Separation and Quantification of Bovine Milk Proteins by Reversed-Phase High-Performance Liquid Chromatography

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Current analytical methods for milk proteins lack the capacity to simultaneously separate and quantify the six major bovine milk proteins and their genetic variants. A method is described that simultaneously separates and quantifies the six major bovine milk proteins. The separation is based on reversed-phase partitioning of the six major milk proteins and several genetic variants of κ-casein, β-casein, and β-lactoglobulin. The described method has for each of the six milk proteins a linear quantitative response, precision (coefficient of variation below 5.1% within days of analysis, and below 7.1% between days of analysis), resolution (over 2.5 between proteins), peak efficiency (theoretical plate numbers between 8 000 and 50 000), and a sample treatment without filtration steps that together with the analysis takes 2 h. The composition of protein from milk of each of 234 cows was determined using the current method, and the results were similar to reference values for milk proteins.

Keywords: *Casein; whey; milk protein; reversed-phase high-performance liquid chromatography; quantification*

INTRODUCTION

Bovine milk typically consists of 3.0−3.5% (w:w) protein, of which 80% is caseins that are defined chemically as the milk proteins that precipitate from solution at pH 4.6 and 20 °C. The bovine casein group consists of α_{S1}-, α_{S2}-, β- and κ-casein, abbreviated as α_{S1}-CN, α_{S2}-CN, β-CN, and κ-CN, respectively, and they occur in the approximate proportions of 4:1:4:1 (w:w:w:w). The whey proteins, which are soluble at pH 4.6, consist mainly of β-lactoglobulin (β-LG) and α-lactalbumin (α-LA) in a ratio of 3:1 (w:w) (Walstra and Jenness, 1984). Immunoglobulins (Ig) and bovine serum albumin (BSA) are also considered to be whey proteins.

Quantification has been done separately for casein and whey proteins by using independent runs of gel electrophoresis (Ng-Kwai-Hang and Kroeker, 1984; Basch et al., 1985), liquid chromatography (Andrews et al., 1985; Guillou et al., 1987; Dumay and Cheftel, 1989; Collin et al., 1991; Visser et al., 1991; Syväoja, 1992; Franzen et al., 1995; Léonil et al., 1995), and capillary electrophoresis (de Jong et al., 1993; Otte et al., 1994; Kinghorn et al., 1995). Simultaneous separation and quantification of the casein and whey proteins has been reported by capillary zone electrophoresis, isoelectric focusing, and high-performance liquid chromatography (HPLC). The method using capillary zone electrophoresis did not report α_{S2}-CN quantification however (de Jong et al., 1993). Moreover, methods using HPLC and isoelectric focusing did not separate α-LA and β-CN B (Visser et al., 1991), α-LA and β-LG (Guillou et al., 1987; Bovenhuis and Verstege, 1989; Nieuwenhuijse et al., 1991; Groen et al., 1994), or κ-CN B and α_{S2}-CN (Parris et al., 1990; Léonil et al., 1995).

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The objective of the current study was to develop and test a method for simultaneous separation and quantification of the six major bovine milk proteins. Such a method should be capable of separating protein variants such as the A and B genetic variants of κ-CN and β-LG that are associated with cheese making properties of milk (van den Berg et al., 1992). A linear quantitative response, precision, peak efficiency, peak resolution, and a protocol for sample preparation without filtration steps that together with the separation could be completed in less than 2 h were desired.

EXPERIMENTAL PROCEDURES

Reagents, Protein Standards, Samples, and Protein Identification. Acetonitrile (Fisher Scientific, Pittsburgh, PA) was of HPLC grade, and water was deionized and distilled. All other chemicals were of analytical grade. BisTris buffer, dithiothreitol (DTT), guanidine hydrochloride (GdnHCl), sodium citrate, trifluoroacetic acid (Sigma, St. Louis, MO), and Preservo Liquid [contains 20% (v:v) 2-bromo-2-nitropropane-1,3-diol in water; D&F Control Systems, San Ramon, CA], were used for separation. Samples of milk from Holstein and Jersey cattle were obtained from the Dairy Breeding Farm and the Dairy Teaching Farm of Iowa State University. Bovine κ-CN, α_S-CN, β-CN, α-LA, β-LG, BSA, IgG, IgM, β-LG_A, and β-LG_B were purchased from Sigma (St. Louis, MO). α_{S2}-CN, purified from bovine milk by the method of Vreeman and van Riel (1990), was used to identify the α_{S2}-CN peak. The genetic variants of the bovine milk proteins were identified by isoelectric focusing with the PhastSystem (Pharmacia, Uppsala, Sweden) as described by Bovenhuis and Verstege (1989).

Sample Preparation. Preservo Liquid was added to fresh milk samples at 1.25:100 (v:v) within 5 min after a sample was taken from a cow to prevent microbial growth. Aliquots containing 500 μL of milk were frozen at −20 °C. A solution containing 0.1 M BisTris buffer (pH 6.8), 6 M GdnHCl, 5.37 mM sodium citrate, and 19.5 mM DTT (pH 7) was added directly to frozen aliquots in a 1:1 ratio (v:v) at room temperature. After thawing, each sample was shaken for 10 s, incubated for 1 h at room temperature, and centrifuged for 5

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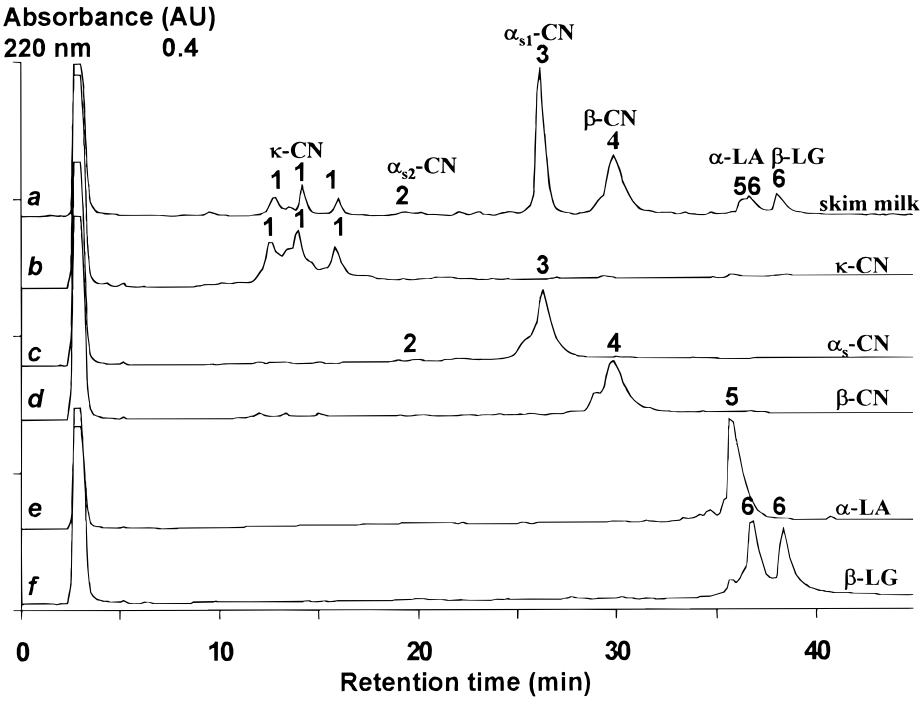

Figure 1. Separation of bovine milk proteins and protein standards by RP-HPLC. Chromatograms of (a) skim milk, (b) κ-CN standard, (c) α_S-CN standard, (d) β-CN standard, (e) α-LA standard and (f) β-LG standard. Sample preparation and conditions are described in Materials and Methods; injection volumes are 50 μL. Identity of peaks: 1, κ-CN; 2, α_{S2}-CN; 3, α_{S1}-CN; 4, β-CN; 5, α-LA; 6, β-LG.

min at 16 000*g* in a microcentrifuge. The fat layer then was removed with a spatula. The remaining solubilized sample was diluted 1:3 (v:v) with a solution containing 4.5 M GdnHCl and solvent A, which consisted of acetonitrile, water, and trifluoroacetic acid in a ratio 100:900:1 (v:v:v; pH 2). The concentration of milk protein in the final diluted solution was approximately 4 mg/mL, whereas the concentration of milk protein in the original milk samples was usually between 30 and 33 mg/mL.

For identification and quantification of milk proteins, a standard was prepared that contained purified bovine milk proteins (over 80% purity for κ-CN, over 85% purity for α_S-CN and α-LA, and over 90% purity for β-CN and β-LG according to Sigma). Concentrations in the standard were 1.5 mg/mL κ-CN, 4.0 mg/mL α_S-CN, 3.0 mg/mL β-CN, 0.5 mg/mL α-LA, and 1.0 mg/mL β-LG. The ratio of α_{S1}-CN to α_{S2}-CN was assumed to be 4:1 (w:w). The standard was prepared as a single batch, and the standard was frozen in aliquots at −20 °C. Aliquots of standards and milk samples were treated the same. The total protein concentration in the final diluted solution of the standard was 1.25 mg/mL, whereas the total protein concentration in original standards was 10 mg/mL. The protein standards were assumed for calculations to be homogeneous because the milk protein standards used had less than 10% contamination from other proteins or minerals (calcium) and no indication of bias for a particular protein (Figure 1, lanes *b*−*f*). To improve the quantitative data for the current method, the standards could be solubilized, purified by RP-HPLC, and then lyophilized and weighed.

Reversed-Phase HPLC (RP-HPLC). The HPLC system consisted of two HPLC pumps (Waters 501), an automatic sample injector (Waters 712), an absorbance detector (Waters 484), and an interface module (Waters, Marlborough, MA). The equipment was controlled by software (Waters 820) that controlled the solvent gradient, data acquisition, and data processing. A filter (pore size: 0.5 μm), followed by a silica-based C-18 RP-HPLC column (250 mm length × 4.6 mm i.d., Microsorb MV C-18, particle size: 5 μm, pore size: 30 nm), was used for protein separation (both from Rainin Instruments, Woburn, MA). All solutions were filtered through a nylon filter (No. 66, i.d.: 46 mm, pore size: 0.2 μm, Rainin Instruments, Woburn, MA).

Chromatographic conditions were as follows:

Solvents. A: Acetonitrile, water, and trifluoroacetic acid in a ratio 100:900:1 (v/v/v). B: Acetonitrile, water, and trifluoroacetic acid in a ratio 900:100:1 (v/v/v).

Total run time. 52 min.

Column temperature. Room temperature.

Flow rate. 1.20 mL/min.

Detection wavelength. 220 nm.

Injection volume of final solution. Milk samples: 5−80 μL (usually 20 μL; in Figure 1: 50 μL). Standard: 20−100 μL.

The solvent gradient program started at 27% of solvent B. A gradient was generated immediately after sample injection by increasing the proportion of solvent B at 2.5%/min (2 min), 0.47%/min (34.9 min), and 0.45%/min (4 min) and then returned linearly to the starting conditions in 2.1 min. Before injection of the next sample, the column was reequilibrated under the starting conditions for 9 min.

Quantitative Determination of Bovine Milk Proteins. Standard curves were developed by measuring peak areas at various known amounts of injected milk proteins. The standard curves were used to calculate the amount of protein represented by peak areas from milk samples of unknown composition. For each 2-day analytical run, or after injection of 42 samples, a new standard curve was developed using six standard aliquots (two at each of 40, 60, or 80 μL injection volume of final solution) and applying simple linear regression.

Calculations for Peak Efficiency and Resolution. The number of theoretical plates (N) and the resolution (R) between peaks were calculated as follows:

$$N = 5.54(V_R/V_{0.5W})^2$$

$$R = 2(V_{R2} - V_{R1})/(V_{W1} + V_{W2})$$

where V_R is the retention volume, $V_{0.5R}$ is the volume of the peak width at half-height, and V_W is the volume of the peak width at baseline (obtained by tangential lines drawn at half-height).

Test for Quantitative Linearity. A sample of fresh milk was divided into 15 aliquots that were injected, three at each of 5, 10, 20, 40 and 80 μL injection volume of the final solution.

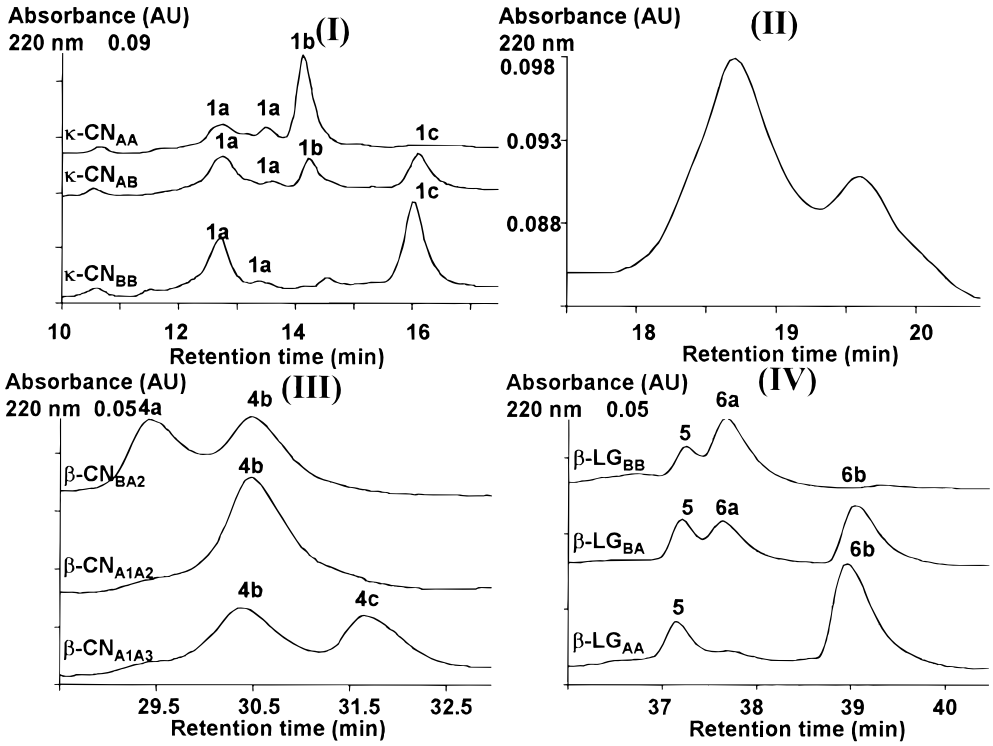

Figure 2. Separation of bovine milk proteins and several genetic variants by RP-HPLC. Chromatograms of bovine milk of cows with (I) κ-CN genetic variants: (a) κ-CN_{AA}, (b) κ-CN_{AB}, (c) κ-CN_{BB}; (II) double peak of α_{S2}-CN; (III) β-CN genetic variants: (d) β-CN_{BA2}, (e) β-CN_{A1A2}, (f) β-CN_{A1A3}; (IV) β-LG genetic variants: (g) β-LG_{BB}, (h) β-LG_{BA}, (i) β-LG_{AA}. Sample preparation and conditions are described in Materials and Methods. Injection volumes are 20 μL. Identity of peaks: 1a, glycosylated κ-CN; 1b, unglycosylated κ-CN_A; 1c, unglycosylated κ-CN_B; 4a, β-CN_B; 4b, β-CN_{A1}, β-CN_{A2}; 4c, β-CN_{A3}; 5, α-LA; 6a, β-LG_B; 6b, β-LG_A.

In an analysis of variance of the 15 values collected for each of the six bovine milk proteins, the total sum of squares was first divided into two parts, one arising from differences in protein amounts injected, and the other arising from differences among replications within amounts injected or experimental error. The test for linearity came from the *F*-test formed by the ratio of the remainder mean square to the experimental error (3 and 10 df; the 80 μL amount for β-LG was omitted because of obvious nonlinearity leading to 2 and 8 df). Low values of this *F* coupled with an indication of nonzero slope described a good linear fit. R^2 values were the portions of the sum of squares obtained from differences in amounts injected that were explained by a linear fit.

Determination of Quantitative Precision. The precision of the method within an analytical run was found by running five consecutive aliquots of the same milk at 20 μL of the final solution and calculating the coefficient of variation (CV, %) of the peak areas (the lower the CV value the better the precision). The precision of the method between analytical runs was found by calculating the CV (%) from stored aliquots of milk that were analyzed at the beginning of each of five 2-day analytical runs (sample injection volume = 20 μL).

Comparison of Milk Protein Composition with Literature Values. The milk protein compositions [(protein weight/total milk protein weight) × 100 = wt %] and their CVs (%) were determined from individual milk samples of 234 cows from the Iowa State University Dairy Breeding Farm (injection volume of the final solution: 20 μL). The amounts of the six major bovine milk proteins in the milk samples were calculated from linear standard curves as described in quantitative determination of bovine milk proteins.

RESULTS AND DISCUSSION

Qualitative Determination of Milk Proteins. Retention times of the major eluted peaks coincide with retention times of the major milk proteins present in the standards. Furthermore, peak areas of the skim milk chromatogram (Figure 1, line *a*) are proportional to known relative abundances of major proteins in bovine milk (Walstra and Jenness, 1984). From these observations, we conclude that the milk proteins eluted in the order: κ-CN, α_{S2}-CN, α_{S1}-CN, β-CN, α-LA, and β-LG (Figure 1).

When pooled milk was analyzed, multiple peaks for κ-CN, β-CN, and β-LG were observed (Figure 1). The sizes of these peaks were proportional to the abundances of milk protein genetic variants in the populations of cattle from which the milk was sampled (Figure 2; Visser et al., 1991). The identification of peaks as genetic variants of proteins was confirmed by comparison with standards that consisted of purified genetic variants of milk proteins [β-LG variants A and B, Figure 2 (IV)] or by isoelectric focusing [κ-CN variants B and A; β-CN variants B, A1 and A2 together, and A3, Figure 2 (I and III)]. Variants B and C of α_{S1}-CN were not separated by the current method or by any other published RP-HPLC method (Nieuwenhuijse et al., 1991; Visser et al., 1991; Groen et al., 1994). The current method resolved several modified forms of milk proteins. κ-CN eluted as four distinct peaks (Figure 1), which consisted of glycosylated and unglycosylated forms of κ-CN_A and κ-CN_B [Figure 2 (I)].

Under the conditions used, the peak efficiencies of the proteins were between 8.6×10^3 for α_{S2}-CN and 46.5×10^3 and 51.5×10^3 for α-LA and β-LG, respectively (Table 1). The peak resolution was 4.4 (between glycosylated κ-CN and unglycosylated κ-CN_A), 4.8 (between unglycosylated κ-CN_A and unglycosylated κ-CN_B), 8.1 (between unglycosylated κ-CN_B and α_{S2}-CN), 8.7 (between α_{S2}-CN and α_{S1}-CN), 3.1 (between α_{S1}-CN and β-CN_B), 1.0 (between β-CN_B and β-CN_{A1A2}, and between β-CN_{A1A2} and β-CN_{A3}), 12.2 (between β-CN_{A3} and α-LA), 2.7 (between α-LA and β-LG_B), and 4.1 (between β-LG_B

Table 1. Precision of Retention Times and Peak Areas and Peak Efficiencies of Bovine Milk Proteins

	within runs[a]		between runs (48 injections apart)[b]		peak efficiency
milk protein	retention time, CV[c] (%)	peak area, CV (%)	retention time, CV (%)	peak area, CV (%)	no. of theoretical plates 10^3
κ-CN[d]	0.30	2.08	1.68	5.33	15.0
α_{S2}-CN	0.08	4.82	1.12	5.72	8.6
α_{S1}-CN	0.18	1.15	1.11	2.48	15.2
β-CN[e]	0.32	2.17	1.45	7.05	9.8
α-LA[f]	0.14	5.02	0.76	5.59	46.5
β-LG[g]	0.21	1.01	0.93	6.65	51.5

[a] Five aliquots of the same milk injected at 20 μL of the final solution. [b] Five aliquots of the same milk injected at the beginning of each of five analysis runs of 48 samples at 20 μL of the final solution. [c] CV, coefficient of variation (%). [d] CN, casein. [e] β-CN coelutes with g-CN. [f] LA, lactalbumin; α-LA coelutes with BSA. [g] LG, lactoglobulin.

and β-LG_A). The peak efficiencies and peak resolution of α-LA and β-LG are similar to values reported for capillary electrophoresis of whey (Otte et al., 1994), whereas, to our knowledge, values have not been reported in the literature for the peak efficiencies and peak resolution of the other milk proteins.

Under the conditions used, Igs were not detected with the described gradient on the basis of comparisons with separate injections that included IgG or IgM or by any other RP-HPLC method that analyzed whole milk (Groen et al., 1994). Immunoglobulins have been observed to coelute, however, with β-CN or after α-LA in milk powder (Parris et al., 1990; Parris and Baginski, 1991), or elute, before, after α-LA, and after β-LG in whey (Resmini et al., 1989).

Under the conditions used, γ-CNs coeluted with β-CN, as reported previously for other RP-HPLC methods that analyzed whole milk (Parris et al., 1990; Strange et al., 1991; Groen et al., 1994). We assumed the same absorption coefficient for γ-CN and β-CN because γ-CNs are proteolytic products of β-CN.

Under the conditions used, BSA coeluted with α-LA, based on comparisons with separate injections that included BSA or any other RP-HPLC method that analyzed whole milk (Groen et al., 1994). BSA has been observed to coelute, however, either with α_{S1}-CN or before α-LA in milk powder (Parris et al., 1990; Parris and Baginski, 1991), elute before α-LA in dried whey (Diosady et al., 1980), or elute either before or after α-LA whey (Resmini et al., 1989; Bican and Spahni, 1991). To obtain quantitative data for BSA and α-LA with the current method, BSA and a-LA could be added to the standard at concentrations of 0.1 mg/mL BSA and 0.4 mg/mL α-LA. The coelution of BSA and α-LA, however, does not significantly affect quantification of proteins in milk because the ratio of BSA to α-LA in milk is primarily affected by infections of and injuries to the mammary gland.

By using chromatography software that could amplify small peaks, we integrated the α_{S2}-CN double peak, as shown in Figure 2 (II), with precision (Table 1) and peak efficiency. The α_{S2}-CN peak in the milk sample and in the α_S-CN standard cannot be seen at the resolution used in Figure 1, however, because of its low abundance and its distribution in more than one peak [Figure 2 (II)]. The α_{S2}-CN double peak could be caused by separation of differently phosphorylated forms of α_{S2}-CN (van Hekken et al., 1990). A change of the detection wavelength from 220 to 210 nm increased the peak height of a_{S2}-CN to a peak height similar to κ-CN and other published RP-HPLC methods (Nieuwenhuijse et al., 1991; Visser et al., 1991; Groen et al., 1994). We preferred as detection wavelength 220 nm over 210 nm because it improved the baseline (less noise) as well as the peak efficiency as well as the resolution of the protein peaks, in particular of α-LA and β-LG_B (Table 1). Despite partial separation of α-LA and β-LG_B [low resolution used in Figure 1, lane a; Figure 2 (IV)], α-LA and β-LG were quantified with sufficient resolution, peak efficiency, precision, and linear quantitative response (Tables 1 and 2). This quantification was possible because the α-LA and β-LG_B peaks were symmetrical (Figure 1, lanes e and f), because we used known amounts of α-LA and β-LG B in the standard and because the chromatography software was capable of high resolution [Figure 2 (IV)].

Table 2. Test for Linearity of Peak Areas of Bovine Milk Proteins[a]

milk protein	range of amounts (μg)	prob. > F[b]
κ-CN[c]	5.5−44.2	0.97
α_{S2}-CN	2.9−22.8	0.82
α_{S1}-CN	5.1−81.9	0.15
β-CN[d]	4.2−66.9	0.22
α-LA[e]	0.5−8.3	0.22
β-LG[f]	1.4−11.3	0.79

[a] A fresh milk sample was divided into 15 aliquots that were injected; three at each of 5, 10, 20, 40, and 80 μL injection volume of the final solution. [b] Test for deviations from linearity (df 3, 10; low values indicate nonlinearity); probability for zero slope of line all <0.01; R^2 all exceed 0.99. [c] CN: casein. [d] β-CN coelutes with γ-CN. [e] LA, lactalbumin; α-LA coelutes with BSA. [f] LG, lactoglobulin.

α_{S2}-CN and β-CN have been reported to carry over from one injection to another (Nieuwenhuijse et al., 1991; Visser et al., 1991). Protein carryover can be minimized by injecting lower amounts of α_{S2}-CN and β-CN (injection volume of 20 rather than 50 μL), or by using a C-4 RP-HPLC column (Jaubert and Martin, 1992). Separation by using a short-chain fatty acid column was not attempted because κ-CN_B and α_{S2}-CN did not resolve on a C-8 RP-HPLC column (Léonil et al., 1995).

Quantitative Determination of Milk Proteins. When the peak areas were plotted as a function of the protein amounts injected, each of the six major bovine milk proteins had a slope that differed significantly from zero, and each was devoid of significant nonlinear components (Table 2). Therefore, each major milk protein increased linearly over the protein ranges indicated in Table 2. R^2 values for each bovine milk protein exceeded 0.99 (Table 2).

CV values for retention times of the six major bovine milk proteins were below 0.35% within analytical runs and below 1.70% between analytical runs (Table 1). CVs for peak areas were below 5.1% within analysis runs and below 7.1% between analysis runs (Table 1). The CV values are similar to those reported in the literature for within-day variation (Collin et al., 1991; Parris and

Table 3. Protein Composition of Individual Samples from 234 Cows[a]

	protein composition		
	current study		literature[b]
milk protein	mean (wt %)[c]	CV[d] (%)	mean (wt %)
κ-CN[d,e]	16.9	13.09	10.7
α_{S2}-CN	8.0	22.50	8.5
α_{S1}-CN	32.2	6.09	32.5
β-CN + γ-CN[f]	28.6	8.13	32.6
α-LA + BSA[g]	3.8	21.58	5.2
β-LG[h]	10.5	22.27	10.4

[a] Concentrations of the six major bovine milk proteins in the milk samples were calculated from linear standard curves as described under Materials and Methods. [b] According to Walstra and Jenness (1984). The column total of 100% is casein proteins + β-LG + α-LA + BSA. [c] wt %, weight percentage on total milk protein. [d] CV, coefficient of variation (%). [e] CN, casein. [f] β-CN coelutes with γ-CN. [g] LA, lactalbumin; α-LA coelutes with BSA. [h] LG, lactoglobulin.

Baginski, 1991; Cifuentes et al., 1993; de Jong et al., 1993; Groen et al., 1994; Franzen et al., 1995; Kinghorn et al., 1995) and between-day variation (Resmini et al., 1989; Cifuentes et al., 1993). We conclude that the method quantifies protein amounts of milk samples with good precision. The quantitative precision was improved by running protein standards every other day to adjust for column-to-column and day-to-day variation (Table 1). The six major bovine milk proteins continued to respond linearly with high precision in repeated analyses of milk protein samples and standards.

General Observations. Milk protein composition as determined by our method was similar to literature values (Walstra and Jenness, 1984) except for κ-CN (Table 3). Our wt % of κ-CN is with 16.9 not unusually high because wt % of κ-CN greater than 16.9 have been reported previously for bovine milk (Strange et al., 1991; Colin et al., 1992; Laurent et al., 1992; Żbikowska et al., 1992). Differences in milk protein composition reported by different investigators can be explained partly by factors such as animal breed, season of the year, and diet composition (McLean et al., 1984; Ng-Kwai Hang et al., 1987).

The lifetimes of the columns were between 500 and 600 chromatographic runs, which is not unusual considering the use of a silica-based column with a mobile phase containing 0.1% trifluoroacetic acid and a pH of 2 (Glajch et al., 1987). Cleaning with high acetonitrile concentrations after each separation of milk proteins or reversing the column flow did not improve the column lifetime significantly.

Concluding Remarks. An RP-HPLC method is described for separation and quantification of the six major bovine milk proteins and several genetic variants. The tested method separates and quantifies the six major bovine milk proteins with a linear quantitative response, precision, peak efficiency, resolution, and a protocol for sample treatment without filtration steps that takes together with the protein separation in less than 2 h. The precision can be improved by running daily protein standards. The results for the protein composition of 234 milk samples were similar to those of other published methods.

ABBREVIATIONS USED

BSA, bovine serum albumin; CN, casein; IG, immunoglobulin; LA, lactalbumin; LG, lactoglobulin; HPLC, high-performance liquid chromatography; DTT, dithiothreitol; GdnHCl, guanidine hydrochloride; RP-HPLC, reversed-phase high-performance liquid chromatography; wt %, weight percent or (weight of protein/weight of total milk proteins) × 100; CV, coefficient of variation.

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