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Detection of Mechanically Recovered Chicken Meat Using Capillary Gel Electrophoresis - A Feasibility Study

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SUMMARY

Mechanically recovered meat (MRM) is recovered by the application of pressure or shear forces to animal bones or poultry carcasses from which the bulk of the meat has been removed. It is used in a wide range of meat products either as a binding agent or as an inexpensive source of meat. Food authenticity issues and the view that products which contain MRM should be labelled to comply with The Food Labelling Regulations (1984) have generated the need for a method of detection of MRM in meat products.

The aim of this work was to test whether a relatively new technique, capillary gel electrophoresis (CGE), could be used to detect MRM in raw, cooked and processed hand deboned meat (HDM) and in mixtures, and if so, what level of MRM could be distinguished from 0% MRM. Results showed:

- CGE using an SDS-sieving matrix was suitable for separating and quantifying proteins extracted from raw and cooked chicken meat.
- In raw chicken meat, peak areas of protein differed significantly between MRM and HDM. That of haemoglobin was higher in MRM, all other peaks were higher in HDM. Detection of MRM in mixtures was dependent on the peak area of haemoglobin and its ratio to other peaks. Mixtures containing 7.5% MRM were significantly different to 0% MRM when using a ratio of the peak area of haemoglobin to 'peak 4' (unknown). Whilst this indicates the potential of the method, variation in MRM needs to be measured and taken into account if this method is to be used for prediction purposes.
- Cooking had an effect on the peak area of all proteins but the range of values for the ratio of peak 1 to peaks 2,3,4,5,6 and 8 was distinct between MRM and HDM. Measurement of the variation of these parameters to look for significant differences is justified.
- Proteins extracted from processed chicken meat (F_0=9-14) were not completely resolved though there were characteristic distinguishing features between MRM

and HDM. One peak which was distinct in processed HDM may provide potential for future methods.

The feasibility of using CGE to detect chicken MRM in raw and possibly cooked meat has been shown. To develop a robust method, further work is required to identify and quantify variables associated with MRM and to use statistical approaches to adjust for the measured uncertainty.

CONTENTS

1. INTRODUCTION

Mechanically recovered meat (MRM) is the finely comminuted product recovered by the application of pressure or shear forces to animal bones or poultry carcasses from which the bulk of the meat has been removed. It is used in a wide range of meat products either as a binding agent or as an inexpensive source of meat.

The UK Food Labelling Regulations (1984) require that when any food or food ingredient has undergone any particular process or treatment, a reference to that process or treatment should be included in the name of the food and/or the list of ingredients on the label where its omission would otherwise mislead the consumer. Those responsible for the enforcement of food law in the UK, the Local Authorities Co-ordinating Body on Food and Trading Standards (LACOTS), believe that this should be applied to MRM. If this is to be achieved, then there is a need for a method of detection of MRM.

The chemical composition of MRM is inherently variable due to the natural variation within and between animal species and cuts of meat, previous treatments of the carcass (trimming, freezing), and the machine type and operating conditions used in the recovery process. Overall, this variability is greater than any differences in the chemical composition of MRM and hand deboned meat (HDM) such that on the basis of chemical composition, they are essentially similar (Meech and Kirk, 1986; Crosland *et al.*, 1995).

Approaches to methods of detection have focused on presence of bone fragments, bone marrow (Field *et al.*, 1978; Stevenson *et al.*, 1992; Savage *et al.*, 1995; Pickering *et al.*, 1995a) and connective tissue (Pickering *et al.*, 1995b) which are likely to be released during the mechanical recovery process. Electrophoretic work (SDS-PAGE) has visually identified protein bands which differ between MRM and HDM in most meat species (beef, pork, chicken, lamb and turkey). Using visual appraisal it was suggested that mixtures incorporating MRM at 5-10% for red meat and 25% for poultry could be differentiated (Savage *et al.*, 1995). However, visual appraisal is

subjective and not suited to compliance testing. It is also difficult to validate the levels of detection. In this work, capillary electrophoresis (CE), a relatively new technique suited to the separation and quantitation of biomolecules, has been used to separate proteins in HDM, MRM and bone marrow, with the objective of identifying differences which could form the basis of a method for the detection of MRM in meat products.

CE systems consist of a narrow bore fused silica capillary with each end held in buffer reservoirs so that the contents of the capillary and reservoirs are identical. A sample is loaded by replacing one of the buffer reservoirs with sample and applying either hydrostatic pressure or an electric field. The buffer reservoir is then returned and an electric field is applied (upto 30kV). Analytes migrate in the electric field with varying mobility and pass through a detector at the opposite end of the capillary. Peak area, height and migration time are recorded. CE is very similar in principle to gel electrophoresis but the fused silica capillary enables heat to be dissipated more readily than in a gel, hence much higher voltages can be applied without adverse heating effects and separations can be achieved in a shorter time. The advantage of CE as compared to SDS-PAGE is the speed of separation with simultaneous quantitation by on-line detection. This negates the time consuming processes of gel preparation, staining and destaining and densitometric analysis for quantitation and avoids exposure to neurotoxic acrylamide monomers.

CE can also be operated in a number of different modes, each utilising different principles to separate components. Three modes have been investigated in this work - capillary zone electrophoresis (CZE), micellar electrokinetic capillary chromatography (MECC) and capillary gel electrophoresis (CGE). CZE and MECC applied to water extracts of chicken meat indicated that there may be differences between the separated components suited to distinguishing between chicken HDM and MRM (Day and Brown, 1993). The use of SDS or urea in the medium used to extract proteins, particularly heat denatured proteins, improves extraction; however, the application of CZE to SDS extracts, as requested by MAFF, resulted in a large unresolved peak of SDS (Day and Brown, 1993). Therefore CGE, a separation mode

which utilises SDS or urea in the electrolyte was used. In this report, the use of CGE is described. Two sieving matrices were compared for their effectiveness in separating chicken meat proteins and ease of use. Of these, one was selected and used to assess quantitatively the variability of potentially discriminating protein peaks in raw, cooked and processed MRM and HDM alone. For raw meat, mixtures of MRM in HDM were used to determine the levels at which MRM can be distinguished.

2. MATERIALS AND METHODS

2.1 Reagents

Milli-Q purified water was used for all the reagents and sample preparation throughout this work. Sodium dodecyl sulphate (SDS), 2-mercaptoethanol, trisodium citrate dihydrate, sodium dihydrogen phosphate and urea (AnalaR grade) were purchased from BDH. Trizma base and DL-dithiothreitol (DTT) were purchased from Sigma. ProSort™ SDS-protein analysis reagent, analysis standards and the reference marker were purchased from Applied Biosystems Ltd. Methylhydroxyethylcellulose (MHEC 35,000) was obtained from Hercules Ltd, Salford, UK.

Sample reduction buffer was prepared by dissolving 73mg of trisodium citrate dihydrate and 38mg of DTT in 37.5ml of 8M urea. The pH was adjusted to 8.0 with 0.1N NaOH and the volume made up to 50ml with water.

Phosphate buffer was prepared by dissolving 69mg of sodium dihydrogen phosphate and 25mg of MHEC in 37.5ml of 8M urea. The pH was adjusted to 2.50 with 4M phosphoric acid and the volume made up to 50ml with water.

Lammeli buffer was prepared using 8ml 0.5M Tris-HCl, pH 6.8, 12.8ml 10% SDS, 3.2ml 2-mercaptoethanol and 40ml water.

2.2 Preparation of samples

2.2.1 Raw, processed and 'cooked' hand deboned chicken meat

Fresh, deboned and skinned chicken breast meat was purchased from a local supermarket. Two chicken breasts were combined to prepare one sample. Twenty samples were prepared using 40 chicken breasts. For each sample both breasts were passed through an Excel mincing machine using a medium grade plate (6mm hole

size) and the mince mixed thoroughly. For raw HDM, five 2g aliquots from each sample were frozen and stored at -18°C until required for use.

For processed HDM, three 150g aliquots were taken from each sample and each processed with 30g 2% sodium chloride solution in U8 lacquered cans. The cans were vacuum seamed and processed at 121°C for 53-54 minutes to give an Fo value of 9.1-13.5. Cans were stored at ambient until required for use.

For 'cooked' HDM two cooking treatments were applied - centre temperature of 70°C for 2 minutes or 100°C for 2 minutes. A thermocouple was located in the centre of a 2.5cm^3 cube of frozen meat by drilling to the centre of the block. The block of meat with the thermocouple held in place was vacuum sealed into a foil pack approximately 11 x 7 cm with a gland to enable connection of the thermocouple. The packs were immersed in a glycerol bath at 71°C (for 70°) and 101°C (for 100°C) and the time taken to achieve the target centre temperature recorded. The samples were held for 2 minutes at this temperature, then removed and placed on ice to cool rapidly. Cooked samples were held at 4°C until extracted. Cooking at each temperature was carried out in duplicate.

2.2.2 Raw, processed and 'cooked' mechanically recovered chicken meat

Twenty 10kg frozen blocks of MRM produced on 20 consecutive days (1st-31st July 1994) by Perimax Meat Company Limited were purchased and stored frozen at -22°C ± 2°C until required for use. Each block was treated as one sample. Approximately 500g of frozen MRM was sawn from each block and allowed to thaw at 4°C overnight. Any drip loss was incorporated into the sample by thorough mixing. Raw, processed and cooked samples and replicates of each were prepared as described for HDM (Section 2.1.2).

2.2.3 Chicken bone marrow

Twelve chicken thighs (meat and bone) were purchased from a local supermarket. After removing the meat, the bones were crushed and the marrow extracted. Marrow from all 12 thighs was pooled to give approximately 1g of sample. This was stored frozen at -18°C until required for use.

2.2.4 Mixtures of MRM in HDM

Mixtures of MRM (0, 5, 7.5,10, 15, 20, 30 and 50%) in HDM were prepared using a sample of HDM (8 chicken breasts minced and mixed as described in Section 2.2.1) and a sample of MRM (250g from each of 2 blocks of MRM, thawed overnight and thoroughly mixed). Appropriate weights of each were mixed to give a final weight of 200g (± 0.1g) per sample. The mixtures were mixed thoroughly using a Moulinex food mixer. The extent of mixing was assessed visually. From each mixture, five 2g aliquots were frozen (setting -18°C) and stored until required for use. The procedure was repeated on five occasions to give 5 batches of mixture (total of 25x2g aliquots of each mixture).

2.3 Extraction procedures for raw and processed meat

2.3.1 SDS extraction

Two approaches to SDS extraction were assessed, one described by Mackie *et al.* (1992) as requested by MAFF, and a modification of this in keeping with the preparation of protein standards for analysis by ProSort™ SDS capillary electrophoresis.

Meat samples were extracted in 2% SDS (raw meat 1:4(w/v), processed meat 1:2(w/v)) by grinding in a Potter-Elverheim hand held homogeniser. The resulting suspension was held in a 60°C water bath for 35 minutes then clarified by centrifugation (11,500g for 5 minutes). For the method of Mackie *et al.* (1992), 1ml

supernatant was diluted with 7.5ml Lammeli sample buffer. For the method using ProSort™, 2.5ml supernatant was diluted with 1ml 5% SDS/5% 2-mercaptoethanol and 1.5ml water. These extracts were heated in a glycerol bath at 100°C for 15 minutes, cooled on ice, then filtered through a 0.45µm membrane filter and stored frozen (-18°C) until required for analysis by CE.

Prior to analysis, a neutral marker was added to the sample. For extracts prepared using the method of Mackie *et al.* (1992), 10µl neutral marker was added to 190µl extract. For the ProSort™ method, 10µl neutral marker and 150µl 0.1% SDS/1% 2-mercaptoethanol was added to 40µl extract. (This was modified when applied to mixtures: 5µl neutral marker and 155µl 0.1% SDS/1% 2-mercaptoethanol was added to 40µl extract).

2.3.2 Urea extraction

Using a pestle and mortar, 2g meat was ground in 4ml sample reduction buffer. The solution was left to stand overnight at 4°C, then centrifuged at 11,500g for 5 minutes. In order to filter the samples prior to CE analysis, raw samples required a further dilution with sample reduction buffer (1:2 v/v) and filtration through Millipore AP20 pre-filters, whereas processed samples did not require further treatment. All samples were filtered through 0.45µm membrane filters prior to analysis by CE.

2.4 Capillary electrophoresis procedures

A Waters Quanta™ 4000 capillary electrophoresis instrument with a Waters Maxima™ 820 data station was used throughout.

2.4.1 CGE with MHEC and urea buffer additives

A hydrophilically coated fused silica capillary column (CElect P1, Supelco) (50µm i.d., total length 57.5cm, effective length 50cm) was conditioned using 0.1N NaOH

for 10 minutes, followed by phosphate buffer, pH 2.5 for 30 minutes. The sample was injected electrokinetically (+5kV) for 30 seconds. Separations were performed with an applied voltage of 20kV (348V/cm). UV detection was at 214nm. Between runs the capillary column was purged with phosphate buffer, pH 2.5 for 20 minutes and washed with Milli-Q water at the end of the day.

2.4.2 CGE with SDS gel-filled capillary system

A fused-silica capillary column (75µm i.d., total length 40cm, effective length 32.5cm) was conditioned using 0.1N NaOH for 10 minutes, followed by Milli-Q water for 5 minutes and then coated with ProSort™ SDS-protein analysis reagent as supplied for 1 hour. The sample was injected into the capillary column electrokinetically (-5 kV) for 20 seconds unless otherwise stated. Separations were performed with an applied voltage of -12kV (300V/cm). UV detection was at 214 nm. Between runs the capillary was purged with ProSort™ SDS-protein analysis reagent for 20 minutes and washed with Milli-Q water at the end of the day.

Protein standards were prepared and analysed according to the method provided with the ProSort™ analysis kit (Applied Biosystems, Perkin-Elmer).

3. RESULTS AND DISCUSSION

3.1 Comparison of separation methods

Whilst some differences between HDM and MRM were seen using water extracts of meat separated by CZE (Day and Brown, 1993), addition of SDS or urea to the medium used for extraction, particularly for heat denatured proteins, may be beneficial. Application of CZE to SDS extracts resulted in a large unresolved peak of SDS (Day and Brown, 1993). In the work reported here capillary electrophoresis methods which incorporate SDS or urea in the electrolyte were used.

3.1.1 Separation by CGE using MHEC and urea buffer additives

CE separations of milk proteins were significantly improved when extracted with urea and separated using a hydrophilically coated column and a low pH (2.5) running buffer containing modified cellulose and urea as compared to CZE (de Jong *et al.*, 1993). Under these conditions, adsorption of proteins onto the capillary wall was suppressed, the urea prevented reassociation of proteins and the modified cellulose behaved as a viscosity agent sieving proteins.

Results of the application of this approach to meat proteins are shown in Figure 1. For raw meat, peak resolution was improved as compared to CZE (Day and Brown, 1993) and more peaks were evident. For processed meat a maximum of two peaks in addition to the neutral marker were present, despite the reduced extraction ratio (1:2w/v) as compared to raw meat (1:4w/v).

A number of practical difficulties were encountered which made this method time consuming and potentially very variable. Column conditioning using the citrate buffer described by de Jong *et al.* (1993) did not result in a steady baseline and if meat, or even milk samples as in the original method, were injected, no peaks were observed. Only by switching to phosphate buffer could samples be run. Buffer preparation was time consuming; solvation of MHEC in the buffer required 2 hours

stirring and slight shifts in either pH (± 0.1) or molarity of the buffer had a marked effect on migration time. Great care was required to use precise volumes of phosphoric acid to achieve the desired pH (2.5) without affecting the molarity. For these reasons this method was not pursued beyond obtaining the data shown in Figure 1.

3.1.2 Separation using SDS ProSort™ reagent

ProSort™ SDS-protein analysis reagent is a non-rigid replaceable sieving matrix in which proteins are separated on the same basis as classical SDS-PAGE. The reagent is an entangled polymer solution containing SDS and a column coating agent. Proteins denatured in SDS acquire the same mass:charge ratio so mobility in a sieving matrix is dependent on size only. Following initial equilibration of a fused silica capillary column with ProSort™ reagent for 1 hour, protein standards were separated (Figure 2a) and reproducible migration times were achieved. Migration times were greater than those quoted by the reagent manufacturer due to a longer effective column length used with the Waters Quanta 4000 CE (32.5cm) instrument as compared to the Model 270-HT ABI CE system (20cm) quoted in the method. The linear relationship between the logarithm of the molecular weight of the protein standards and the relative migration time still held (Figure 2b). The peak area of the myosin standard was variable and on occasions was not seen. Repeated freeze thaw cycles of the stock solution may have caused insolubility of the protein. For this reason, myosin was omitted from the standard curve.

3.1.3 Comparison of extraction methods

Meat proteins were extracted using two methods differing in SDS and mercaptoethanol concentration, and final weight to volume extraction ratio. The same CGE separation method was applied to both extracts. For raw HDM and MRM, the extraction procedure had little effect on the numbers of peaks in the electropherogram (Figure 3). Resolution appeared slightly better for the 'Mackie' extraction method.

This is likely to be due to the lower concentration of sample injected, the final extraction ratio of the injected sample being 1:31.6 (w/v) for this method compared with 1:16 for the 'ProSort' method. Levels of protein in the extracts were not determined as SDS at the levels present is known to interfere with the assay (Bio-rad, 1992).

For processed HDM and MRM, the 'Mackie' extraction method resulted in very few peaks whereas the 'ProSort' method gave a discernible overall shape in the electropherogram but without baseline resolution of peaks (Figure 4). Again the difference may be due to concentration, the final extraction ratio of the methods being: 'Mackie' 1:15.8(w/v) and 'ProSort' 1:8 (w/v).

As the 'ProSort' extraction method provided information to compare both raw and processed MRM and HDM, this was used for all further work.

3.2 Discrimination between HDM and MRM

Initially samples of 100% HDM and 100% MRM (both raw and processed) were analysed to determine whether there were significant differences between them which justified the further analysis of mixtures.

3.2.1 Raw meat - comparison of HDM, MRM and bone marrow

Comparisons of electropherograms of raw HDM and MRM did not indicate unique peaks to either sample type (Figure 5) but there were significant differences ($P<0.05$) in peak area (selected peaks are labelled 1-9 in Figure 5), between the two meat types, both for absolute peak area and when normalised with respect to the neutral marker (Figure 6). Normalisation reduced the relative standard deviation (%RSD) of peak areas in HDM, except for peak 5 (Table 1), and therefore increased the magnitude of differences between the two meat types (Table 2, see t statistic).

Table 1 The effect of normalisation of peak areas on the variation in raw HDM and raw MRM

Peak No.	%RSD HDM		%RSD MRM	
	Peak area	Normalised peak area	Peak area	Normalised peak area
Marker	13.06	0.00	13.65	0.00
1	25.16	18.00	16.75	16.09
2	25.20	18.54	12.96	14.73
3	22.92	17.69	13.41	11.80
4	18.28	13.32	12.11	16.07
5	7.66	11.04	13.85	14.73
6	18.56	13.40	18.16	19.39
7	15.47	11.20	13.53	18.30
8	15.13	10.90	28.20	26.61
9	13.33	12.17	27.23	28.07

Table 2 Comparison of peak areas in raw HDM and raw MRM (two sample t-tests) before and after normalisation with respect to the neutral marker. (t = t statistic, P = probability)

Peak no.	Peak area				Normalised peak area			
	HDM	MRM	t	P	HDM	MRM	t	P
Marker	6249	6675	-1.26	0.220	-	-	-	-
1	12183	23659	-8.31	0.000*	1.93	3.57	-8.92	0.000*
2	20913	13335	4.77	0.000*	3.32	2.02	6.69	0.000*
3	27164	19151	4.17	0.001*	4.33	2.89	6.06	0.000*
4	39004	21019	8.30	0.000*	6.24	3.19	11.04	0.000*
5	85347	73620	3.54	0.002*	13.81	11.12	4.32	0.000*
6	28706	15870	7.46	0.000*	4.59	2.40	10.12	0.000*
7	13445	9255	6.09	0.000*	2.16	1.41	7.65	0.000*
8	17227	13896	2.58	0.017*	2.76	2.09	3.90	0.001*
9	86616	69427	2.84	0.010*	13.96	10.46	3.78	0.001*

* $P < 0.05$. - = not applicable as peak areas normalised relative to the neutral marker

Normalisation had little effect on the %RSD of peaks in MRM (Table 1). For most peaks, peak area was greater in HDM than MRM, the exception being peak 1 (Figure 6).

MRM is considered to differ from HDM due to the potential for inclusion of components from bone marrow. During the mechanical recovery process, the high pressure or shear forces applied can cause release of marrow into the meat. Extraction and separation of bone marrow components using the same method applied to MRM, showed a major peak with same relative migration time as peak 1 (Figure 7). The molecular weight of peak 1, determined using a calibration curve similar to that in Figure 2, was 15,700Da, of the order of a subunit of haemoglobin (64,500/4 kDa). Haemoglobin was reported to be the fastest migrating band in SDS-PAGE studies of MRM (Field *et al.*, 1978; Savage *et al.*, 1995) though the intensity of this band was reported to be less evident in chicken than beef MRM (Savage *et al.*, 1995).

Other named proteins, which SDS-PAGE electrophoresis has suggested differ between MRM and HDM, are actin and myosin (Field *et al.*, 1978; Stevenson *et al.*, 1992). From the molecular weight of these proteins (43kDa and 205kDa respectively) and the calibration curves, peak 5 is likely to be actin and peak 9 myosin (see Figure 5). Both were present at significantly higher levels in HDM than MRM (Table 2) which is in agreement with other workers.

Whilst these data suggest that the peak area of all 9 peaks can be used to distinguish between 100% HDM and 100% MRM, absolute differences will be affected by factors that influence protein levels in the bird and in the extract prepared for analysis. The use of peak ratios may enable such variation to be overcome. As peak 1 was the only peak which was greater in MRM than HDM, a ratio of peak 1: peak x (any of the other peaks) will give the greatest difference between HDM and MRM. Using two-sample t-tests to compare peak ratios in HDM and MRM, significant differences were observed in all cases and the value of the t-statistic was increased as compared to using peak area alone (Table 3).

Table 3 Comparison of peak ratios in raw HDM and raw MRM (two sample t-tests). (T = t statistic, P = probability)

Peak ratio	HDM	MRM	t	P
pk1/pk2	0.59	1.78	-22.65	0.000*
pk1/pk3	0.45	1.24	-14.39	0.000*
pk1/pk4	0.31	1.13	-16.47	0.000*
pk1/pk5	0.14	0.32	-11.94	0.000*
pk1/pk6	0.42	1.51	-17.25	0.000*
pk1/pk7	0.90	2.59	-11.43	0.000*
pk1/pk8	0.70	1.77	-10.16	0.000*
pk1/pk9	0.14	0.36	-9.53	0.000*

The largest t-statistic was achieved for the ratio of peak 1/ peak 2. Interestingly, Meech and Kirk (1986) concluded that of the chemical parameters available for the detection of MRM, the ratio of haemoglobin to myoglobin or total haem 'would probably be best'. The proximity of peak 2 to peak 1 (haemoglobin) with respect to migration time and the molecular weight of peak 2 (approximately 17,500kDa), suggest this is likely to be myoglobin (MW sperm whale myoglobin is 17,200kDa (Werner *et al.*, 1993)).

In mixtures where MRM is added to HDM, the area of peak 1 would be expected to increase with % MRM and the peak area of other peaks would be expected to decrease. Hence the ratio of peak 1/ peak x should be greater in samples containing MRM than in those containing HDM alone. This hypothesis is tested in Section 3.3.

3.2.2 Processed meat

Twenty processed samples of each meat type were analysed. Electropherograms showed an unresolved peak within which there was some characteristic features (Figures 8 and 9). This is similar to the analysis of processed meat by SDS-PAGE

which resulted in smearing of the background with fewer distinctive bands (Savage *et al.*, 1995). Here, migration times were variable between samples and to enable comparison, only data from peak start to peak end were plotted on an equivalent time scale (Figures 8 and 9). Despite the lack of baseline resolution of peaks, HDM and MRM had distinctive patterns which enabled electropherograms to be distinguished visually. The variation in migration time limited assignment of equivalent peaks in HDM and MRM and the use of pattern recognition techniques. Generally the pattern could be divided into two main areas of peaks separated by a valley. This was most distinctive for HDM due to a large peak (labelled A, Figure 8). MRM followed a similar trend but there was not a clearly distinctive peak of the same size relative to peaks around it (Figure 9).

Despite the lack of clearly resolved peaks and the difficulty of assigning corresponding peaks in MRM and HDM, an attempt was made to quantitatively compare them using peak A alone; the last peak in the electropherogram, labelled peak B (Figures 8 and 9), selected on the basis of ease of assignment in both meat types; and a ratio of peak A/ peak B. Peak area determination, the preferred method for quantitation, was not meaningful due to the lack of resolution so peak height was used (Table 4).

Table 4 Comparison of selected peaks in processed HDM and MRM. Results of two-sample t-tests.

(For HDM n=12, MRM n=14)

Attribute	HDM			MRM			Statistic	
	Mean	SD	%RSD	Mean	SD	%RSD	t-value	P
Peak A[a]	3,256	727	22.3	1119	478	42.7	10.98	0.000
Peak B[a]	398	116	29.1	339	135	39.8	1.48	0.15
pkA/pkB	8.65	2.4	27.7	3.29	0.63	19.1	9.65	0.000

[a] peak height

Normal probability plots showed a normal distribution of the two data sets. Two-sample t-tests comparing the equivalence of the mean peak heights and the peak ratio showed they each differed significantly between MRM and HDM except for peak B (Table 4). Despite this statistical significance, the subjective nature of peak assignment did not, in our opinion, justify the analysis of mixtures of processed HDM and MRM. However, future work to isolate and identify peak A in processed HDM may be warranted. It is not clear whether this is a heat resistant component in HDM or if it is generated on processing. If the observed difference were substantiated, antibodies raised to this component and another as an internal standard may form the basis of a rapid detection method for processed chicken MRM in HDM.

3.2.3 Cooked meat

This section of work was requested by MAFF after a review of data for raw and processed meat. The objective was to carry out initial testing on moderately heat treated samples as opposed to processed samples to determine whether peaks which differed between raw MRM and HDM would still be present after cooking. Only two samples of HDM and MRM were used for each of the 2 cooking treatments.

The cooking procedures were standardised and the time and temperature profile of the samples recorded using a thermocouple (Table 5). Differences in the temperature of samples at the start of heating did not have a great effect on the time taken to reach the target temperature.

Heating at 70°C and 100°C did not result in the same degree of denaturation as 'processing' as peaks present in the raw meat were still discernible after these more moderate heat treatments (Figures 10 and 11). Similar observations were made for cooked versus processed treatments when proteins were separated by SDS-PAGE (Savage *et al.*, 1995). The same nine peaks selected in electropherograms of raw meat (labelled in Figures 10 and 11) were used to assess the effects of cooking on proteins in HDM and MRM. When comparing absolute peak areas, heat treatment did not result in a consistent trend between duplicates. Normalisation of peak areas with

respect to the neutral marker removed these inconsistencies and was therefore used to compare the effects of heating on HDM and MRM (Figure 12). Heating reduced the peak area of all peaks except that the trend for peaks 1 and 2 differed between HDM and MRM. Further replication is needed to substantiate the trends observed, but in HDM peak 1 (haemoglobin) was unaffected at 70°C and reduced at 100°C whereas in MRM it was reduced at 70°C but no further reduction occurred at 100°C. Peak 2 (myoglobin) in MRM was greater after heating at 100°C than in the unheated meat. Myoglobin is one of the more heat stable sarcoplasmic proteins and is considerably more heat stable when extracted than *in situ* (Bernofsky *et al.,* 1959).

Table 5 Summary of time and temperature profiles during cooking

	70°C				100°C			
	HDM1	HDM2	MRM1	MRM2	HDM1	HDM2	MRM1	MRM2
Temp. at start (°C)	14	6	15	6	5	3	18	8
Time to target temp. (min)	33.6	31	26.5	37.4	19.5	18.5	19.1	40
Time at target temp. (min)	3.1	2.4	2.5	2.4	2.5	2.2	2.6	NR
Max. temp. (°C)	71	70	70	70	100	100	100	99

NR not recorded

The effect of heating on the peak area ratio of peak 1 to each of the other peaks differed between HDM and MRM (Table 6). In HDM, after heating at both 70°C and 100°C, the ratio increased relative to that for the raw meat, whereas in MRM there was not a clear trend. The ratios of peak 1 to peaks 7 and 9 lie within the same range in MRM and HDM so could not be used to distinguish MRM from HDM; for all other peak ratios, further replication is required to measure the variation to determine whether they would be suitable to distinguish MRM from HDM.

These initial data do indicate that heating has an effect on the peak area of proteins and will therefore affect the detection of MRM in cooked meat using this approach. Further replication is required to determine whether this will affect the suitability of this method to detect MRM.

Table 6 The effect of heating at 70°C and 100°C for 2 minutes on the area of peak 1 (haemoglobin) and the ratio of peak 1 to each of the other selected peaks.
(Mean *normalised peak area* and peak area ratio for duplicate samples)

Treatment	HDM			MRM		
	raw	70°C	100°C	raw	70°C	100°C
peak 1	*2.47*	*2.48*	*1.89*	*5.54*	*4.19*	*4.11*
pk1/pk2	0.77	1.36	1.51	2.82	2.77	1.92
pk1/pk3	0.63	1.09	1.00	1.98	1.95	2.59
pk1/pk4	0.48	0.75	0.60	1.83	1.60	2.10
pk1/pk5	0.16	0.27	0.26	0.50	0.44	0.59
pk1/pk6	0.61	0.93	0.94	2.28	2.37	2.97
pk1/pk7	1.18	1.58	1.90	1.87	2.10	2.64
pk1/pk8	0.78	2.38	1.77	2.62	2.62	3.82
p1/pk9	0.17	0.93	0.79	0.61	0.84	1.53

Italics indicate peak area

3.3 Analysis of mixtures of raw MRM in HDM

A range of mixtures of MRM in HDM (0, 5, 7.5, 10, 15, 20, 30, 50% MRM) were prepared five times from five batches of meat. One sample of each mixture from each batch was analysed by CGE (SDS-ProSort). Peak area data were extracted for the selected peaks (labelled 1 to 9 in Figure 5) to determine whether the presence of MRM in a mixture could be detected and at what level it could be distinguished from 0% MRM.

Two-way analysis of variance was used to look for differences between batches and mixtures. When using absolute peak area, peaks 1, 2, 3, 4, 5 and 8 differed significantly ($P<0.05$) between batches (Table 7) but by normalising with respect to the neutral marker, the batch effect was removed for all peaks except peak 1. Ratios of peak 1 to each of the other peaks did not remove the difference between batches (Table 7) though the ratio peak1/ peak 2 (haemoglobin to myoglobin) did have a

higher F-ratio (9.7) as compared to peak 1 alone (7.2) suggesting that the variation in peak 2 does account for some but not all of the variation in peak 1 between batches.

Table 7 Results of two-way analysis of variance (P values) to determine whether there were significant differences* ($P<0.05$) between batches or mixtures of raw MRM in HDM.

Peak number	Absolute peak area		Normalised peak area		Peak ratio	Peak ratios	
	Batches	Mixtures	Batches	Mixtures		Batches	Mixtures
Marker	0.249	0.594	-	-	-	-	-
1	0.000*	0.000*	0.000*	0.000*	-	-	-
2	0.024*	0.302	0.287	0.627	pk1/pk2	0.000*	0.000*
3	0.009*	0.440	0.072	0.530	pk1/pk3	0.000*	0.000*
4	0.044*	0.273	0.271	0.271	pk1/pk4	0.000*	0.000*
5	0.04*	0.142	0.318	0.193	pk1/pk5	0.000*	0.000*
6	0.064	0.404	0.218	0.380	pk1/pk6	0.000*	0.000*
7	0.053	0.212	0.230	0.343	pk1/pk7	0.000*	0.000*
8	0.036*	0.233	0.167	0.408	pk1/pk8	0.000*	0.000*
9	0.229	0.344	0.154	0.348	pk1/pk9	0.000*	0.000*

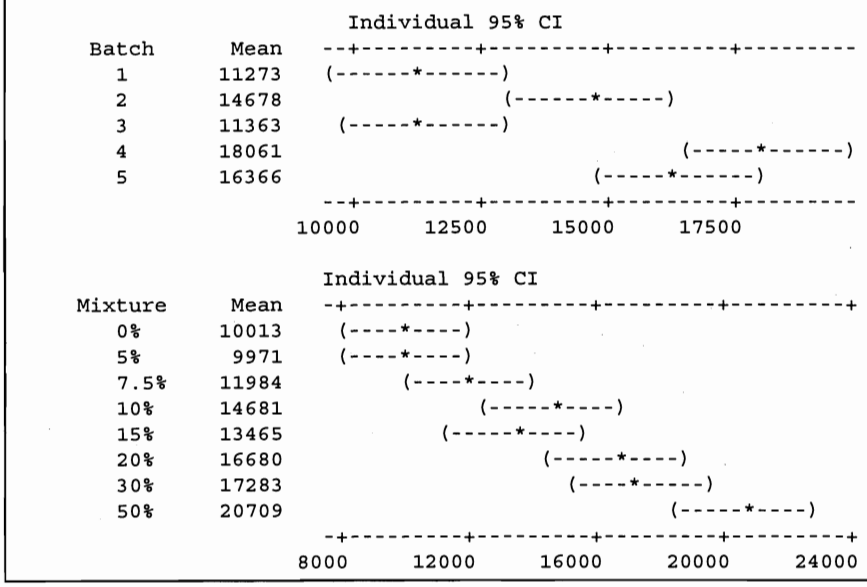
When preparing mixtures, frozen blocks of MRM were thawed and considerable 'drip-loss' occurred. Haemoglobin (peak 1) is the most likely pigment to be present in the drip loss. Whilst this 'drip liquid' was mixed into the MRM before use, uneven mixing into the main mass of the meat and differences in the degree of incorporation between batches may have introduced variability which would be independent of other less soluble proteins. This could be tested by making multiple analyses on one batch.

Despite the differences between batches, significant differences between mixtures ($P<0.05$) were detected for peak 1 (Table 7). Both absolute and normalised peak areas increased with % MRM in the mixture such that mixtures containing greater than 20% MRM were significantly different from 0% MRM (Table 8). Mixtures of 5, 7.5, and 15% MRM were not significantly different from 0%.

Table 8 Use of the area of peak 1 to determine differences between batches and mixtures - results of two-way analysis of variance

Analysis of Variance for pk 1

Source	DF	SS	MS	F	P
Batch	4	290670400	72667600	13.48	0.000
Mixture	7	494762336	70680336	13.11	0.000
Error	28	150983056	5392252		
Total	39	936415808			

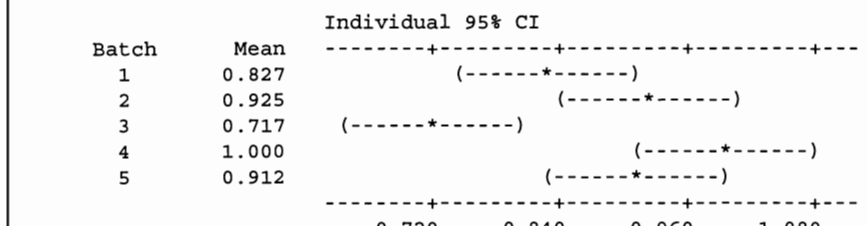


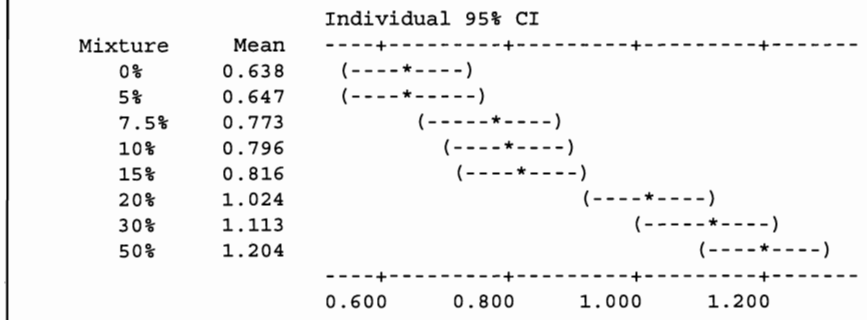
By taking the ratio of peak 1 to each of the other peaks, significant differences between mixtures for all peak ratios were observed (Table 7). The highest F-ratio was achieved for peak 1/peak 4 (23.3). Using this parameter, mixtures containing greater than 7.5% MRM were significantly different from 0% MRM (Table 9). The ratio peak 1/ peak 2 was the only ratio to show a consistent increase in ratio with percentage MRM in the mixture (Table 9). For this parameter, mixtures containing more than 20% MRM were significantly different ($P<0.05$) from 0% MRM.

Table 9 Differences between batches and mixtures determined using peak area ratios peak 1/peak 2 and peak 1/peak 4 - results of two-way analysis of variance

Analysis of Variance for pk1/pk2

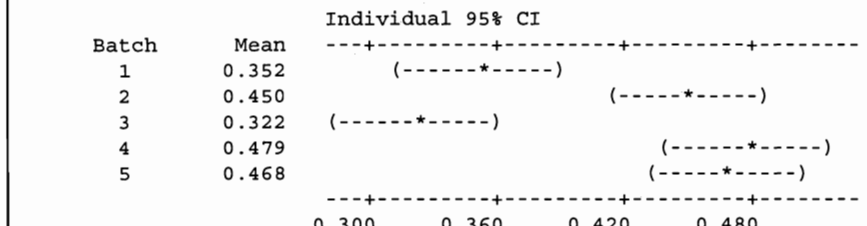
Source	DF	SS	MS	F	P
Batch	4	0.37435	0.09359	7.19	0.000
Mixture	7	1.57789	0.22541	17.31	0.000
Error	28	0.36468	0.01302		
Total	39	2.31693			

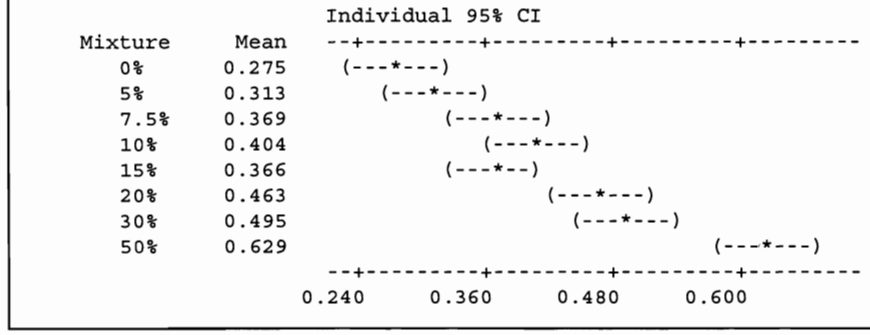




Analysis of Variance for pk1/pk4

Source	DF	SS	MS	F	P
Batch	4	0.166263	0.041566	15.23	0.000
Mixture	7	0.444524	0.063503	23.27	0.000
Error	28	0.076407	0.002729		
Total	39	0.687194			





Whilst these data suggest that there is potential for this technique to detect MRM in raw meat mixtures, the variability in the data indicates that for prediction purposes, they should be treated with caution (see Figure 13). Further work is required to measure and quantify the variables associated with MRM in raw chicken meat products and in the use of statistical approaches to adjust for the measured uncertainty for a robust method for the detection of MRM in meat products to be developed.

An alternative approach to data analysis in which all nine peaks were combined was Principal Component Analysis (PCA). PCA was used to examine the relationship between the peak area of the nine selected peaks measured in the five batches of mixtures of MRM in HDM. This statistical procedure looks for correlations between variables (in this case the 9 peaks) and uses them to derive fewer, new, independent variables, principal components (PC's), which maximise the variation in the original data. For these data, 92% of the variation was described by the first 3 principal components. The principal component scores for PC1 and PC2 are shown in Figure 14. In such plots, samples which lie close together are similar, whilst the further apart the samples are, the greater is the difference between them. Figure 14 shows some separation in both dimensions (PC1 and PC2) but PC2 gave separation in terms of mixture - those mixtures containing higher % MRM having positive values, those with lower % MRM having negative values. The loading which each of the individual peaks contributes to PC1 and PC2 is shown in Figure 15. Values of loadings can range between 1 and -1, indicating positive and negative correlation respectively; values of zero indicate no correlation. No one particular peak contributed to PC1, whereas peak 1 and peaks 4 and 6 contributed to PC2 (Figure 15) indicating that these peaks, particularly peak 1, were the greatest contributors to the separation of the mixtures seen in Figure 14.

In previous studies an indication of the variation in the data has not been given for the detection of MRM in a sample. Whilst the variation here is high (Figure 13), it is perhaps not surprising in the light of the inherent variability of MRM (Meech and Kirk, 1986; Crosland *et al.*, 1995). Indications are that similar variability affects other detection methods. SDS-PAGE studies indicated protein bands useful for

distinguishing MRM and HDM (chicken was the most difficult of the species used to distinguish) and estimates of levels that could be detected were based on visual comparisons between mixtures.

When running unknown mixtures it was possible to distinguish incorporation at 25% MRM but below this mixtures were incorrectly identified (0% and 10% MRM being mis-identified) (Savage *et al.*, 1995). Similarly using an immunological method, samples containing less than 50% MRM were incorrectly identified (0% being estimated as 50%, 5% as 30%, and 50% and 100% as greater than 50%) (Pickering *et al.*, 1995a).

Whilst the natural variability of MRM is likely to be responsible for much of the variation, there are aspects of the CGE method which could be improved to reduce the experimental variability. Advances in CE instrumentation have introduced changes which will reduce variability (e.g. temperature control of the column) and another commercial source of SDS sieving matrix has become available which, in a one-off test, gave improved resolution though at the expense of total run time.

Both single and multivariate analysis of the data indicated the importance of peak 1 (haemoglobin) to detecting MRM in mixtures. The use of haemoglobin level alone may be limited as variations in the level in bone marrow are known to occur as a result of age and physiological condition (Field *et al.*, 1978) and it could be envisaged that the addition of blood to HDM products could result in incorrect identification as MRM. Use of a ratio of haemoglobin to other meat proteins (e.g. peak 2 or peak 4) and quantification of the variables associated with MRM should enable these limitations to be overcome and CGE has been shown to be a technique suited to the separation and quantitation of proteins in raw meat. If CGE is to be used as a method for the detection of MRM in meat products, further work, using the knowledge gained here for experimental design, is needed to measure and quantify the variables associated with MRM in raw meat products (e.g. preparation of mixtures, age of the bird, cut of meat, mechanical recovery process, effect of mixed species, binding agents used in meat products) and in the use of statistical approaches to adjust for the measured uncertainty.

4. CONCLUSIONS

Separation of chicken meat proteins by CGE was achieved more readily using an SDS-sieving matrix than with MHEC. The major difference between both raw and cooked HDM and MRM was the peak area of haemoglobin (peak 1) and the ratio of peak 1 to each of the other 8 peaks used for quantification. In raw meat each of these parameters enabled the presence of MRM in a mixture to be detected. Using the ratio of peak 1 to peak 4, mixtures containing greater than 7.5% MRM were significantly different from 0% MRM.

In meat cooked at 70°C and 100°C for 2 minutes, most of these parameters still differed between MRM and HDM but further replication is needed to measure the variation to determine if the two meat types can be distinguished.

In processed meat, protein peaks were not resolved though there were characteristic features which distinguished MRM and HDM. These could not be quantified due to variable migration time and lack of resolution.

The feasibility of using capillary gel electrophoresis to distinguish MRM from HDM and to detect MRM in mixtures has been demonstrated using chicken meat which, for other methods, has been the most difficult meat species. To develop this method, further work is needed to identify and measure sources of variation and if necessary to use statistical approaches to adjust for these uncertainties. This approach is justified for both raw and cooked meat.

5. ACKNOWLEDGEMENTS

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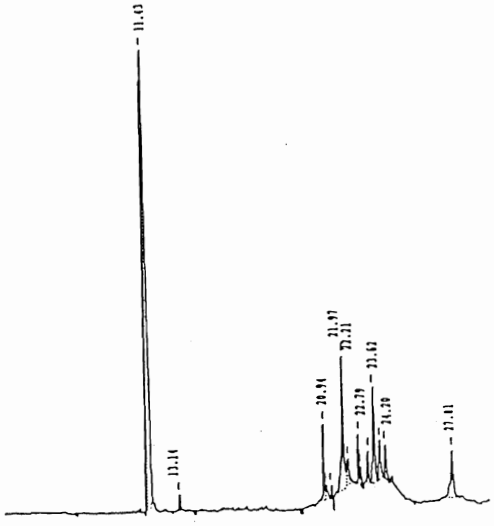
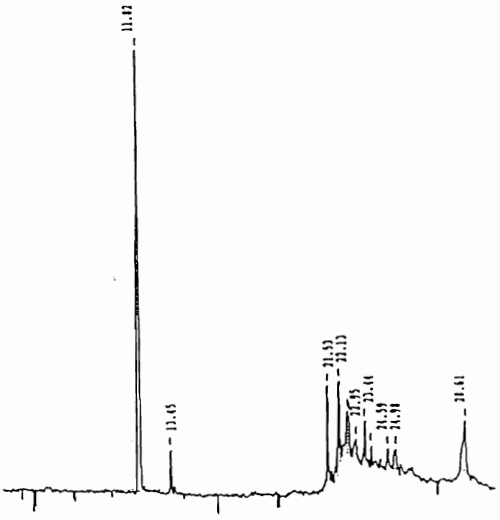
APPENDIX - Figures

Figure 1 Electropherograms of HDM and MRM meat proteins extracted with 8M urea and separated using CGE with MHEC and urea as buffer additives.
Sample injected electrokinetically (5kV for 30s) on to a CElect P1 capillary (57.5cm total length, 50cm effective length, 50μm i.d.). Electrolyte: 10mM phosphate buffer, pH 2.5 containing 6M urea and 0.05% MHEC. Separation performed at 350V/cm Detection at 214nm.

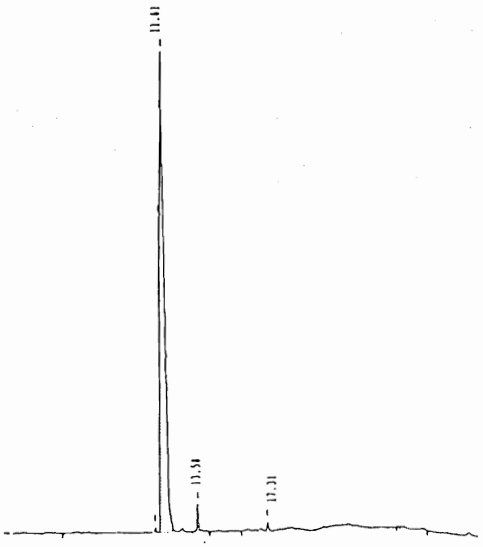
(a) raw HDM



(b) raw MRM



(c) processed HDM



(d) processed MRM

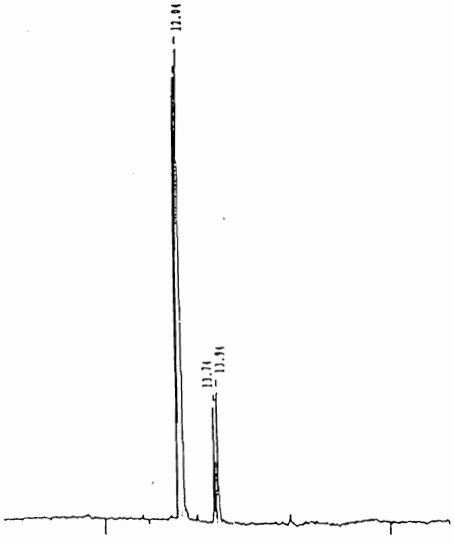
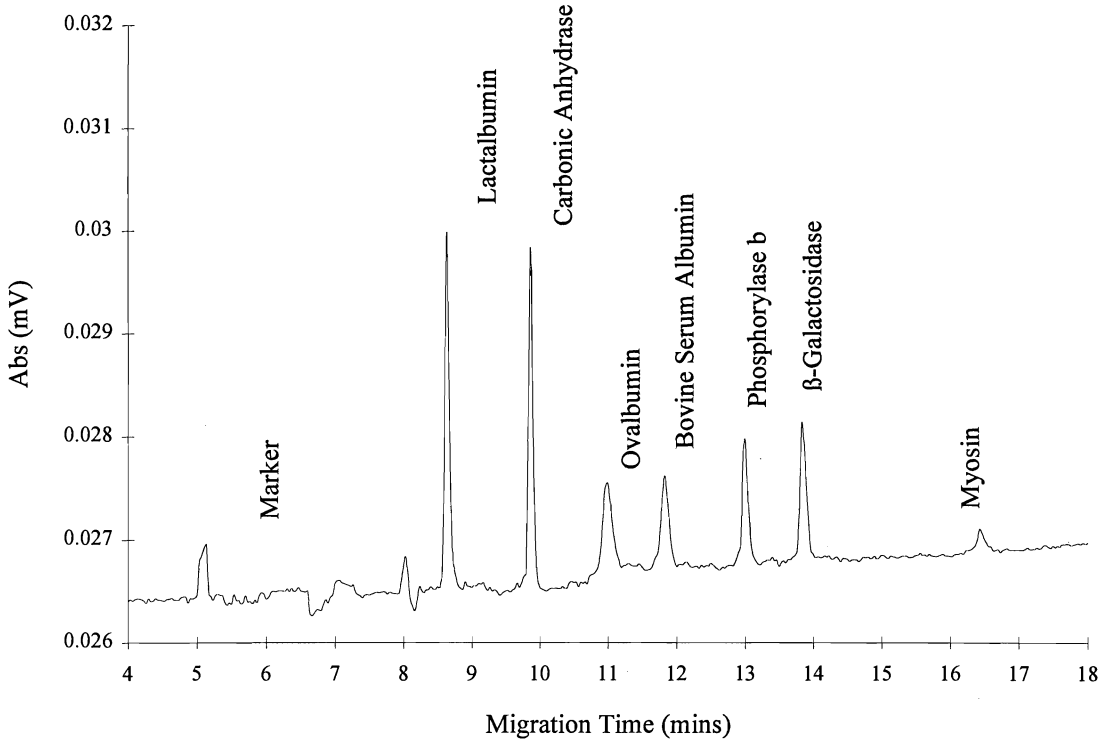


Figure 2 Analysis of protein standards using ProSort™ SDS-protein analysis reagent. (a) Electropherogram showing separation of standard proteins. Standards injected electrokinetically (-5kV for 20s) on to a fused silica capillary (40cm total length, 32.5cm effective length and 75µm i.d.) filled with ProSort™ SDS-protein analysis reagent. Separation at 300V/cm. Detection at 214nm.



(b) Standard curve used to calculate molecular weights. (Relative migration time calculated relative to marker)

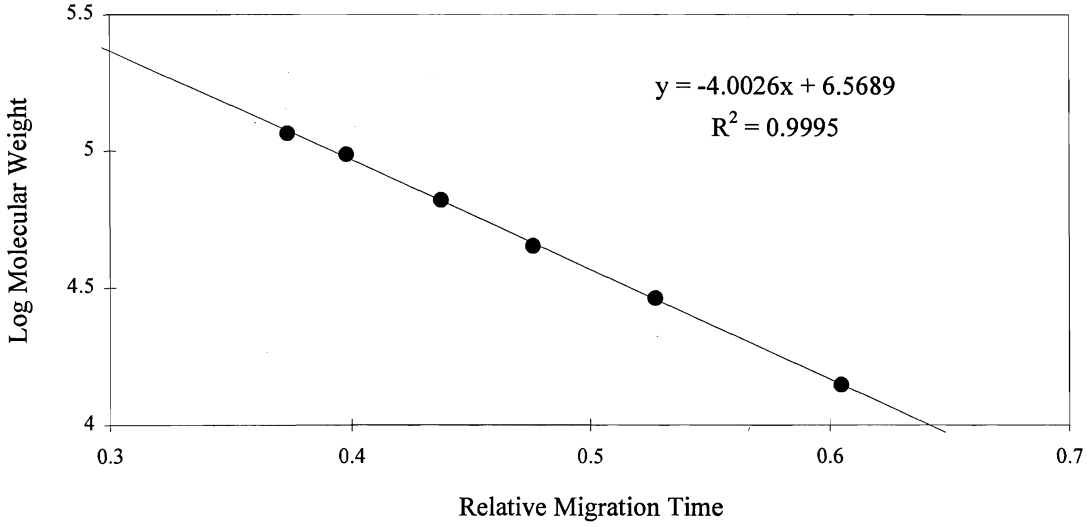


Figure 3 The electropherograms of HDM and MRM raw meat extracted by (a) the 'Mackie' method (2% SDS diluted with Lammeli buffer) and (b) 'ProSort' method (2% SDS diluted with 5% SDS/5% mercaptoethanol).
Sample injected electrokinetically (-5kV for 10s) on to a fused silica capillary (40cm total length, 32.5cm effective length and 75μm i.d.) filled with ProSort™ SDS-protein analysis reagent. Separation at 300V/cm. Detection at 214nm.

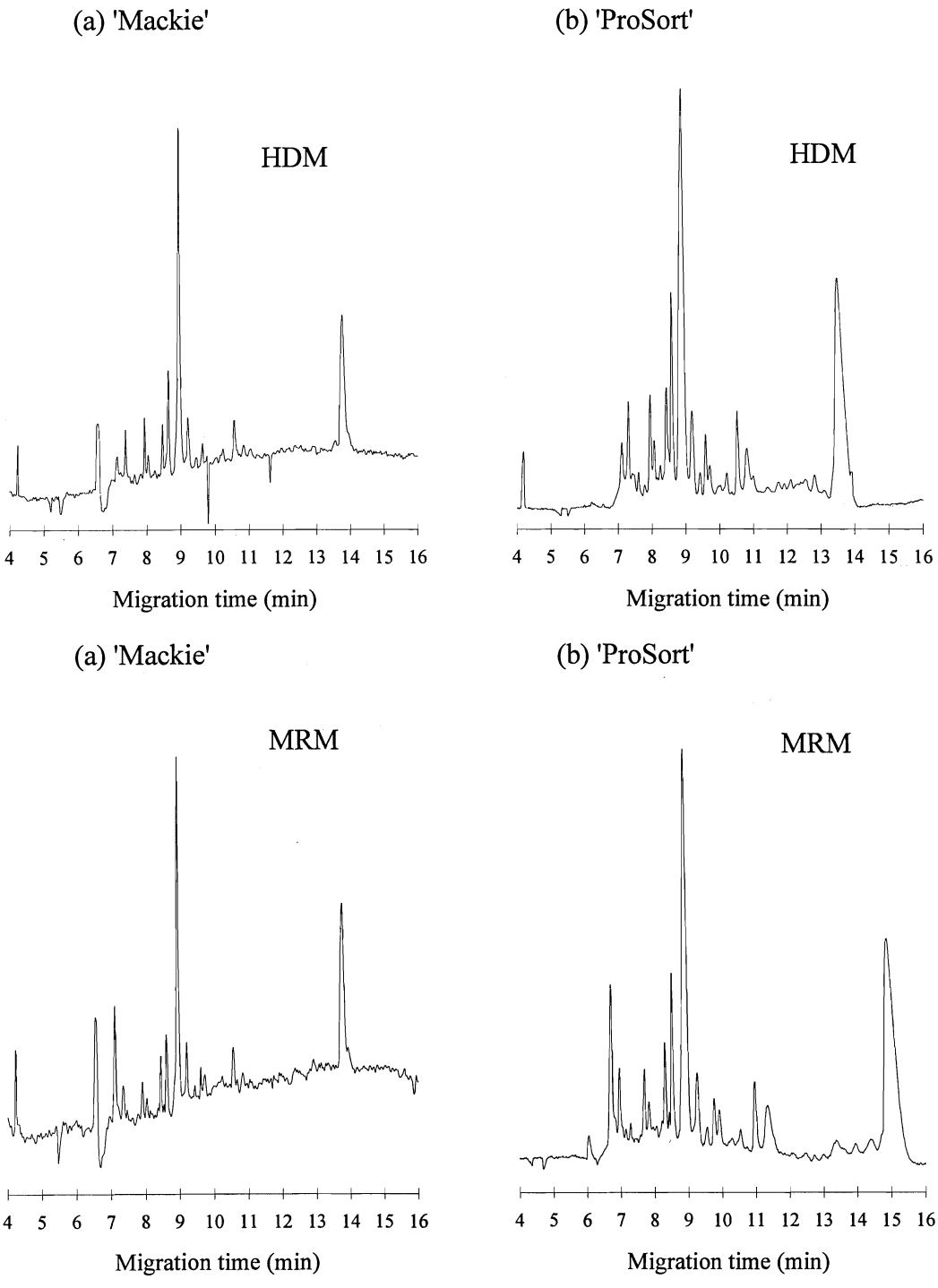


Figure 4 The electropherograms of HDM and MRM processed meat extracted by (a) the 'Mackie' method (2% SDS diluted with Lammeli buffer) and (b) 'ProSort' methods (2% SDS diluted with 5% SDS/5% mercaptoethanol).
Sample injected electrokinetically (-5kV for 10s) on to a fused silica capillary (40cm total length, 32.5cm effective length and 75μm i.d.) filled with ProSort™ SDS-protein analysis reagent. Separation at 300V/cm. Detection at 214nm.

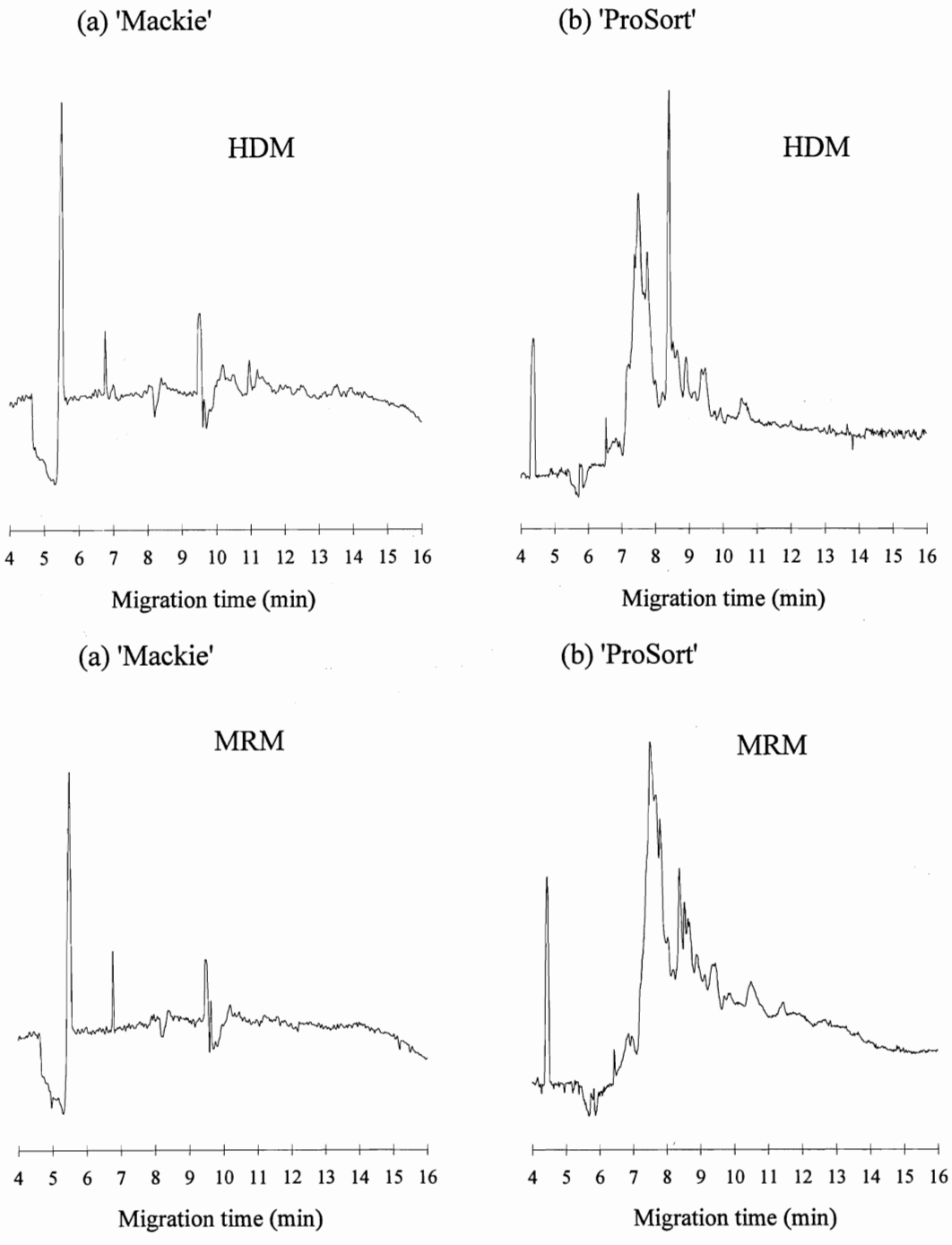
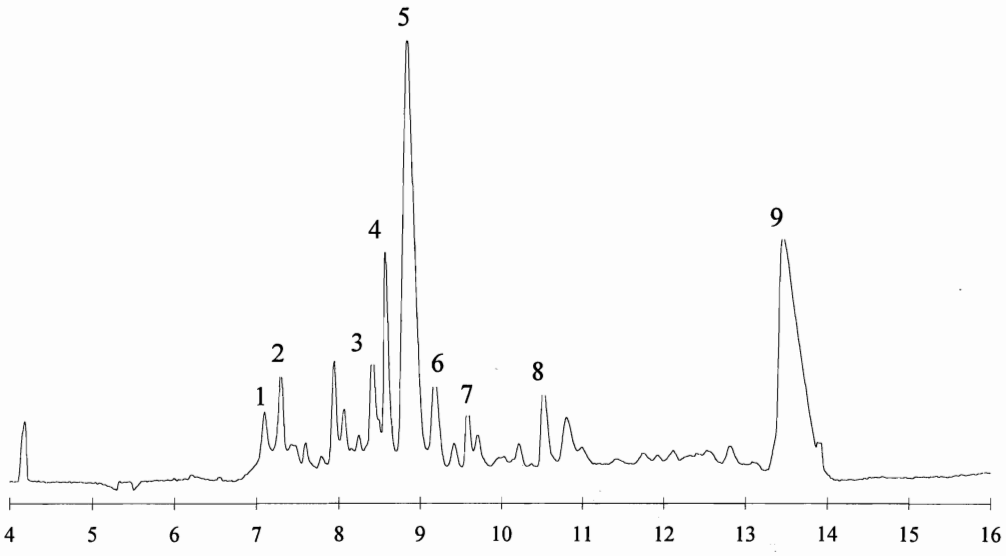


Figure 5 Electropherograms of raw HDM and MRM extracted with 2% SDS and diluted with 5% SDS/5% 2-mercaptoethanol.

Sample injected electrokinetically (-5kV for 10s) on to a fused silica capillary (40 cm total length, 32.5cm effective length and 75 μm i. d.) filled with ProSort™ SDS-protein analysis reagent. Separation at 300V/cm. Detection at 214nm. Well resolved and adequately integrated peaks labelled 1-9. Peak 1 - haemoglobin; peak 2 - myoglobin; peak 5 - actin; peak 9 - myosin.

(a) HDM



(b) MRM

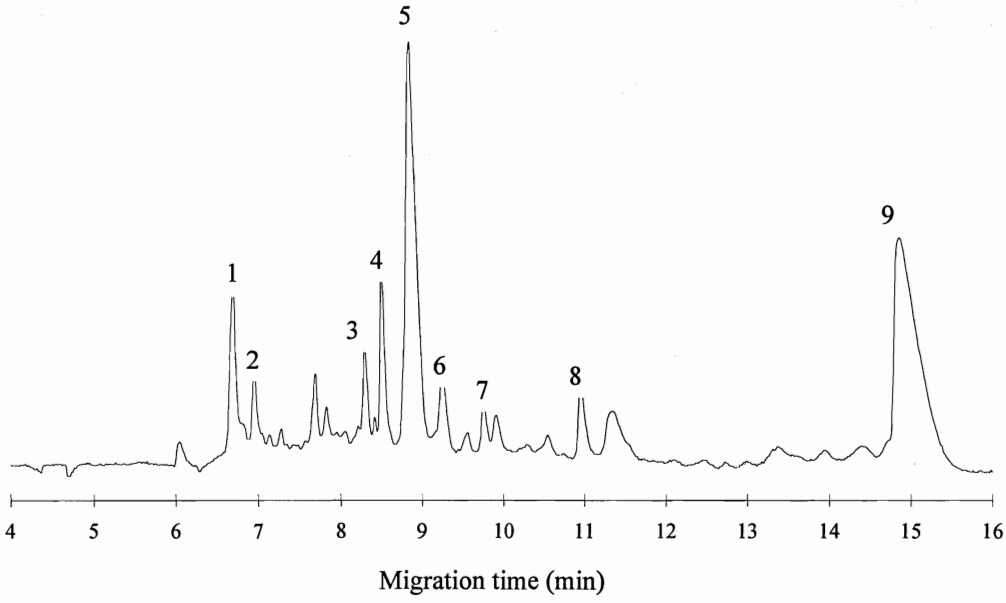


Figure 6 Comparison of selected peak areas in HDM and MRM (a) absolute peak areas, (b) normalised peak areas. Bars represent mean peak area ± standard error of mean (SEM). * indicates a significant difference ($P<0.05$) between the two meat types. HDM MRM

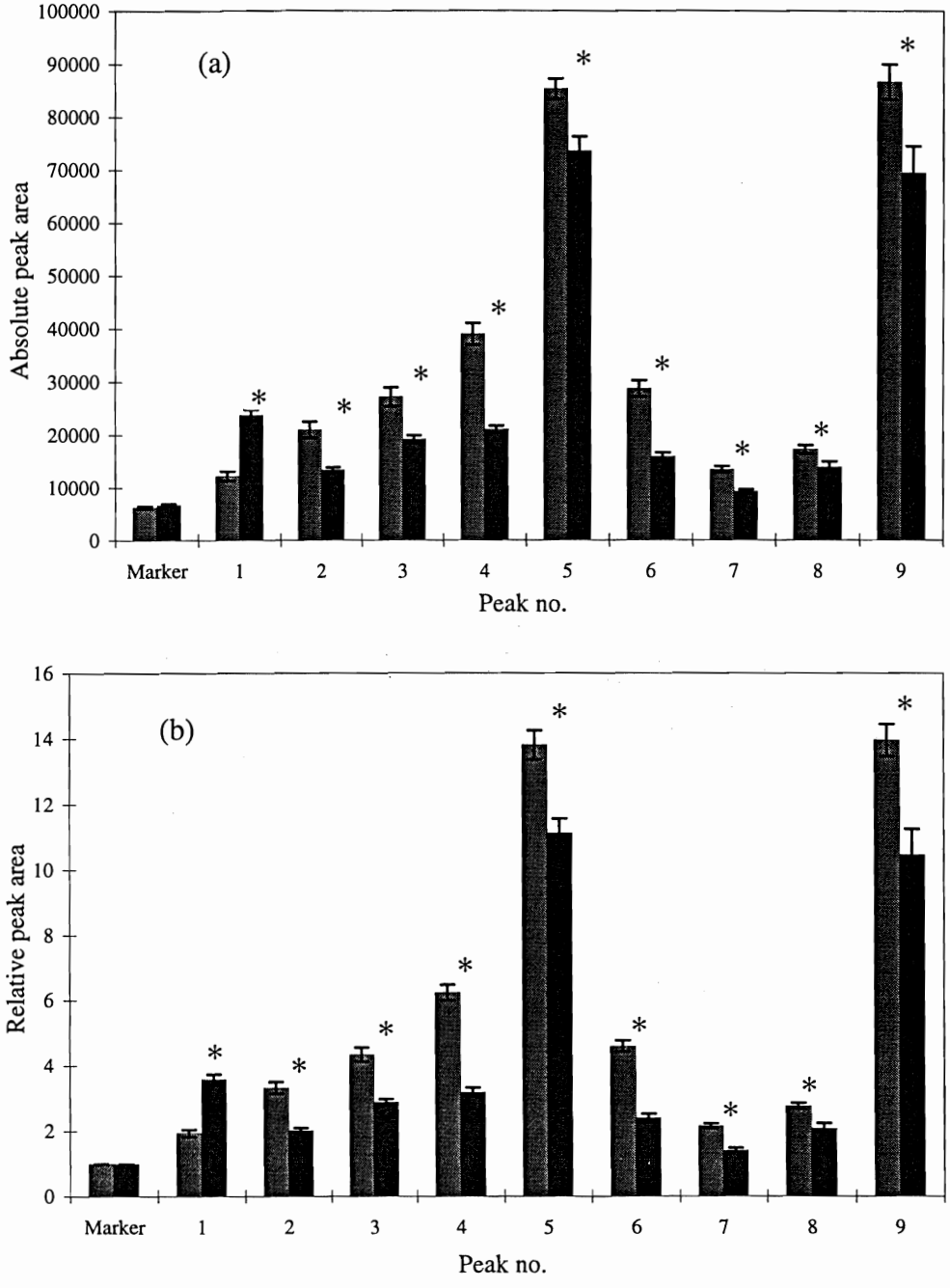


Figure 7 Electropherogram of chicken bone marrow extracted in 2% SDS and diluted with 5% SDS/5% mercaptoethanol.
Sample injected electrokinetically (-5kV for 10s) on to a fused silica capillary (40cm total length, 32.5cm effective length and 75µm i.d.) filled with ProSort™ SDS-protein analysis reagent. Separation at 300V/cm. Detection at 214nm.

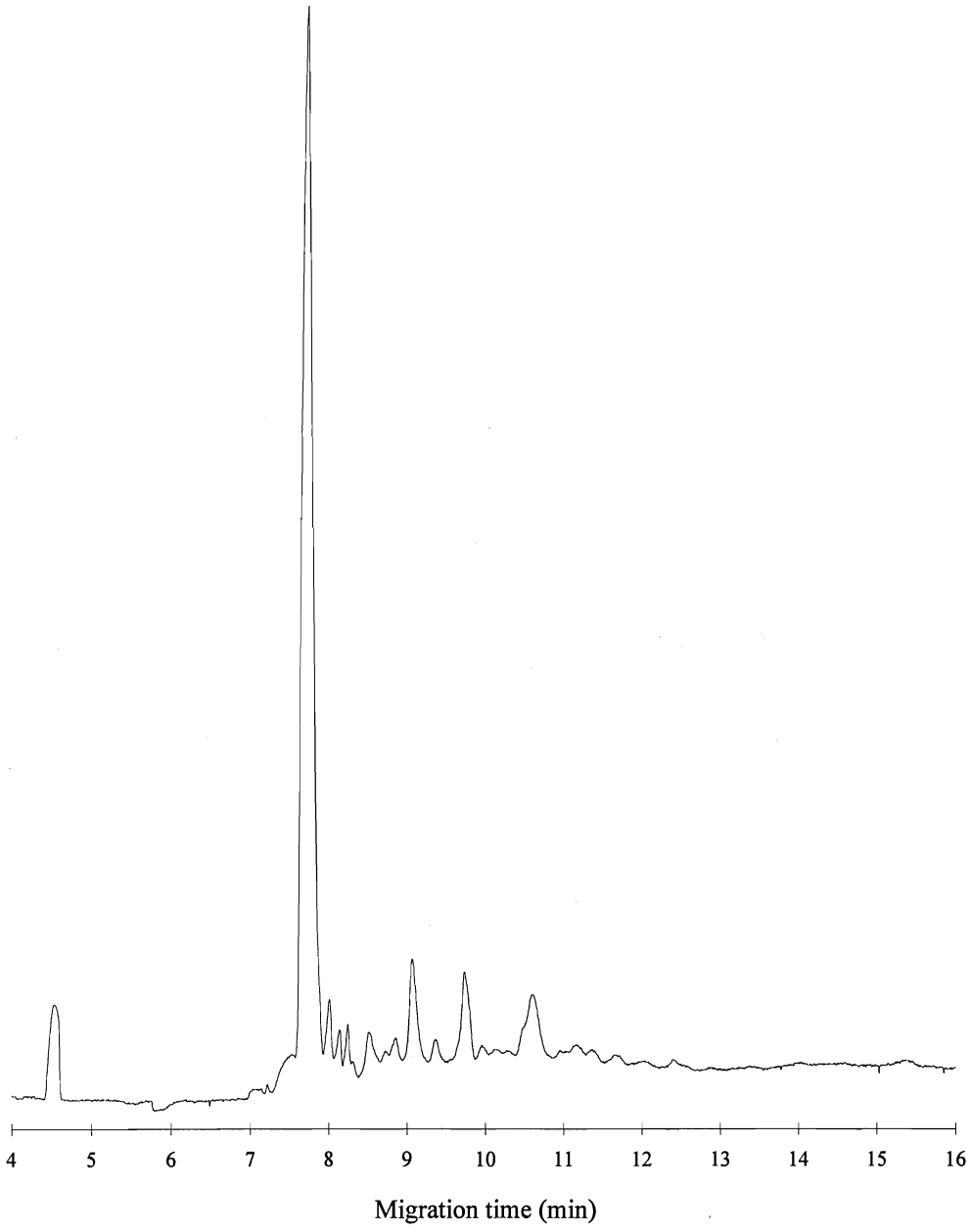


Figure 8 Electropherograms of processed HDM. Data from peak start to peak end extracted from electropherograms to facilitate comparison when looking for recognisable patterns. Peaks labelled A and B were used to look for differences between HDM and MRM.

Sample injected electrokinetically (-5kV for 20s) on to a fused silica capillary (40cm total length, 32.5cm effective length and 75µm i.d.) filled with ProSort™ SDS-protein analysis reagent. Separation at 300V/cm. Detection at 214nm.

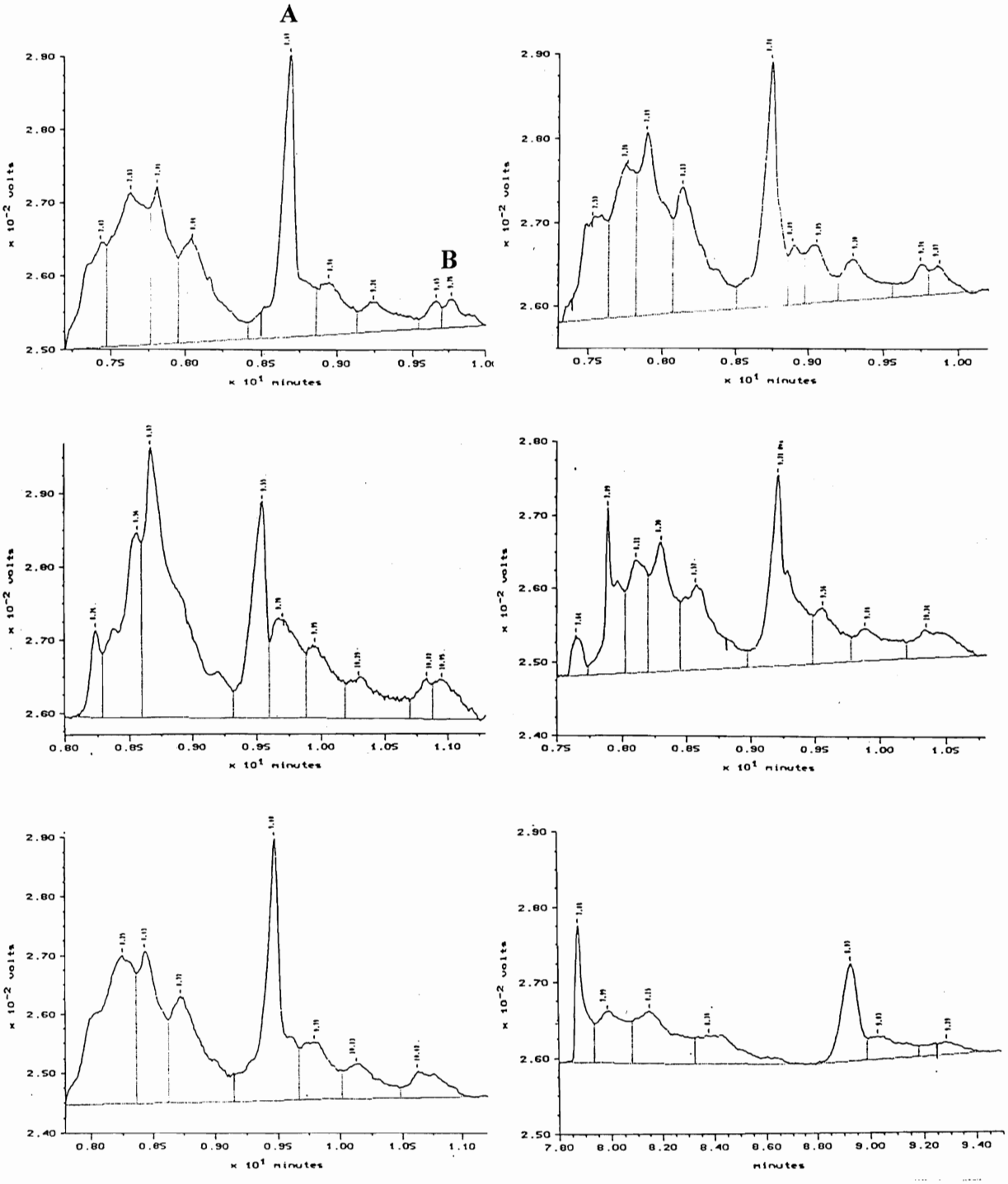


Figure 8 - continued.

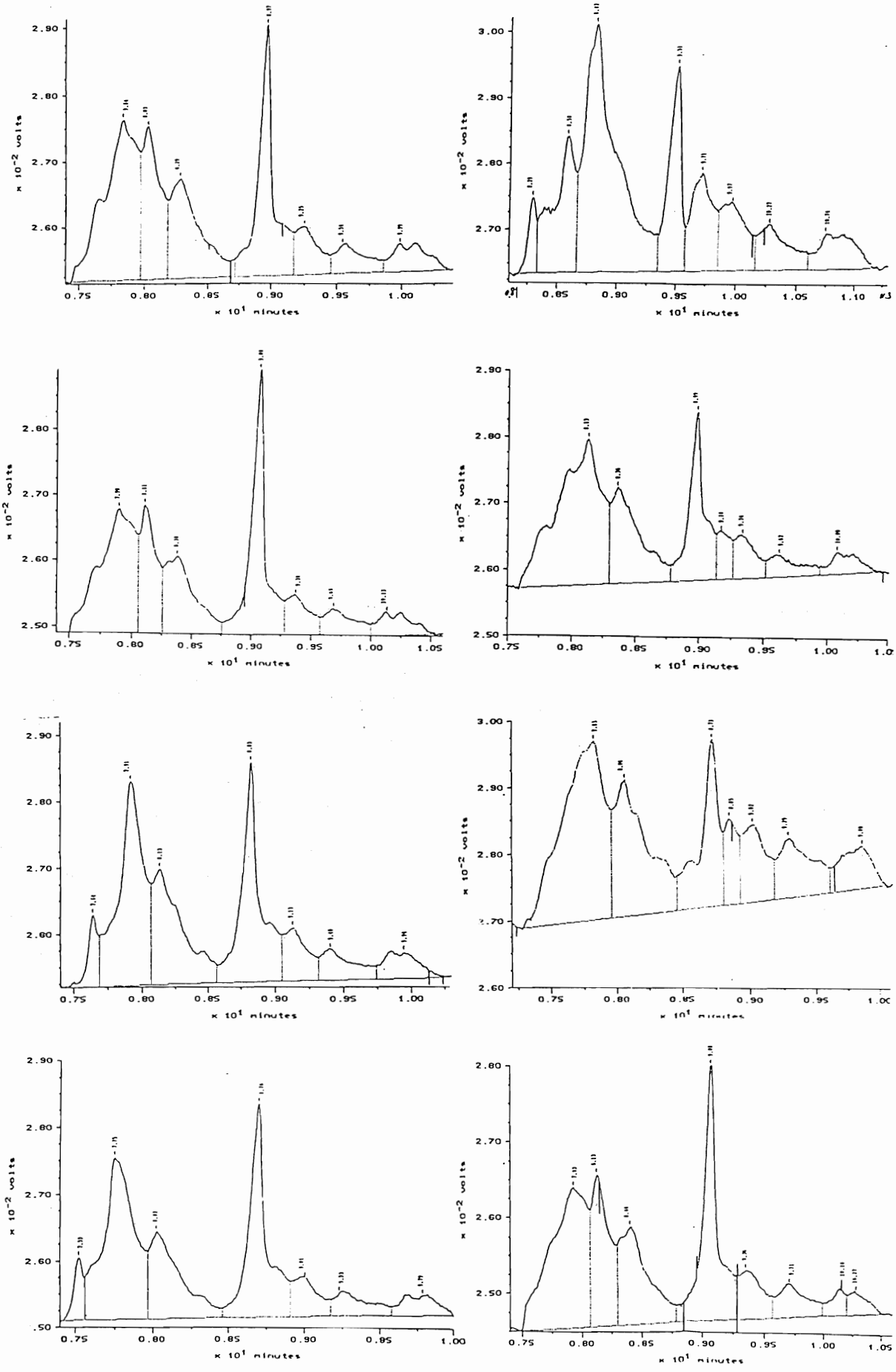


Figure 8 - continued.

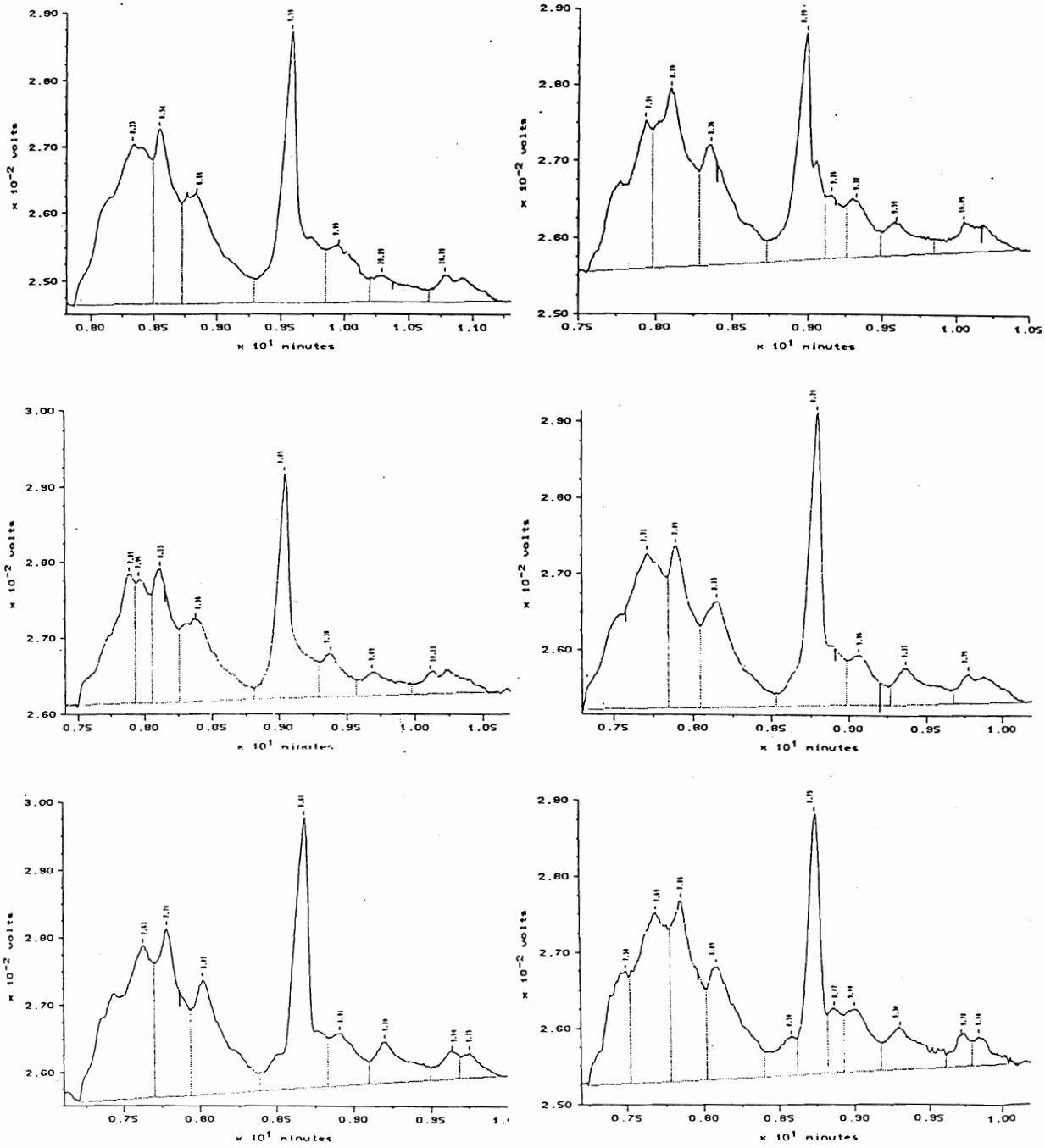


Figure 9 Electropherograms of processed MRM. Data from peak start to peak end extracted from electropherograms to facilitate comparison when looking for recognisable patterns. Peaks labelled A and B were used to look for differences betweeen HDM and MRM.

Sample injected electrokinetically (-5kV for 20s) on to a fused silica capillary (40cm total length, 32.5cm effective length and 75µm i.d.) filled with ProSort™ SDS-protein

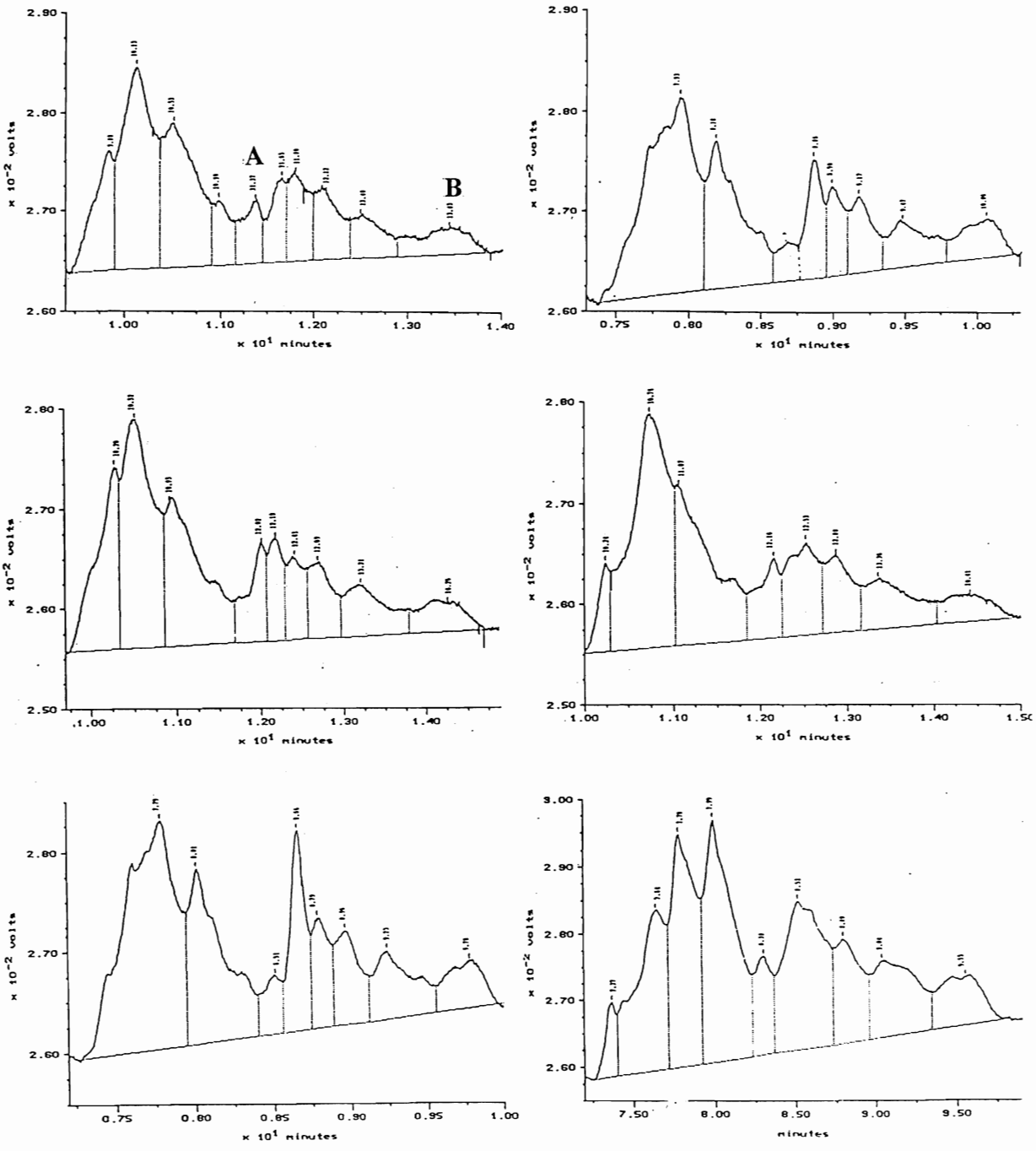


Figure 9 - continued.

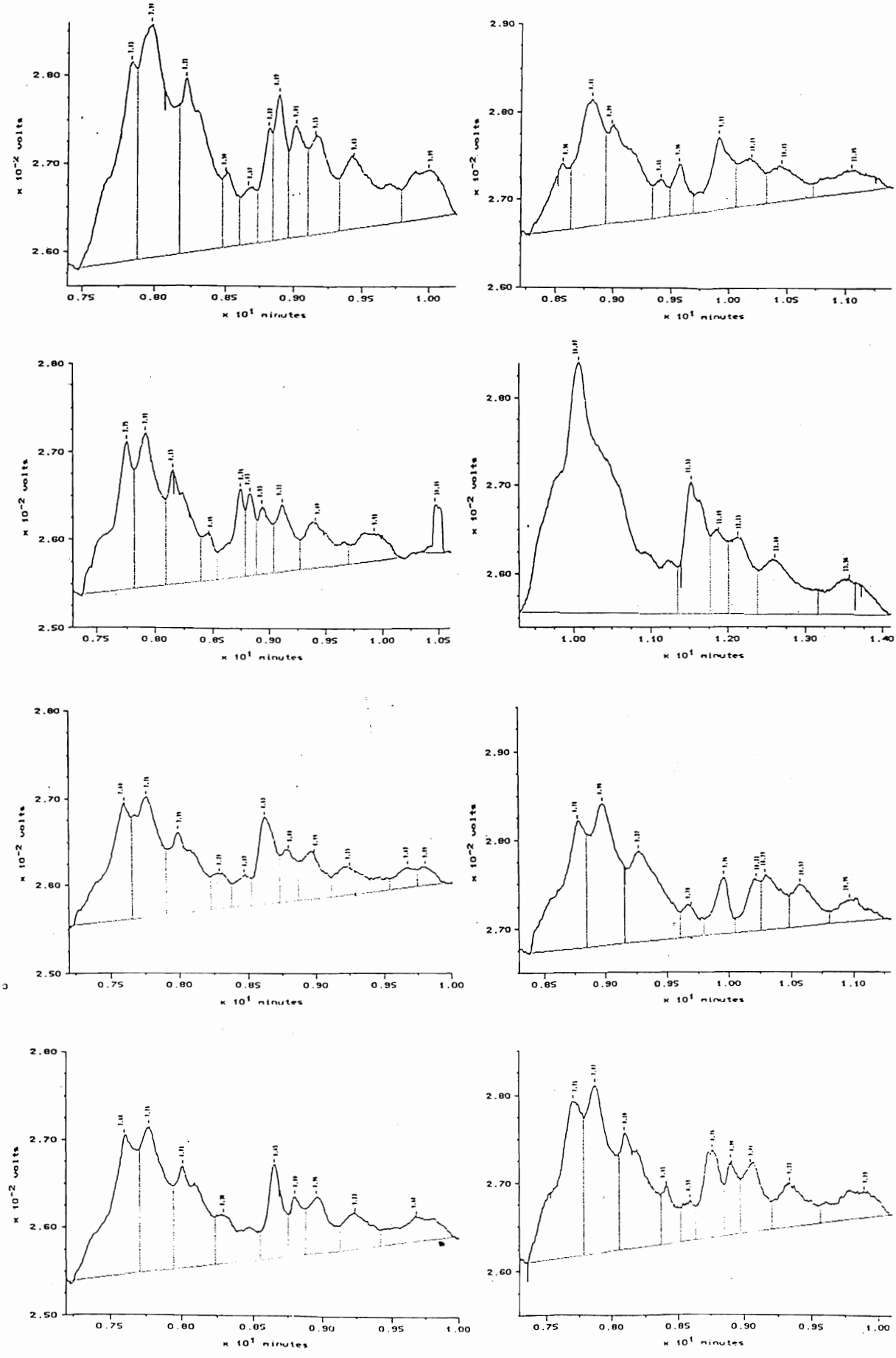


Figure 9 - continued.

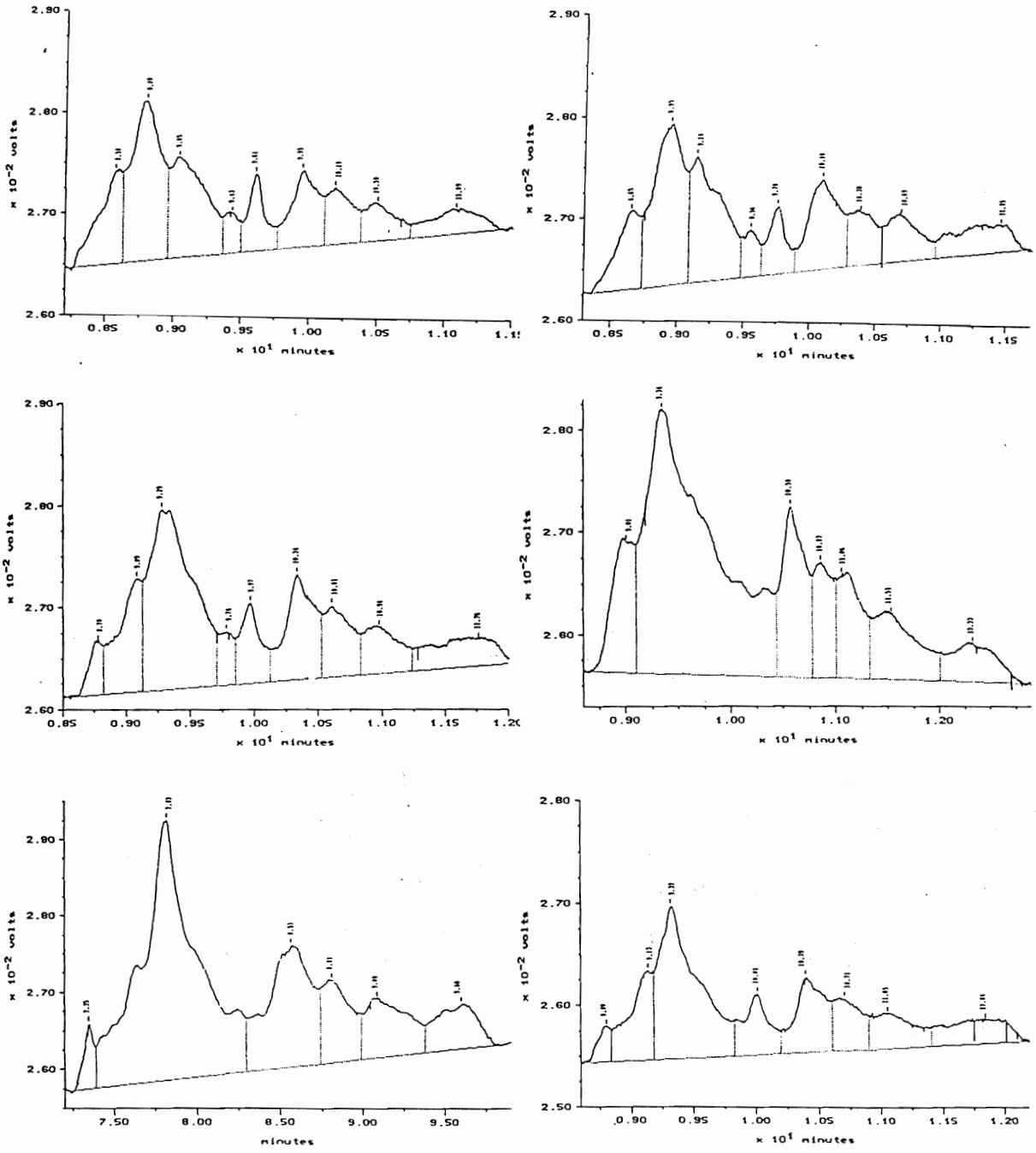


Figure 10 Comparisons of electropherograms of HDM before and after cooking. Sample injected electrokinetically (-5kV for 10s) on to a fused silica capillary (40cm total length, 32.5cm effective length and 75µm i.d.) filled with ProSort™ SDS-protein analysis reagent. Separation at 300V/cm. Detection at 214nm.

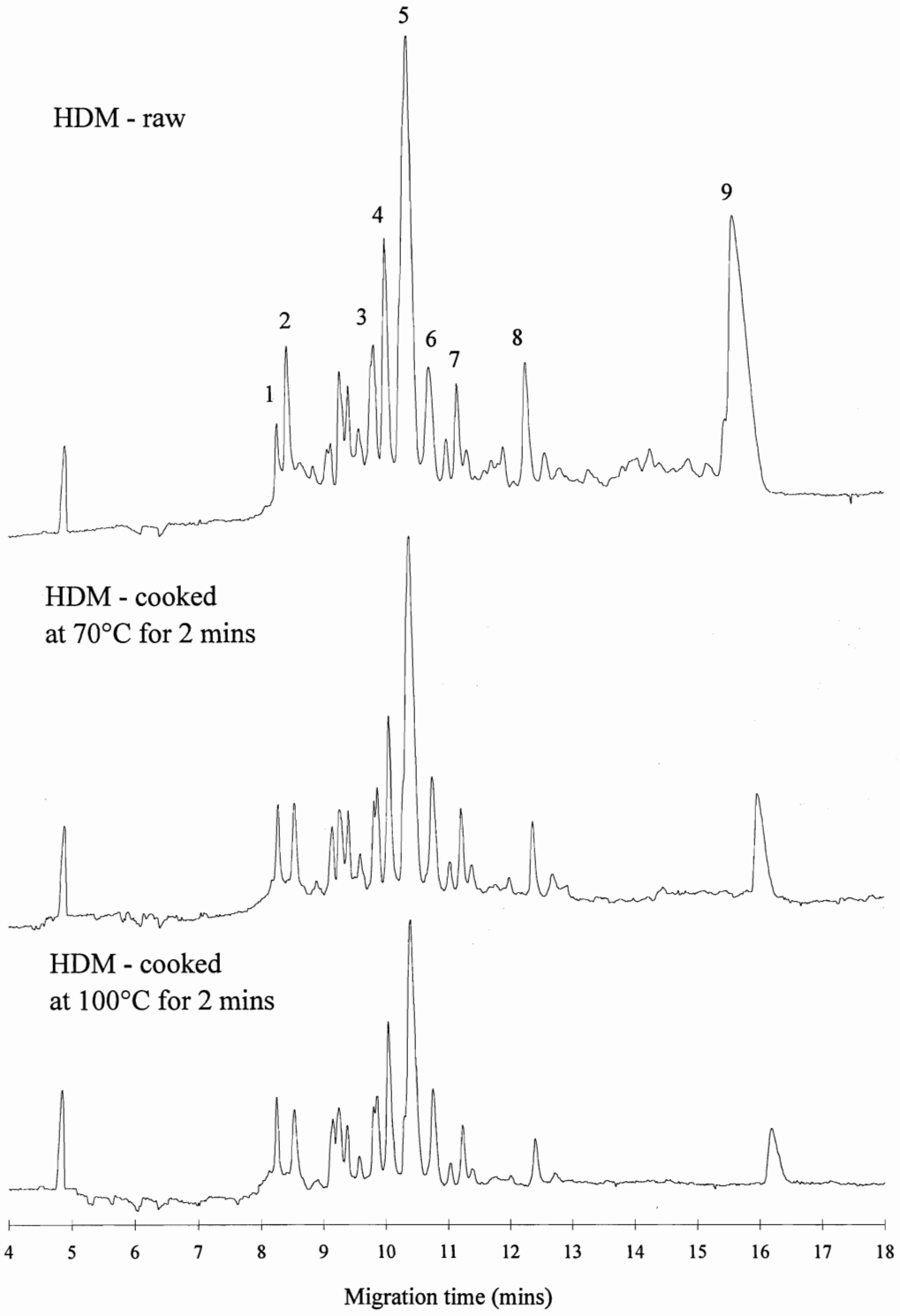


Figure 11 Comparisons of electropherograms of MRM before and after cooking. Sample injected electrokinetically (-5kV for 10s) on to a fused silica capillary (40cm total length, 32.5cm effective length and 75µm i.d.) filled with ProSort™ SDS-protein analysis reagent. Separation at 300V/cm. Detection at 214nm.

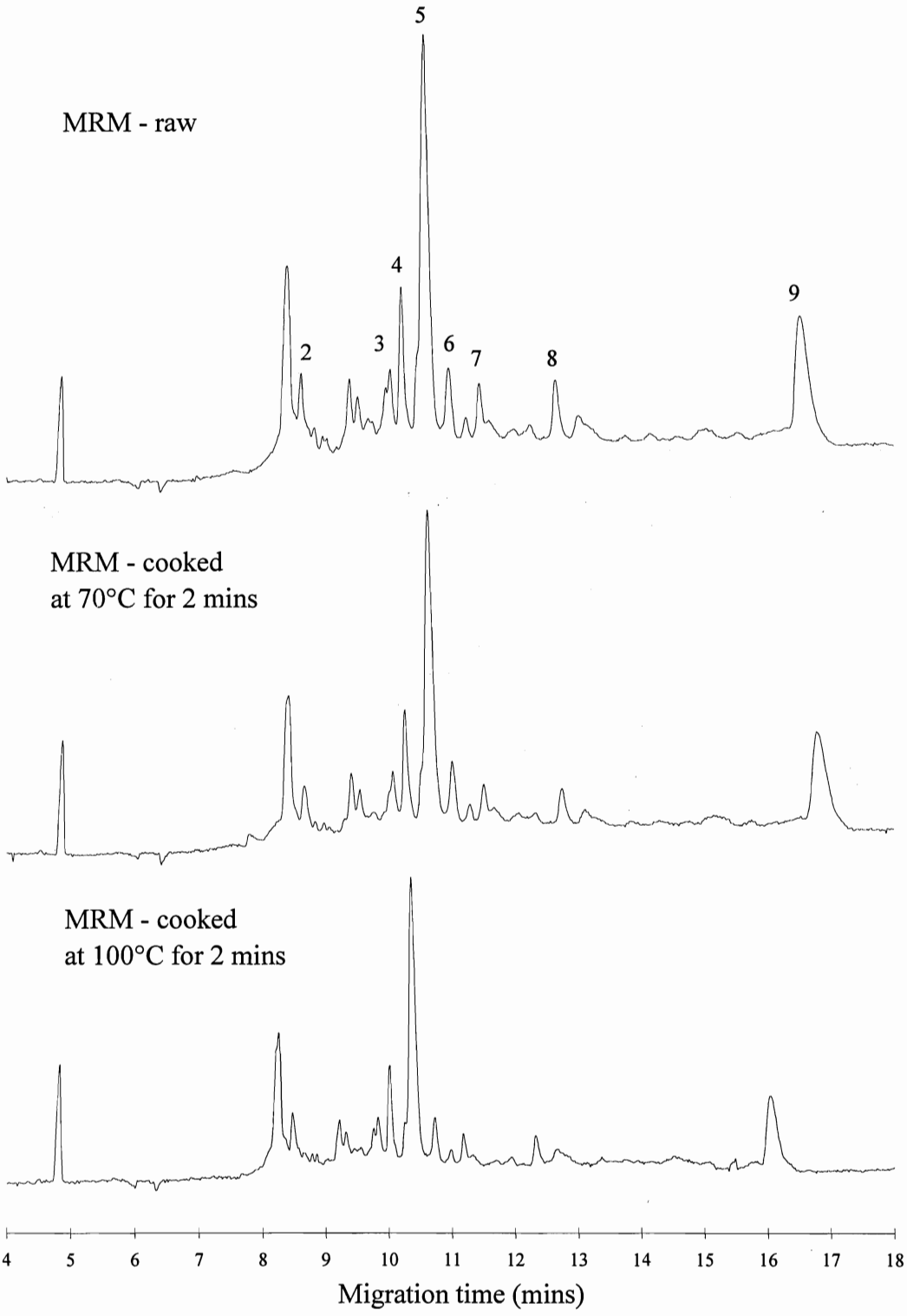
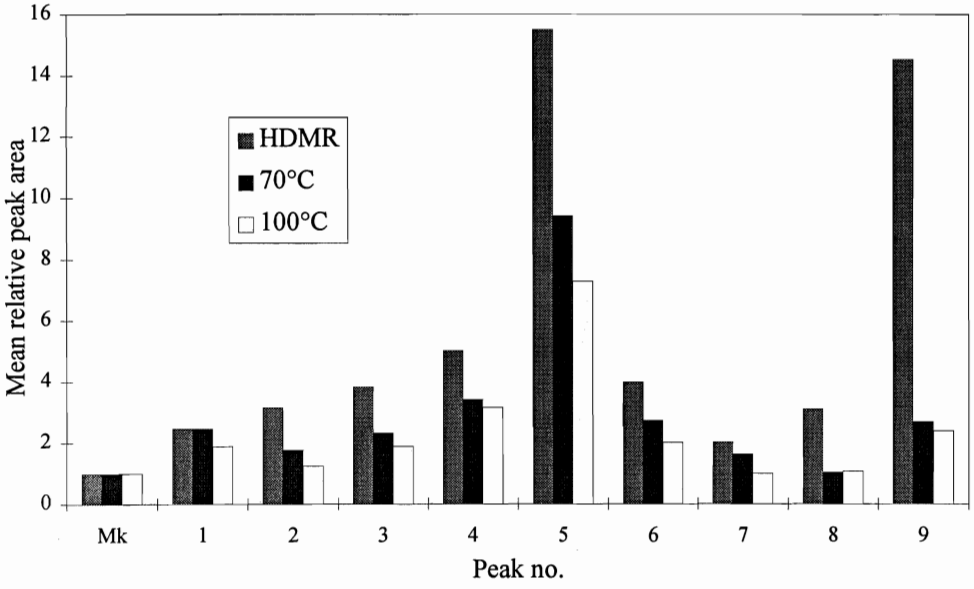


Figure 12 Comparison of relative peak areas of HDM and MRM before and after cooking at 70°C and 100°C for 2 minutes. (Peak numbers correspond to those in Figures 10 and 11). (Peak area is mean of 2 samples)

(a) Effect of cooking on HDM



(b) Effect of cooking on MRM

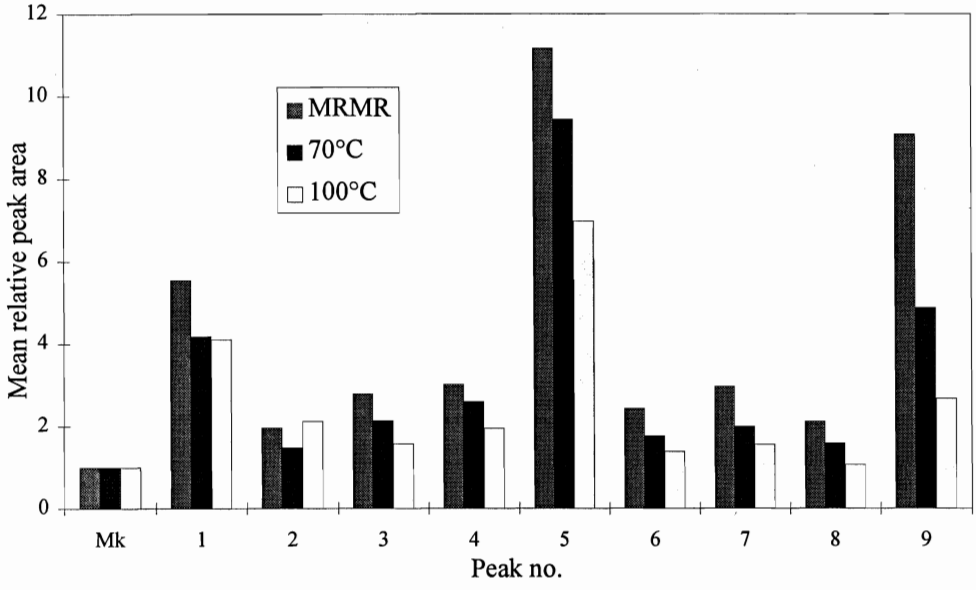


Figure 13 Relationship between %MRM in a mixture and the peak area ratio peak 1/ peak 2, to indicate the variation within the data set

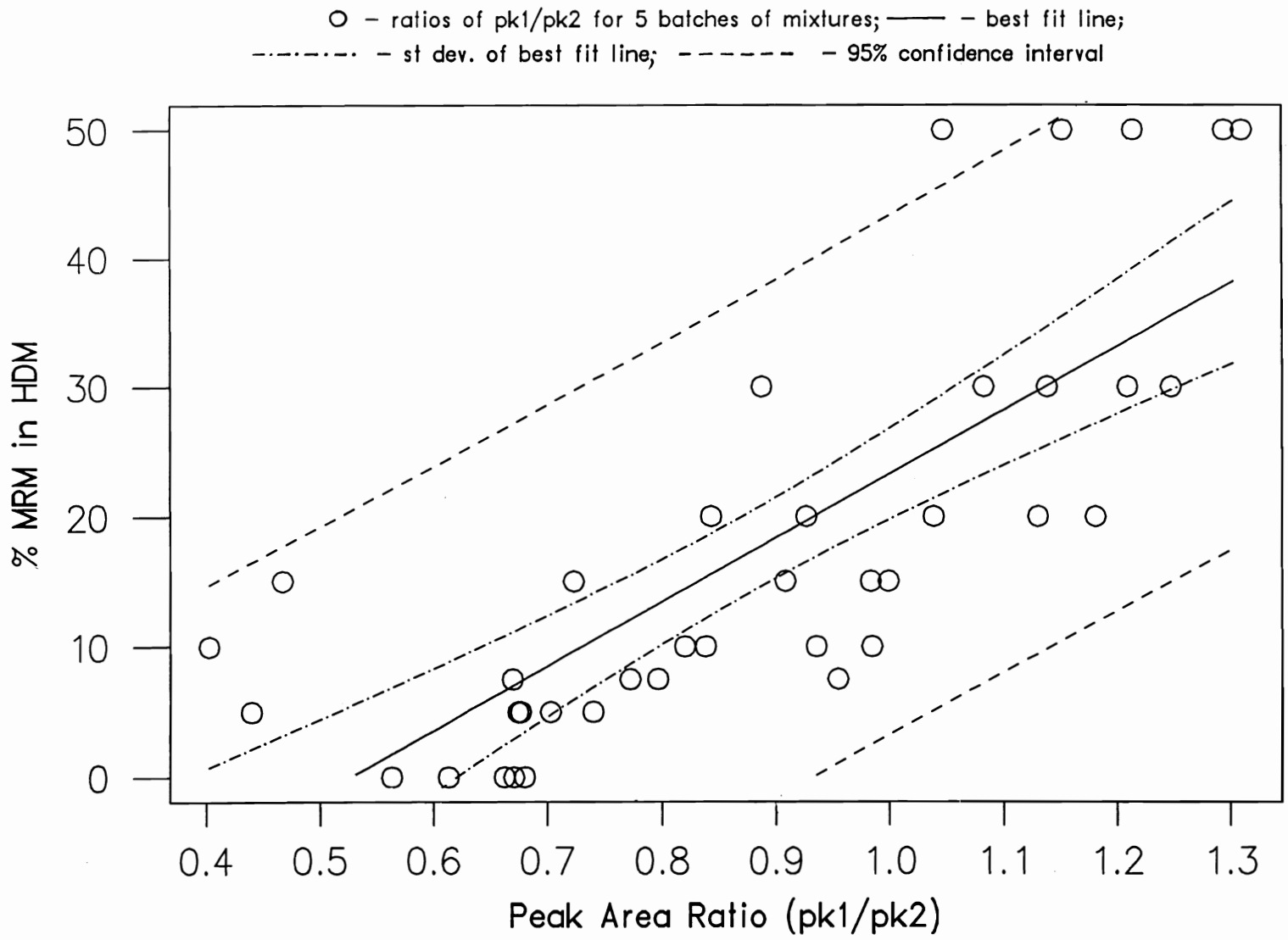


Figure 14 Principle component plot of mixtures of MRM in HDM. Each sample represented by %MRM and batch number (e.g. 50%b5 - 50%MRM mixture from batch 5)

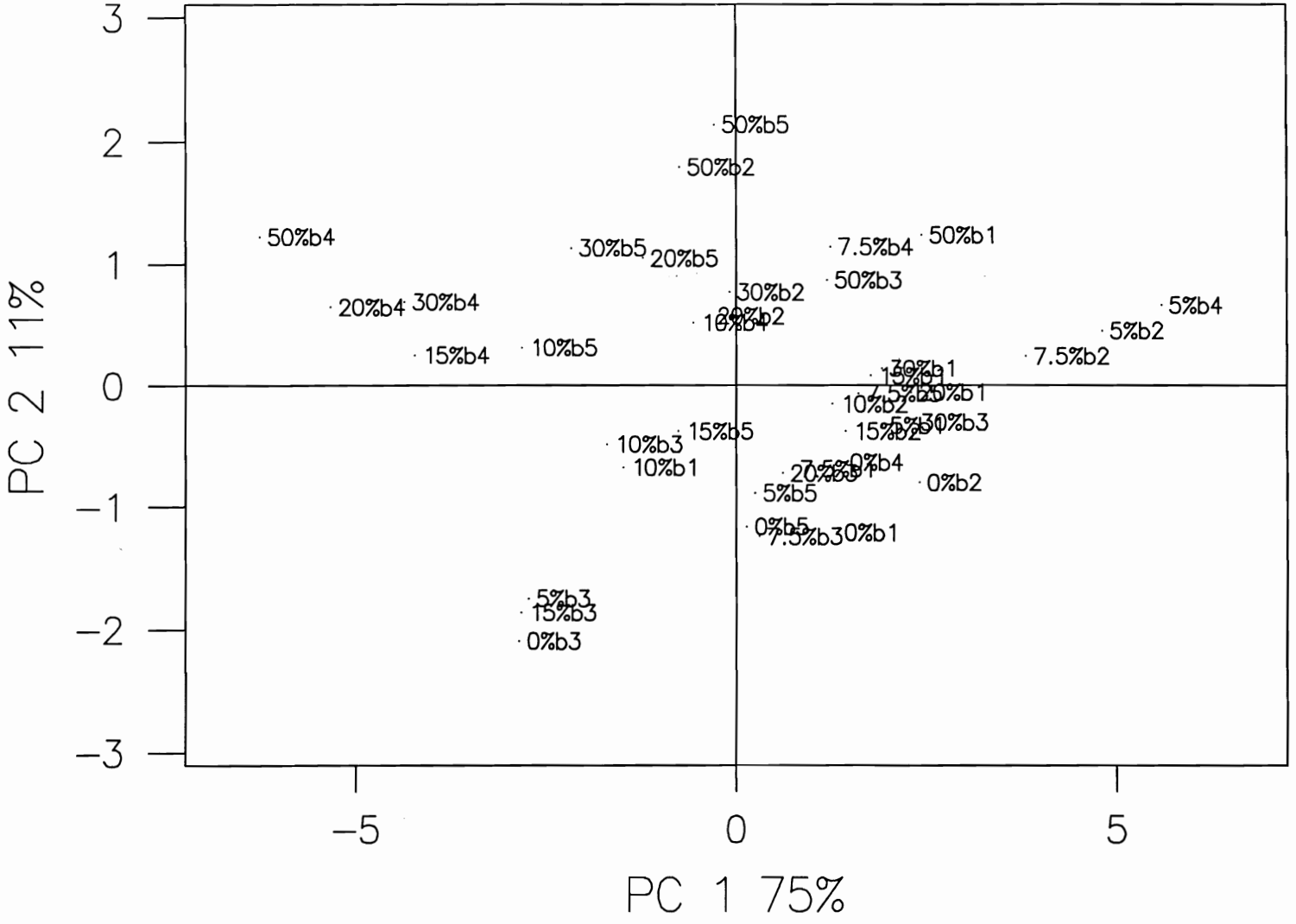


Figure 15 Principle component plot to show loadings for each of the nine peaks for PC1 andPC2 (P1 indicates peak 1 etc.)