Sample Preparation Affects Separation of Whey Proteins by Reversed-Phase High-Performance Liquid Chromatography

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Measured concentrations of whey proteins in single milk samples often differ by more than 20% when analyzed by different separation methods. In the current study, we examined the effects of using guanidine hydrochloride or urea at zero and 8.5 h after sample preparation and the effects of using dithiothreitol or 2-mercaptoethanol on the separation of bovine milk proteins by reversed-phase high-performance liquid chromatography. Treatment with guanidine hydrochloride or urea resulted in similar separation of milk proteins when samples were injected immediately after preparation. Separation was repeatable over 8.5 h for samples prepared with guanidine hydrochloride, whereas the resolution of α-lactalbumin and β-lactoglobulin decreased for samples treated with urea. Treatment with dithiothreitol improved the resolution of α-lactalbumin and β-lactoglobulin in comparison with treatment with 2-mercaptoethanol. Quantitation of whey proteins is more reliable when milk samples are treated with dithiothreitol and guanidine hydrochloride than when treated with 2-mercaptoethanol or urea.

Keywords: *Casein; whey; milk protein; reducing agent; chaotropic agent; reversed-phase high-performance liquid chromatography*

INTRODUCTION

Bovine milk proteins can be quantified by many different separation methods. Several studies have compared different separation methods and have reported up to 20% differences in concentrations of individual proteins from the same milk sample (Dumay and Cheftel, 1989; Collin et al., 1991; Hollar et al., 1991; Strange et al., 1991; Cifuentes et al., 1993; Law, 1993; Kinghorn et al., 1995). The source of these differences, which could be caused by differences in separation methods or sample preparation, have not been examined. Because milk proteins interact with each other as well as with numerous chemical and physical agents, a comparison of different reducing and chaotropic agents is warranted.

Bovine milk contains 3.0−3.7% protein; 80% of milk protein consists of caseins that are defined chemically as proteins that precipitate from solution at pH 4.6 at 20 °C, and the remaining 20% are whey proteins that are soluble at pH 4.6. The bovine casein group consists of α_{S1}-, α_{S2}-, β-, and κ-casein (α_{S1}-CN, α_{S2}-CN, β-CN, and κ-CN, respectively) and are secreted in approximate proportions of 4:1:4:1 (w:w). The whey proteins consist mostly of β-lactoglobulin (β-LG) and α-lactalbumin (α-LA) in a ratio of 3:1 (w:w) (Walstra and Jenness, 1984).

Studies using reversed-phase high-performance liquid chromatography (RP-HPLC) have shown that, in samples prepared with urea, the β-CN peak area decreases relative to the peak areas of the whey proteins as the time interval between sample preparation and injection increases (Groen et al., 1994). To our knowledge, guanidine hydrochloride (GdnHCl) has only been used as a chaotropic agent for separation of milk proteins by Bobe et al. (1998). Studies using RP-HPLC have shown that the type of reducing agent used in the sample preparation affects the separation of α-LA and β-LG. When no reducing agent was used, α-LA eluted between α_{S1}-CN and β-CN (Parris et al., 1990; Visser et al., 1991). When 2-mercaptoethanol (2ME) was present, α-LA coeluted either with β-LG (Parris et al., 1990) or with β-CN (Jeurnink and de Kruif, 1995) or eluted between α_{S1}-CN and β-CN (Visser et al., 1991). In the presence of dithiothreitol (DTT), however, α-LA either coeluted with β-LG (Nieuwenhuijse et al., 1991; Groen et al., 1994) or eluted before β-LG (Bobe et al., 1998). When DTT or no reducing agent was used, β-LG eluted as two separate peaks in samples containing β-LG A or B (Nieuwenhuijse et al., 1991; Visser et al., 1991; Groen et al., 1994; Bobe et al., 1998), whereas additional β-LG peaks eluted when 2ME was used (Visser et al., 1991; Jeurnink and de Kruif, 1995). Our objective was to determine the effect of sample preparation and time interval between sample preparation and injection on the subsequent separation of milk proteins by RP-HPLC.

EXPERIMENTAL PROCEDURES

Reagents and Samples. Acetonitrile (Fisher Scientific, Pittsburgh, PA) was of HPLC grade, and water was deionized and distilled. All other chemicals, which were BisTris buffer, DTT, GdnHCl, 2ME, sodium citrate, trifluoroacetic acid, and urea (Sigma, St. Louis, MO) were of analytical grade. Samples of bovine milk were obtained from the Dairy Teaching Farm of Iowa State University. Standards of bovine κ-CN, α_S-CN, β-CN, α-LA, and β-LG (Sigma) were used to identify the respective peaks (Figures 1 and 2) as described by Bobe et al. (1998). α_{S2}-Casein, purified from bovine milk samples by the method of Vreeman and van Riel (1990), was used to distinguish the α_{S2}-CN peak from the α_{S1}-CN peak (Figures 1 and 2; Bobe et al., 1998). α-Lactalbumin−β-lactoglobulin (α-LA−β-LG) was identified on a 12.5% T gel by SDS−urea polyacryl-

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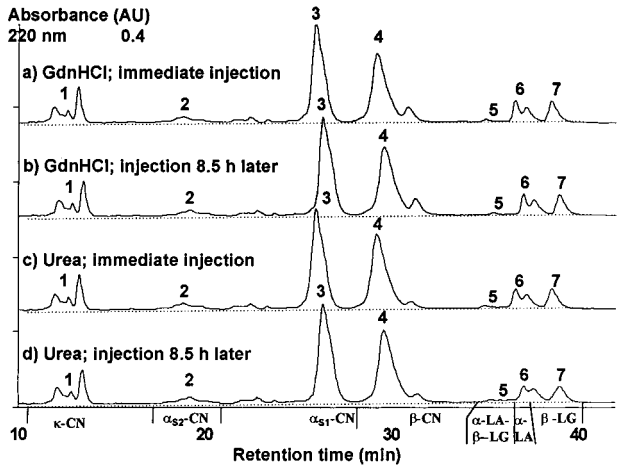

Figure 1. Separation of bovine milk proteins by RP-HPLC using different chaotropic agents at different time intervals between sample preparation and injection. Chromatograms of a bovine milk sample (phenotype: κ-CN_{AA}; α_{S2}-CN_{AA}; α_{S1}-CN_{BB}; β-CN_{A2A2}; α-$LA_{AA;}$ β-LG_{AB}) using GdnHCl as chaotropic agent and injecting (a) immediately after sample preparation or (b) 8.5 h later or using urea as chaotropic agent and injecting (c) immediately after sample preparation or (d) 8.5 h later. Details of sample preparation and separation conditions are described under Experimental Procedures. Injection volumes were 50 μL. Peak area starts and ends and peaks used for peak height determination are shown: 1 = κ-CN; 2 = α_{S2}-CN; 3 = α_{S1}-CN; 4 = β-CN; 5 = α-LA–β-LG; 6 = α-LA; 7 = β-LG.

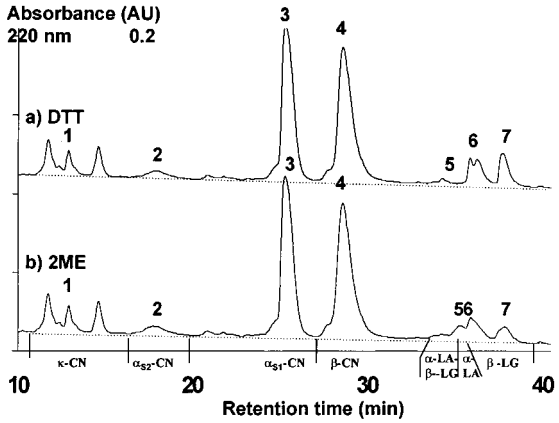

Figure 2. Separation of bovine milk proteins by RP-HPLC using different reducing agents. Chromatograms of a bovine milk sample (phenotype: κ-CN_{AB}; α_{S2}-CN_{AA}; α_{S1}-CN_{BB}; β-CN_{A2A2}; α-$LA_{AA;}$ β-LG_{AB}) treated either with (a) DTT or (b) 2ME as reducing agent. Details of sample preparation and separation conditions are described under Experimental Procedures. Injection volumes were 50 μL. Peak area starts and ends and peaks used for peak height determination are shown: 1 = κ-CN; 2 = α_{S2}-CN; 3 = α_{S1}-CN; 4 = β-CN; 5 = α-LA–β-LG; 6 = α-LA; 7 = β-LG.

amide gel electrophoresis (SDS = sodium dodecyl sulfate) with a PROTEAN II xi vertical electrophoresis cell (BIO-RAD, Hercules, CA) as described by Bollag and Edelstein (1991). The α-LA–β-LG peak was detected only in samples containing α-LA alone or in mixtures with β-LG. The genetic variants of the major bovine milk proteins were identified by isoelectric focusing with the PhastSystem (Pharmacia, Uppsala, Sweden) as described by Bovenhuis and Verstege (1989).

Sample Preparation. In experiment 1, the peak areas and peak heights of individual major bovine milk proteins were compared when either urea or GdnHCl was used as the chaotropic agent. Furthermore, effects of the time interval between sample preparation and injection on peak areas and heights of the major milk proteins were examined. Fresh milk samples from five cows were each divided into two aliquots and then assigned randomly to two treatments. The milk samples were collected and prepared on the same day, and each sample was prepared separately before injection. For the GdnHCl group, a solution containing 0.1 M BisTris buffer, 5.37 mM sodium citrate, 19.5 mM DTT, and 6 M GdnHCl (pH 7) was added directly to the GdnHCl assigned aliquots in a 1:1 ratio (v:v) at room temperature. For the urea group, a solution containing 0.1 M BisTris buffer (pH 6.8), 5.37 mM sodium citrate, 19.5 mM DTT, and 8 M urea (pH 7) was added directly to the urea-assigned aliquots in a 1:1 ratio (v:v) at room temperature. Each sample was shaken for 10 s, incubated for 1 h at room temperature, and centrifuged for 5 min at 16000*g* in a microcentrifuge. The fat layer then was removed with a spatula. For the GdnHCl group, the remaining solubilized GdnHCl assigned samples were 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 the ratio 100:900:1 (v:v:v; pH 2). For the urea group, the remaining solubilized urea assigned samples were diluted 1:3 (v:v) with a solution containing 6 M urea 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. A 50 μL aliquot of each sample was injected twice at 8.5 h apart under the reversed-phase HPLC conditions, as described by Bobe et al. (1998).

In experiment 2, the peak areas and peak heights of individual major bovine milk proteins were compared when either DTT or 2ME was used as the reducing agent. Fresh milk samples from five cows were each divided into two aliquots and then assigned randomly to the two treatments. The milk samples were collected and prepared on the same day, and each sample was prepared separately before injection. For the DTT group, a solution containing 0.1 M BisTris buffer, 5.37 mM sodium citrate, 19.5 mM DTT, and 6 M GdnHCl (pH 7) was added directly to the DTT assigned aliquots in a 1:1 ratio (v:v) at room temperature. For the ME group, a solution containing 0.1 M BisTris buffer, 5.37 mM sodium citrate, 38.4 mM 2ME, and 6 M GdnHCl (pH 7) was added directly to the 2ME assigned aliquots in a 1:1 ratio (v:v) at room temperature. Each sample was shaken for 10 s, incubated for 1 h at room temperature, and centrifuged for 5 min at 16000*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. A 50 μL aliquot of each sample was injected under the reversed-phase HPLC conditions, as described by Bobe et al. (1998). The sample preparation for milk samples of the GdnHCl group in experiment 1 and the DTT group in experiment 2 was the same. Differences in peak areas and peak heights are caused by the fact that the milk samples were from different cows and that the day of sampling and analysis were different. As demonstrated previously, the same method can be used for quantification of milk proteins from peak areas by using standard curves of purified proteins (Bobe et al., 1998).

Statistical Methods. In experiment 1, the statistical model is a split-plot design with treatment (urea or GdnHCl; 1 degree of freedom (df) and cow (4 df) as whole-plot factors and time (0 or 8.5 h; 1 df) and the time × treatment interaction (1 df) as subplot factors. The tests for treatment differences came from the *F*-test formed by the ratio of the treatment mean square to the treatment × cow interaction mean square, which is the MSE for treatment (1 and 4 df), as shown in Tables 1 and 2. The tests for time and time × treatment interactions came from the *F*-test formed by the ratio of the time mean square or time × treatment mean square to the residual MSE, which is the MSE for time and time × treatment (1 and 4 df), as shown in Tables 1 and 2. Differences

Table 1. Effect of Chaotropic Agent and Time Interval between Sample Preparation and Injection on Peak Areas (V × s) of Bovine Milk Proteins[a]

	treatment (trt)								
	guanidine HCl time (h)		urea time (h)		MSE[b]		prob > F^c		
milk protein	0	8.5	0	8.5	trt	time; trt × time	trt	time	trt × time
κ-CN[d]	9.92	9.85	8.91	9.08	0.99	0.04	0.11	0.58	0.24
α_{S2}-CN	3.67	3.28	3.32	3.42	0.17	0.10	0.61	0.39	0.17
α_{S1}-CN	30.76	30.75	27.92	28.67	9.79	0.20	0.15	0.14	0.13
β-CN	28.54	29.33	26.73	27.22	4.88	1.58	0.12	0.32	0.80
α-LA−β-LG[e]	1.20	1.16	1.45	1.85	0.03	0.006	<0.01	<0.01	<0.01
α-LA	3.12	2.93	3.16	2.90	0.30	0.06	0.99	0.10	0.77
β-LG	7.68	7.70	7.84	7.17	0.58	0.10	0.61	0.08	0.07

[a] Fresh milk samples from five cows were divided into two aliquots, and either GdnHCl or urea was used as the chaotropic agent. Each sample was injected twice, 8.5 h apart. Details for sample preparation and separation conditions are described in Experimental Procedures. Injection volumes were 50 μL. Peak area starts and ends are shown in Figure 2. [b] MSE: mean square error(trt) = cow × trt (df 4); mean square error(time; trt × time) = residual (df 8). [c] Probability for treatment differences. The statistical model contains treatment, cow, and treatment × cow as whole plot independent variables and time and time × treatment as subplot variables. Effects of treatment and cow (results not shown) were tested by using cow × treatment as the error term. Effects of time and treatment × time were tested by using the residual error term with 4 df. [d] CN: casein. [e] α-LA−β-LG: complex of α-lactalbumin and β-lactoglobulin.

Table 2. Effect of Chaotropic Agent and Time Interval between Sample Preparation and Injection on Peak Heights (mV) of Bovine Milk Proteins[a]

	treatment (trt)								
	guanidine HCl time (h)		urea time (h)		MSE[b]		prob > F^c		
milk protein	0	8.5	0	8.5	trt	time; trt × time	trt	time	trt × time
κ-CN[d]	83.0	79.2	72.6	73.1	60.70	5.78	0.08	0.19	0.11
α_{S2}-CN	36.8	38.4	35.5	37.3	7.44	6.23	0.38	0.19	0.96
α_{S1}-CN	591.9	573.1	549.8	533.3	1841.75	253.55	0.10	0.07	0.88
β-CN	340.6	343.5	331.1	324.7	820.75	131.30	0.33	0.75	0.41
α-LA−β-LG[e]	21.0	18.4	22.1	22.2	0.65	1.23	<0.01	0.07	0.06
α-LA	137.5	127.9	136.1	107.0	278.31	47.28	0.21	<0.01	0.03
β-LG	180.5	167.8	175.1	136.4	387.56	90.91	0.10	<0.01	0.04

[a] Fresh milk samples from five cows were divided into two aliquots, and either GdnHCl or urea was used as the chaotropic agent. Each sample was injected twice, 8.5 h apart. Details for sample preparation and separation conditions are described in Experimental Procedures. Injection volumes were 50 μL. Peaks used for peak height determination are shown in Figure 2. [b] MSE: mean square error(trt) = cow × trt (df 4); mean square error(time; trt × time) = residual (df 8). [c] Probability for treatment differences. Statistical model contains treatment, cow and treatment × cow as whole plot independent variables and time and time × treatment as subplot variables. Effects of treatment and cow (results not shown) were tested by using cow × treatment as the error term. Effects of time and treatment × time were tested by using the residual error term with 4 df. [d] CN: casein. [e] α-LA−β-LG: complex of α-lactalbumin and β-lactoglobulin.

Table 3. Effect of Reducing Agent on Peak Areas and Heights of Bovine Milk Proteins[a]

	peak area (V × s)				peak height (mV)			
	treatment				treatment			
milk protein	DTT	2ME	MSE[b]	prob > F[c]	DTT	2ME	MSE[b]	prob > F[c]
κ-CN[d]	8.90	9.10	0.26	0.56	98.1	98.2	37.86	0.97
α_{S2}-CN	1.83	2.27	0.005	<0.01	18.3	21.4	3.71	0.06
α_{S1}-CN	23.09	23.80	0.71	0.25	440.0	441.2	86.07	0.85
β-CN	23.66	24.11	0.34	0.29	278.9	288.3	49.42	0.10
α-LA−β-LG[e]	1.25	3.47	0.11	<0.01	7.9	45.9	14.25	<0.01
α-LA	1.57	1.85	0.14	0.30	86.8	65.9	95.63	0.03
β-LG	6.69	4.25	0.29	<0.01	124.4	59.9	123.72	<0.01

[a] Fresh milk samples from five cows were divided into two aliquots, and either 19.5 mM DTT or 38.4 mM 2ME was used as the reducing agent. Details for sample preparation and separation conditions are described in Experimental Procedures. Injection volumes were 50 μL. Peak area starts and ends and peaks used for peak height determination are shown in Figure 1. [b] MSE: mean square error (df 4). [c] Probability for treatment differences. The statistical model contains treatment and cow as independent variables. [d] CN: casein. [e] α-LA−β-LG: complex of α-lactalbumin and β-lactoglobulin.

at the probability of $P \leq 0.05$ were considered significant. In experiment 2, the statistical model is a factorial design with treatment (2ME or DTT; 1 df) and cow (4 df) as factors. The tests for treatment differences came from the *F*-test formed by the ratio of the treatment mean square to the mean square error (MSE; 1 and 4 df), as shown in Table 3.

RESULTS AND DISCUSSION

Chaotropic Agent. Urea and GdnHCl were compared as chaotropic agents for preparation of milk samples. With both agents, the six major milk proteins separated and eluted in the same order (Figure 1). The use of the two chaotropic agents resulted in similar peak areas and peak heights of milk proteins when samples were injected immediately after preparation (Tables 1 and 2). Time-dependent changes for whey proteins were detected, however, only in samples that were treated with urea, whereas no time-dependent changes were detected for the casein proteins. The time-dependent changes occur primarily in the first 6 h after sample preparation, as described previously by Groen et al. (1994). Peak heights of α-LA and β-LG in urea-treated samples were decreased significantly when samples were injected at 8.5 h rather than immediately after

preparation (Figure 1c,d; Table 2). No time-dependent changes in peak height (Table 2) nor in peak area (Table 1) of α-LA and β-LG were detected in samples that were treated with GdnHCl. The area of the α-LA−β-LG peak increased significantly when urea-treated samples were injected at 8.5 h rather than immediately after preparation, whereas no changes in peak area occurred when GdnHCl was used (Table 1). Moreover, the α-LA and β-LG peaks were sharper in milk samples treated with GdnHCl than in milk samples treated with urea at 8.5 h after sample treatment (Figure 1c,d), thereby improving the sensitivity of peak area integration.

Exposure to urea could have influenced the structures of whey proteins in a time-dependent manner, thereby affecting separation and quantification of milk proteins. The number of free sulfhydryl groups of β-LG and BSA decreased over time in a 6 M solution of urea under reducing conditions (Xiong and Kinsella, 1990). Exposure to cyanate present in urea caused modifications of free sulfhydryl groups of β-LG (Stark et al., 1960). Both could explain the decreased β-LG and α-LA peak areas under our experimental conditions when milk samples were treated with urea. The presence of GdnHCl rather than urea, however, improves separation and quantification of β-LG and α-LA because GdnHCl does not cause time-dependent changes in peak areas of whey proteins by RP-HPLC.

Reducing Agent. DTT and 2-ME were compared as reducing agents for preparation of milk samples. The concentrations of reducing agents were similar to those used previously by others for milk protein separation by RP-HPLC (Parris et al., 1990; Nieuwenhuijse et al., 1991; Visser et al., 1991, Groen et al., 1994; Jeurnink and de Kruif, 1995). The six major milk proteins were separated in both solutions, and the elution order was unaffected by the choice of reducing agent (Figure 2).

The presence of reducing agents, however, affected the order of protein elution. α-LA eluted after β-CN in the presence of reducing agents, whereas α-LA eluted before β-CN in the absence of reducing agent, as previously shown by Parris et al. (1990). Moreover, the choice of reducing agent affected the peak areas and peak heights of milk proteins. Peak heights of α-LA and β-LG and the peak area of β-LG were significantly greater for samples treated with 1.95 mM DTT than with 3.84 mM 2-ME (Figure 2; Table 3). The peak area and height of the α-LA−β-LG peak was significantly lower in samples treated with DTT rather than with 2-ME (Table 3). Moreover, the α-LA and β-LG peaks were resolved more clearly in milk samples treated with DTT than with 2-ME (Figure 2). Additionally, in the presence of DTT, a shoulder peak was absent that had eluted directly before the α-LA peak when 2-ME was used (Figure 2). Time-dependent changes were detected, however, only in samples that were treated with 2ME. The peaks of α-LA−β-LG, α-LA, and β-LG did not resolve in 2ME-treated samples injected several hours after preparation, and therefore the peak areas and peak heights could not be determined (results not shown). We conclude that the presence of DTT rather than 2ME improves separation and quantification of β-LG and α-LA because the sensitivity and the accuracy of the peak area integration of the whey proteins were improved by the use of DTT rather than 2-ME during sample denaturation.

The formation of an α-LA−β-LG peak depends on conditions of sample preparation and is influenced differentially by the presence of DTT and 2-ME during sample preparation. Reduction of sulfhydryl groups, shifting of disulfide bridges, structural modifications, and aggregation of whey proteins were proposed to explain the appearance of the α-LA−β-LG peak (Parris et al., 1990) that increased after heating of milk (Parris et al., 1990; Nieuwenhuijse et al., 1991; Jeurnink and de Kruif, 1995). Several other physical and chemical treatments induce α-LA−β-LG association, such as electroreduction (Bazinet et al., 1997), and concentrated solutions of urea (Xiong and Kinsella, 1990). 2ME has a lower redox potential than does DTT (Jocelyn, 1987). Under our separation conditions at pH 2.0, the reducing activity of both DTT and 2ME is minimal (Jocelyn, 1987) and α-LA partially aggregates (Kronman and Andreotti, 1964; Kronman et al., 1964). Disulfide exchanges between β-LG and α-LA and aggregation of whey proteins are therefore likely to have occurred under our experimental conditions, in particular in 2ME treated samples, which could explain changes in peak areas and heights in response to different reducing agents.

The peak areas and peak heights of α_{S2}-CN were greater in milk treated with 2ME than in milk treated with DTT (Table 3), which can be explained by differences in size and/or polarity of the two tested reducing agents (Rasmussen and Petersen, 1991). The disulfide bridges of α_{S2}-CN are more stable in the presence of 0.04 M dithioerythritol than in the presence of 0.6 M 2ME (Rasmussen and Petersen, 1991). Differences in protein separation that were detected among chaotropic and reducing agents are unlikely to have been caused by metal ions in milk because sufficient citrate was added as a chelating agent in the preparation of samples. Reducing conditions, therefore, affect the separation of major milk proteins.

Concluding Remarks. The selection of reducing and chaotropic agents and the time interval between sample preparation and injection affected separation of the major milk proteins in their analysis by RP-HPLC. The resolution of α-LA and β-LG decreased in samples treated with urea over the 8.5 h between sample preparation and injection, whereas separation of milk proteins is repeatable in samples treated with GdnHCl. Substitution of DTT for 2ME improved the resolution of α-LA and β-LG. Therefore, separation of whey proteins is more precise after sample preparation in DTT and GdnHCl than in 2ME or urea. Disulfide exchanges within and between the whey proteins are likely to have been influenced by the choice of sample preparation conditions. Therefore we recommend avoiding the use of 2ME or urea for whey protein separation.

ABBREVIATIONS USED

CN, casein; LA, lactalbumin; LG, lactoglobulin; GdnHCl, guanidine hydrochloride; DTT, dithiothreitol; 2ME, 2-mercaptoethanol; RP-HPLC, reversed-phase high-performance liquid chromatography.

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