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RAPID ANALYSIS OF BOVINE MILK PROTEINS BY FAST PROTEIN LIQUID CHROMATOGRAPHY

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SUMMARY

The separation of bovine milk proteins by fast protein liquid chromatography has been investigated by ion-exchange chromatography on Mono Q and Mono S columns and by gel filtration on a column of Superose 12. The four major casein components (α_{s1}, α_{s2}, β and κ) as well as the minor γ-caseins were generally well separated on the Mono S column with urea-containing buffers at pH 3.8 in as short a time as 7 min, although there was considerable overlap between α_{s1}- and α_{s2}-casein peaks. Peak area measurements indicated that the four caseins α_{s1}, α_{s2}, β and κ were present in total casein in the approximate proportions of 3.0:0.5:3.4:0.9, in good agreement with other literature values. Whey proteins were not separated on the Mono S column, but were all well resolved by rapid analysis on the Mono Q column at pH values between 6 and 8 in buffers free of urea or 2-mercaptoethanol. Both urea and 2-mercaptoethanol were required for casein analyses on the Mono Q column, but all the casein components were then separable over a broad pH range (5.0–11.0). While urea levels of 4.5–8.0 *M* and pH values of 7.0 to 8.0 were most generally useful, the resolution of some components was affected by urea concentration or pH, so conditions may have to be modified for specific analysis problems. The caseins were too similar in size to be separated on the Superose 12 column but high-speed gel filtration in as little as 15 min separated all the whey proteins well, molecular weight values obtained being in good agreement with literature values.

INTRODUCTION

Early methods for the analysis and preparation of milk proteins relied upon salt and organic solvent fractionation (reviewed by McKenzie[1]). These gave way to paper and then to the much higher resolution starch gel electrophoresis[2] for analytical purposes and to ion-exchange chromatography[3–5] for larger scale preparative separations. Starch gel electrophoresis in turn rapidly gave way to the use of polyacrylamide gel techniques[6] for protein analysis, and although the highest resolution methods employing two-dimensional separations[7] or isoelectric focusing[7] have been applied to milk proteins, simple single-dimension separations remain much the most widely used for this purpose[8,9]. Column chromatography on hydroxyapatite[10], gel

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filtration and various electrophoretic methods have been used for the small scale preparation of a few milligrams of individual milk proteins but undoubtedly the method of choice for larger scale separations is ion-exchange chromatography on DEAE-cellulose columns[11].

Separations by gel filtration and ion-exchange chromatography by conventional low-pressure methods are relatively time consuming, often taking 24 h or more if the best resolution is needed. While the advent of more recent column materials, such as Sephacryl, enabled columns to be run faster, so that separation times could be reduced to 3–4 h, the most important advance has been in the application of high-performance liquid chromatography (HPLC) techniques to protein separations. The ability to obtain quantitative measurements in a few minutes in this way is especially valuable when following the course of reactions which may alter the protein composition or state (*e.g.*, chemical modification, enzymic hydrolysis, thermal denaturation etc). The HPLC separation of milk whey protein by size exclusion on TSK gel columns[12–14], by reversed-phase chromatography[12,15] and by ion-exchange[16] have been reported, while caseins have been separated by reversed-phase chromatography[12,15] and using a column of a DEAE derivative of the TSK gel matrix[16].

The latest developments in high-speed protein analysis, however, are embodied in the various columns manufactured by Pharmacia. This paper describes the rapid separation of bovine milk proteins using ion-exchange chromatography on the Mono Q and Mono S columns and size-exclusion chromatography (gel filtration) on a Superose 12 column.

EXPERIMENTAL

Total casein, in which it is known that the α_{s1}, α_{s2}, β- and κ-casein components occur in the very approximate ratio 3:1:3:1[17], was prepared by isoelectric precipitation from skimmed fresh raw bulk bovine milk[11]. Total whey protein, prepared from the filtrate (acid whey) fraction of such milk, and the individual α-lactalbumin (α-la), β-lactoglobulin A (β-lg A) and β-lactoglobulin B (β-lg B) whey proteins, purified by ion-exchange chromatography on DEAE-cellulose, were kindly supplied by Dr. R. L. J. Lyster (FRI, Reading, U.K.) who also provided a sample of colostrum immunoglobulin (mostly IgG). The individual α_{s1}, α_{s2}, β- and κ-caseins were also prepared by DEAE-cellulose ion-exchange chromatography[11]. Bovine serum albumin (BSA) was purchased from Sigma and from Armour Labs.

The apparatus used was the Pharmacia fast protein liquid chromatography (FPLC) system fitted with either the Mono S HR 5/5 cation-exchange column, the Mono Q HR 5/5 anion-exchange column or the Superose 12 HR 10/30 gel filtration column.

With both ion-exchange columns sample solutions generally contained 5–10 mg protein per ml and sample volumes of 100–500 μl were applied but very much smaller amounts of only a few μg can easily be analyzed if desired. We began elution programmes with the passage of 3 ml of salt-free buffer before the start of the sodium chloride gradient. Since the "void volume" (the volume of buffer required after the start of sample injection before unadsorbed material began to be eluted from the bottom of the column) was about 0.75 ml, this ensured that at least 3 void volumes

of buffer followed the sample onto the column to elute unretarded protein before the gradient began. This initial 3 ml of buffer was followed by 20 ml or sometimes 25 ml of a gradient of increasing sodium chloride concentration to 0.3 or 0.35 *M* followed by 3–4 ml of a "high salt" wash with buffer containing 0.35 or 0.5 *M* sodium chloride and finally, by 3–4 ml of salt-free buffer to re-equilibrate the column in readiness for the next sample. Unless stated otherwise a flow-rate of 1 ml/min was used throughout as this was found to give slightly better resolution than higher flow-rates (*e.g.* 2 ml/min), while reducing the rate to 0.5 ml/min gave only a marginal further improvement in resolution at a cost of much longer analysis times. Rather than reducing flow-rate, resolution between selected components could generally be improved more effectively by making the salt gradient more shallow for that portion of the run and maintaining the flow-rate.

For the Superose 12 gel filtration column the volume of sample solution injected was kept down to 100 μl, or for best resolution to 50 μl, and flow-rates between 0.25 (for highest resolution) and 1.0 ml/min (for rapid asnalysis) were used. With gel filtration there is of course no requirement for buffer salt gradients so routinely we employed a 0.05 *M* or 0.1 *M* Tris–HCl buffer pH 7.0 containing 10 m*M* sodium azide as preservative and 0.5 *M* sodium chloride to provide a high ionic strength environment and prevent any possible ionic interactions between proteins and the gel matrix.

The identities of the eluted peaks were established by chromatographing under the same conditions samples of highly purified individual milk proteins. Identities were confirmed by preparative runs in which 500 μl samples containing 10 mg protein per ml were applied to the ion-exchange columns or 100 μl samples containing 20 mg/ml to the Superose 12 column. The experiments were performed under otherwise identical conditions to the purely analytical runs except that fractions of 0.5 ml were collected throughout. Components in the eluted fractions were then identified by polyacrylamide gel electrophoresis (PAGE) using T = 12.5%, C = 5% gels containing 4.5 *M* urea for casein analysis[11] or without urea for whey protein identification.

RESULTS AND DISCUSSION

Separations on Mono S

All the major milk proteins have isoelectric points between about 4.5 and 5.5[17] so that as well as the commonly employed fractionation on anionic exchange materials such as DEAE-cellulose at pH values above 5.5 (usually in the range of pH 7–8), they should also be separable below pH 4.5 on cationic materials. The separation of caseins in 50 m*M* sodium formate buffer pH 3.8 containing 8 *M* urea and 0.01 *M* 2-mercaptoethanol on the Mono S column is shown in Fig. 1. The profile represents the separation of the components of total casein in a 0–0.3 *M* sodium chloride salt gradient taking only 20 min. Almost as good results were obtained by increasing the pump speed to 2 or 3 ml/min, so that the total run time including washes at the start and end of the gradient was only 10 min.

In Fig. 1 the major peak eluting after approximately 9 min was β-casein while the other major peak at about 18.5 min was α_{s1}-casein. There was no evidence from runs with purified samples that the α_{s2} caseins (a group of proteins sharing a common polypeptide chain but with varying degrees of phosphorylation) separated from one

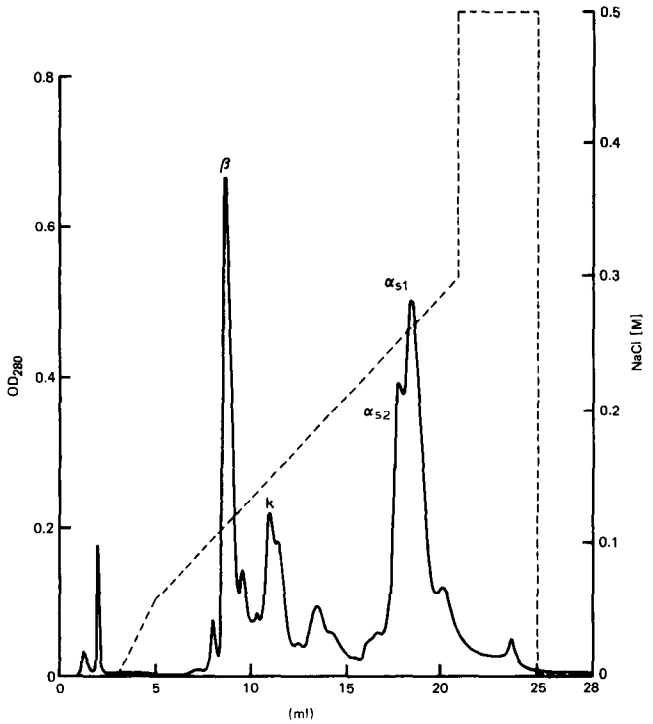

Fig. 1. The FPLC separation of caseins on the Mono S column. A 500-μl sample of total casein (10 mg/ml) dissolved in 50 m*M* sodium formate buffer pH 3.5 containing 8 *M* urea and 10 m*M* 2-mercaptoethanol was applied and the column eluted at a flow-rate of 1 ml/min using a 0–0.3 *M* NaCl gradient in this buffer beginning 3 ml after sample application. Peaks: β = β-casein; κ = κ-caseins; α_{s2} = α_{s2}-caseins; α_{s1} = α_{s1}-caseins.

another under these conditions and they eluted as a single zone at about 17.5 min. There was very considerable overlap with the α_{s1}-peak even if the salt gradient was made more shallow in this part of the run and although in the experiment shown in Fig. 1 α_{s2}-casein resolved as a leading shoulder to the α_{s1} peak, more usually α_{s2}-casein was merely concentrated towards the leading edge of the peak without clearly resolving from it. The region between the β and α_{s1} peaks was occupied by a number of smaller peaks of κ-casein and other minor components, probably γ-caseins. The major κ-casein peak eluted after 11 min, but this protein is a glycoprotein with variable carbohydrate composition and the peak pattern is more complex than that of the other caseins. It is best identified by chymosin (rennet) treatment which specifically hydrolyses a Phe–Met bond to give *para*-κ-casein and a glycomacropeptide. The *para*-κ-casein was found to elute after approximately 13 min under the conditions of Fig. 1. Taking the extinction values, $E^{1\%}_{1cm}$ = 10.0, 10, 4.6 and 10.5 for α_{s1}-, α_{s2}-, β- and κ-caseins respectively[17], peak areas showed that these four proteins occurred in the approximate proportions of 3:0.5:3.4:0.9 respectively (taking α_{s1}-casein as 3), although there must be some uncertainty due to the difficulty of effectively resolving α_{s2}-casein from α_{s1}-casein and in ensuring the inclusion of all the area that should be attributed to κ-casein components. In other work (unpublished) we have found these

conditions to be useful for resolving the genetic variant β-casein B from β-casein A_1 or A_2, but A_1 cannot be separated from A_2 in this way.

Chromatography of the whey proteins under similar conditions to those shown in Fig. 1 gave only two large peaks, the first at about 16 min corresponding to α-la and the second at 20 min being β-lg. The two genetic variants of β-lg (β-lg A and β-lg B) which are easily separated on the Mono Q column were not resolved and the β-lg peak itself was unsymmetrical and partially overlapped the α-la peak. Purified BSA gave a broad peak at about 21 min and an even wider peak from about 22 to 26 min (the gradient was extended to at least 0.35 *M* sodium chloride for these experiments), while Ig G did not adhere to the column. In the absence of urea and mercaptoethanol only purified β-lg gave a well-defined peak. Thus the Mono S column was not judged suitable for the rapid analysis of whey proteins.

Separations on Mono Q

Casein components can be separated on the Mono Q column in a variety of buffer systems over the pH range of at least 5.0 to 11.0, as long as urea is incorporated to disaggregate and unfold the casein molecules. Since Mono Q contains quaternary ammonium groups the column can be run satisfactorily at much higher pH than is possible when ion exchangers with DEAE groups are used, and the polyimine matrix itself is also stable at strongly alkaline pH values. This could theoretically be helpful for κ-casein measurement because the heterogeneity due to variable carbohydrate and especially sialic acid content is confined to the glycomacropeptide portion of the molecule and all variants give the same *para*-κ-casein following treatment with chymosin. The isoelectric point of *para*-κ-casein is about 9.4 which is very different to those of the other caseins (4.5–5.5) so by running columns at pH values above 9.4 all casein constituents should adhere to the column, greatly facilitating area measurements of eluted peaks. Unfortunately although *para*-κ-casein did bind to the column at high pH values, the elution position always coincided with one of the γ-casein peaks. Resolution of the other casein components was better at pH values closer to neutrality however, so a compromise must be chosen and a typical rapid separation at pH 7.0 is shown in Fig. 2. The first group of minor peaks consists of the γ-caseins and a number of minor unidentified components, all of low electrophoretic mobility, which are well separated from one another. Resolution between the various κ-casein species was excellent (Fig. 2) with virtually no overlap with the γ-caseins and only the relatively minor κ-casein component adhering most strongly to the Mono Q column overlapped with unidentified minor proteins eluting just before β-casein. The major region of compromise therefore is in the α-casein zones. The principal peak is α_{s1}-casein, but this is not usually well resolved from the α_{s2}-caseins. Better separation between these two proteins can be achieved by chromatography on the less polar DEAE type of ion-exchange material[11,16]. They can be separated to some extent on the Mono Q column, however, but the separation is dependent not on pH over the range 5–11 but on urea concentration, with very little separation in 8M urea and α_{s2}-caseins clearly beginning to be eluted ahead of α_{s1}-casein as the urea level is reduced to 4.5 *M* (Fig. 2) or 3.0 *M*, below which adequate disaggregation of casein complexes cannot be assured. α_{s0}-casein resolved as a small peak (Fig. 2) on the side of the α_{s1}-casein peak when 8 *M* urea was used in the buffers and the separation from the main α_{s1}-peak was best at high pH (11.0), at which pH

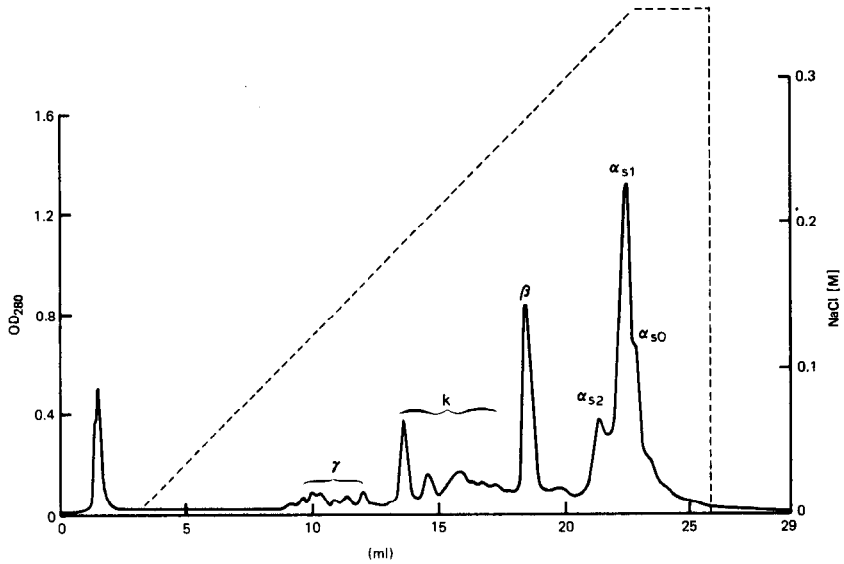

Fig. 2. The FPLC separation of caseins on the Mono Q column. A 200-μl sample of total casein (10 mg/ml) dissolved in 20 m*M* Tris–HCl buffer pH 7.0 containing 4.5 *M* urea and 10 m*M* 2-mercaptoethanol was applied and the column eluted at 1 ml/min using a 0–0.35 *M* NaCl gradient in this same buffer. Peaks: γ = γ-caseins; κ = κ-caseins; β = β-casein; α_{s2} = α_{s2}-casein; α_{s1} = α_{s1}-casein; α_{s0} = α_{s0}-casein.

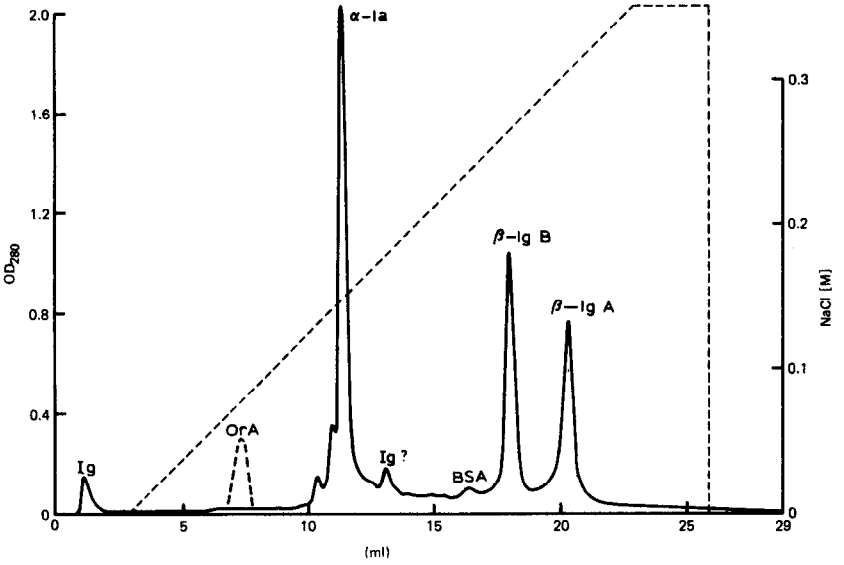

Fig. 3. The FPLC separation of whey proteins on the Mono Q column. A 200-μl sample of total whey protein (10 mg/ml) dissolved in 20 m*M* Tris–HCl buffer pH 7.0 was applied and the column eluted with a 0–0.35 *M* NaCl gradient in this buffer at a flow-rate of 1 ml/min. Peaks: Ig = immunoglobulin; α-la = α-lactalbumin; BSA = bovine serum albumin; β-lg B = β-lactoglobulin B; β-lg A = β-lactoglobulin A. When undialysed whey was analysed with the same elution programme a peak (rather variable in size) of orotic acid (OrA) was eluted in the position shown by the dashed peak.

separations in the γ- and κ-casein regions were less good. In buffer containing low urea levels (*e.g.* 4.5 M) α_{s0}-casein was not resolved from the main α_{s1}-peak.

Whey proteins were very well separated under the same conditions as those used for the caseins when urea and mercaptoethanol were omitted from the buffers (Fig. 3). A number of minor peaks eluting on either side of the α-la peak remain to be identified, but α-la, BSA and the two genetic variants of β-lg were completely separated. The principal Ig peak did not adhere to the column and was eluted in the column void but a minor component may be responsible for the small peak eluting just after α-la (Fig. 3). When freshly prepared undialysed whey was analysed on the column, a peak identified as orotic acid was eluted before α-la (see Fig. 3), after about 7.2 ml with this elution programme. Unfortunately when urea was added to buffers the whey proteins were poorly resolved so we have not yet been successful in finding conditions that will simultaneously suit both whey proteins and caseins, which therefore still have to be analysed in two separate runs.

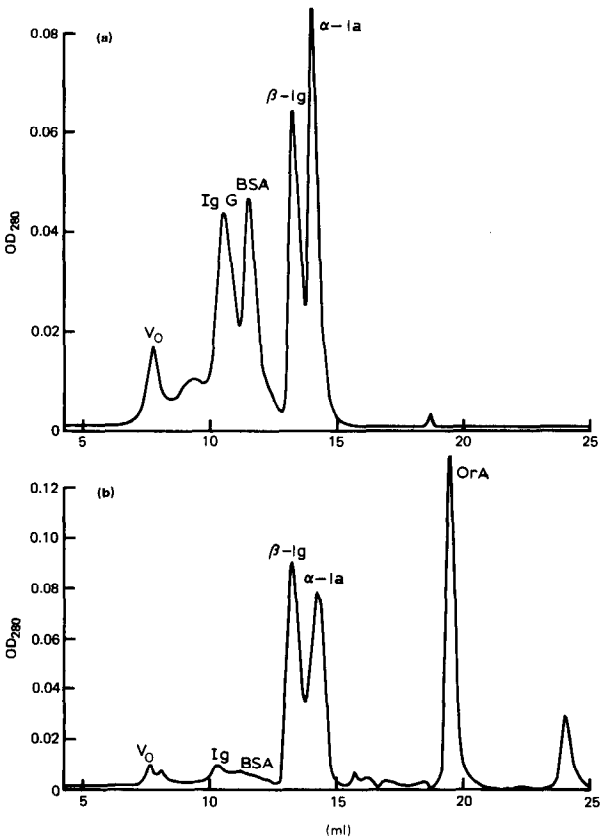

Fig. 4. FPLC separation of whey proteins by gel filtration on the Superose 12 column. (a) Separation of 100 μl of a solution of standard proteins, immunoglobulin G (Ig G; 0.8 mg/ml), bovine serum albumin (BSA; 0.4 mg/ml), β-lactoglobulin A + β-lactoglobulin B (β-lg; 0.4 mg/ml) and α-lactalbumin (α-la, 0.2 mg/ml). Column was run at a flow-rate of 0.5 ml/min in 0.1 *M* Tris–HCl pH 7.0 buffer containing 0.5 *M* NaCl and 10 m*M* NaN_3. (b) Separation of 50 μl fresh acid whey under the same conditions except that flow-rate was 1.0 ml/min.

Separations on Superose 12

The caseins are too similar in size to be separated when aqueous buffers or buffers containing urea are employed. In a 5 *M* urea-containing Tris–HCl buffer of pH 7.5, either with or without added 10 m*M* 2-mercaptoethanol, only a single broad peak eluting at a volume corresponding to a molecular weight of about 105 000 with a small amount of material at the void volume (V_0) was obtained. In urea-free buffer, the apparently identical two peaks were obtained but with a larger proportion of the material at V_0.

Separations of whey proteins were very satisfactory, however. Fig. 4a shows the separation of a standard mixture of the four major bovine whey proteins, IgG, BSA, β-lg and α-la. Elution positions corresponded to apparent molecular weights of 133 000 (160 000), 67 000 (66 290), 31 200 (36 500) and 17 200 (14 175), respectively, the generally accepted literature values[17] being in parentheses. The small peak at the column void volume (7.80 ml) was contributed either by aggregated material or high molecular weight impurities in the IgG sample used. Application of a sample of acid whey applied directly to the column gave the separation shown in Fig. 4b. Area measurements led to concentration values of IgG = 0.51 mg/ml, BSA = 0.56 mg/ml, β-lg A + β-lg B = 3.25 mg/ml (the genetic variants being similar in size are not separated by gel filtration) and α-la = 1.25 mg/ml, in good agreement with literature values[17,18] showing that recovery from the column was essentially complete. A major peak eluted after 19.7 ml was identified as orotic acid and a further smaller peak of low molecular weight due to UV-absorbing salts, vitamins etc was seen at the total column volume (24.3 ml). Both these peaks were removed by dialysis and were not seen when samples of redissolved precipitated whey proteins were analysed.

CONCLUSIONS

Although the caseins can be separated by ion-exchange chromatography on either the Mono S column at pH 3.8 or on the Mono Q column at pH values from about 5.0 to 11.0, as long as urea is incorporated in the buffers, for most routine analyses the latter at pH values in the region of 7–8 is likely to be most effective. Urea levels must be sufficiently high to prevent casein aggregation but otherwise are not critical unless the Mono Q column is being used for resolving α_{s2}-casein (which is better resolved on the less polar DEAE type of ion-exchange matrix), in which case urea levels should be kept low (*e.g.* 3.0–4.5 *M*). Urea-containing buffers are not satisfactory for separation of whey proteins which are well-separated in non-urea buffers at pH values from 6 to 8 on the Mono Q column and on the Superose column, the former also separating the genetic variants of β-lg very well and the latter being ideal for molecular weight measurements. With the ion-exchange columns, using flow-rates of 3 ml/min enables good separations to be achieved in 6–7 min, while running the Superose 12 column at 1 ml/min results in a good separation of protein components in 15 min, the total column run time being 25 min. Naturally lower flow-rates give slightly better resolutions and we routinely use 1 ml/min with the Mono S and Mono Q columns and 0.25 or 0.5 ml/min with the Superose column. Resolution on the ion-exchange columns can also be enhanced with only a slight penalty in terms of analysis time by making salt gradients more shallow in regions where closely spaced peaks are eluted.

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