTS

Effects of extraction buffers

Typical chromatograms from the HPLC analysis of pork and beef samples are shown in Fig. 1(a) and (b). These chromatograms were derived when the optimal buffer (0.4 M NaCl, pH 6.0) was used for protein extraction. When the other 14 buffers were used, the chromatographic profile for pork or beef were similar to these figures; however, the areas under each peak varied. The extracts of pork and beef produced different chromatographic patterns but only the area under peak 1 (actomyosin/myosin: retention time of approximately 9 min) was significantly ($p \leq 0.05$) affected by NaCl and

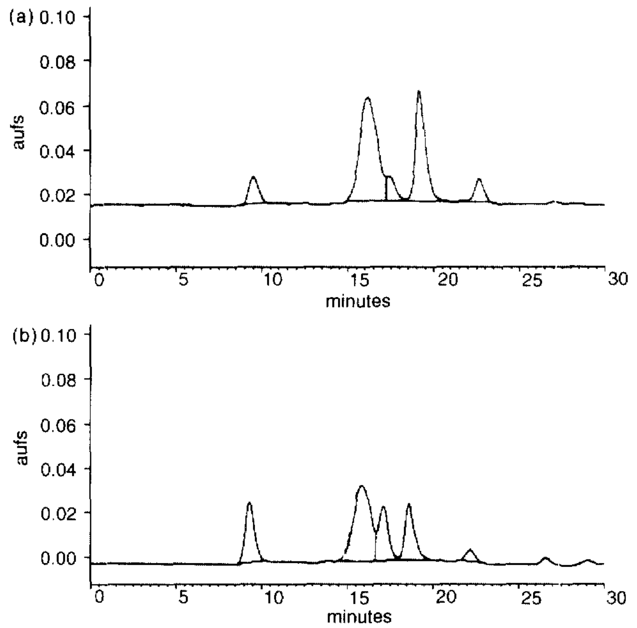

Fig. 1. Typical chromatogram for pork (a) or beef (b) longissimus muscle extracted with 0.4 M NaCl MES buffer, pH of 6.0 eluted from a Bio-Gel 40XL column.

pH in the extraction buffer for either pork or beef extracts. While other proteins in the chromatogram were affected by salt and pH, these followed the same general pattern observed for myosin.

There were a few samples in this study where salting-out occurred. With pork, this occurred for one animal at pH 7.0, in 0.5 M NaCl and 0.6 M NaCl. Results from this pork sample were not included in the dataset since the extract was of a viscous consistency unsuitable for HPLC analysis but samples obtained from three other animals from the same source were not effected. With beef, precipitation occurred with pH 7.0 and 0.6 M NaCl (two animals). These samples were not analyzed by HPLC because of their viscous consistency and were treated as missing data in statistical analysis.

Response surfaces were generated to show the effect of salt and pH on the area of peak 1 (actomyosin/myosin) for pork and beef. The response surface analysis for the pork extract (Fig. 2(a)) predicted an increase in peak area with increasing NaCl and pH levels until the NaCl level of between 0.475 and 0.6 M and pH of between 6.33 and 7.0, where the area achieved a maximum and then decreased. Simultaneously, increases in pH resulted in increased peak area to a maximum of between pH 6.33 and 7.0. The estimated maximum was 0.56 M NaCl and pH 7.05. The interaction between pH and NaCl was found to be statistically significant ($p \leq 0.10$), as were the quadratic effects of pH ($p \leq 0.10$) and of salt ($p \leq 0.05$) on peak area. A response surface generated for beef samples (Fig. 2(b)), demonstrated a similar increase in the area of peak 1 (actomyosin/myosin) with increasing NaCl concentration to a maximum between 0.475 to 0.6 M. An increase in the pH level also resulted in an increase in peak area, but no significant interaction was found between pH and NaCl. A linear effect of pH and salt on the peak area was found to be significant ($p \leq 0.10$); however, the quadratic effect was not statistically significant for pH and approached significance

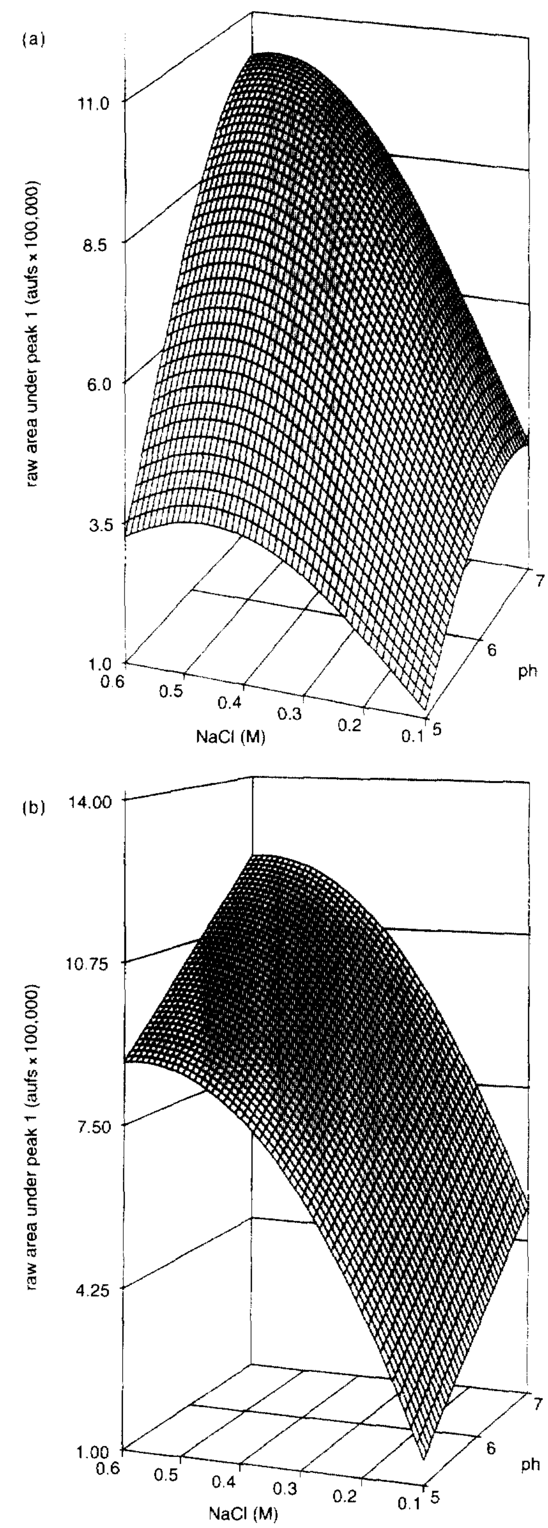

Fig. 2. Response surface showing the effect of NaCl concentration and pH on the area of peak 1 (actomyosin/myosin) of the pork (a) or beef (b) extract.

($p \leq 0.12$) for NaCl. The quadratic term for NaCl was, therefore, included in the regression model because it reduced the overall error. The maximum value for NaCl was estimated to be 0.58 M. Since the effect of pH increased linearly, the maximum for pH at the NaCl maximum could not be determined.

Chromatographic conditions

The criteria used for selecting the best flow rate was a chromatographic profile that yielded the best resolution (separation of peaks), and sharp peaks. The chromatograms of the gel filtration standard analyzed at the three KCl concentrations are shown in Fig. 3. The first standard peak (protein aggregates and thyroglobulin) was significantly affected by flow rate. Peak width decreased and peak sharpness increased as the flow rate increased.

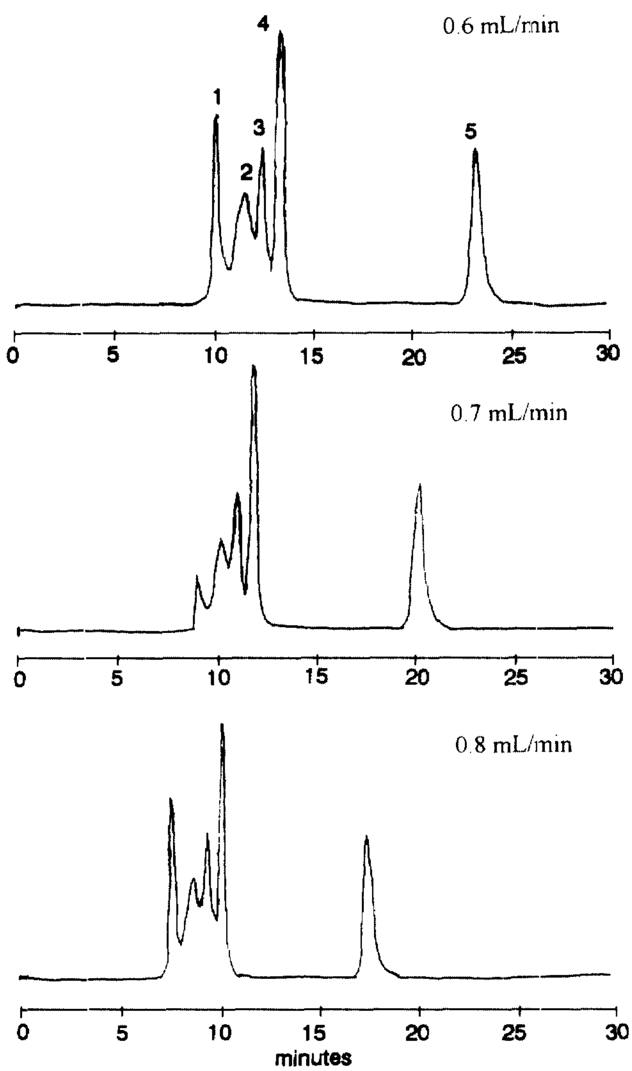

Fig. 3. Chromatograms of a gel filtration standard analyzed at three different flow rates: 0.6, 0.7 and 0.8 ml min^{-1}, at pH 6.8 using 0.50 M KCl. The peaks were identified as follows: peak 1 = protein aggregates and thyroglobulin, peak 2 = gamma globulin, peak 3 = ovalbumin, peak 4 = myoglobin and peak 5 = vitamin B-12.

Table 2. Amount of protein (μg) under the chromatographic peaks that composed the myosin light chains standard[a]

KCl (M)	peak 1[b]	peak 2[b]	peak 3[b]	peak 4[b]
0.30	35.20	55.05	57.84	45.75
(SEM)[c]	(2.58)	(3.86)	(4.34)	(9.88)
0.40	32.44	48.36	53.21	54.09
(SEM)	(3.21)	(4.46)	(7.76)	(16.30)
0.50	26.69	42.77	58.92	63.49
(SEM)	(0.99)	(0.64)	(5.31)	(7.12)
overall SEM	3.03	4.27	5.39	11.10

[a]There was an initial contaminating myosin peak which was not quantitated.
[b]The amount of protein represented by the peak is an average of three injections. 200 μg was the total amount of protein injected.
[c]Standard error of the mean.

At a flow rate of 0.8 ml min^{-1}, resolution of the early peaks was decreased.

A protein separation generated at pH 5.95 with flow rate of 0.7 ml min^{-1} and 0.5 M KCl, was not different from the chromatogram generated at 0.7 ml min^{-1}, 0.5 M KCl and pH 6.8. Variation in KCl level did not affect peak sharpness or separation (chromatograms not shown); however, the last peak of the myosin light chains standard tended to increase, as a percentage of total area, as KCl concentration increased (Table 2).

Repeated injections of the myosin, myosin light chains and troponin standards resulted in a statistically significantly linear relationship between concentration and peak area ($p \leq 0.05$) as shown in Table 3. The mass recovery of repeated injections of pork and beef (muscle and organ) was 98% and 114%, respectively.

Chromatograms for pork extracts analyzed after different storage times are shown in Fig. 4, with details in Table 4. The most notable change was the increase and subsequent decrease of peak 1/2. Peak 1 (actomyosin) and 2 (myosin) were statistically analyzed together because they did not always separate into discrete peaks. The combined peak increased in area from time zero to approximately 10 h, then decreased slightly and plateaued after 24 h. At approximately the same time that actomyosin and myosin reached maximum concentration, myosin light chains reached a minimum (10.2 and 9.1 h for peaks 4 and 5, respectively). Peaks 5 and 6 (both myosin light chains) were also added together because they did not always separate into discrete peaks. Approximately 3 h earlier α-actinin (7.26 h) and

Table 3. Linearity of myosin, myosin light chains and troponin absorbance with increasing protein concentration

Protein	Regression equation	R^2
Myosin	$y = 8314x - 28333$	0.9965
Myosin light chains	$y = 2750x + 45027$	0.9795
	$y = 5955x - 186542$	0.9984
	$y = 23373x - 1501194$	0.9641
Troponin	$y = 11422x - 33322$	0.9993

Table 4. Regression analysis of change in peak areas during storage of the protein extracts (p-values are provided, $p < 0{\cdot}05$ is significant)

Peak no.	Peak identity	Average retention time (min)[a]	Quadratic response	Minimum[b] (hours)	Maximum[b] (hours)
1/2	Actomyosin	7.95 (0.01)	0.0025	—	10.10
	Myosin	8.51 (0.02)			
3	Troponin	11.10 (0.01)	0.4556	—	—
4	Myosin light chain myoglobin	12.25 (0.01)	0.0009	10.20	—
5/6	Myosin light chains	14.42 (0.05)	0.0013	9.10	—
		15.01 (0.02)			
7	α-Actinin	19.79 (0.02)	0.0020	7.26	—
8	30 Kd peptide (possibly breakdown from troponin-T)	24.63 (0.06)	0.0116	7.69	—
9	Unknown peptide	32.24 (0.08)	0.3891	—	—

[a]Standard error of the mean retention time is recorded in brackets.
[b]Minimum and maximum of the quadratic response of the peak area.

the 30 KDa peptide (7.69 h) had reached their minimum. After the maximum peak area was reached for myosin and actomyosin at 10 h, this peak (1/2) decreased.

Typical chromatograms of fresh, raw beef, lamb, veal, pork and turkey are shown in Figs 5 and 6, respectively. Lamb was distinguished from veal, pork and beef by a larger area under peak 1 (actomyosin/myosin), peak 4 (myosin light chain), peak 5 (myosin light chain) and peak 7 (α-actinin). Lamb also had a larger area under peaks 1 (actomyosin/myosin) and 4 (myosin light chains) than pork. Lamb samples had more detectible protein under peaks 1 (actomyosin/myosin), 3 (troponin) and 7 (α-actinin) than beef. Peaks 8 (30 Kd) and 10 (unknown) were found in beef, pork and veal but not in lamb and therefore could be used to distinguish between these species. Beef and veal have very similar patterns except that peak 4 (myosin light chains) is larger in beef. Peak 9 (unknown) appears in beef and veal samples but not pork samples and could be used to identify these species.

Extracts of turkey breast muscles were also characterized and these chromatograms were compared to pork and beef extracts (Fig. 6). Both pork and beef can be distinguished from turkey with peaks 4 (myosin light

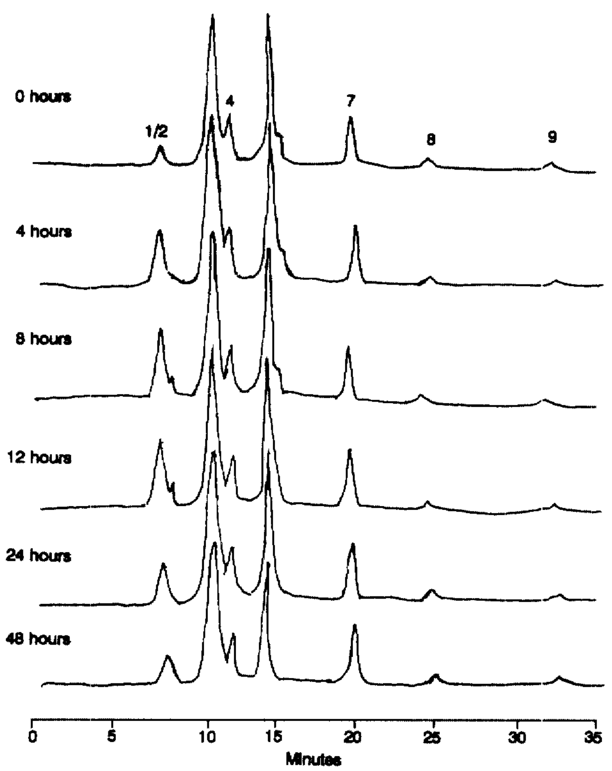

Fig. 4. Chromatograms of a pork sample extract injected 0, 4, 8, 12, and 48 h after protein extraction was complete. The peaks were identified as follows: p1/2 = actomyosin + myosin, p3 = troponin, p4 = a myosin light chain, p5/6 = myosin light chains, p7 = α-actinin, p8 = 30 Kd possibly from troponin-T and p9 = unknown peptide. The mobile phase used was 0.4 M KCl with a 3000PW×1 column.

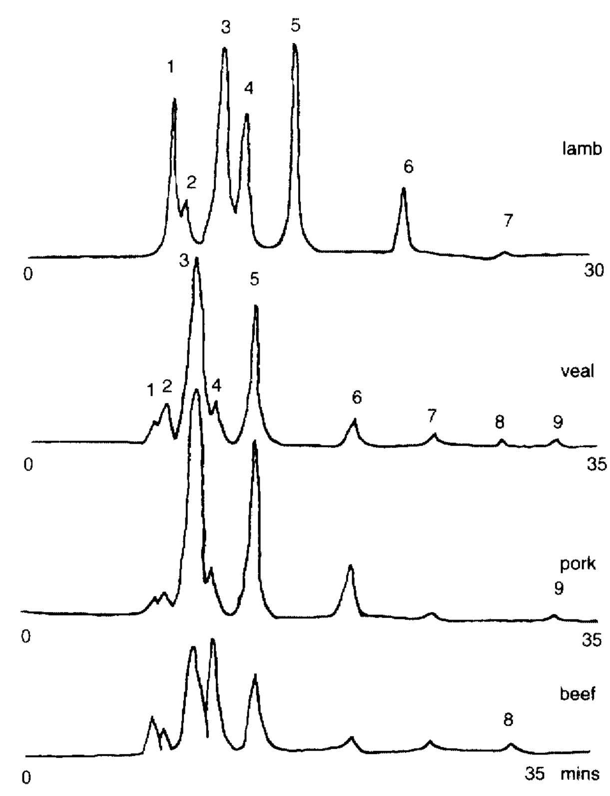

Fig. 5. Typical chromatograms of fresh, raw: lamb, veal, pork and beef LM. The mobile phase used was 0.4 M KCl with a 3000PW×1 column.

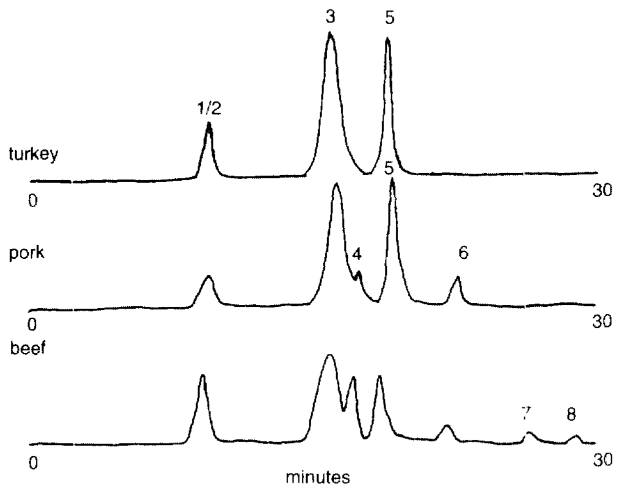

Fig. 6. Typical chromatograms of fresh, raw: turkey breast, pork LM and beef LM. Mobile phase used was 0.5 M KCl with a 40×1 column.

chains) and 6 (myosin light chains) for pork, and peaks 4 (myosin light chains), 6 (myosin light chains), 7 (α-actinin), and 8 (30 Kd) of beef chromatograms. Also, the area of peak 4 (myosin light chain) varied with turkey the lowest, pork intermediate and beef the highest. The area of peak 1/2 (actomyosin/myosin) varied between all three species as well.

DISCUSSION

Developing a method for regular use in monitoring meat adulteration is important to prevent situations where religious, economic, or health aspects are a concern. The overall objective of this research was to develop a method that was simple, quick, did not denature proteins and was quantitative. Several steps were involved in the adaptation of this method. Initially, it was necessary to optimize the conditions of protein extraction from a variety of muscle samples. The quantity of myofibrillar protein that can be extracted from a meat sample is dependent on the pH and ionic strength of the extraction buffer (Richardson and Jones, 1987) as well as the sample to extraction buffer ratio and homogenizing and centrifugation conditions (Lan *et al.*, 1993). Proteins can be damaged or lost during these preparation steps, thereby influencing subsequent analysis. An interaction between pH and salt in the extraction buffer has also been shown to influence protein extraction (Richardson and Jones, 1987) and solubility (Eisele and Brekke, 1981; Samejima *et al.*, 1985; Foegeding, 1987). Most processed meat products (emulsified and comminuted) contain 2.5 to 3% NaCl usually applied in a 0.6 M NaCl solution (Eisele and Brekke, 1981). However, these conditions may not be optimal for extraction of the muscle proteins for further characterization by HPLC.

Once protein extraction was optimized, our next goal was to carefully examine the chromatographic conditions through manipulation of flow rate, pH and ionic strength and to maximize the resolution and sharpness of protein fractions of meat samples. By increasing the flow rate, an economic advantage can be gained through decreased separation time and increased resolution of peaks; however, if the flow rate is too high, the peaks may overlap and resolution can be lost (Synder and Kirkland, 1979). Additionally, if the flow rate is too low, samples may diffuse and peaks become broad and rounded (Synder and Kirkland, 1979). Another component of the chromatographic analyses was the optimization of the salt concentration and pH of the running buffer. A salt concentration above 0.1 M is generally used with size exclusion chromatography to prevent ionic interactions with the column packing; however, if the concentration is too high (0.3 M) it can invoke hydrophobic interactions between the sample and the column (Anon, undated). Both pH and ionic strength affect protein conformation and aggregation in solution (Willis, 1991) and may have interactive effects on protein solubility (Eisele and Brekke, 1981; Samejima *et al.*, 1985; Foegeding, 1987). Therefore, it was necessary to optimize these two factors at a level that both solubilizes the sample and prevents secondary interactions between the sample and the column. The final stage in the method development process was verification of linearity in sample response, mass recovery, column efficiency and sample extract stability.

The optimum pH and salt combination for the extraction of myofibrillar proteins was determined in a variety of muscle species. From the response surfaces analyses, the estimates of the maximum value for NaCl and pH, for both pork and beef, suggests that a pH of 7.0 and 0.6 M NaCl would be the optimal values. Similar values were used for extracting salt soluble proteins (Gaska and Regenstein, 1982; Gordon and Barbut, 1992) and for preparation of actomyosin (Galluzzo and Regenstein, 1978*a*). Lan *et al.* (1993) found that extraction of proteins from beef muscles at a pH of 6.0 and 0.6 M NaCl was most effective for protein extractability but increasing the pH to 6.5 resulted in a significant decline. With chicken breast and thigh, Xiong and Brekke (1991) found that a solution of 0.6 M NaCl at pH 6.00 or pH 5.75 was most effective for protein extractability. Similarily, Foegeding (1987) found protein solubility increased with increased salt level (0.25 to 0.5 M NaCl pH 6.0) but no effect was found at pH 5 or 7. Samejima *et al.* (1992) found that rabbit and pig skeletal and cardiac myofibrils had a maximum solubility at pH of 6.0, using a 0.6 M NaCl solution. Overall, the literature suggests pH 6.0 to be the optimum value for muscle protein extraction. Our analyses suggested a slightly higher theorectical pH for pork and beef muscle extraction. However, problems were encountered. Salting out occurred at pH 7.0, 0.5 M NaCl and pH 7.0, 0.6 M NaCl with the pork sample, and at pH 7.0, 0.6 M NaCl for some beef samples. It was necessary to modify the theoretical optima to reflect these phenomena and therefore the recommended extraction buffer consists of NaCl at 0.4 M and pH 6.0.

The extracted supernatant, which contains mainly salt soluble proteins and sarcoplasmic proteins (Prusa and Bowers, 1984; Froning and Sackett, 1985), was used for all analyses. The pellet consisting of fibre, fibril and stromal proteins; primarily collagen and elastin (Schut, 1978), was discarded. Reproducible chromatograms and acceptable column lifetime were obtained for all samples analysed. Quantitation was linear and, based upon the mass recovery, measured the amount of protein in the peaks directly. The area under individual peaks was adversely affected by storage of the extract for more than 10–12 h and care must be taken to ensure analysis of prepared samples prior to this time.

Two different columns were compared during these studies and the most representative chromatograms were included for each. In general, there was a greater resolution of both the high and low molecular weight protein peaks with the 3000PW×1 column but the 40×1 column could be used to effectively distinguish between very different species. Although the chromatographic patterns were similar between different muscles, within species, there were several unique proteins and the percentage area under several peaks varied (for a detailed explanation, see Toorup *et al.*, 1998). A similar result was also found by Ashoor *et al.* (1988) when using reverse phase chromatography but quantitation with size exclusion chromatography HPLC requires less interaction between the proteins and the column, and therefore, should be more accurate than the complex and indirect methods that were used in the past.

The methods developed during this study should prove to be quite useful for detection of meat adulteration or separation of muscle proteins. It should be quite simple for commercial laboratories to adapt this method for routine scanning of prepared meat products and in this way the method can be used to protect against consumer fraud. In addition, it will be possible to apply this method to the study of muscle metabolism, ageing, protein accretion etc. Studies of functional overload of muscles, particularly cardiac muscle, as produced through training or stress may make this method useful to both human and animal physiologists.

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