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Comparison of capillary electrophoresis with traditional methods to analyse bovine whey proteins

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Abstract

The separation of the four major whey proteins by sodium dodecyl sulphate (SDS)–capillary gel electrophoresis (CGE) is described. Whilst commercially purified whey proteins could be analysed using the recommended protocol, the more complex nature of an acid whey and a reconstituted whey protein concentrate (WPC) powder necessitated considerable refinement of the CGE sample buffer. Individual whey proteins in the acid whey and WPC samples were then also separated and quantitated using capillary zone electrophoresis, polyacrylamide gel electrophoresis (PAGE) and HPLC methods and the results were compared. The values obtained for α-lactalbumin (α-Lac) and β-lactoglobulin (β-Lg) were consistent throughout the various methods, although size-exclusion HPLC, SDS-PAGE and SDS-CGE could not separate the two β-Lg variants or the glycosylated form of α-Lac from the β-Lg. There was considerable variation in the values for the bovine serum albumin and immunoglobulin determined by the different methods and it was concluded that none of the methods could satisfactorily quantitate all four whey proteins.

1. Introduction

For humans bovine milk is an excellent source of essential nutritional components. These include proteins, present at 3–3.5% (w/v) of which the whey proteins comprise 0.5–0.7% (w/v) [1]. Whey proteins are classified as milk proteins which are soluble at pH 4.6 and 20°C and include β-lactoglobulin (β-Lg), α-lactalbumin (α-Lac), bovine serum albumin (BSA) and immunoglobulins (Ig) [2]. Also present are the minor proteins lactoferrin (LF), lactoperoxidase, proteose-peptone components (PP), glycomacropeptide and protein components of the milk fat globular membrane [3]. The four major whey proteins represent a diverse group of globular proteins and their properties are listed in Table 1.

The analysis of the whey proteins has traditionally been performed by polyacrylamide gel electrophoresis (PAGE) [native and sodium dodecyl sulphate (SDS)] and HPLC which provide information on purity, molecular mass and microheterogeneity. These methods have been extensively discussed in a number of recent reviews [4,5]. The advent of capillary electrophoresis has resulted in the development of similar techniques but in a capillary format which can be quicker, automated with on-line detection and which require very small sample and buffer volumes [6]. To date, capillary zone electrophoresis (CZE) has predominantly been used to separate the whey proteins. Most research has centered on the separation of only α-Lac and the

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Table 1
Physical properties of the whey proteins[a]

Whey protein	Molecular mass	p*I*	Concentration in whey (mg/ml)	Total whey protein (%)
Major				
β-Lg	18 300	5.4	2.0–3.0	50
α-Lac	14 200	4.4	0.6–1.7	12
BSA	66 000	5.1	0.2–0.4	5
IgG	150 000	5.5–8.3	0.5–1.8	10
Minor				
LF	80 000	7.5	0.1	1
PP	4000–40 000	–	1.4	23

[a] See Refs. [1,2].

two variants of β-Lg (A and B) in uncoated [7] and coated [8,9] capillaries, although Otte et al. [10] have used uncoated capillaries and low pH buffers to separate the four major whey proteins.

In the present study a capillary gel electrophoresis (CGE) method has been developed to analyse whey proteins. This method and a recently developed CZE method [11] have then been used in conjunction with a number of traditional analytical techniques to quantitate the four major whey proteins in a liquid whey sample and a reconstituted whey protein concentrate (WPC) powder. The results were then compared and the suitability of using just one of these techniques to quantitate all the whey proteins is discussed.

2. Experimental

2.1. Materials

All buffers and reagents were of Analar grade or better. The commercially purified whey proteins α-Lac, β-Lg A, β-Lg B, BSA and IgG were supplied by Sigma (St. Louis, MO, USA). These are referred to in the text as the whey protein standards. Water was purified by reverse osmosis followed by deionization (Milli-Q, Millipore, Bedford, MA, USA).

2.2. Whey and WPC samples

Acid whey, prepared by the acid precipitation of casein from skim milk at pH 4.6, and WPC, prepared by ultrafiltration/difiltration, evaporation and spray drying acid whey, were obtained from a New Zealand commercial whey processing site. A liquid sample of WPC was also obtained immediately prior to evaporation and drying.

2.3. α-Lactalbumin and glycosylated-α-lactalbumin samples

An α-Lac/glycosylated-α-Lac (glyco-α-Lac) preparation was made using the method of Mailliart and Ribadeau-Dumas [12]. The final preparation was essentially free of β-Lg but contained some BSA and IgG. Glyco-α-Lac was produced from the sample above by separating the α-Lac from the glyco-α-Lac using a size-exclusion HPLC column (Beckman Ultraspherogel-SEC 3000). The glyco-α-Lac eluted before the α-Lac and, although there was a small amount of overlap between the two peaks, fractions were collected so as to avoid this overlap.

2.4. Deglycosylation of glyco-α-Lac

PNGase F (New England Biolabs, Beverly, MA, USA) was used to cleave the N-linked carbohydrate moiety from the asparagine residue in glyco-α-Lac. A volume of 50 μl of a 2-mg/ml solution of glyco-α-Lac was mixed with 10 μl of Biolabs 10x denaturing buffer and 19 μl of Milli-Q water. The protein in the glyco-α-Lac sample was then denatured by heating to 100°C for 10 min. After cooling, 10 μl of Biolabs reaction

buffer, 10 μl of 10x NP-40 (Biolabs) and 1 μl Biolabs PNGaseF enzyme were added and incubated at 37°C for 1 h. Following this deglycosylation step the sample was analysed by SDS and native PAGE.

2.5. Ultrafiltration/diafiltration of whey sample

A 5-ml sample of acid whey was ultrafiltered to 0.5 ml in an Amicon Micro-UF System (Model 8MC, Beverly, MA, USA) using a 1000 nominal molecular mass cut-off polysulphone membrane. This was then diafiltered with two 4.5-ml volumes of sample buffer before the volume was made up to the original 5 ml with CGE sample buffer.

2.6. Capillary electrophoresis

CE was performed on an Applied Biosystems 270A-HT CE system (Foster City, CA, USA) using a PE Nelson 900 series interface and a PE Nelson Turbo Chrom 3.3 software package (Cupertino, CA, USA) for data acquisition and handling, respectively.

2.7. HPLC

The HPLC system consisted of two pumps (Waters Model 6000A), an automatic injector (Waters WISP 7108), a Waters 490 absorbance detector and a Waters Millenium 2010 data acquisition and manipulation system. Prior to use all buffers were filtered (0.45 μm cellulose acetate, Millipore) and degassed. All samples were filtered (0.45 μm Sunvial nylon/polypropylene filter).

2.8. Capillary gel electrophoresis

A ProSort SDS-Protein Analysis Kit (Applied Biosystems) was used for CGE. The manufacturer's protocol was followed, except the reduced sample buffer was 2% SDS–5% 2-mercaptoethanol (2ME) and the non-reduced sample buffer was 2% SDS. The following whey protein concentrations were used to construct standard curves: α-Lac, 0.05–0.24 mg/ml; β-Lg A and β-Lg B, 0.09–0.45 mg/ml; BSA, 0.03–0.13 mg/ml and IgG, 0.02–0.10 mg/ml. Acid whey and WPC were prepared at a 1:9 dilution and 1 mg/ml respectively.

2.9. Capillary zone electrophoresis

CZE was performed as described by Kinghorn *et al.* [11] using an uncoated capillary (72 cm total length, 50 cm effective length and 50 μm I.D.), a 10-m*M* phosphate, pH 7.4 sample buffer and a 150-m*M* sodium borate, pH 8.5, plus 0.05% Tween 20 separation buffer. Whey protein standards, acid whey and WPC were prepared in sample buffer as described above (Section 2.8).

2.10. Size-exclusion HPLC

A Beckman Ultraspherogel-SEC 3000 column (300 × 7.5 mm I.D., 5 μm bead diameter, Beckman, Fullerton, CA, USA) connected in series with a Beckman Ultraspherogel guard column (40 × 7.5 mm I.D., 5 μm bead diameter) were used to separate the whey proteins. The flow-rate was 1 ml/min using a buffer of 0.05 *M* sodium sulphate–0.02 *M* sodium dihydrogen orthophosphate, pH 6.8. Proteins were detected by absorbance at 280 nm and the total run time was 16 min. Whey protein standards (α-Lac, β-Lg A, β-Lg B, BSA and IgG) in the range 2–40 μg were used to construct standard curves. The acid whey was diluted 1:6 in sample buffer and the WPC made up to 2 mg/ml in Milli-Q water.

2.11. Ion-exchange HPLC

The anion-exchange column MonoQ (Pharmacia, Uppsala, Sweden) was used for the ion-exchange chromatography. Samples were introduced on to the column in buffer A (20 m*M* piperazine and 1 m*M* $CaCl_2$, pH 5.5) and eluted with a gradient of buffer B (Buffer A plus 1 *M* NaCl) according to the method of Humphrey and Newsome [13]. Detection was by absorbance at 214 nm and 280 nm with a total run time of 28 min. The whey protein standards, whey and WPC were prepared as described above (Section 2.10), except IgG was not included in the whey

protein standard as this protein does not bind to the MonoQ column.

2.12. IgG affinity HPLC

Bovine IgG was measured by affinity HPLC on a Pharmacia Hi-Trap Protein G column. Samples were injected on to the column in buffer A (50 m*M* sodium dihydrogen phosphate, pH 6.5) and eluted with a gradient of buffer B (50 m*M* glycine, pH 2.5) according to the protocol described in Table 2. Proteins were detected by absorbance at 280 nm with a 7-min total run time. A standard curve was constructed using 4–40 μg bovine IgG. Whey was diluted 1:5 and WPC was prepared at 2 mg/ml in buffer A.

2.13. Native PAGE

Native, non-denaturing polyacrylamide gels were prepared according to Andrews [14] with a 4% stacking gel and a 15% separating gel. The resolving gel buffer was 0.375 *M* Tris–HCl (pH 8.8), the stacking gel buffer was 0.125 *M* Tris–HCl (pH 6.8) and the reservoir buffer was 0.025 *M* Tris–0.192 *M* glycine (pH 8.8). Slab gels of 0.75 mm were prepared and run using the Mini Protean II apparatus (Bio-Rad Labs., Richmond, CA, USA). Protein staining was performed with 0.05% Coomassie Brilliant Blue R-250 (Bio-Rad Labs.) in 25% isopropanol, 10% glacial acetic acid for 1 h.

Destaining was with 10% isopropanol, 10% glacial acetic acid for 2 h. Protein bands were quantitated using a laser scanning computing densitometer (Molecular Dynamics Model 300A, Sunnyvale, CA, USA). Volumes of 10 μl of either whey protein standards (α-Lac, β-Lg A, β-Lg B: 0.04–0.22 mg/ml; BSA and IgG: 0.02–0.12 mg/ml), acid whey (1:9 dilution in sample buffer) or WPC (1 mg/ml) were loaded on to each lane.

2.14. SDS-PAGE

The procedure of Laemmli [15] was employed for SDS-PAGE using a 4% stacking gel and a 15% separating gel. Protein staining, destaining and quantitation was as described above (Section 2.13). Standard concentrations were: α-Lac (0.04–0.23 mg/ml), β-Lac, (0.09–0.46 mg/ml); BSA (0.02–0.12 mg/ml) and IgG (0.02–0.12 mg/ml) with 10 μl being loaded. Acid whey and WPC were prepared in sample buffer at a 1:9 dilution and at 1 mg/ml, respectively.

3. Results and discussion

3.1. Capillary gel electrophoresis

The separation and quantification of the whey standards and whey samples by capillary gel electrophoresis are shown in Fig. 1. Although a commercial kit (ProSort SDS-Protein Analysis Kit, Applied Biosystems) was used, a number of modifications were required before satisfactory results were obtained. The recommended sample buffer (0.28% SDS–1% 2ME) resulted in excellent resolution of the whey protein standards with the exception of the two β-Lg variants which migrated as a single peak (results not

Table 2
Gradient conditions for protein G affinity HPLC

Time (min)	Flow-rate (ml/min)	A (%)	B (%)	Gradient
0	1.0	100	0	–
0.5	1.0	100	0	–
1.0	2.0	100	0	Linear
1.5	2.0	0	100	–
4.0	2.0	0	100	Linear
5.0	1.0	100	0	–
7.0	1.0	100	0	–

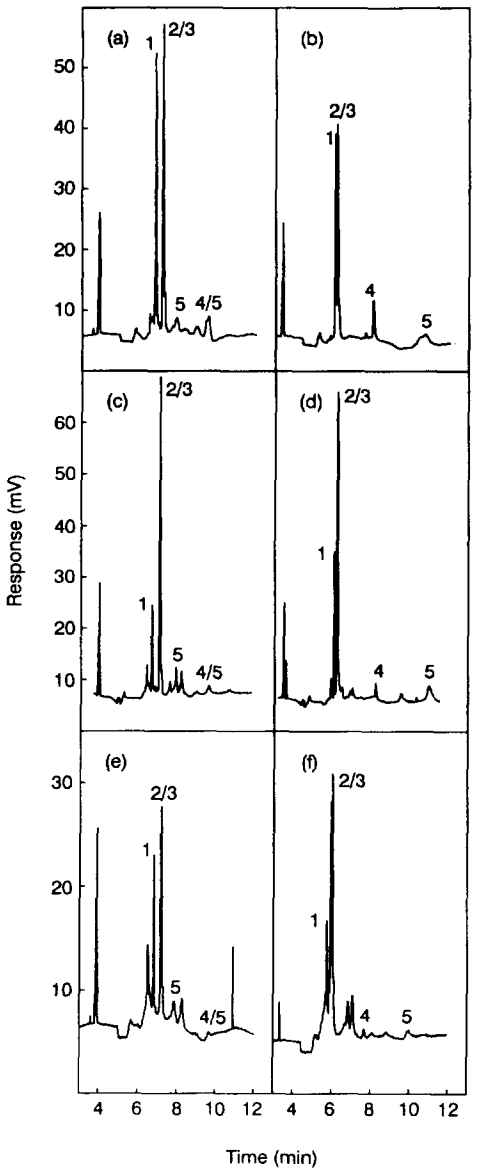

Fig. 1. Capillary gel electrophoresis of whey protein standards, acid whey and WPC was performed under reduced (a), (c) and (e), and non-reduced (b), (d) and (f) conditions respectively. Electrophoresis was performed as described in the Experimental section. Peaks: 1 = α-Lac; 2 = β-Lg A; 3 = β-Lg B; 4 = BSA; 5 = IgG.

shown). When this system was used with the acid whey sample, however, a white precipitate formed. After this precipitate was removed from the sample by centrifugation and filtration the subsequent whey protein values were considerably lower than expected from the results of other techniques. This precipitate was probably a sodium or potassium-dodecyl sulphate complex which on aggregation also precipitated out some of the whey protein.

A sample of whey was ultrafiltered and diafiltered against the sample buffer to decrease both its potassium ion concentration and its overall ionic strength. Table 3 shows that after this treatment the values for both α-Lac and β-Lg were restored to the expected levels as ascertained by SDS-PAGE.

Another possible reason for the low values for α-Lac and β-Lg in the acid whey samples was that if the initial ionic strength of the whey was higher than that of the whey protein standards then less whey would be introduced on to the capillary by the electrokinetic injection. This effect would presumably have been negated by the ultrafiltration/diafiltration step and may be circumvented by using vacuum injection.

As the use of ultrafiltration/diafiltration is not practical when assaying large numbers of samples, the sample buffer conditions were therefore modified to prevent the formation of this precipitate. A sample buffer of 2% SDS, containing either 5% 2ME for a reduced system or no 2ME for a non-reduced system, together with an 1:6–1:10 dilution of the whey sample was selected to prevent any precipitate formation. Under these conditions, the reduced and non-reduced buffer systems produced different separation profiles for the whey protein standards (Fig. 1a and b).

Using the reducing buffer baseline separation of α-Lac and β-Lg was achieved but the BSA and IgG peaks were not as well defined. The disulphide bonds in the IgG protein were disrupted resulting in peaks corresponding to the heavy and light chain subunits. The heavy chain subunit peak overlapped with the BSA peak. When the BSA and IgG standards were loaded at lower concentrations to mimic the protein ratios observed in whey quantification of the two proteins was very difficult.

In the non-reduced buffer systems, BSA and IgG were more highly resolved and gave discrete peaks. Unexpectedly, however, there was de-

Table 3
Effect of whey sample ultrafiltration/diafiltration (UF/DF) on analysis by capillary gel electrophoresis

Sample	α-Lac (mg/ml)	β-Lg (mg/ml)
Acid whey	0.32	1.28
Retenate after UF/DF	0.52	3.17
Permeate after UF/DF	0	0
Analysis by SDS-PAGE	0.50	3.10

Sample preparation and methods used were as described in the Experimental section.

creased separation between the α-Lac and β-Lg peaks. When run under non-reducing conditions β-Lg would be expected to be in the dimer form and hence have a higher apparent molecular mass (approximately 36 600, cf. 18 300 for the monomer) which would result in greater separation from α-Lac. The reason for this anomalous behaviour is not presently known.

An electrophoretic pattern similar to that observed for the whey protein standards was obtained when the acid whey was separated using the buffer system described above (Fig. 1c and d). There were also a number of other peaks present which were most probably low-molecular-mass proteose peptone fractions and small amounts of casein protein. One problem with these buffer systems, however, was that at the whey sample dilution required to prevent precipitation, the levels of both BSA and IgG were close to the detection limits, which made quantification of these proteins difficult.

With WPC (Fig. 1e and f), although the general electrophoretic protein pattern was similar to that obtained with the whey protein standards, there were a number of differences. Whilst the α-Lac levels were similar to those measured in the corresponding whey sample the α-Lac–β-Lg protein ratio was considerably higher which implied that there was a loss of β-Lg material during the processing of the whey to WPC. This loss could be accounted for by an increase in other 215 nm-absorbing material which took the form of an extra peak eluting before α-Lac at approximately 6.5 min with the reduced buffer and 6.0 min with the non-reduced buffer, and two extra peaks eluting after the β-Lg peak. This latter material was assumed to be denatured and aggregated β-Lg as any β-Lg polymerisation would have been dissociated by the SDS and reducing buffers.

There was also a considerable amount of background material eluting under the α-Lac–β-Lg peaks which made quantification difficult. This material may be Amadori rearrangement products of the α-Lac and β-Lg proteins which could be produced during evaporation and drying [16].

3.2. Capillary zone electrophoresis

The separation of the whey proteins in the three samples by CZE is shown in Fig. 2. As described previously [11] there was excellent resolution of the whey protein standards (Fig. 2) although baseline separation was not achieved between the two variants of β-Lg and BSA. There was also high resolution of the different

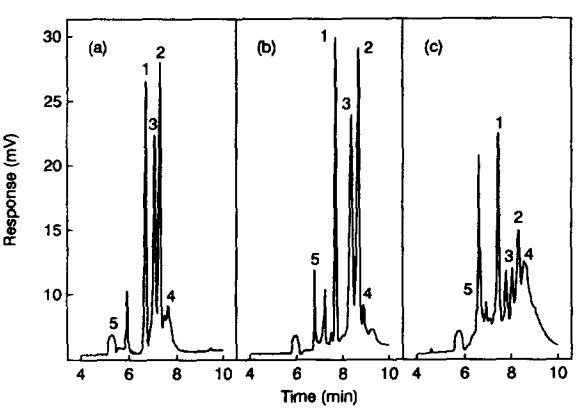

Fig. 2. Capillary zone electrophoresis of (a) whey protein standards, (b) acid whey, (c) WPC. Conditions for capillary electrophoresis as in Experimental. Peaks as described in Fig. 1.

whey proteins with the acid whey sample (Fig. 2b) although in this instance there were also a number of other minor peaks between IgG and α-Lac and β-LgA and BSA, respectively. There was a change in the retention times with the acid whey sample which was presumed to be caused by whey matrix effects (minerals, ionic strength, pH, ions, and lactose) on the electroosmotic force and/or on the protein–capillary wall interactions. These factors all contributed to making quantitation of the BSA peak more difficult. A whey sample was spiked with BSA standard to verify the position of the BSA peak (data not shown).

The separation of the whey proteins in WPC by CZE (Fig. 2c) proved to be more difficult with an extra peak overlapping the IgG peak, the loss of resolution of the β-Lg variant peaks, the emergence of additional peaks adjacent to the β-Lg peaks, and a subsequent lack of resolution of the BSA peak from these additional peaks. These changes were attributed to heat-induced chemical modifications in the protein structure during the evaporation/drying stage of processing as discussed in the previous section. This was verified by comparing the electropherograms of WPC immediately prior to evaporation and drying but after ultrafiltration and diafiltration (Fig. 3a) to the final dried WPC powder (Fig. 3b). Although the WPC before drying was highly concentrated it still had a protein peak profile similar to both the whey protein standards and the whey sample. There was, however, a loss of resolution of the β-Lg peaks by the evaporation stage of processing. This was presumed to be due to the partial polymerisation of the β-Lg molecules which was brought about by their high concentration. This polymerisation was even more evident after drying (Fig. 3b) with the increased change in the peak profile presumed to be due to a combination of the high protein concentration, high temperature (above 65–70°C) and possible high pH (above pH 7) experienced by the sample during evaporation and drying. The poor resolution and unknown/unverified peaks made quantification very difficult and methods to overcome this problem are currently being investigated.

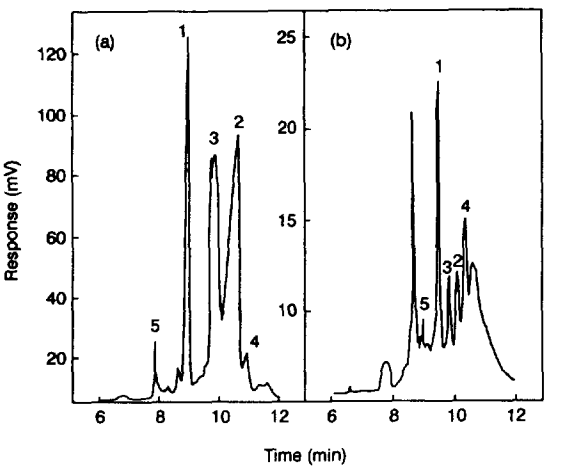

Fig. 3. Capillary zone electrophoresis of WPC (a) before and (b) after drying. Conditions for capillary electrophoresis as described in the Experimental section. Peaks as in Fig. 1. Concentrated WPC prior to drying was diluted 1:100 in sample buffer.

3.3. Native PAGE

The separation of whey protein standards, an acid whey and a WPC by native PAGE is shown in Fig. 4. The separation is based on both the charge and size of the different whey proteins such that the two main β-Lg variants (A and B) are separated and that α-Lac runs above these two β-Lg bands even though it has a smaller molecular mass.

BSA migrates as a well defined band although if there are any caseins present in the sample, these run as a broad smear which covers the BSA band. The high molecular mass of the IgG proteins restricted their entry into the gel such that they appear as a band at the interface between the stacking and running gels and are therefore difficult to quantitate. There may also be other high-molecular-mass material at this interface.

The acid whey (Fig. 4, lane 3) and WPC (Fig. 4, lane 4) samples show similar band patterns to the whey protein standards (Fig. 4, lanes 1, 2, 5 and 6) although the bands in the WPC were not as sharp. In both samples the BSA band was considerably weaker than the α-Lac and β-Lg

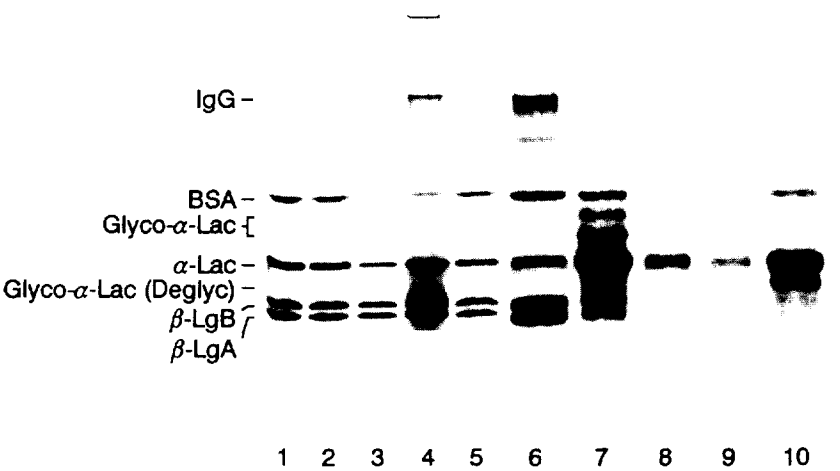

Fig. 4. Native PAGE of whey standards and samples. Gels were run and samples prepared as in Experimental. Lanes 1, 2, 5, 6 = whey protein standards; 3 = acid whey; 4 = WPC; 7 = α-Lac/glyco-α-Lac preparation; 10 = deglycosylated α-Lac/glyco-α-Lac preparation. The whey protein standards are identified on the left.

bands due to its lower concentration and the IgG appeared as a smear centered around the stacking/running gel interface. There was also considerable distortion of the β-Lg bands in the WPC sample. Whilst this sample had a high protein loading on the gel the most probable reason for the anomalous behaviour was chemical modifications of the β-Lg protein during the WPC processing as previously discussed. There was also some WPC protein material which did not enter the stacking gel from the sample well. This high-molecular-mass material was assumed to be aggregates produced during the WPC processing.

In addition to the four major whey proteins there are also a number of minor bands. These include two bands between α-Lac and BSA which have been identified as two species of glycosylated α-Lac. This was verified by the analysis of an α-Lac/glyco-α-Lac preparation (Fig. 4, lane 7) and is in agreement with the results of Hopper and McKenzie [17]. After deglycosylation of the α-Lac/glyco-α-Lac sample (Fig. 4, lane 10) the two glyco-α-Lac bands disappeared and were replaced by one band which ran just below the α-Lac band. The deglycosylated α-Lac was presumed to have a different migration pattern to α-Lac because when the carbohydrate moiety is cleaved from the Asn side chain in an N-linked glycoprotein the amino group is also removed to convert the Asn to an Asp. The concomitant change in the overall charge of the α-Lac molecule is reflected in the faster relative migration.

3.4. SDS-PAGE

The electrophoresis patterns of the whey protein standards, the acid whey and the WPC samples after separation by reduced SDS-PAGE are shown in Fig. 5. As this technique separates proteins by molecular mass differences only, the two β-Lg variants co-migrate as a single band above α-Lac. The presence of the reducing agent 2-Mercaptoethanol results in the cleavage of the disulphide bonds in IgG into its heavy and light chains which run just below BSA and above β-Lg, respectively. This method results in good separation of the whey protein standards (Fig. 5, lanes 1, 2, 5, 6 and 9) although both the heavy

Fig. 5. SDS-PAGE of whey standards and samples. Gels were run and samples prepared as in Experimental. Lanes 1,2,5,6 and 9 = whey protein standards; 3 = acid whey; 4 = WPC; 7 = purified glyco-α-Lac; 8 = deglycosylated glyco-α-Lac; and 10 = PNGase F. The whey protein standards and PNGase F bands are identified on the left.

and light chains of IgG are reasonably diffuse making quantification more difficult.

The acid whey (Fig. 5, lane 3) and the WPC sample (Fig. 5, lane 4) displayed similar protein band profiles to the whey protein standards with the exception of the light chain of IgG which occurred as a series of smeared bands running just below the IgG light chain standard. There were also a number of light bands above the IgG light chain which were assumed to be casein protein and also some higher molecular mass material which ran above the BSA band. In the WPC sample the β-Lg band did not have the same distortion problem as observed with the native gel. This was probably because any small changes, e.g. deamidation, would not significantly alter the molecular mass.

An α-Lac/glyco-α-Lac preparation was separated by SDS-PAGE to determine where glyco-α-Lac runs using this system (Fig. 5, lane 7). The glyco-α-Lac co-migrated with the β-Lg which, on quantification, would result in an overestimate of the amount of β-Lg and an underestimate of the amount of α-Lac. When this sample was deglycosylated (Fig. 5, lane 8) the α-Lac band increased and the β-Lg/glyco-α-Lac band decreased. The deglycosylated bands were more diffuse on electrophoresis due to the different buffers used in the enzymatic deglycosylation with PNGase F. A band corresponding to this protein was also observed (Fig. 5, lane 10).

3.5. PAGE Limitations

Whey proteins in both whey and WPC samples can be measured by PAGE methods subject to the following criteria. Native PAGE can be used to determine levels of α-Lac, β-Lg A and β-Lg B as there is no interference by glyco-α-Lac though the α-Lac level may be low if glyco-α-Lac is not also measured. BSA can also be quantitated providing caseins are not present. IgG proteins can not be quantitated. SDS-PAGE can be used to quantitate BSA and IgG (using the heavy chain) but the presence of glyco-α-Lac should be considered when determining α-Lac and β-Lg levels. Whilst reduced SDS-PAGE measures total protein the native PAGE only measures undenatured, non-aggregated protein.

The accuracy of both PAGE methods is dependent on staining/destaining protocols and on reproducible densitometry. Differences of up to 10% have been observed between individuals quantitating the same protein bands on any given gel (unpublished results).

3.6. Size-exclusion HPLC

Fig. 6 shows the separation of whey proteins by size-exclusion HPLC. Whilst there was near baseline separation for the whey protein standards (Fig. 6a), with the whey and WPC samples (Fig. 6b and c) the resolution between the β-Lg A/B and α-Lac peaks was not as good. This was due to: (i) the high concentration of β-Lg required to enable simultaneous identification and quantification of the BSA and IgG proteins; (ii) the high extinction coefficient of α-Lac which enlarged that peak, and; (iii) the presence of glyco-α-Lac in the whey and WPC samples which migrated as a shoulder on the trailing edge of the β-Lg A/B peak.

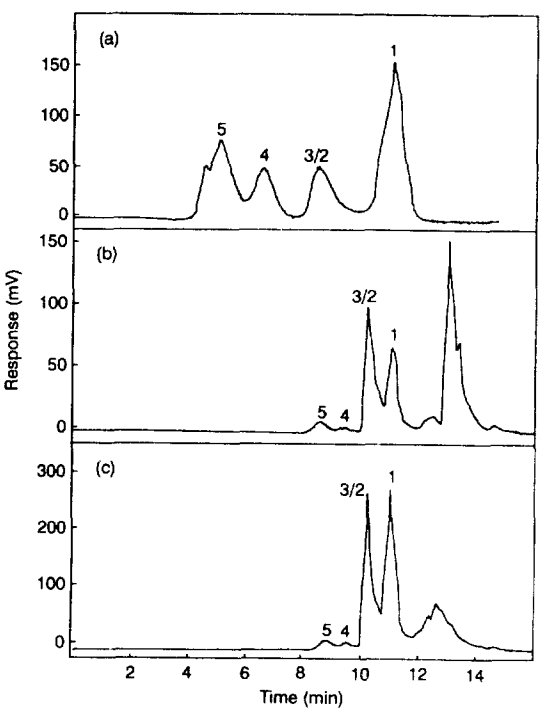

Fig. 6. Size-exclusion HPLC of (a) whey protein standards, (b) acid whey, (c) WPC. Conditions for chromatography as in Experimental.

This lack of resolution could not be readily overcome by using a higher sample dilution as the β-Lg A/B retention time was dependent on its concentration such that the retention time increased with decreasing β-Lg A/B concentration [18]. These factors need to be considered when quantifying β-Lg and α-Lac.

Other characteristics of size-exclusion HPLC included the co-migration of the A and B variants of β-Lg due to their similar molecular masses and the appearance of two peaks within the IgG standard. As observed previously with CGE there was also a number of lower molecular mass peaks in the whey and WPC samples. These were presumed to be attributable to the presence of the proteose-peptone fraction, orotic acid and hippuric acid [19]. Lastly, if there are any casein proteins present in the sample these co-migrate with the BSA and IgG peaks in the form of an ill-defined peak making quantification of BSA and IgG impossible.

3.7. Ion-exchange HPLC

Separation according to charge was performed using an anion-exchange column (Fig. 7). This resulted in the separation of the two β-Lg variants and good baseline separation of all the whey protein standards (Fig. 7a) with the exception of IgG which did not bind to the column at pH 5.5. With the acid whey and WPC samples, however (Fig. 7b and c), although the two β-Lg variants were separated the BSA peak was not very well defined, running into the leading edge of the much larger β-Lg B peak. It also co-elutes with orotic acid which made quantitation at 214 nm impossible.

The α-Lac peak in acid whey and WPC was also not as well resolved as the α-Lac standard and with the WPC the retention time increased slightly from 3.6 to 4.3 min. This was presumed to be caused by changes to the α-Lac molecular structure during processing as discussed previously.

Overall it was possible to measure both β-Lg A and B variants and α-Lac by anion-exchange

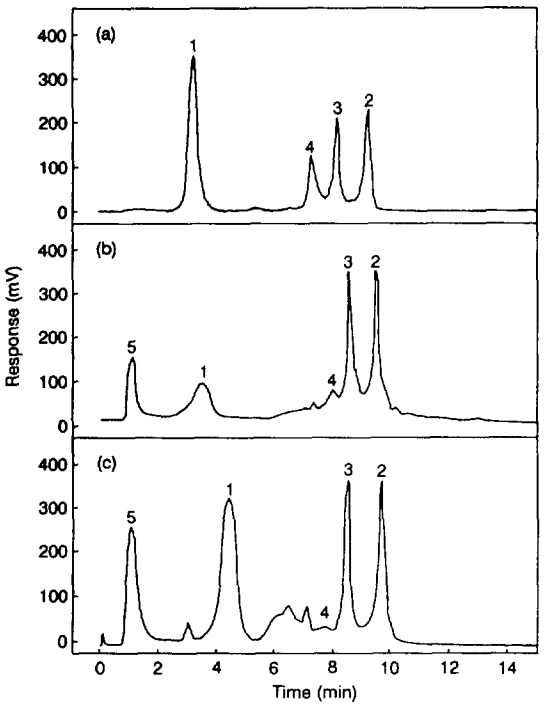

Fig. 7. Ion-exchange HPLC of (a) whey protein standards, (b) acid whey, (c) WPC, on a Pharmacia MonoQ anion exchange column. Conditions for chromatography as in Experiment. Peaks as described in Fig. 1.

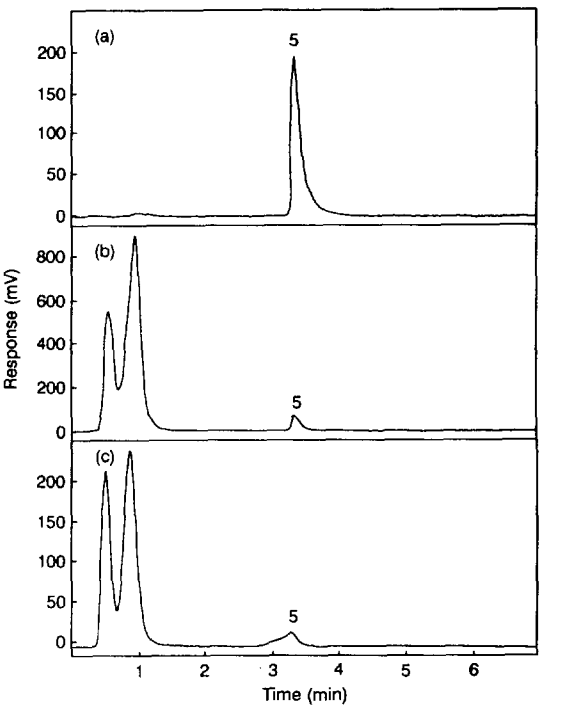

Fig. 8. Affinity HPLC of bovine IgG in (a) whey protein standards, (b) acid whey, (c) WPC, on a Pharmacia Protein G Hi-Trap column. Conditions for chromatography as in Experimental. Peaks as described in Fig. 1.

chromatography but quantification of BSA was difficult and it was not possible to quantitate IgG as it was not known what other proteins in whey besides IgG did not bind to the column.

3.8. *Affinity HPLC*

The affinity ligand protein G was used to specifically bind bovine IgG molecules (Fig. 8). All other proteins were eluted in the void volume of the column. As IgG is the predominant immunoglobulin in bovine milk [20] this matrix gave a very specific measure of the amount of bovine immunoglobulins present. It was also able to give an indication of the structural state of the IgG protein as the binding is a specific interaction between the F_c region on the immunoglobulin heavy chain and a binding site of the protein G molecule. This is demonstrated by the shapes of the IgG peak in the different samples. The standard (Fig. 8a) has a very well defined IgG peak with a sharp leading edge and only slight tailing. The IgG peak in the acid whey (Fig. 8b) is similar although with a slight loss of peak shape whilst the WPC (Fig. 8c), which has undergone more processing/denaturation by ultrafiltration, diafiltration, evaporation and drying, has a poorly defined peak shape which makes quantification more difficult.

3.9. *Quantification of acid whey and WPC*

The concentration of each whey protein in the acid whey and the WPC was determined by the different assay methods using five-point standard curves for each individual whey protein (Tables 4 and 5). As mentioned previously each analytical method had individual characteristics which resulted in variation in the results between the different assays.

Table 4
Analysis of acid whey using different methods

Separation method		Whey protein (mg/ml)			
		α-Lac	β-Lg	BSA	IgG
PAGE	Native	0.61	2.78[a]	0.26	nd[b]
	SDS	0.50	3.10	0.35	0.17[c]
HPLC	SEC	0.55	3.10	nd	0.17
	IEX	0.60	2.90	0.44	nd
	Affinity	na[d]	na	na	0.49
CE	Free zone	0.56	2.82	0.12	0.26
	Gel[e]	0.50	2.93	0.32	0.48

Sample and methods used were as described in the Experimental section. Gels, chromatograms and electropherograms are shown in Figs. 1–8.
[a] Combination of β-LgA and β-LgB.
[b] nd = Not able to be determined.
[c] Determined from IgG heavy chain.
[d] na = Not applicable.
[e] Non-reduced.

For the acid whey sample there was good agreement between the assays for both α-Lac (range 0.50 to 0.61 mg/ml) and β-Lg (range 2.78 to 3.10 mg/ml). No allowance was made for the presence of glycosylated-α-Lac which may account for the lower α-Lac levels and the higher β-Lg levels in the SDS-PAGE, size-exclusion HPLC and CGE results. The largest variation occurred amongst the BSA (range 0.12 to 0.44 mg/ml) and IgG (range 0.17 to 0.49 mg/ml) results. These variations were due principally to the low concentration of these proteins in the whey which made accurate quantitation more difficult and also to their heterogeneous nature.

In comparison to the whey sample there was wide variation in all the whey protein values of the WPC sample when they were assayed by the different methods. As mentioned previously when discussing the individual assays there were considerable problems differentiating and quantifying the individual whey proteins in the WPC, presumably because of chemical modifications to the proteins during processing. This was evident from the blurred bands for the WPC proteins on the SDS- and native-gels and from the loss of resolution with HPLC and CE.

With respect to the individual whey proteins the α-Lac and β-Lg values were between 47 to 64 mg/g and 128 to 157 mg/g respectively and the differences in the values observed between the different assays could not be as readily explained by the ability of the individual assay methods to differentiate the glyco-α-Lac moiety from the other whey proteins as was observed with the acid whey sample. As with the whey sample the BSA and IgG values in the WPC were not very consistent, again probably a reflection of the low concentration and microheterogeneity of these proteins in the WPC.

Table 5
Analysis of WPC using different methods

Separation method		Whey protein (mg/g powder)			
		α-Lac	β-Lg	BSA	IgG
PAGE	Native	52	129[a]	20	nd[b]
	SDS	47	151	26	17[c]
HPLC	SEC	51	139	14	19
	IEX	61	140	21	nd
	Affinity	na[d]	na	na	17
CE	Free zone	56	134	28	12
	Gel[e]	64	135	14	6

Sample and methods were as described in the Experimental section. Gels, chromatograms and electropherograms are shown in Figs. 1–8.
[a] Combination of β-LgA and β-LgB.
[b] nd = Not able to be determined.
[c] Determined from IgG heavy chain.
[d] na = Not applicable.
[e] Non-reduced.

3.10. Comparison of CE assays and other techniques

When comparing the CE free zone and gel results with those obtained using the other analytical techniques, the CE results were generally within the ranges of the other results and followed their trends. Thus capillary gel electrophoresis results for α-Lac and β-Lg were low and high respectively as was observed for both the PAGE-SDS and size-exclusion HPLC methods. There was also more variation in the results with both BSA and IgG. The reproducibility of the CE results was as good as those for HPLC and better than could be attained by PAGE (results not shown).

4. Conclusions

Two new CE-based methods (GCE and CZE) have been used to quantitate the four major whey proteins in both liquid whey samples and reconstituted WPC powder. These were then compared with other methods to measure the whey proteins. Overall none of the assays could provide all the results for the few major whey proteins. For liquid whey samples the main obstacles against achieving this goal appeared to be the large differences in concentration, extinction coefficients and microheterogeneity of the different whey proteins and in the ability of the different assays to measure either native or total whey protein.

Whilst the range of results for α-Lac and β-Lg was reasonably narrow over the different assays there was considerable variation between the results for BSA and IgG. The variation within the α-Lac and β-Lg results may be decreased by differentiating out and allowing for the glycosylated-α-Lac fraction. To achieve this the extinction coefficient and standard curves will need to be determined for glycosylated α-Lac. This will form the basis of future work in the area.

The large variations in the results for the BSA and IgG concentrations may be overcome by analysing samples at both a high and a low concentration and to have separation methods in which the BSA and IgG proteins are well resolved from the α-Lac and β-Lg proteins at high concentration levels. Under these constraints capillary gel electrophoresis appeared to be the most promising method for analysing whey proteins although the two β-Lg variants were not separated by this method and a sample preparation method which avoided precipitations at low whey dilutions will have to be developed.

For WPC powders, however, there also appeared to be problems with changes to the protein structure brought about by the different processing steps. More research is required on both sample preparation and on the extent of protein denaturation and how this is reflected by the different assays.

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