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Quantification of the major bovine whey proteins using capillary zone electrophoresis ☆

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Abstract

Bovine whey comprises four major protein groups [α-lactalbumin, β-lactoglobulin variants A and B, bovine serum albumin and immunoglobulin (specifically IgG)] which have a diverse range of molecular masses, p*I* values, number of phenotypic variants and subunit compositions. The development of a capillary zone electrophoresis method to separate these whey proteins is described. Initially separation of the individual whey proteins was evaluated using a number of different buffer systems with pK_a values above pH 7. At buffer pH values greater than 7, protein–capillary wall interactions were minimized as the majority of the whey proteins had a net negative charge because their p*I* values are in the range pH 4–6. A wide range of buffer additives (organic modifiers and surfactants) were also added to alter the chemistry of the separation, to further block protein–capillary wall interactions and to thus optimize the resolution of the different protein peaks. A sample buffer/separation buffer system was developed which eliminated an initial solvent trough that coincided with the IgG peak. This made it possible to quantify the IgG protein. Optimum resolution and analysis time (10 min) for the four whey proteins was achieved with a sample buffer consisting of 10 m*M* phosphate, pH 7.4 and a separation buffer consisting of 150 m*M* sodium borate, pH 8.5 containing 0.05% Tween 20. This method was successfully used to separate a mixture of commercially purified whey proteins and to separate and quantitate the individual whey proteins in an acid whey sample.

1. Introduction

Bovine whey comprises four major protein groups which have a diverse range of molecular masses, p*I* values, number of phenotypic variants, subunit compositions and degrees of post-translational modification (Table 1). This presents particular problems for their separation and quantification. A number of methods [3–5] have already been published on the capillary zone electrophoretic separation of whey proteins. These have, however, focused primarily on the separation of α-lactalbumin (α-Lac) and the two most common variants of β-lactoglobulin (β-LgA and β-LgB).

As whey is increasingly being used to develop a range of sophisticated protein products it has become necessary to accurately quantify all the major whey protein components including both the bovine serum albumin (BSA) and the immunoglobulin (Ig) fractions. These latter two proteins are more difficult to characterize in

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Table 1
Physical characteristics of the major whey proteins[a]

Whey protein	M_r	p*I*	Concentration in whey (mg/ml)	Extinction coefficient (280 nm)
α-Lac	14 200	4.4	0.6–1.7	20.1
β-Lg	18 300	5.4	2.0–3.0	9.7
BSA	66 000	5.1	0.2–0.4	6.6
IgG	150 000	5.5–8.3	0.5–1.8	14.0

[a] Refs. [1,2].

whey because of their low concentrations, their microheterogeneity and, in the case of IgG, its high p*I* values [6].

In the present investigation a capillary zone electrophoresis (CZE) method which had been developed previously [7] to measure α-Lac and β-LgA, β-LgB and β-LgC, was developed further to also separate both BSA and IgG from a mixture of commercially purified whey proteins and to separate and quantitate the liquid whey proteins in whey samples.

2. Experimental

2.1. Materials

All buffers and buffer additives were of analytical grade or better and were obtained from either BDH Chemicals (Poole, UK) or Sigma (St. Louis, MO, USA). The purified whey proteins α-Lac, β-LgA, β-LgB, BSA and IgG were supplied by Sigma and were called the whey protein standards. Water was purified by reverse osmosis followed by deionization (Milli-Q, Millipore, MA, USA).

Simulated milk ultrafiltrate (SMUF) was prepared by the method of Jenness and Koops [8] and consisted of potassium dihydrogen phosphate, 1.58 g/l; tripotassium citrate, 1.20 g/l; trisodium citrate, 2.12 g/l; dipotassium sulphate, 0.18 g/l; calcium chloride, 1.32 g/l; magnesium chloride, 0.65 g/l; potassium carbonate, 0.30 g/l and potassium chloride, 0.6 g/l. The SMUF was equilibrated to pH 6.6 with KOH.

2.1.1. Whey sample

Acid whey was produced in the laboratory by acid precipitation of casein from skim milk at pH 4.6 using mineral acid. This casein was removed by centrifugation and the whey was filtered (0.45 μm) to remove any residual casein precipitate and fat.

2.1.2. Buffers

The buffers described in Table 2 were used throughout this study.

2.1.3. Buffer additives

The following organic modifiers and surfactants were used as buffer additives; ethanolamine, diethanolamine, triethanolamine, Triton X-100, glycerol, polyethyleneglycol 600, Nonidet P-40, polyvinylalcohol and Tween 20.

2.2. Capillary electrophoresis

Capillary zone electrophoresis was performed on an Applied Biosystems 270A-HT CE system (Foster City, CA, USA) using a PE Nelson 100 Series interface and a PE Nelson TurboChrom 3.3 software package (Cupertino, CA, USA) for data acquisition and analysis, respectively. The uncoated capillary (72 cm total length, 50 cm effective length and 50 μm I.D.) was supplied by Applied Biosystems. Samples were loaded at the anode using vacuum injection (17 kPa) for 10 s. The separation voltage was 20 kV with detection at 215 nm. Between injections the capillary was flushed for 2 min (68 kPa) consecutively with 0.1 *M* NaOH, Milli-Q water and buffer to retain separation reproducibility.

Table 2
Properties of various buffer systems[a]

Buffer	Common name	pH range	Buffer pH used
Sodium tetraborate/HCl	Sodium borate	7.80–10.60	8.5
N-Tris (hydroxymethyl)methyl-3-amino-propanesulphonic acid	TAPS	7.55–9.55	8.5
N,N-Bis(2-hydroxyethyl)glycine	Bicine	7.35–9.35	8.5
N-Tris(hydroxymethyl)methylglycine	Tricine	7.15–9.15	8.0
N-2-Hydroxyethylpiperazine-N-propanesulphonic acid	EPPS	7.10–9.10	8.0
N-2-Hydroxyethylpiperazine-N-ethanesulphonic acid	HEPES	6.55–8.55	7.5
N-Tris(hydroxymethyl) methyl-2-aminoethanesulphonic acid	TES	6.50–8.50	7.5
3-(N-Morpholino) propanesulphonic acid	MOPS	6.15–8.15	7.5
Imidazole/HCl	Imidazole	6.20–7.80	7.5
1,3-Bis[tris(hydroxymethyl)-methylamino] propane	Bistris propane	5.80–7.80	7.4
Sodium dihydrogen phosphate/disodium hydrogen phosphate	Phosphate	5.00–8.00	7.4
Simulated milk ultrafiltrate	SMUF	6.6	6.6
Bis(2-hydroxyethyl)imino-tris-(hydroxymethyl)methane	Bistris	5.50–7.50	6.0
2-(N-Morpholino)ethanesulphonic acid	MES	5.15–7.15	6.0
Disodium citrate/HCl	Citrate	1.20–5.00	4.6

[a] See Ref. [9].

3. Results and discussion

3.1. Initial separation

Whey protein standards were initially prepared in Milli-Q water and separated using a 150 mM sodium borate buffer at pH 8.5 containing 0.1% ethanolamine and 0.1% Tween 20 (Fig. 1) as this buffer system had been used previously to

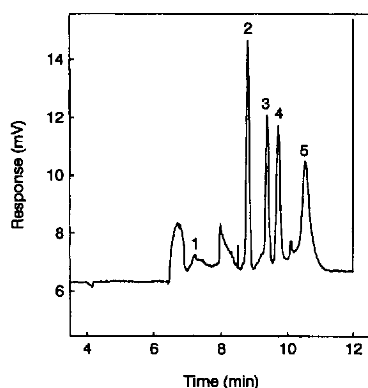

Fig. 1. Initial separation of whey protein standards. α-Lac, β-LgA, β-LgB, BSA and IgG were prepared at 0.2 mg/ml in Milli-Q water. The separation buffer was 150 mM sodium borate, pH 8.5 containing 0.1% ethanolamine and 0.1% Tween 20. Peaks: 1 = IgG; 2 = α-Lac; 3 = β-LgB; 4 = β-LgA; 5 = BSA.

successfully separate α-Lac, β-LgA and β-LgB (unpublished results). The elution order of the individual whey proteins was IgG, α-Lac, β-LgB, β-LgA and lastly BSA. This order was determined both by spiking the combined whey protein standard and by running each whey protein separately. Although this initial method resulted in baseline separation of α-Lac, β-LgB and β-LgA there was interference of the IgG peak by the solvent front material and both the IgG and BSA peaks were not as highly resolved as were the peaks for the other whey proteins. This latter problem became more pronounced when the whey protein standards were prepared in a similar ratio to that observed in a typical whey sample as both IgG and BSA are present in whey at lower concentrations than α-Lac and β-Lg.

3.2. Effect of using the same buffer type for both the separation buffer and the sample buffer

A number of different separation buffer and sample buffer systems were then surveyed together with a range of organic and polymeric modifiers to determine the optimal conditions to separate all the major whey proteins. The principal requirements for the method were that both

IgG and BSA should be able to be separated and quantitated at the levels observed in whey samples and in the presence of the other whey proteins.

The effects of using the same buffer type for both the sample buffer and the separation buffer are shown in Fig. 2. As well as the biological buffers sodium borate and imidazole, a range of

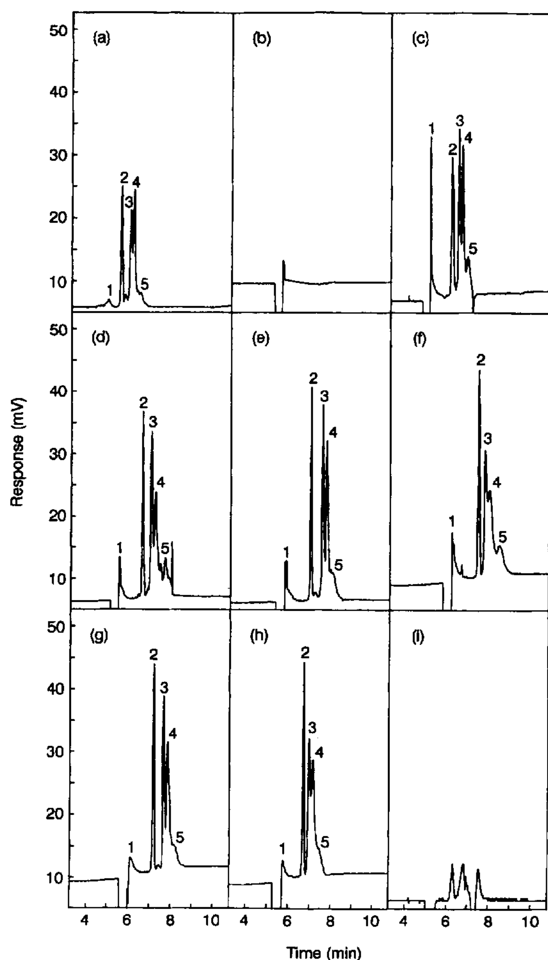

Fig. 2. Effect of buffer type on separation of whey protein standards. Buffers were all 150 m*M*. (a) Sodium borate, pH 8.5 plus 0.05% Tween 20; (b) Bicine, pH 8.5; (c) Tricine, pH 8.0; (d) TAPS, pH 8.5; (e) MOPS, pH 7.5; (f) TES, pH 7.5; (g) EPPS, pH 8.0; (h) HEPES, pH 7.5; (i) imidazole, pH 7.5. Whey protein standards were prepared in the corresponding buffer (10 m*M*) and with the following composition: α-Lac, 0.16 mg/ml; β-LgA, 0.20 mg/ml; β-LgB, 0.24 mg/ml; BSA, 0.07 mg/ml; IgG, 0.06 mg/ml. Peaks were as in Fig. 1.

Good buffers [10] were tested as these have both good buffering capacity and low specific conductance, thus allowing high ionic strengths whilst maintaining reasonable conductivity and heating effects. A pH value close to the pK_a of the buffer was chosen for each buffer system. The sample buffers were also used at a lower concentration than the separation buffers to encourage stacking [11] of the samples and thus to enhance the resolution.

Elution patterns similar to those obtained using the initial separation conditions were observed for all the buffers except Bicine, pH 8.5 and imidazole, pH 7.5. There was very little 215 nm absorbing material with both the VBicine and imidazole buffers which indicated that under these conditions either the protein was interacting with the capillary wall and not eluting or there was non electro-osmotic flow. The latter was unlikely as there was a solvent front trough between 5 and 6 min for both systems and there were some peaks in the electropherogram of the imidazole buffer.

Similar solvent troughs were present in all the other buffer systems except sodium borate, pH 8.5 containing 0.05% Tween 20. In each case these troughs coincided with the IgG peak and therefore interfered with its identification and quantitation. This concurrent elution was caused by the high p*I* value of the IgG proteins and may have been circumvented by increasing the buffer pH. However, a compromise had to be made between increasing the buffer pH to affect separation of the IgG from the solvent peak and the deleterious effect this may have on the separation of the other whey proteins which have lower p*I* values.

Of the other whey proteins there was baseline separation of α-Lac in every buffer system. This was presumed to be due primarily to the difference in p*I* value of α-Lac when compared to β-LgA, β-LgB and BSA (Table 1). The α-Lac peak was also the sharpest which meant that, together with its high extinction coefficient, it was the highest peak in the electropherograms

In contrast to this the separation of β-LgA and β-LgB highlighted the differences in the separation capabilities of the different buffer systems.

These ranged from very poor separation in the case of TES, pH 7.5 through to almost baseline separation for the MPS, pH 7.5 and EPPS, pH 8.0 buffers. The separation of β-LgA and β-LgB may have been optimized further by changing the pH values of the various buffers. (See Table 2 for buffer abbreviations).

Lastly, BSA eluted immediately after, and generally as a shoulder of, the β-LgA peak. The BSA peak was therefore very poorly resolved as a result of the combination of its low concentration in whey, its low extinction coefficient, its similar p*I* to that of β-LgA and finally the microheterogeneity observed within the BSA molecules. This microheterogeneity is proposed to be due to post-translational modification of amino acid side chains and/or disulphide isomerization [11]. Under the current conditions TAPS, pH 8.5, Tricine, pH 8.0 and TES, pH 7.5 afforded the best separation of BSA of the buffer systems examined.

Overall, the sodium borate buffer was selected for further study as there was no solvent front to interfere with the IgG peak and the separation of α-Lac was excellent. Other separation variables were then changed to try to improve the separation of the other whey protein components.

3.3. Effect of sample buffer

The introduction of the whey protein standards to the capillary in a buffer system different from the separation buffer [12] and at a lower concentration than the separation buffer was examined to further optimise the separation of the whey proteins, in particular the IgG and BSA fractions (Fig. 3). As well as the buffers used in the previous section other buffers and also SMUF were tested.

In all cases the use of sodium borate as the separation buffer eliminated the initial solvent front making analysis of IgG possible. There were, however, other anomalous peaks which were attributed to the interaction of the different buffer types, e.g. peaks in MOPS, TES and MES at approximately 8 min. Whilst the general pattern of whey protein separation remained similar to that observed in Fig. 1, there were some obvious differences which were presumed to be caused by the different sample buffers.

The best separation was obtained with a sample buffer of 10 m*M* phosphate at pH 7.4. All five whey protein standards were separated although baseline separation was still not attained for β-LgA, β-LgB and BSA. In addition, two other minor peaks were observed: the first occurring between α-Lac and β-LgB at approximately 6.3 min and the second occurring between β-LgA and BSA at approximately 6.9 min. On running the individual whey standards these minor peaks were shown to be impurities in the α-Lac and β-LgA standards respectively.

Of the other sample buffer systems imidazole, MOPS, TES, Bistris propane and Tricine also produced good separations although in each case there were either extra peaks present or the overall resolution was not as high as with the phosphate sample buffer. The remaining buffers resulted in either uncharacteristic electropherograms or in electropherograms that indicated only low amounts of 215 nm absorbing material which suggested considerable protein–protein and protein–capillary wall interactions. Included amongst these buffers was the SMUF buffer, which was examined to determine whether undiluted whole milk or whey samples could be introduced on to the capillary and to also give an indication of any possible matrix effects likely to be experienced with these samples. Fig. 3 shows that, with SMUF sample buffer, although the protein elution pattern was similar to that of Fig. 1, the peaks were not as well resolved especially with respect to β-LgA, β-LgB and BSA.

3.4. Effect of separation buffer

From the electropherograms in the previous section 10 m*M* phosphate, pH 7.4 was selected as the sample buffer for future method development. The various buffers used in the initial survey were then retested using the phosphate sample buffer (Fig. 4). Once again, a solvent trough and peak was present with all the buffers except sodium borate, which made quantitation of IgG impossible. In most instances however, the use of a phosphate sample buffer did im-

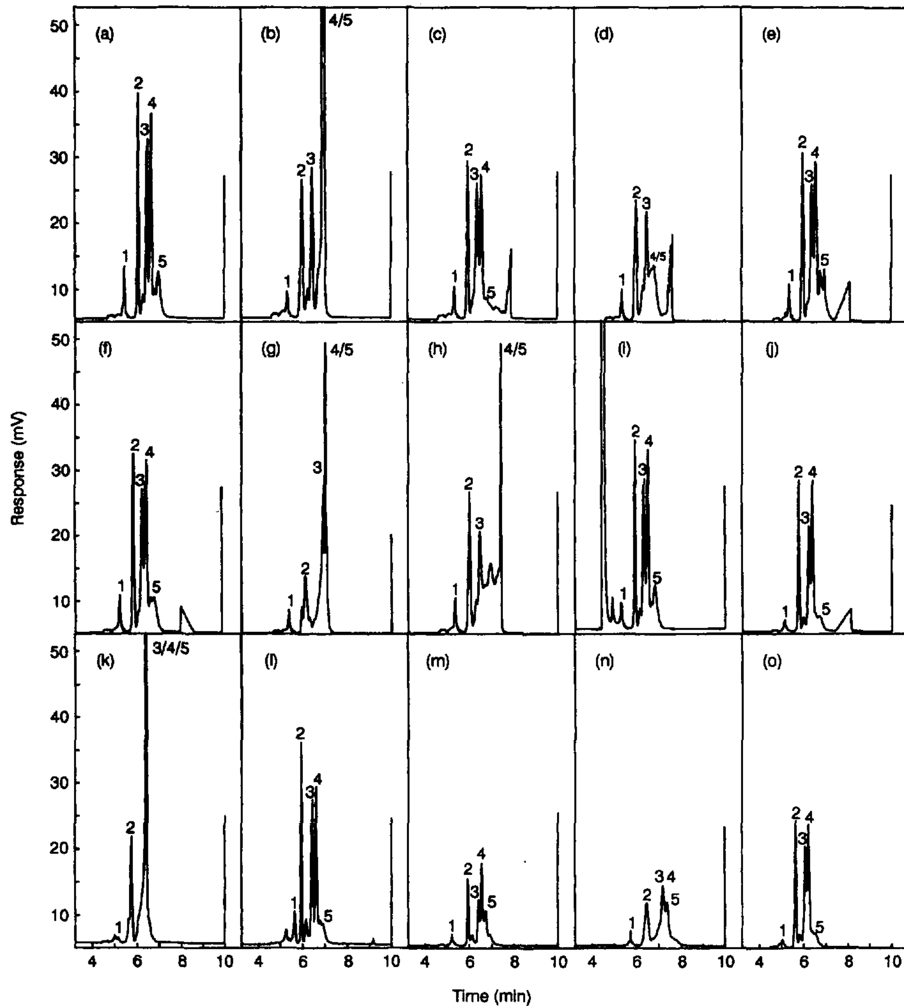

Fig. 3. Effect of sample buffer on separation of whey protein standards. Separation buffer was 150 m*M* sodium borate, pH 8.5 plus 0.05% Tween 20. All sample buffers were 10 m*M*. Whey protein standards and electropherogram peaks were as described in Fig. 1. Sample buffers: (a) phosphate, pH 7.4; (b) Bicine, pH 8.0; (c) Tricine, pH 8.0; (d) TAPS, pH 8.5; (e) MOPS, pH 7.5; (f) TES, pH 7.5; (g) EPPS, pH 8.0; (h) HEPES, pH 7.5; (i) imidazole, pH 7.5; (j) MES pH 6.0; (k) Bistris, pH 6.0; (l) Bistris propane, pH 7.4; (m) citrate, pH 4.6; (n) SMUF, pH 6.6; (o) sodium borate, pH 8.5 plus 0.05% Tween 20.

prove the resolution of the β-LgA, β-LgB and BSA peaks.

It was therefore decided to use a final buffer system consisting of a sample buffer of 10 m*M* phosphate, pH 7.4 and a separation buffer of 150 m*M* sodium borate, pH 8.5 containing 0.05% Tween 20.

3.5. Effect of modifiers

The addition of modifiers to CZE buffers to improve the separation of complex mixtures of sample components by altering the electro-osmotic flow and/or the analyte–capillary wall interactions has been well documented [13,14]. A

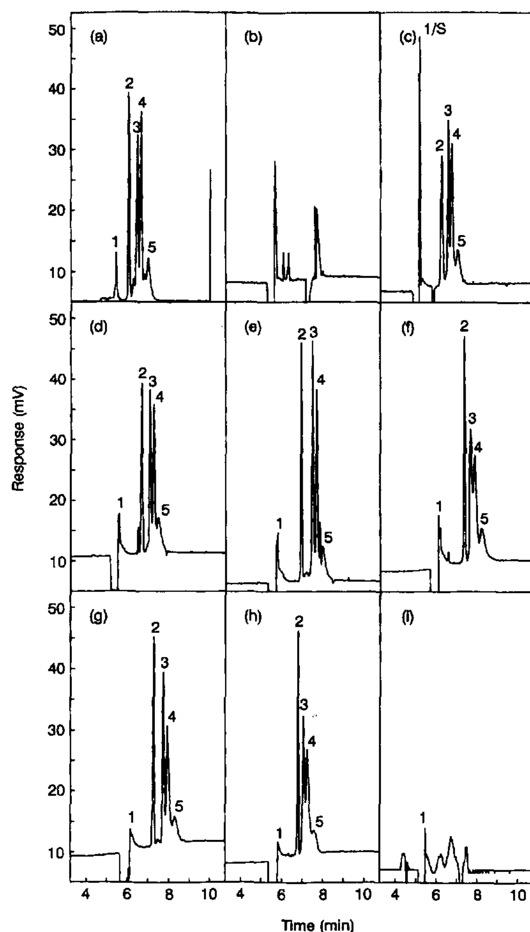

Fig. 4. Effect of separation buffer on separation of whey protein standards. Separation buffers, whey protein standard concentrations and electropherogram peaks were as described in Fig. 2. Standards were prepared in 10 mM phosphate, pH 7.4.

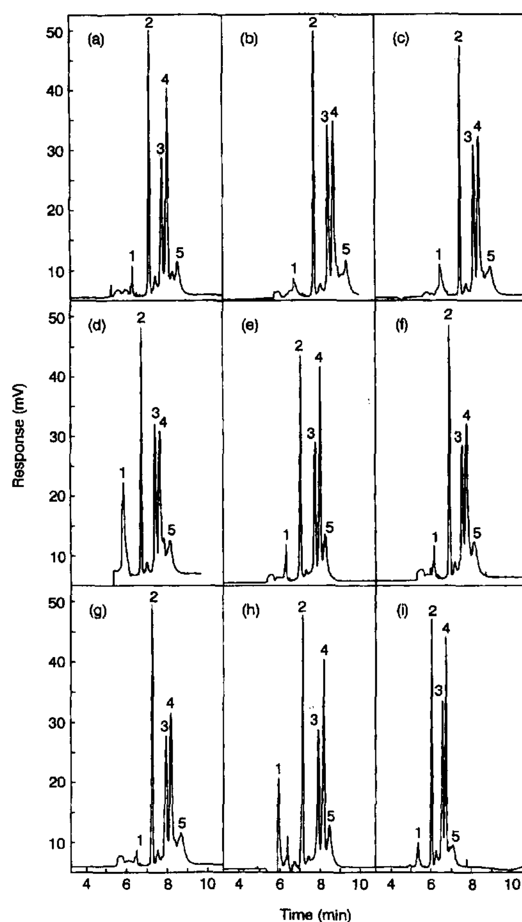

Fig. 5. Effect of modifiers on the separation of whey protein standards. Separation buffer was 150 mM sodium borate, pH 8.5; Sample buffer was 10 mM phosphate, pH 7.5. Whey protein standards were prepared in sample buffer and electropherogram peaks were as described in Fig. 1. Modifiers were all added at 0.1%: (a) Tween 20; (b) ethanolamine; (c) diethanolamine: (d) triethanolamine: (e) Triton X-100; (f) glycerol; (g) polyethyleneglycol 600; (h) Nonidet P-40; (i) polyvinylalcohol.

range of modifiers (organic, surfactant and amino) were examined in the present study with the primary aim of improving the separation of the β-LgB, β-LgA and BSA peaks. Preliminary work had shown that similar resolution of the whey protein standards was achieved if the 0.1% ethanolamine and 0.1% Tween 20 modifiers included in the initial separation buffer (Fig. 1) were replaced by only 0.05% Tween 20 (results not shown).

The electropherograms in Fig. 5 show that the modifier used initially, Tween 20, resulted in the best resolution of the whey proteins. Whilst most of the other modifiers altered the overall separation pattern, they were not able to match the resolution of β-LgB, β-LgA and BSA achieved by Tween 20.

The effect of different concentrations of Tween 20 showed that, whilst similar separations

were achieved with both 0.05 and 0.1% of this modifier, with lower concentrations (0.02 and 0.01%) the peaks were not as well resolved (data not shown).

3.6. Final method

Based on the results presented above the optimum conditions for the separation and quantitation of whey proteins by CZE on an uncoated capillary involved sample preparation in a 10 m*M* phosphate buffer, pH 7.4 and separation with a 150 m*M* sodium borate buffer, pH 8.5 containing 0.05% Tween 20. Between runs the capillary was washed with 0.1 *M* NaOH and then re-equilibrated with separation buffer.

The separation of whey protein standards and of an acid whey using this method is shown in Fig. 6. The proteins eluted after 5 min and separation was completed within 10 min. The elution profile of the acid whey mimicked that of the whey standards although there were more protein peaks eluting around the IgG peak and the overall retention times were slightly longer. This was attributed to the different ionic strength of the whey sample but did not affect the overall separation. The relatively smaller amounts of both BSA and IgG in comparison to α-Lac and β-Lg may result in some quantitation problems although in both instances the peaks were discrete and well resolved.

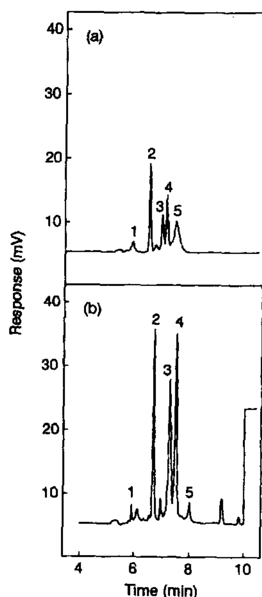

Fig. 6. Separation of (a) whey standards and (b) acid whey. Separation buffer was 150 m*M* sodium borate, pH 8.5 plus 0.05% Tween 20. Whey protein standards were prepared in 10 m*M* phosphate, pH 7.4 at the following concentrations; α-Lac, 0.08 mg/ml; β-LgA 0.10 mg/ml; β-LgB, 0.12 mg/ml; BSA, 0.04 mg/ml; IgG, 0.03 mg/ml. Acid whey was prepared as described in Experimental and then diluted 1:10 with 10 m*M* phosphate, pH 7.4. Peaks were as in Fig. 1.

4. Conclusion

A CZE method for the separation of the major whey proteins in bovine whey has been successfully developed. An extensive survey of buffer systems showed that the best resolution of the major whey proteins was achieved with a low ionic strength phosphate sample buffer and a higher ionic strength sodium borate run buffer. The difference in ionic strength and pH of the two buffers was presumed to improve protein separation by encouraging initial stacking of the sample. Resolution was also enhanced by the inclusion of the non-ionic surfactant Tween 20.

The main advantage of the present method over previously published methods for separating whey proteins was that both BSA and IgG could also be separated and quantitated. This was demonstrated for both whey protein standards and an acid whey.

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