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Simultaneous separation and quantitation of the major bovine whey proteins including proteose peptone and caseinomacropeptide by reversed-phase high-performance liquid chromatography on polystyrene–divinylbenzene

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Abstract

A precise, sensitive and reliable RP-HPLC method was developed to enable not only unequivocal determination of α-lactalbumin and β-lactoglobulin in bovine whey samples, but also simultaneous measurement of proteose peptone, caseinomacropeptide, bovine serum albumin and immunoglobulin G. The optimised method on the Resource RPC column allowed separation of the proteins in 30 min and could be applied to the analysis of soluble proteins in a variety of commercial and laboratory whey products. Furthermore, some qualitative information on protein heterogeneity and quality could be derived from the RP-HPLC analyses with additional data available from on-line electrospray mass spectrometry. Within- and between-day repeatability over a wide range of concentrations was excellent (RSD≤5%) for all proteins except immunoglobulin G and bovine serum albumin where RSD was 7–10%. Analysis of grouped data from whey protein concentrate and whey protein isolate samples gave a limit of detection of ≤0.3% powder mass and a limit of quantitation of ≤1.0% powder mass for all proteins except immunoglobulin G. Limits of detection and quantitation were 0.6% and 2.0%, respectively, for this protein. Quantitative data obtained by the RP-HPLC method compared very favourably with data obtained by alternative methods of whey protein analysis.

Keywords: Whey; Food analysis; Proteins; Proteose peptone; Caseinomacropeptide

1. Introduction

With the increasing diversification of markets for dairy products and in particular the push for value added products based largely on nutritional and bioactive properties of individual whey proteins or peptides, the search for commercially viable ways to fractionate these proteins has intensified. The need to monitor these isolations (process control) coupled with greater stringencies in product specifications (quality control), has necessitated a requirement for improved analytical capabilities, particularly those which are time and cost effective and yield the maximum data from a single assay. There is already

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an abundance of literature concerning whey protein analysis. Methods include gel electrophoresis [1,2], liquid chromatography (for review see Ref. [3]), capillary electrophoresis [4–6] and immunochemical detection [7] (see also review of Grappin and Ribadeau-Dumas [8]).

By far the most used method for whey protein analysis is liquid chromatography. In particular high-performance liquid chromatography (HPLC) has become one of the main techniques in the dairy industry as it combines versatility, short analysis time and high resolution with increasingly sophisticated automation and superior column performance. There are a large number of methods described for ion-exchange, gel permeation, hydrophobic interaction and reversed-phase (RP) HPLC (for reviews see Refs. [3,9]), but no one method is suitable for all the whey proteins and the method of choice is usually based on one or two particular proteins of interest.

RP-HPLC has the advantage that it can be coupled directly to electrospray ionisation mass spectrometry (ESI-MS) and appears to give a more complete separation of the major whey proteins with good recovery, suitable for quantitation. Resmini et al. [10] used RP-HPLC on a C_8 column to quantify whey proteins in raw bulk milk and pasteurised milk processed under different conditions. Although they were able to measure α-lactalbumin (α-lac), β-lactoglobulins (β-lg) A and B, bovine serum albumin (BSA), immunoglobulin G (IgG) and a proteose peptone (PP) fraction, the PP fraction eluted as multiple peaks which were not well resolved from the solvent front. Saito et al. [11] analysed cheese whey using a C_{18} column and observed caseinomacropeptide (CMP) eluting as five partially resolved peaks followed by PP (multiple unresolved peaks), BSA, α-lac and β-lg. The latter three proteins were not sufficiently resolved to allow quantitation. Similarly, Léonil and co-workers [12,13] used both C_8 and C_{18} RPC columns to separate CMP, PP, α-lac and the two β-lg genetic variants. In this case, the two aglyco-CMP variants were resolved and were able to identified by ESI-MS, but the method was not suitable for measurement of CMP as the glyco species were not resolved from the solvent front. PP5 (β-casein-5-phosphate 1-105/107) originating from β-caseins A1 and A2 was identified by ESI-MS in the PP peak.

The objective of this study was to develop and validate a RP-HPLC method for simultaneous quantitative analysis of the bovine whey proteins α-lac, β-lg, BSA, PP, IgG and CMP. Collectively, these proteins constitute virtually the entire protein mass of sweet and acid wheys. Earlier work had established the use of the Resource RPC column as an analytical tool for measurement of the major whey proteins α-lac, β-lg, BSA and IgG in different whey types [14]. The Resource RPC column differs from conventional alkyl-bonded silica RP matrices in that it is an underivatised polystyrene–divinylbenzene matrix consisting of rigid monodisperse 15 μm beads. The material has excellent chemical and pH stability and has proved, in our hands, to be quite robust with an approximate lifetime of 2000 injections, most of which were commercial samples of whey protein concentrate (WPC) or whey protein isolate (WPI). Furthermore, there is no requirement for filtration of samples prior to analysis. In this work, method development was aimed at maximising resolution of the major whey proteins including PP and CMP, and validating reliable and accurate quantitation of these proteins in liquid whey samples, and WPC and WPI powders.

2. Experimental

2.1. Materials

Acetonitrile (MeCN; far UV grade) and trifluoroacetic acid (TFA; HPLC grade) were from BDH (Poole, UK). Q Sepharose Fast Flow and Sephadex G-75 were from Pharmacia Biotech (Uppsala, Sweden). All other buffers and reagents were analytical grade or better. Aqueous buffers (eluents) were filtered through 0.45-μm cellulose acetate membranes (Millipore, Bedford, MA, USA) and degassed prior to use. Water was purified by reverse osmosis followed by deionisation (Milli-Q, Millipore).

2.2. Whey protein standards

Commercially purified bovine whey protein standards (α-lac, β-lg A, β-lg B, BSA and IgG) were purchased from Sigma (St. Louis, MO, USA). Gly-

cosylated (glyco-)-α-lac was prepared in the laboratory as described by Kinghorn et al. [6]. Total PP (TPP) was prepared according to the method of Andrews and Alichanidis [15] using fresh bulk skim milk obtained from The New Zealand Dairy Research Institute, and a commercial sample of pasteurised homogenised milk. The latter was defatted by centrifugation at 1500 *g* for 20 min at 25°C and removal of the fat layer. PP5 was isolated from mineral acid WPC (commercial source) by anion-exchange chromatography on Q Sepharose Fast Flow at pH 4.7, followed by rechromatography of the PP5-containing fractions on the same anion exchanger at pH 8. Final purification was accomplished by gel filtration on Sephadex G-75 as described by Sorensen and Petersen [16]. Mixed variant (A/B) CMP was prepared by the method of Coolbear et al. [17]. Purifications were monitored by gel electrophoresis and identity confirmed by N-terminal sequencing and amino acid analysis, and in the case of PP5 and CMP, ESI-MS.

2.3. Whey samples

Single batches of lactic, mineral and cheese WPC and a batch each of WPI prepared by either ion-exchange (IX) or microfiltration (MF) of cheese whey were obtained from commercial sources. Mineral acid and rennet wheys were made in the laboratory from fresh bulk skimmed milk which had been pasteurised. Mineral acid whey was prepared by isoelectric precipitation of casein at pH 4.6 and 50°C using sulphuric acid and rennet whey was prepared using New Zealand Standard strength rennet (60 RU/ml; 32 μl per 100 ml milk) to coagulate the caseins. Whey samples were stored in small aliquots at −18°C and thawed on the day of use.

2.4. Gel electrophoresis

Non-denaturing and sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) were performed as described previously using whey protein standards at similar concentrations [6]. WPC and WPI samples prepared as for RP-HPLC (see Section 2.9) were diluted in sample buffer to 0.35 mg/ml and 0.2 mg/ml, respectively. Whey was diluted 1 in 10 with sample buffer. Load volume of samples was 10 μl. Quantitation of protein bands on SDS gels stained with Coomassie Brilliant Blue was by laser scanning computer densitometry as also described previously [6].

2.5. Radial immunodiffusion

Bovine IgG was measured by radial immunodiffusion (RID) using polyclonal-based radial immunodiffusion kits (The Binding Site, UK).

2.6. HPLC

The HPLC system consisted of a Waters 2690 Alliance Separation Module (Waters, Milford, MA, USA) interfaced with a Waters 486 MS tunable absorbance detector and a Waters Millenium 32 data acquisition and manipulation system. Queued samples were refrigerated at 5°C.

2.7. IgG affinity HPLC

Bovine IgG was measured by affinity HPLC on a Hi-Trap Protein G column as previously described [6].

2.8. Mono S cation-exchange HPLC

CMP was measured by the Mono S HPLC method of Léonil and Mollé [18] with a slight modification of the gradient to reduce the run time to 15 min.

2.9. RP-HPLC

A 1-ml Resource RPC column (Pharmacia Biotech) was operated at room temperature (RT) and at a flow-rate of 1 ml/min. The column was equilibrated in 80% solvent A (0.1%, v/v, TFA in Milli-Q water) and after sample injection a 1-min isocratic period was applied followed by a series of linear gradients to 100% solvent B (0.09%, v/v, TFA, 90%, v/v, MeCN in Milli-Q water) as follows: 1–6 min, 20–40% B; 6–16 min, 40–45% B; 16–19 min, 45–50% B; 19–20 min, 50% B; 20–23 min, 50–70% B; 23–24 min, 70–100% B. The column was re-equilibrated after a 1-min hold at 100% B by a 2-min linear gradient to 20% B followed by an

isocratic period of 3 min. Detection was by absorbance at 214 nm and total run time was 30 min. Stock solutions of individual protein standards were prepared at approximately 5–6 mg/ml in Milli-Q water and stored at −20°C. Concentrations were determined by absorbance at 280 nm (α-lac, β-lg, BSA and IgG) or 214 nm (CMP) using published extinction coefficients [17,19–21], or by quantitative amino acid analysis (PP5 and TPP). Mixed calibration standards were prepared freshly from the stock solutions on the day of use to give working concentrations of 0.6–0.7 mg/ml (α-lac, β-lg A, β-lg B, CMP) or 0.3–0.4 mg/ml (PP5, BSA, IgG). These concentrations were weighted to the expected proportional concentrations of each protein in whey. Eight-point standard curves were constructed from the mixed standard using injection volumes of 10–100 μl.

For liquid whey samples and reconstituted powders, sample size and concentration were adjusted to bring the likely range of protein values within the linear portion of the standard curve. Whey was diluted threefold with water. Powder samples were made to approximately 5 mg/ml in water and stirred gently for 2 h at RT after which they were diluted to ~1.5 mg/ml (WPI) or ~2 mg/ml (WPC) in water. Sample injection volumes were 100 μl. Prior to RP-HPLC analysis, all samples were centrifuged in a microfuge at 16 000 *g* for 3 min to remove any insoluble material.

2.10. Amino acid analysis

Amino acids were determined using pre-column derivatisation followed by RP-HPLC. Samples were hydrolysed with 6 *M* HCl for 23 h at 110°C under a nitrogen atmosphere. The resulting amino acids were derivatised with Waters AccQ.Fluor reagent (6-aminoquinolyl-*N*-hydroxysuccinimidylcarbamate) and separated using RP-HPLC on a Waters AccQ.Tag C_{18} amino acid analysis column using a Waters 2690 Alliance HPLC system connected to a Waters 470 fluorescence detector and a Waters Millenium 32 data acquisition and manipulation system. α-Aminobutyric acid was used as an internal standard.

2.11. ESI-MS

The mass spectrometer was a triple quadropole Perkin-Elmer Sciex (Thornhill, Canada) Model API 300 equipped with an atmospheric pressure electrospray ion source and interfaced with a Mac LC Tune 2 data acquisition and manipulation system. The raw data were interpreted using Mac BioMultiview 1.3.1 software. Samples introduced on-line from RP-HPLC were scanned in the positive ion mode with orifice, ring and ion spray voltages set at 30, 140–280 and 5500 V, respectively. Each scan was acquired over the mass-to-charge (m/z) range 200–3000, with a step size of $m/z=0.2$, a dwell time of 0.5 ms and a scan time of 7.3 s. Samples introduced by infusion were scanned in the negative ion mode. The sample was dissolved in 50% (v/v) aqueous MeCN to a concentration of approximately 50 pmol/μl and introduced into the electrospray source at a flow-rate of 5 μl/min using a syringe infusion pump (Harvard Apparatus, Quebec, Canada) connected to the electrospray source by a fused-silica capillary of 100 μm I.D. Orifice, ring and ion spray voltages were −25, −140 to −280 and −4900 V, respectively. Identities of whey proteins were assigned by comparison of experimental molecular masses with theoretical molecular masses derived from the Swissprot. data base.

3. Results and discussion

HPLC conditions were optimised for mobile phase composition, gradient, sample size and concentration and flow-rate. Details of the selected protocol are given in the Experimental section. PP5 was initially chosen as a standard for evaluating PP as it represents the major component of this fraction [16,22].

3.1. Separation of whey protein standards

Fig. 1A–F shows the RP-HPLC elution patterns of the standard whey proteins using the conditions and gradient described in the Experimental section. Elution was monitored at 214 nm to enable detection of CMP. Detection at this wavelength also has the advantage that it improves the sensitivity of response

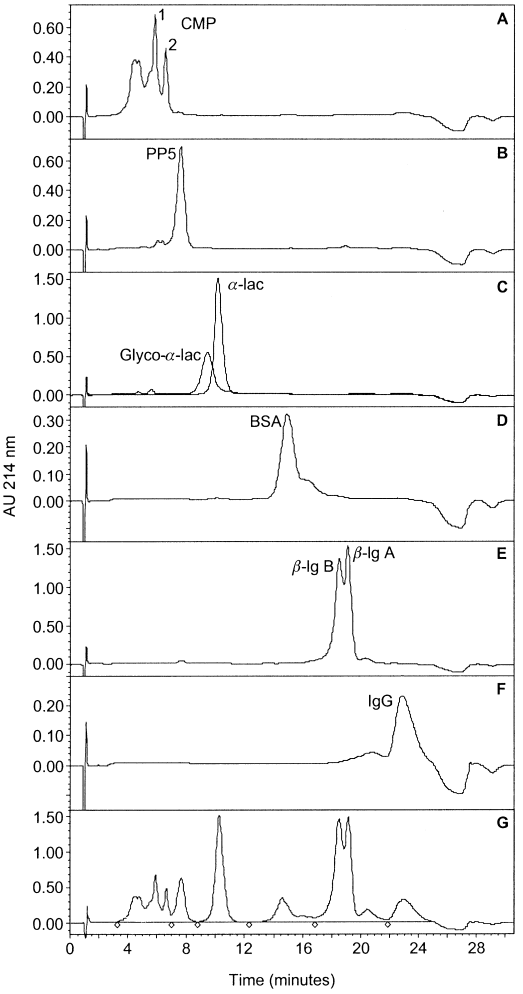

Fig. 1. RP-HPLC chromatograms of individual (A–F) and mixed (G) whey protein standards. Injection volume was 60 μl, protein concentrations were 0.6–0.65 mg/ml (α-lac, β-lg A, β-lg B, CMP) or 0.3 mg/ml (PP5, BSA, IgG, glyco-α-lac). Peaks 1 and 2 in profile A refer to aglyco CMP, A and B variants, respectively. Conditions for RP-HPLC as described in Experimental.

for proteins such as PP5 and BSA which have a low absorbance at 280 nm. The profile of the mixed standard (Fig. 1G) shows that the individual proteins were baseline resolved from each other, enabling straightforward integration. The order of elution of the major whey proteins resembled that obtained by Resmini et al. [10] using a C_8 RPC column. However, in the latter case, PP eluted as an ill-defined series of peaks which were not clearly differentiated from the solvent front and CMP was not detected.

The resolution of CMP species in our RP-HPLC system was similar to that observed previously on C_{18} RP-HPLC matrices [17,18]. Specifically, the two major peaks corresponding to the aglyco components of the two principal genetic variants (A and B) of CMP (Fig. 1A, peaks 1 and 2, respectively) eluted after the less well resolved glyco-CMP components. ESI-MS data obtained on-line from RP-HPLC gave molecular masses of 6787 and 6755, respectively, for the aglyco A and B CMP peaks but spectra obtained from the fused peaks corresponding to glycosylated material were too complicated to interpret. Nevertheless, the partial resolution is useful in that it can give some qualitative information on the heterogeneity of CMP.

The commercial α-lac standard (Fig. 1C) was almost exclusively non-glycosylated as shown by non-denaturing PAGE (results not shown but see Kinghorn et al. [6]) and an M_r of 14 177 was obtained from on-line ESI-MS. However, as glycosylated α-lac can constitute up to 15% of the total α-lac in whey [14], it was important to establish its elution pattern. The RP-HPLC profile for a sample of glyco-α-lac purified in the laboratory is also shown in Fig. 1C. The glycosylated protein eluted slightly earlier than aglyco-α-lac but was discrete from the PP5 peak (Fig. 1B).

BSA eluted as a main peak with a small, rather ill-defined shoulder which was included in peak integrations for quantitative analysis (Fig. 1D). Similar behaviour of commercial BSA in RP-HPLC separations has been observed previously and ascribed to BSA aggregates [23,24]. In particular, it has been noted that BSA occurs as a split peak in RP-HPLC systems if a shallow gradient is used during elution [24].

Only partial separation of the two genetic variants of β-lg was realised under the selected elution conditions (Fig. 1E). Although these components have been resolved in other RP-HPLC systems [10,25], manipulation of the gradient to effect their resolution in our system resulted in loss of resolution for BSA and IgG. A very small peak eluting on the tail of the β-lg A peak was attributed to β-lg dimers as M_r values of 36 554, 36 724 and 36 639 were

found under this peak by ESI-MS. These values correspond, respectively, to the theoretical molecular masses for the β-lg A and β-lg B homodimers and the β-lg A/B heterodimer.

Molecular mass data could not be obtained on-line for the PP5 peak (Fig. 1B), but a separate infusion of the standard using the negative ion mode gave an M_r of 12 178 which aligns with the theoretical M_r of 12 177 for β-casein A2 1-105. The PP fraction constitutes up to 13% of the whey protein complement but values vary widely [22]. Van Boekel and Crijns reported an average value in rennet whey of 0.3 mg/g (5% of true whey protein), estimated from protein nitrogen analysis of the classic heat-soluble fraction [22]. PP5 and PP component 3 (PP3) are the major components of the PP fraction, contributing approximately 50% and 30%, respectively [16,22,26]. To validate the use of PP5 as a calibration standard for PP, TPP fractions were prepared from both fresh and commercially available milks and their RP-HPLC elution patterns established. As shown in Fig. 2, both batches gave very similar profiles with the majority of the material eluting in the same position as the PP5 standard and accounting for >70% of the collective TPP peak area. Preliminary studies [27] have shown that PP3 elutes fractionally ahead of PP5 but is incorporated into the main PP (PP5) peak. Small peaks eluting earlier in the region of CMP elution probably represented highly hydrophilic minor components such as β-casein-4-phosphate 1-28 (PP8F), other casein peptides and osteopontin. With respect to calibration, RP-HPLC UV response factors (214 nm) for PP5 and TPP, calculated from quantitative amino acid data, were closely similar. Thus PP5 is a useful standard for quantitation of the hydrophobic component of TPP. Additionally, PP5, PP3 and TPP all have virtually identical Kjeldahl factors [22] enabling calibration using protein nitrogen values. The profiles of TPP additionally monitored at 280 nm (Fig. 2) are interesting in that they clearly show material eluting as two partially resolved peaks immediately after the PP5 peak. Based on elution time and the comparatively higher extinction at 280 nm, this material was most probably glycosylated and non-glycosylated α-lac. Small amounts of α-lac and β-lg commonly occur in such PP preparations [15]. Thus measurement of material under the PP5 peak may yield a better estimation of true PP, as residual α-lac and β-lg are excluded from the analysis. Furthermore, Paquet et al. [28] have shown that the same main components are found in the PP fraction regardless

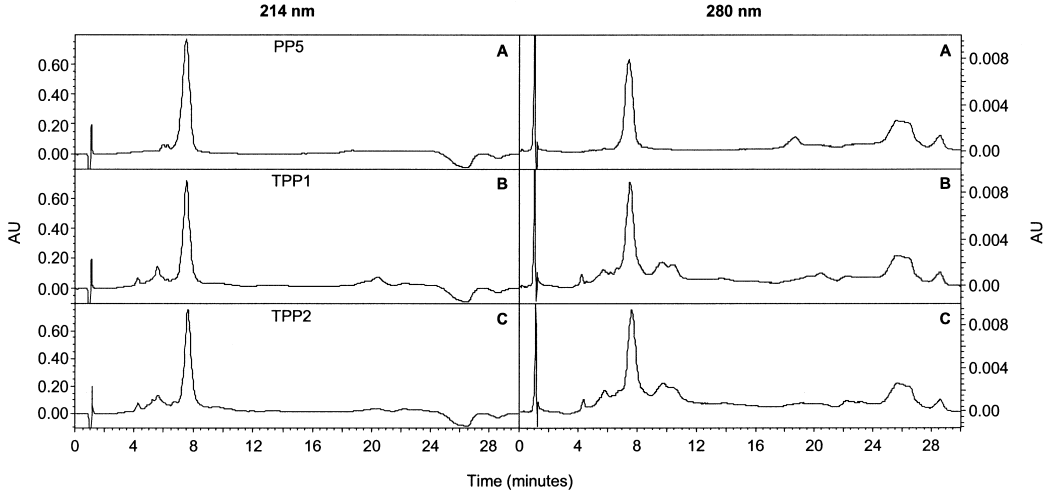

Fig. 2. RP-HPLC chromatograms of (A) PP5 standard, (B) and (C) TPP monitored at 214 nm and 280 nm. TPP1 was from fresh bulk skim milk and TPP2 was from commercial pasteurised homogenised milk. Injection volume was 60 μl, PP5 concentration was 0.3 mg/ml and TPP concentration was 0.5 mg/ml.

of the mode of extraction or the nature of the milk. Our RP-HPLC results with two different TPP preparations confirm this.

3.2. Precision

The resolution and peak shapes of the whey protein standards were consistent throughout the mass range of the standard curve (see profile 2, Fig. 3A and profile 3, Fig. 3B). Also shown in Fig. 3A is a blank (50 μl water injection) run prior to a set of mixed standards. This shows a slight baseline rise in the region of IgG elution (~22 min). This was typical and was likely due to the elution of small amounts of TFA which adsorbed to the matrix in the polar solvent and eluted in the increasingly organic phase [29]. Although this effect could be minimised by further reduction of TFA in solvent B, doing so brought about a larger negative peak at the end of the gradient which compromised re-equilibration time.

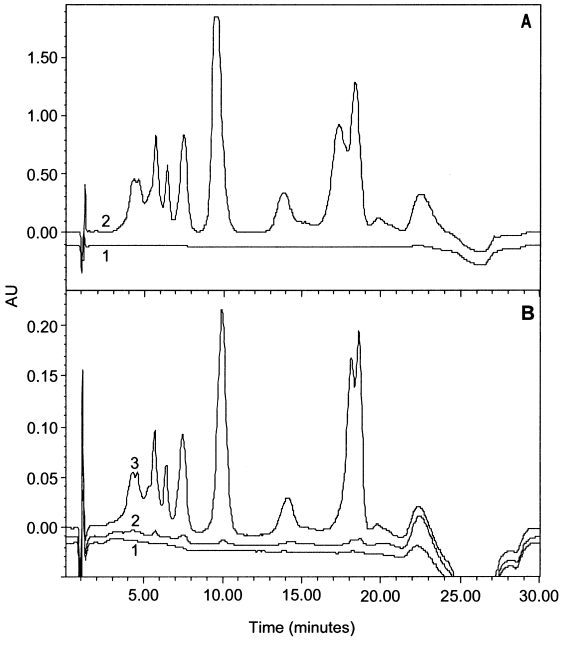

Fig. 3. RP-HPLC chromatograms of (A) 50 μl water blank (profile 1) and mixed whey standard, 100 μl injection (profile 2); (B) 50 μl water blank run prior to (profile 1) and following (profile 2) a set of mixed whey standards, the 10 μl injection of which is illustrated (profile 3).

Carry over between samples was minimal and could be monitored where necessary by running blanks. Fig. 3B shows chromatograms of blank runs inserted prior to (profile 1), and following (profile 2), a set of eight injections of mixed standard (10 μl through to 100 μl). Although carry over for most of the whey proteins amounted to less than 10% of peak area of the lowest standard (10 μl, profile 3), the contribution to the IgG peak was, however, significant and equivalent to a 10 μl injection of this standard. Apparent carry over of IgG was dependent to some extent on the IgG content of the prior sample but did not appear to be cumulative beyond the level illustrated. This suggests that there was some small, saturable but non-specific adsorption of IgG to the syringe, injector or column. For critical determination of IgG, it is therefore recommended that a blank be run prior to sample analysis and that the analysis be performed in duplicate.

Comparison of the elution profiles of the mixed standard (60 μl) injected over 10 consecutive days showed that while all profiles were generally similar, there was some day to day variation in elution times and peak shapes, especially for BSA and the β-lg A and B variants which eluted in the very flat portion of the gradient (not shown). This may have been due to slight day-to-day variations in solvent composition (solvents were made freshly each day) or minor fluctuations in ambient temperature. However, the relative standard deviation (RSD) for retention time, taken from mid-peak, was $<2\%$ for each protein. With regard to temperature, the column was operated at RT (~20°C) as it was found that higher operating temperatures did not significantly improve resolution.

There was a linear response between protein mass and UV absorbance at 214 nm for all standards over the calibration range studied. For the purpose of quantitation, standard curves were constructed using a line fit forced through zero (Millenium 32 software) and in all cases except for IgG, the correlation coefficient for eight points was $r>0.99$. Although line fits for IgG were generally excellent ($r>0.99$), the occasional standard curve gave a line fit with $r<0.99$. The reason for this is not clear but may relate to non-specific adsorption effects as discussed above. Blanks were, however, run routinely prior to and following sets of standard curve injections.

The response factors, or slopes of standard curves, were highly reproducible between runs. The variation in slope over a series of 10 standard curves run over 10 days was ≤2% for each protein standard with the exception of IgG, where slope variation was closer to 7%. All standards gave a similar UV response at 214 nm with the exception of α-lac which gave a significantly higher response (~30% greater than the mean). Visser et al. [30] have also commented that α-lac has a considerably higher UV response at 220 nm than β-lg. This was presumed to be due to the higher aromatic amino acid content of α-lac.

3.3. Sample analysis

Representative RP-HPLC chromatograms of commercial whey powders and laboratory prepared whey are shown in Fig. 4. Peak integration markers are also indicated on the profiles. For whey and WPI samples, the resolution of individual proteins was closely similar to the standards. However, in all three WPC powders analysed, β-lg eluted as a broad, ill-defined peak which appeared to contain additional components. This pattern of β-lg resolution has previously been observed in RP-HPLC separations of commercial WPC samples [31–33]. Both Parris and Baginski [34] and Pellegrino et al. [33] used RP-HPLC to monitor the effects of heat treatments on whey protein denaturation and found new peaks eluting ahead of β-lgs A and B in high heat samples. These changes have been ascribed to modification of the protein by heat induced aggregation and lactosylation. In this study, on-line ESI-MS of the β-lg peaks from RP-HPLC of mineral acid and lactic WPC not only revealed molecular masses for unmodified β-lg A and β-lg B, but also β-lg species with adducts of 324 molecular mass units, corresponding to covalent linkage of lactose units [13,35]. In mineral acid WPC, up to three adducts were found for β-lg A and up to two for β-lg B, while for lactic WPC only the single adduct was detected for both variants. Based on relative intensities of spectral peaks, singly lactosylated β-lg was the predominant form in mineral acid WPC, and was apparent in even greater quantity than the native protein. However, in the lactic WPC sample, unmodified β-lg was predominant. Molecular mass data could not be obtained for β-lg from cheese WPC due to poor ionisation. Léonil et al. [13] have attributed poor ionisation of β-lg to irreversible polymerisation. β-Lgs A and B with one lactose adduct were found in both MF- and IX-WPI, and in both rennet whey and acid whey, but in relatively low amounts. Interestingly, lactosylation of β-lg was also evident in the commercial standard. Single lactose adducts were observed for both β-lg A and B, in both cases eluting in the region of the chromatogram defined for that

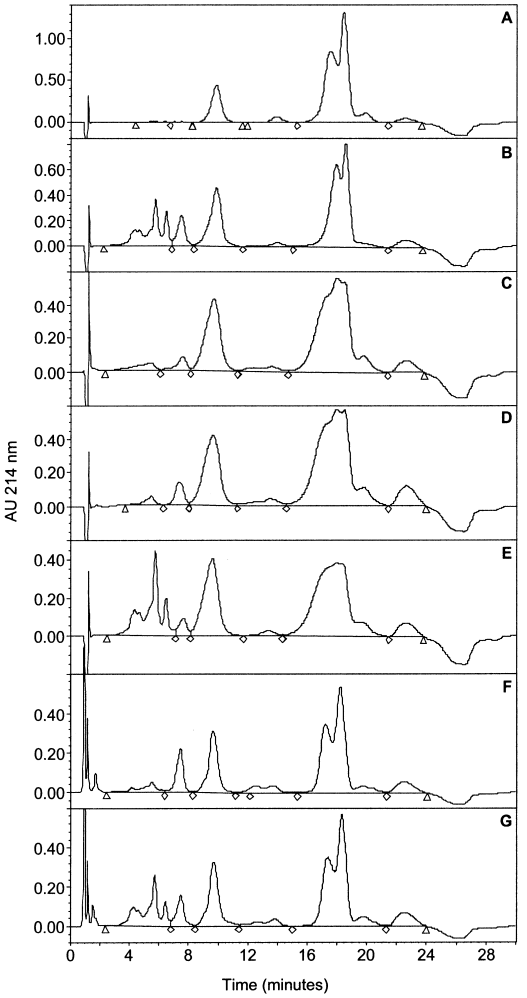

Fig. 4. RP-HPLC chromatograms of (A) IX-WPI, (B) MF-WPI, (C) lactic WPC, (D) mineral WPC, (E) cheese WPC, (F) mineral acid whey, (G) rennet whey. Sample concentrations were 1.5 mg/ml (WPI) and 2.0 mg/ml (WPC); whey samples were diluted threefold, injection volume was 100 μl. Baseline and integration markers are indicated on each profile.

variant. Léonil et al. [13] have shown that covalent attachment of lactose can occur during mild heat treatment and that it is progressive with time. This suggests that the extent of lactosylation in industry samples is related to process conditions. However, Burr et al. [35] have postulated that lactosylation may occur naturally as they found lactosylated β-lg in non-heat treated whey isolated by ultracentrifugation.

The β-lg dimer peak was present in all samples but molecular mass data for this peak could not be obtained readily from on-line ESI-MS, with the exception of the IX-WPI sample, where M_r values for the heterodimer (36 637) and A and B homodimers (36 724 and 36 553, respectively) were observed. The presence of β-lg dimers eluting in this position has also been observed in other commercial whey powders [27].

In sample chromatograms from mineral acid and rennet whey, a leading shoulder was observed on the α-lac peak, while in the WPC samples this peak was broadened and skewed. It is likely that this shoulder was contributed to in part by naturally occurring glycosylated α-lac species, since these elute just ahead of aglyco-α-lac and were also observed in these samples on non-denaturing gels (not shown). Pellegrino et al. [33] also reported peak shape changes for α-lac in RP-HPLC profiles from WPCs and non-fat dry milks and observed new components eluting ahead of α-lac in high heat samples. As with β-lg, they assigned this to lactosylation. In the mineral acid WPC, M_r values of 14 501 and 14 823 were detected in addition to an M_r of 14 176 for unmodified α-lac (identified in all samples). These additional M_r values correspond to α-lac with one and two lactose adducts, respectively and based on relative spectral intensity these species were present in significant proportions in the sample. The ability of the RP-HPLC method to measure total α-lac as the sum of both glycosylated and non-glycosylated protein confers an advantage over other quantitative methods such as SDS–PAGE, where measurement of α-lac is not unequivocal due to co-migration of glyco-α-lac with β-lg [6].

In those samples expected to contain significant amounts of CMP, i.e., MF-WPI, cheese WPC and rennet whey, the RP-HPLC profiles of CMP (Fig. 4B, E and G) were qualitatively similar to that of the standard. However, differences in the relative proportions of CMP components were apparent. For example, both cheese WPC and rennet whey appeared to contain proportionally more aglyco A variant CMP. In all three samples, molecular masses for the aglyco-monophosphate A and B variants were found under the designated peaks (data not shown). In the cheese WPC sample, an additional M_r of 3394 was found under the aglyco-A peak. This was possibly a chymosin hydrolysis product of CMP [36]; for example, the κ-casein peptide 109-140 (A variant), which is in the CMP portion of the molecule, has a theoretical M_r of 3394. Small amounts of material eluting in the region of CMP were seen in those samples which had not been subjected to rennet hydrolysis (lactic and mineral WPC, mineral whey; Fig. 4C, D, F). While some of this may have been CMP derived from psychrotrophic bacterial enzyme hydrolysis [37], the remainder was likely to have been PP material since minor components of this fraction eluted in this position (see Fig. 2). The implications of this for CMP quantitation are discussed below. For those samples containing very low amounts of PP and CMP, e.g., IX-WPI, differentiation of the PP5 peak from the CMP region was not straightforward; however, for integration purposes reference was made to the elution positions of the standards.

BSA eluted as a discrete peak in the IX-WPI and cheese WPC samples (Fig. 4A and E). However, for all other samples, an additional peak was observed eluting ahead of but not wholly resolved from BSA, either as an ill-defined shoulder (e.g., lactic WPC, Fig. 4C) or an equivalent peak (mineral acid whey, Fig. 4G). Since BSA can form aggregates which behave differently from the monomer on RP-HPLC [23,24], this peak was included in BSA quantitations.

3.4. Quantitation of whey proteins

Protein concentration was calculated from integrated peak areas using the response factors determined with the standards. β-Lg concentration was determined from the total peak area inclusive of genetic variants, dimer peaks, chemically modified or otherwise denatured material. In those cases where resolution of individual whey proteins was not quite baseline, a vertical drop was used from the lowest

point of the valley between them. The results are given in Table 1 and are presented either on an individual concentration basis (whey) or as % powder mass (WPC and WPI). Table 1 also lists sample data obtained from alternative methods of whey protein analysis which were used as standards of comparison.

3.4.1. α-Lac and β-lg

For WPI, WPC and whey samples, the protein distributions and concentrations as determined by RP-HPLC were generally consistent with literature values. Levels of α-lac were similar for all WPC and WPI samples and correlated well with values obtained from SDS–PAGE, a typical example of which is shown in Fig. 5. For WPI and WPC samples, β-lg levels determined by RP-HPLC were lower than those found by SDS–PAGE. For the most part this can be explained by the widely recognised fact that, unlike SDS–PAGE, RP-HPLC methods measure only soluble protein [6,34,38]. Heat denaturation of whey proteins and associated formation of insoluble protein aggregates can occur to varying degrees depending on whey processing conditions, thus leading to loss of protein measurable by RP-HPLC. In particular, β-lg is the most heat labile whey protein [39]. In fact Bican and Spahni [40] have demonstrated the applicability of RP-HPLC to measurement of thermal degradation of β-lg, and Kim et al. [38] have shown that β-lg determined by RP-HPLC can be correlated with WPC functionality. Additionally, analyses of β-lg by SDS–PAGE may

Table 1
RP-HPLC determination of whey proteins in bovine whey, WPI and WPC samples

Sample		α-Lac	β-Lg	BSA	IgG	PP	CMP	Total	TN·6.38	Recovery (% TN)
IX-WPI	Mean[a]	10.8 (10.53*)[b]	70.66 (76.46*)	2.59 (3.33*)	3.25 (2.71*) (4.17†) (4.83‡)	0.34	0.43 (0.54)	88.07	89.3[c]	99
	SD	0.13	0.93	0.21	0.33	0.04	0.03	0.89		
	RSD (%)	1.18	1.32	8.21	10.29	12.46	6.59	1.01		
MF-WPI	Mean	12.72 (10.61*)	40.55 (50.70*)	1.17 (1.47*)	3.54 (2.47*) (ND†) (4.07‡)	5.09	16.79 (16.75)	79.86	84.9	94
	SD	0.30	0.42	0.28	0.27	0.12	0.14	0.80		
	RSD (%)	2.33	1.03	24.34	7.68	2.32	0.82	1.00		
Lactic WPC	Mean	11.73 (11.52*)	40.05 (47.40*)	2.03 (2.11*)	3.35 (3.55*) (1.88†) (ND‡)	1.87	2.3 (1.48)	61.32	75.3	81
	SD	0.35	0.55	0.21	0.31	0.14	0.11	1.23		
	RSD (%)	2.95	1.38	10.61	9.22	7.67	4.60	2.00		
Mineral acid	Mean	11.92 (11.28*)	43.23 (50.79*)	2.21 (2.19*)	5.59 (3.96*) (6.46†) (ND‡)	2.50	1.39 (0.63)	66.84	75.9	88
WPC	SD	0.25	0.35	0.21	0.40	0.12	0.06	0.85		
	RSD (%)	2.13	0.81	9.64	7.11	4.78	4.45	1.27		
Cheese WPC	Mean	10.91 (10.50*)	30.64 (40.05*)	1.13 (2.02*)	3.34 (4.68*) (3.73†) (ND‡)	1.50	13.15 (13.90)	60.67	75.3	81
	SD	0.25	0.16	0.13	0.24	0.07	0.15	0.76		
	RSD (%)	2.25	0.51	11.13	7.14	4.94	1.18	1.25		
Acid whey	Mean	0.76 (0.59*)	3.12 (3.28*)	0.15 (0.19*)	0.37 (0.31*) (ND†) (0.47‡)	0.45	0.25 (0.22)	5.10	ND	–
	SD	0.01	0.03	0.06	0.05	0.01	0.02	0.18		
	RSD (%)	1.40	1.10	38.10	13.90	2.20	9.20	3.90		
Rennet whey	Mean	0.79 (0.70*)	3.23 (3.83*)	0.12 (0.23*)	0.48 (0.40*) (ND†) (0.53‡)	0.31	1.16 (1.39)	6.09	ND	–
	SD	0.01	0.03	0.03	0.03	0.01	0.01	0.13		
	RSD (%)	1.30	0.80	22.4	7.10	2.30	1.00	2.10		

[a] Mean values expressed as % powder mass (WPI, WPC) or mg/ml (whey); data from 30 separate determinations.
[b] Values in parentheses refer to analysis by other methods; () Mono S HPLC, (*) SDS–PAGE, (†) RID, (‡) Protein G HPLC; analyses performed in duplicate.
[c] TN, Total protein was measured as protein nitrogen determined by Kjeldahl analysis using a conversion factor of 6.38 for whey proteins. ND, Not determined.

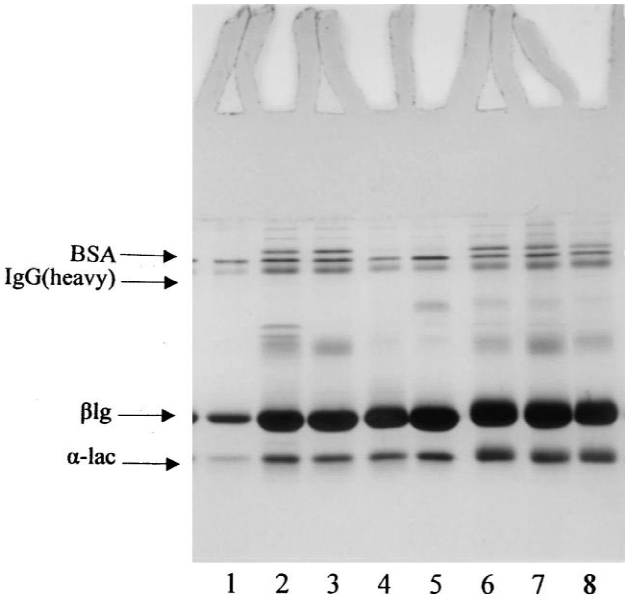

Fig. 5. SDS–PAGE (reducing) of WPC, WPI and whey samples. Lanes: 1=mixed whey protein standard (at highest dilution); 2=rennet whey; 3=mineral acid whey; 4=MF-WPI; 5=IX-WPI; 6=lactic WPC; 7=mineral acid WPC; 8=cheese WPC. The whey protein standards are identified on the left. Conditions for SDS–PAGE as described in Experimental.

be distorted since PP5 migrates very closely with β-lg on SDS gels (not shown) and is included, along with glyco-α-lac, in β-lg quantitations. It should be noted that in the present study, attempts to bring about complete solubilisation of whey proteins in powder samples using agents such as urea and guanidine hydrochloride proved ineffective.

For acid and rennet whey, the RP-HPLC method yielded higher estimates for α-lac than SDS–PAGE. In fresh whey samples where the proteins are predominantly undenatured, RP-HPLC gives a truer measure of α-lac as the sum of both glycosylated and non-glycosylated protein, since on SDS–PAGE glyco-α-lac co-migrates with β-lg and is therefore excluded from α-lac estimations [6].

3.4.2. BSA

BSA measured by RP-HPLC gave similar values to those obtained by SDS–PAGE for most samples, but assay variability was greater than for α-lac and β-lg as reflected in the higher RSD values. This greater variability probably related to the low concentrations of BSA being measured as well as the history and heterogeneity of the sample. BSA has proven to be notoriously difficult to assay with accuracy and many anomalies both in resolution and quantitation of this protein have been observed [6,14,23]. Indeed, Norris et al. [14] assayed BSA in various whey types by 15 different methods and reported RSD values of up to 64% relative to the mean value.

3.4.3. IgG

Results for IgG quantitation by RP-HPLC were consistent with data obtained from SDS–PAGE, RID and Protein G HPLC determinations. Given the inherent difficulties in determining IgG by the RP-HPLC method, and indeed the general difficulties in assaying IgG by any method [14], the RSD values were very acceptable, generally being within 10% of mean.

3.4.4. PP

In acid whey, the level of PP measured as material under the PP5 peak was approximately 9% of true whey protein (calculated as the sum of all the whey protein values exclusive of CMP). This value is well within the range of values reported in the literature

[22] and agrees well with an average value of 10.5% found in mineral acid whey [10]. Although acid and rennet whey samples were generated from the same milk source, the lower PP value obtained for rennet whey probably resulted from some loss of PP into the casein coagulum during renneting [22]. However, the PP content of 0.31 mg/ml agreed well with previously reported values for rennet whey [22].

Values for PP in WPC and WPI samples varied from ~0.3 to 5% of powder mass (2–6% of total protein) and these were dependent to some extent on the processing history of the sample. The RSD values for PP analysis on the whole showed good precision with the highest variability being observed at very low concentration of this component.

3.4.5. CMP

Concentrations of CMP determined by RP-HPLC showed excellent agreement with those obtained by the Mono S HPLC method and values in rennet whey were closely similar to reported values [18]. For those samples containing significant amounts of CMP (MF-WPI, cheese WPC, rennet whey) assay reproducibility was excellent, while at lower concentrations of CMP, RSD was still within 10%. In some samples with low concentrations of CMP (lactic and mineral acid WPC), the RP-HPLC method gave somewhat higher values than those obtained by the cation-exchange method. This is probably due to the fact that small amounts of hydrophilic PP co-elute with CMP on RP-HPLC and are included in peak integrations. Nevertheless, the RP-HPLC results give some useful information even at these low levels of CMP.

3.4.6. Protein recovery

For WPC and WPI samples, the total protein recovered from RP-HPLC is shown in Table 1 as a percentage of total protein determined by protein nitrogen analysis. In the WPC samples, protein recovered as soluble protein from RP-HPLC was 81–88% of total protein, while in the WPI samples virtually all the protein was recovered as soluble protein.

3.5. Repeatability

WPC and WPI samples were analysed for within-day ($n=5$) and between-day ($n=5$ on six consecutive days) precision and accuracy. Table 2 shows within- and between-day repeatability for each of the whey proteins, calculated from grouped data. For all proteins except IgG, the between-day variability was higher than within-day but the RSD remained $<3\%$ for CMP, PP, α-lac and β-lg. Although precision for BSA analysis decreased markedly between-days, overall variation was still $\leq 10\%$. Accuracy of IgG determinations appeared to be independent of the day of analysis as shown by the similar within- and between-day RSD values (7–8%).

To determine the minimum levels at which the whey proteins could be reliably detected and quantitated by the RP-HPLC method, further statistical analysis was performed to obtain the limits of detection (LODs) and quantification (LOQs) for each

Table 2
Within- and between-day repeatability of the RP-HPLC determination of bovine whey proteins in WPI and WPC samples (combined data)

	Accuracy[a]	CMP	PP	α-Lac	BSA	β-Lg	IgG
Within-day	SD	0.03	0.02	0.03	0.05	0.16	0.20
($n=5$)	RSD (%)	0.30	0.20	0.60	3.30	0.40	8.30
Between-day	SD	0.11	0.11	0.28	0.23	0.57	0.27
($n=30$)[b]	RSD (%)	1.10	3.00	2.60	10.0	1.40	7.00
LOD		0.03	0.03	0.10	0.03	0.30	0.60
LOQ		0.30	0.10	0.30	0.10	1.00	2.00

[a] Values are expressed as SD and RSD relative to the mean values determined as % powder mass.
[b] Six separate days.

protein [41]. As shown in Table 2, LOD values were 0.03–0.6% of powder mass while LOQ values were generally within 1% of powder mass, giving reliability of assay at relatively low protein concentrations. The higher LOQ for IgG (2%) probably reflects the general difficulties associated with assay of this protein as well as the problems with baseline and non-specific adsorption effects observed in measurement of IgG by this method.

4. Conclusions

In conclusion, a RP-HPLC method is presented for the determination of α-lac, β-lg, BSA, PP, CMP and IgG in bovine whey, WPC and WPI powders. The method is rapid, reliable and sensitive and gives very good accuracy and precision for measurement of the whey proteins in all sample types. Furthermore, the method is a measure of soluble, and therefore functional, protein. An additional advantage conferred over other methods such as SDS–PAGE and size-exclusion HPLC is that determination of α-lac and β-lg is unequivocal. Some qualitative information on protein heterogeneity and quality may also be derived from the RP-HPLC analyses, and further information can be readily obtained by on-line mass spectrometry.

The described method may have wide application in the routine analysis of whey, fractionated whey streams and whey protein powders produced by dairy industries. Further development of the method to enable analysis of minor basic whey protein components in fractionated whey streams is currently ongoing.

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