Serum Protein and Casein Concentration: Effect on pH and Freezing Point of Milk with Added CO₂¹

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ABSTRACT

The objective of this study was to determine the effect of protein concentration and protein type [i.e., casein (CN) and serum protein (SP)] on pH (0°C) and freezing point (FP) of skim milk upon CO₂ injection at 0°C. CN-free skim milks with increasing SP content (0, 3, and 6%) and skim milks with the same SP content (0.6%) but increasing CN content (2.4, 4.8, and 7.2%) were prepared using a combination of microfiltration and ultrafiltration processes. CO₂ was injected into milks at 0°C using a continuous flow carbonation unit (230 ml/min). Increasing SP or CN increased milk buffering capacity and protein-bound mineral content. At the same CO₂ concentration at 0°C, a milk with a higher SP or a higher CN concentration had more resistance to pH change and a greater extent of FP decrease. The buffering capacity provided by an increase of CN was contributed by both the CN itself and the colloidal salts solubilized into the serum phase from CN upon carbonation. Skim milks with the same true protein content (3%), one with 2.4% CN plus 0.6% SP and one with 3% SP, were compared. At the same true protein content (3%), increasing the proportion of CN increased milk buffering capacity and protein-bound mineral content. Milk with a higher proportion of CN had more resistance to pH change and a greater extent of FP decrease at the same carbonation level at 0°C. Once CO₂ was dissolved in the skim portion of a milk, the extent of pH reduction and FP depression depended on protein concentration and protein type (i.e., CN and SP).

(Key words: carbon dioxide, casein, freezing point, pH, serum protein)

Abbreviation key: CCP = colloidal calcium phosphate; CN2.4, CN4.8, and CN7.2 = skim milks with,

respectively, 2.4, 4.8, and 7.2% casein in the fat-free portion and 0.6% serum protein in the fat-free, casein-free portion; **FP** = freezing point; **MF** = microfiltration; **NCN** = noncasein nitrogen; **NPN** = nonprotein nitrogen; **SP** = serum protein; **SP0**, **SP3**, and **SP6** = skim milks with, respectively, 0, 3, and 6% serum protein in the fat-free portion and 0% casein; **TN** = total nitrogen; **TP** = true protein.

INTRODUCTION

The addition of low levels of CO₂ is used widely in commercial practice to extend the shelf life of cottage cheese due to the ability of low concentrations of CO₂ (e.g., 400 ppm) to inhibit growth of gram-negative spoilage organism and mold (Hotchkiss and Lee, 1996). Added CO₂ can effectively control growth of psychrotrophic bacteria in raw milk and finished dairy products during refrigerated storage (King and Mabbitt, 1982; Rashed et al., 1986; Hotchkiss et al., 1999). Dissolved CO₂ increases the lag phase and generation time of microorganisms (Daniels et al., 1985) due to a direct effect of the CO₂ and not due simply to a oxygen displacement. The dairy industry is interested in expanding the use of CO₂ technology, seeking applications for shelf life extension in other dairy foods and identifying benefits of CO₂ addition other than its antimicrobial effect. In order to find new opportunities to use the CO₂ technology in the dairy industry, the impact of CO₂ addition on the chemistry of milk components needs to be thoroughly understood.

When CO₂ is dissolved in milk, milk pH decreases (Ma et al., 2001; Ma and Barbano, 2002). Although the antimicrobial effect of CO₂ is not pH-dependent (King and Mabbitt, 1982), many dairy processing steps are pH-dependent (Smits and van Brouwershaven, 1980; Corredig and Dalgleish, 1996; Beaulieu et al. 1999). The extent of pH reduction is related to the amount of CO₂ dissolved, hydrated, and protonated in the aqueous phase of a food and, thus, depends on the intrinsic properties of the aqueous phase, such as buffering capacity and initial pH (Gill, 1988; Devlieghere et al., 1998).

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Milk is a buffered system, and the major buffering components are soluble phosphates, colloidal calcium phosphate (CCP), citrate, bicarbonate, and proteins (SriLaorkul et al., 1989; Lucey et al., 1993; Singh et al., 1997). Milk proteins, including both CN and serum proteins (SP), with their acid and basic side groups, produce a buffering effect. Serum protein, because of its low concentration in milk, contributes relatively less to the buffering capacity of milk compared with CN (SriLaorkul et al., 1989). At the same CO₂ concentration, milk with a higher buffering capacity is expected to exhibit a greater resistance to pH change. Upon CO₂ addition, a decrease in milk pH is also accompanied by a progressive solubilization of CCP and other colloidal salts from casein micelles into the serum phase (Dalglish and Law, 1989; Gevaudan et al., 1996; Law and Leaver, 1998). The increase in the concentration of solutes in the serum phase of milk, together with carbonic acid (H₂CO₃) and its dissociation products (H⁺, HCO₃⁻, and CO₃²⁻) leads to decreases in milk freezing point (FP; Ma et al., 2001).

Microfiltration (MF) and UF processes have allowed the concentration and separation of milk fat, protein, lactose, and other minor components in milk. The separated CN and SP retain their native state in milk and have high functionality (Britten and Pouliot, 1996). The processing characteristics of MF and UF concentrated milk have been investigated, especially within the context of cheese making (Brandsma and Rizvi, 1999; Neocleous et al., 2002a, 2002b). In addition, the properties of fluid milk products with modified protein concentration (Quiñones et al., 1997 and 1998) and with CN:SP ratio other than the 4:1 ratio found in typical milk have been studied (Patocka et al., 1993; Beaulieu et al., 1999; Barbano et al., 2000). In order to expand the use of CO₂ in the dairy industry and explore the use of CO₂ as a possible processing aide, a basic understanding of how milk protein influences pH decrease and FP depression of milk with added CO₂ is important. Changes in pH and freezing point of milk and milk concentrates will have impacts on the functionality of milk during subsequent thermal, freezing, or fermentation processes that may be beneficial in some cases and problematic in others. The objective of this study was to measure the effect of protein concentration and protein type (i.e., CN and SP) on pH (0°C) and FP of skim milk upon CO₂ injection at 0°C.

MATERIALS AND METHODS

Milk Formulation

Microfiltration and ultrafiltration. Each replication was completed in a 6-d period. On d 1, raw skim milk (800 kg, Cornell Dairy plant) was HTST (74°C for

16s), pasteurized, and fractionated using a pilot scale, uniform transmembrane pressure MF system (Tetra Alcross M7 Pilot Plant Type, Tetra Pak, Denmark). The MF system was equipped with ceramic Membralox membranes with a nominal pore diameter of 0.1 μm and an effective surface area of 1.7 m². The MF process was carried out at 50°C with a transmembrane pressure of 0.22 to 0.28 bar. The inlet retentate pressure was approximately 4.2 bar, and outlet pressure was 2.3 bar. The flow rates of permeate and retentate were 90 and 45 l/h, respectively. For 5 h continuously, the MF system produced a 3X retentate that was used directly in milk formulation on d 4 and a permeate that was further processed using a UF system on d 2.

On d 2, permeate from the MF process was fractionated at 49°C ± 3°C in a plate-and-frame UF system (model Dorr-Oliver Iopor Series 'S'; Amicon, Beverley, MA) to achieve a final SP concentration of about 10 to 11%. The UF system was equipped with 21 S-10 polysulfone membrane plates with a mean molecular weight cutoff of 10,000 Da and an effective surface area of 0.067 m² per plate. The UF inlet pressure was 3.1 bar, and outlet pressure was 1.1 bar.

Formulation and batching. Five ingredients were used to formulate milks of different protein concentration and protein type: MF retentate from skim milk, UF retentate from MF permeate, UF permeate from MF permeate, raw cream from the same batch of whole milk as the skim milk used for MF on d 1, and lactose (Lactose monohydrate TG 207, EM Industries, Inc., NJ; 5% moisture). Composition analysis of the above ingredients, except lactose, was completed on d 3.

The MF retentate, the UF retentate and UF permeate from MF permeate, and the raw cream were analyzed for TS (AOAC, method number 990.20; 33.2.44), fat (AOAC, method number 989.05; 33.2.26 for milk; AOAC, method number 995.18; 33.3.18 for cream; and Marshall, 1993, number 15.8B for skim milk), lactose (AOAC, method number 984.15; 33.2.24), total nitrogen (TN; AOAC, method number 991.20; 33.2.11), and non-protein nitrogen (NPN; AOAC, method number 991.21; 33.2.12). Noncasein nitrogen (NCN; AOAC, method number 998.05; 33.2.64) was analyzed for the MF retentate and the raw cream. For the UF retentate and UF permeate from MF permeate, NCN was assumed to be equivalent to TN because no CN was present (based SDS-PAGE, data not shown). All nitrogen results were expressed as a protein equivalent using a conversion factor of 6.38. True protein (TP) and CN were calculated as (TN – NPN) × 6.38 and (TN – NCN) × 6.38, respectively. SP was calculated as the difference between TP and CN, and for the UF retentate and UF permeate from MF permeate, SP was equal to TP (i.e., CN = 0%).

Table 1. The ANOVA models used for the analysis of pH (0°C) and freezing point (FP) data for experiments 1 and 2.

Independent variable	df for pH	df for FP	Analyzed as	Error term
Replicate	1	1	Block	
Treatment ¹	2	2	Fixed effect	Replicate × treatment
Replicate × treatment	2	2	Interaction	
CO ₂ linear	1	1	Continuous variable	E
CO ₂ quadratic	1	...	Continuous variable	E
CO ₂ linear × treatment	2	2	Interaction	E
CO ₂ quadratic × treatment	2	...	Interaction	E
Error (E)	24	27		

¹For experiment 1, the treatment is serum protein concentration: 0, 3, and 6%. For experiment 2, the treatment is CN concentration: 2.4, 4.8, and 7.2%.

On d 4, the compositions of the ingredients were used to calculate the amount of each ingredient needed to achieve the desired level of CN, SP, and lactose in milks used in the current study. Six batches of milk (6 kg each) with different protein composition were prepared on d 4 and stored in 4-L plastic containers at 4°C. The actual composition of each milk was confirmed by analysis after preparation. Fat, TS, ash, calcium, and lactose were determined using, respectively, Mojonnier (AOAC, method number 989.05; 33.2.26), oven-drying (AOAC, method number 990.20; 33.2.44), ashing oven (AOAC, method number 945.46; 33.2.10), atomic absorption (Brooks et al., 1970), and enzymatic (AOAC, method number 984.15; 33.2.24) methods. The CN and SP contents were determined by the Kjeldahl methods as described above.

Carbonation and analysis. On d 5, milks were carbonated at 0 to 1°C in a lab-scale continuous flow inline CO₂ injection system (Ma and Barbano, 2003). At this injection temperature, any milk fat present in the sample would be mostly solid and would not dissolve CO₂ (Ma and Barbano, 2003), so any variation in the background fat content of the milk would not influence the results. The injection system was a countercurrent stainless steel tubular heat exchanger (internal diameter = 0.5 cm). The entire heat exchanger was cooled by circulating ice water (0 to 1°C). Milk was pumped through the system by a peristaltic pump (Amicon LP-1 pump, Beverly, MA with Cole-Palmer Masterflex® 7015-81 pump head, Vernon Hills, IL) and CO₂ (beverage grade) was injected into milk through a stainless steel tube (internal diameter = 0.08 cm) inserted through a Tee-fitting perpendicular to the milk flow immediately after the feed pump. The residence time of the milk in the heat exchanger was approximately 60 s. Six different carbonation levels ranging from 0 (control) to 1800 ppm were achieved by adjusting the flow rate of CO₂ while keeping the flow rate of milk constant (230 ml/min). After carbonation, milk was stored in 40-ml plastic vials (Capitol Vial Corp., Ful-

tonville, NY) overnight at 0 to 1°C to allow equilibration and analyzed on d 6 for pH (0°C), CO₂ concentration (Ma et al., 2001), and FP (AOAC, method number 990.22; 33.2.04). Freezing point was determined using a cryoscope (Model 4D3, Advanced Instruments, Inc., Norwood, MA). Before pH measurement, milk samples were left in ice water for at least 2 h. The pH probe (model HA 405 DXK-58/120 combination pH probe; Mettler Toledo, Columbus, OH) was calibrated with pH 7.13 and 4.01 buffers (Fisher Scientific, Fair Lawn, NJ) at 0 to 1°C.

Experimental Design and Statistical Analysis

Experiment 1: effect of SP concentration on skim milk pH and FP. The effect of SP concentration on pH (0°C) and FP of skim milk with various levels of added CO₂ (0 to 1800 ppm) was determined. The experiment was replicated two times using different batches of fresh milk. Three fat-free (ca., 0% fat), CN-free (0% CN) milks with increasing SP concentration (i.e., 0, 3, and 6%) were prepared, with the goal of having a constant background concentration of lactose. The three milks were designated as **SP0**, **SP3**, and **SP6**, respectively, and were formulated using only three ingredients: the UF retentate from MF permeate, the UF permeate from MF permeate, and lactose.

Each milk had a target concentration of 5.3% lactose in its fat-free, CN-free, and SP-free portion. For SP0, which had 0% fat, 0% CN, and 0% SP, its lactose content on a total sample weight basis was equal to 5.3%. The lactose concentrations in SP3 and SP6 on a whole sample weight basis were calculated to be, respectively, $5.3 \times (100 - 3)\% = 5.14\%$ and $5.3 \times (100 - 6)\% = 4.98\%$. Because of the water displacement effect due to the presence of SP, this formulation method kept the lactose concentration the same in the total background water phase of the three milks regardless of their SP concentration in the total sample. Each milk was carbonated at 0 to 1°C to contain six different CO₂ concen-

Table 2. Mean (n = 2) composition, initial CO₂ concentration, pH at 0°C, and freezing point (FP) of the three milks with increasing serum protein (SP) concentration in experiment 1.

Parameters	SP0	SP3	SP6
CO ₂ , ppm	40	38	38
pH at 0°C (lsd = 0.07)	6.74 ^c	6.87 ^b	6.94 ^a
FP, °H ¹ (lsd = 0.046)	-0.537	-0.557	-0.580
Total solids, % (lsd = 0.47)	6.03 ^c	8.98 ^b	11.90 ^a
Calcium, mg/100 g milk (lsd = 9.6)	27.6 ^c	43.9 ^b	59.2 ^a
Ash, % (lsd = 0.03)	0.47 ^c	0.56 ^b	0.64 ^a
NPN ² , % (lsd = 0.04)	0.19 ^b	0.22 ^{ab}	0.24 ^a
CN in the fat-free portion, %	0.00	0.00	0.00
SP in the fat-free, CN-free portion, % (lsd = 0.14)	0.05 ^c	3.02 ^b	5.98 ^a
Lactose in the fat-free, CN-free, and SP-free portion, % (lsd=0.29)	5.24	5.30	5.36

^{a,b,c}Means within the same row with different superscripts differ ($P < 0.05$).

¹°H = degrees Hortvet.

²NPN = nonprotein nitrogen $\times$ 6.38.

trations ranging from 0 (control) to 1800 ppm. The pH (0°C), CO₂ concentration, and FP of the three milks were determined.

Experiment 2: effect of CN concentration on skim milk pH and FP. The effect of CN concentration on pH (0°C) and FP of skim milk with various levels of added CO₂ (0 to 1800 ppm) was determined. The experiment was replicated two times using different batches of fresh milk. Three skim (ca., 0% fat) milks with increasing CN content in the fat-free portion (i.e., 2.4, 4.8, and 7.2%) were prepared. The milks were designated as **CN2.4**, **CN4.8**, and **CN7.2**, respectively, and were formulated using five ingredients: the MF retentate from skim milk, the UF retentate from MF permeate, the UF permeate from MF permeate, the raw cream, and lactose. The MF retentate from skim contained a low concentration of fat (e.g., 0.15 to 0.30%). Cream was used as an ingredient to keep the background fat level constant in all treatments. Each milk had a target concentration of 0.60% SP in its fat-free and CN-free portion and a target concentration of 5.3% lactose in its fat-free, CN-free, and SP-free portion. The CN2.4 milk simulated the composition of a typical skim milk. The calculation method used to set the formulation targets for SP and lactose concentrations on a whole sample weight basis in each milk was similar to the method described for experiment 1, except in this case, percent CN was considered in the calculation. Each milk was carbonated to contain six different CO₂ concentrations ranging from 0 (control) to 1800 ppm. The pH (0°C), CO₂ concentration, and FP of the three milks were determined.

Statistical analysis. When CO₂ is injected into cold milk, CO₂ does not dissolve in the solid fat portion of the milk, and the extent of pH reduction is related to the amount of CO₂ dissolved in the skim portion of the milk (Ma and Barbano, 2003). Therefore, for data analysis, the concentration of CO₂ in each milk was

standardized to the concentration of CO₂ in the fat-free portion by correcting for fat content, i.e., CO₂ in the fat-free portion = CO₂ in the whole milk sample/[1 minus (percent fat/100)] (Ma and Barbano, 2003). All of the CO₂ concentrations presented in this paper were CO₂ concentrations in the fat-free portion of milk.

The ANOVA models used for analysis of data from experiments 1 and 2 are shown in Table 1. The concentration of CO₂ was treated as a continuous variable. A quadratic term of CO₂ concentration was included in the ANOVA model for the pH data but not for the FP data, as suggested from previous research (Ma et al., 2001; Ma and Barbano, 2003). Because the concentration of CO₂ was treated as a continuous variable in the ANOVA model (Table 1), any model terms involving CO₂ concentration would be correlated. For example, the CO₂ linear and the CO₂ quadratic or the CO₂ quadratic and CO₂ quadratic $\times$ treatment would be correlated. Distortion of the ANOVA by multicollinearity of these terms was resolved by centering the CO₂ data using a mathematical transformation of the data for CO₂ concentration (Glantz and Slinker, 2001). The transformation was done by subtracting the mean CO₂ concentration from each of the individual CO₂ concentrations and using these "mean-centered" data in the statistical analysis. This type of linear transformation minimized correlation between the model terms involving CO₂ concentration but did not alter the mathematical nature of the statistical testing (Glantz and Slinker, 2001).

Regression analysis was used to produce equations to predict pH (0°C) and FP of milks in experiments 1 and 2 at various levels of CO₂. A quadratic term of CO₂ concentration was used in the regression for pH but not for FP. For estimation of the regression parameters (i.e., intercepts and slopes), the nontransformed CO₂ concentrations were used. All statistical analyses were done using SAS (Version 8.02, 1999-2001).

Table 3. Sum of squares (SS) and probabilities (*P*) from the ANOVA of pH (0°C) and freezing point (FP) data in experiment 1. The treatment was serum protein (SP) concentration: 0, 3, and 6%.

Independent variable	pH at 0°C SSM ¹ = 3.361, Model R ² = 0.997		FP SSM = 0.0280, Model R ² = 0.997	
	SS ²	<i>P</i>	SS	<i>P</i>
Replicate	0.039		0.0024	
Treatment (i.e., SP concentration)	0.518	<0.01	0.0144	<0.05
Replicate × treatment	0.002		0.0005	
CO ₂ linear	2.415	<0.01	0.0099	<0.01
CO ₂ quadratic	0.075	<0.01	...	...
CO ₂ linear × treatment	0.033	<0.01	0.0001	<0.01
CO ₂ quadratic × treatment	0.013	<0.01	...	...
Error (E)	0.011		0.0001	

¹SSM = Sum squares of model.²SS = Sum squares of individual factors.

RESULTS AND DISCUSSION

Experiment 1: Effect of SP Concentration on Skim Milk pH and FP

Composition. Mean (*n* = 2) compositions of SP0, SP3, and SP6 are shown in Table 2. The three milks had increasing SP content in the fat-free and CN-free portion but similar lactose content in the fat-free, CN-free, and SP-free portion, and similar FP (Table 2). NPN increased slightly with increasing SP content, but the differences were small (Table 2). A low level of measured SP content (0.05%, Table 3) in SP0 was probably caused by low molecular weight peptides present in the UF permeate and trace amounts of SP. The calcium present in SP0 was soluble calcium. The increase in calcium with increasing SP content reflected the additional calcium that was bound to SP (Table 2). With every 1% increase of SP concentration, the concentration of calcium increased about 5.3 mg/100 g milk. A similar level of SP-bound calcium was observed by Britten and Pouliot (1996). Due to increases in calcium and possibly other minerals that were bound to SP, increasing SP also led to an increase in ash content (Table 2).

Without carbonation, the concentration of CO₂ in SP0, SP3, and SP6 were similar (Table 2). In the noncarbonated milks, pH at 0°C increased with increasing SP concentration. Without protein, the major buffering components in milk are soluble phosphate (*pK*_a = 6.6) and bicarbonate (*pK*_a = 6.4; Singh et al., 1997).

pH at 0°C. Treatment (i.e., SP concentration) had a significant impact on milk pH at 0°C, and the ANOVA terms for treatment (*P* < 0.01), CO₂ linear × treatment (*P* < 0.01), and CO₂ quadratic × treatment (*P* < 0.01) were significant (Table 3). The effect of treatment can be seen in Figure 1: at the same CO₂ concentration, the extent of pH reduction was less in milk with a higher

Figure 1. pH (0°C) of milks with 0% (■, □), 3% (●, ○), and 6% (▲, △) serum protein upon CO₂ addition. The solid and open symbols represent data from replication 1 and 2, respectively. The dotted lines are the least-square second order polynomial regression lines for each serum protein level.

Table 4. Regression coefficients for the prediction of pH (0°C) and freezing point (FP) upon carbonation for milks with three serum protein (SP) levels (i.e., SP0, SP3, and SP6) and the three casein levels (i.e., CN2.4, CN4.8, and CN7.2). X = concentration of CO₂ in ppm.

	SP					
	pH at 0°C			FP, °H ¹		
	SP0	SP3	SP6	SP0	SP3	SP6
Intercept	6.77	6.90	6.96	-0.5348	-0.5541	-0.5763
X	-11.30×10^{-4}	-7.91×10^{-4}	-6.66×10^{-4}	-2.93×10^{-5}	-3.47×10^{-5}	-3.94×10^{-5}
X ²	3.40×10^{-7}	1.75×10^{-7}	1.30×10^{-7}	...	...	...
R ²	0.99	0.99	0.99	0.98	0.99	0.99
	Casein					
	pH at 0°C			FP, °H		
	CN2.4	CN4.8	CN7.2	CN2.4	CN4.8	CN7.2
Intercept	7.01	7.00	6.99	-0.5463	-0.5572	-0.5658
X	-7.52×10^{-4}	-5.88×10^{-4}	-4.72×10^{-4}	-4.19×10^{-5}	-4.55×10^{-5}	-4.82×10^{-5}
X ²	1.8×10^{-7}	1.17×10^{-7}	0.70×10^{-7}	...	...	...
R ²	0.99	0.99	0.99	0.99	0.99	0.99

¹°H = degrees Hortvet.

SP concentration. The resistance to pH change in a higher SP milk demonstrated its higher buffering capacity.

SP0 consisted primarily of water, lactose, and soluble milk salts (Table 2). Thus, the buffering components in SP0 were mainly soluble milk salts, such as phosphate and bicarbonate. Increasing SP concentration to 6% enhanced the ability of milk to resist pH change upon CO₂ addition at 0°C (Figure 1). The increase in the ability of milk to resist pH change or the increase in buffering capacity was more when SP was increased from 0 to 3% than when SP was increased from 3 to 6% (Figure 1). Second-order polynomial regression lines predicting pH (0°C) for SP0, SP3, and SP6 at different CO₂ concentrations are plotted in Figure 1, and the regression coefficients are shown in Table 4.

Freezing point. The ANOVA (Table 3) found a significant influence of treatment (i.e., SP concentration) and CO₂ linear by treatment interaction, indicating a significant difference in FP depression among SP0, SP3, and SP6 upon CO₂ addition. The decrease in FP upon CO₂ addition was a linear effect, and the impact on FP was greater at a higher SP concentration (Table 4 and Figure 2). In contrast to the direction of pH change with increasing SP content, at the same CO₂ concentration, the extent of FP decrease was greater in milk with a higher SP content. Regression coefficients shown in Table 4 were calculated using the observed FP. In Figure 2, only the change of FP was plotted, that is, each regression line was shifted to have a zero intercept on the y-axis while keeping the slope the same (Ma et al., 2001). The reason for doing such correction was that only the slope of the regression line or the change of FP was of interest. Increasing SP concentration resulted in

a more negative slope for change of FP upon CO₂ addition (Table 4 and Figure 2).

FP is a colligative property of a solution and is directly related to the molality of all the solute particles present in the solution. In SP0, no CN, SP, or protein-bound minerals were present. The FP decrease in SP0 would be contributed by (1) the solubilization of CO₂ and the dissociation of its hydrated form, carbonic acid (H₂CO₃) into H⁺, HCO₃⁻, and CO₃²⁻ (only trace amount at milk pH) and (2) possible increase in soluble solute concentration due to shifts in the degree of association of soluble mineral aggregates. The more negative slopes for FP depression observed for the SP3 and SP6 compared with SP0 (Figure 2 and Table 4) could be due to the release of SP-bound minerals, such as calcium, into the serum phase and an increase in the formation and dissociation of carbonic acid at a constant CO₂ concentration.

Experiment 2: Effect of CN Concentration on Skim Milk pH and FP

Composition. Mean (n = 2) compositions of CN2.4, CN4.8, and CN7.2 are listed in Table 5. The three milks had increasing CN concentration but similar SP content (ca. 0.6%) in the fat-free, CN-free portion, similar lactose content (ca. 5.25%) in the fat-free, CN-free, SP-free portion, and similar FP (Table 5). In the CN2.4 milk, the calcium (104.2 mg/100 g milk) and the ash (0.71%) contents were comparable to those of skim milk (Swaisgood, 1985). As would be expected, increasing CN content resulted in an increase in calcium and ash content in milk (Table 5). For every 1% increase of CN concentration, the concentration of calcium increased

Figure 2. Change of freezing point (FP) in milks with 0% (■, □), 3% (●, ○), and 6% (▲, △) serum protein upon CO₂ addition. The solid and open symbols represent data from replication 1 and 2, respectively. The dotted lines are the least-square linear regression lines for each serum protein level.

about 32.7 mg/100 g milk. Typically, the total calcium concentration in milk is 30 mM, of which 20 mM is associated with casein micelle (Holt, 1997). Assuming a 2.4% CN concentration in milk (Swaisgood, 1985), CN-bound calcium is estimated to be about 33 mg/g CN. Therefore, the increase in calcium in milk was mostly contributed by colloidal calcium bound to CN. The calcium that was bound to CN was about 6.1 times the amount of calcium that was bound to SP (i.e., 5.3 mg/g SP).

Without carbonation, the pH (0°C) of CN2.4 (pH 6.97), CN4.8 (pH 6.96), and CN7.2 (pH 6.96) were similar (Table 5). Due to the presence of ionizable side groups and bound minerals, milk proteins, both CN and SP, can raise the pH of a UF permeate. The pH of the UF permeate from skim milk with no SP or CN was 6.74 at 0°C (Table 2). The pH of milk permeate will

increase to an equilibrium level at which a further increase of milk protein concentration will not cause a further increase in milk pH. The equilibrium pH was probably already reached for CN2.4 and, therefore, increasing CN content further did not affect milk pH at 0°C (Table 5). This was in contrast to observations from experiment 1, where increasing SP from 0 to 6% continuously increased pH to a level similar to the pH of CN2.4 (Table 2). The protein concentration at which an equilibrium pH was reached in milk depended on the type of protein being added. This is not surprising because of the differences in protein ionizable side groups and the differences in protein bound minerals between CN and SP.

pH at 0°C. The ANOVA found (Table 6) a significant influence of treatment (i.e., CN concentration) on the pH of milks with different CO₂ concentrations. Statistical terms (Table 6) treatment, CO₂ linear $\times$ treatment, and CO₂ quadratic $\times$ treatment were all significant ($P < 0.01$). The effect of CN concentration can be seen in Figure 3: at the same CO₂ concentration, the extent of pH reduction was less in milk with a higher CN concentration. This is similar to the effect observed for SP concentration. A milk with a higher CN concentration showed a greater resistance to pH change, indicating its higher buffering capacity. The higher the CO₂ concentration, the greater the difference in pH (i.e., significant interaction effects) among CN2.4, CN4.8, and CN7.2 (Figure 3).

Casein is a major contributor to the buffering capacity of milk (Srilaorkul et al., 1989; Lucey et al., 1993), thus increasing CN concentration is expected to increase the buffering capacity of milk. Increasing CN concentration also leads to higher concentration of CCP that is associated with the native CN micelles. The presence of CCP is unique to CN. Decreasing milk pH causes a progressive solubilization of micellar CN, CCP, and other colloidal salts from CN micelles into the serum phase (Dagleish and Law, 1989; Law and Leaver, 1998). Dissociated CN and colloidal salts in the water phase of milk provide negatively charged groups, such as phosphates, that can be combined with hydrogen ions produced by the dissociation of H₂CO₃, further providing resistance to the pH reduction effect of CO₂.

Freezing point. Treatment (i.e., CN concentration) had a significant effect on milk FP, and the statistical terms (Table 6) for treatment and CO₂ linear $\times$ treatment were significant ($P < 0.01$). FP decreased linearly with increasing CO₂ concentration ($P < 0.01$), and at the same CO₂ concentration, FP depression was greater for milk with a higher CN concentration (Figure 4). The regression slope for CN2.4 was similar to a previously reported value for skim milk (-4.207×10^{-5} , Ma and Barbano, 2002). Increasing CN content produced a

Figure 3. pH (0°C) of milk with 2.4% (■, □), 4.8% (●, ○), and 7.2% (▲, △) CN upon CO₂ addition. The solid and open symbols represent data from replication 1 and 2, respectively. The dotted lines are the least-square second order polynomial regression lines for each CN level.

more negative slope for FP depression upon CO₂ addition (Table 4 and Figure 4).

With the lowering of milk pH due to CO₂ addition, micellar calcium-phosphate and other colloidal salts begin to dissociate, contributing to an increased concentration of solute in the serum phase of milk (Dalgleish and Law, 1989; Gevaudan et al., 1996). Based on results from both experiments 1 and 2, in CN2.4, CN4.8, and CN7.2, the decrease of FP could be contributed by four sources of increasing solute concentration: (1) carbonic acid and its dissociation products, (2) shifts in the degree of association of soluble mineral aggregates, (3) solubilized SP-bound minerals, and (4) solubilized CN-associated CCP and other colloidal minerals.

Figure 4. Change of freezing point (FP) in milks with 2.4% (■, □), 4.8% (●, ○), and 7.2% (▲, △) CN upon CO₂ addition. The solid and open symbols represent data from replication 1 and 2, respectively. The dotted lines are the least-square linear regression lines for each CN level.

Impact of the Aqueous Phase Properties on Milk pH and FP

Protein type. Both the SP3 and CN2.4 had 3% TP in the fat-free portion of the milk (Tables 2 and 5). Instead of 3% SP in SP3, there were 2.4% CN plus 0.6% SP in CN2.4. The higher calcium and ash content in the CN2.4 milk compared with the SP3 milk was expected because of the higher level of bound minerals associated with CN. Upon carbonation, CN2.4 exhibited more ($P < 0.05$) resistance to pH decrease than SP3 (Figures 1 and 3). Therefore, the buffering capacity of CN2.4 was greater than that of SP3, even though the two milks had the same TP content (i.e., 3%). Upon carbonation, there was a greater ($P < 0.05$) extent of FP depression for the CN2.4 milk than the SP3 milk as reflected by a more negative slope for the FP depression curve for the CN2.4 milk (Figures 2 and 4). Thus, with increasing CO₂ concentration, the increase in solute concentration

Table 5. Mean ($n = 2$) composition, initial CO₂ concentration, pH at 0°C, and freezing point (FP) of the three milks with increasing CN concentration in experiment 2.

Parameters	CN2.4	CN4.8	CN7.2
CO ₂ , ppm	64	66	66
pH at 0°C (lsd = 0.03)	6.97	6.96	6.96
FP, °H ¹ (lsd = 0.038)	-0.551	-0.561	-0.570
Total solids, % (lsd = 2.70)	9.86 ^b	12.35 ^{ab}	14.80 ^a
Calcium, mg/100 g milk (lsd = 9.99)	104.2 ^c	178.7 ^b	252.5 ^a
Ash, % (lsd = 0.08)	0.71 ^c	0.93 ^b	1.14 ^a
NPN ² , %	0.20	0.20	0.20
CN in the fat-free portion, % (lsd = 0.09)	2.40 ^c	4.71 ^b	6.99 ^a
SP in the fat-free, CN-free portion, % (lsd = 0.29)	0.60	0.60	0.64
Lactose in the fat-free, CN-free, and SP-free portion, % (lsd = 0.22)	5.27	5.25	5.24

^{a,b,c}Means within the same row with different superscripts differ ($P < 0.05$).

¹°H = degrees Hortvet.

²NPN = nonprotein nitrogen $\times$ 6.38.

in the skim portion of milk was greater in CN2.4 than in SP3. Changes in buffering capacity and mineral content due to a change in protein type at the same protein concentration had an influence on the extent of pH decrease and FP depression produced in milk when CO₂ was added.

Validity of using pH as an estimate of CO₂ concentration. Currently, low levels of CO₂ are added to some dairy foods to inhibit microbial growth. The antimicrobial effect of CO₂ is related to the amount of CO₂ dissolved in the aqueous phase of a food (Fernandez et al., 1997; Devlieghere et al., 1998) and is independent of the pH reduction effect of CO₂ (King and Mabbitt, 1982). The correlation between milk pH and CO₂ concentration has been used as an indirect method for CO₂ determination in milk (King and Mabbitt, 1987). Such correlation is only valid for milks with a similar fat content and a similar skim portion composition. The temperature of CO₂ injection, milk fat content, and milk fat type can affect how much of the total dissolved CO₂ will be dissolved in the skim portion of milk (Ma and

Barbano, 2002). Once CO₂ is dissolved in the skim portion, the buffering capacity of the components in the skim portion will affect the extent of pH decrease (Figures 1 and 3). Therefore, when the buffering capacity of the skim portion of milk is changed, either by an increase of protein concentration or by an alteration of CN to SP ratio, a simple pH measurement does not provide an accurate estimate of the amount of CO₂ dissolved in the skim portion. Therefore, if CO₂ is going to be used in a dairy food as an antimicrobial agent and a specific target concentration of CO₂ is needed, then CO₂ concentration should be measured directly and not estimated by decrease in pH.

Using CO₂ as a processing aide. In addition to the use of CO₂ as an antimicrobial agent, another application of the CO₂ technology in the dairy industry could be the use of CO₂ as an acidulant to decrease milk pH and to release protein bound minerals, such as calcium, into the serum phase. For example, preacidification of milk with CO₂ in cheesemaking has been explored (McCarney et al., 1995; St-Gelais et al., 1997; Cham-

Table 6. Sum of squares (SS) and probabilities (P) from the ANOVA of pH (0°C) and freezing point (FP) data in experiment 2. The treatment was CN concentration: 2.4, 4.8, and 7.2%.

Independent variable	pH at 0°C SSM ¹ = 1.613 Model R ² = 0.996		FP SSM = 0.0285, Model R ² = 0.996	
	SS ²	P	SS	P
Replicate	0.025		0.0015	
Treatment (i.e., CN concentration)	0.061	<0.01	0.0036	<0.05
Replicate $\times$ treatment	<0.001		0.0001	
CO ₂ linear	1.493	<0.01	0.0200	<0.01
CO ₂ quadratic	0.037	<0.01	...	...
CO ₂ linear $\times$ treatment	0.014	<0.01	0.0001	<0.01
CO ₂ quadratic $\times$ treatment	0.013	<0.01	...	...
Error (E)	0.007		0.0001	

¹SSM = Sum squares of model.

²SS = Sum squares of individual factors.

pagne et al., 1998). Using CO₂ as a processing aide is promising because once the desired and irreversible changes in milk are achieved, CO₂ can be removed from the final product by applying a vacuum. In the case of cheesemaking, CO₂ is simply lost during further processing. Another attractive aspect for using CO₂ is that CO₂ is a GRAS (Generally Recognized As Safe) food additive (21CFR184.1240, 2001).

Upon CO₂ addition, there is a solubilization of protein-bound minerals as a result of pH reduction, and an increase in the level of solubilized minerals can either cause problems or create benefits during dairy processing, depending on the situation. For example, a lower pH and an increased soluble calcium of milk due to CO₂ addition may increase fouling in a heat exchanger during pasteurization of milk. Decreased pH and increased SP concentration have been shown to increase the extent of thermal aggregation, especially β -LG and CN during heating (Smits and van Brouwershaven, 1980; Corredig and Dalgleish, 1996; Beaulieu et al. 1999). Heating-induced association between SP and CN modifies the physiochemical properties of the CN micelles, and the modified CN micelles may exhibit different behavior in dairy food systems compared with the native CN micelles.

As the dairy industry starts to bring various processing technologies and ingredients together to create novel products, it is important to understand and control the behavior of milk-derived beverages with altered protein level, protein type, and mineral content created through membrane technology. The knowledge gained in the current study may facilitate future research on CO₂ as a processing aide to achieve favorable changes in the physiochemical properties of dairy foods.

CONCLUSIONS

Increasing SP or CN content increased milk buffering capacity and protein-bound mineral content. At the same added CO₂ concentration at 0°C, a milk with a higher SP or a higher CN content had more resistance to pH change and a greater extent of FP depression. At constant TP content, milk with a higher proportion of CN had a higher buffering capacity and provided more resistance to pH decrease and a larger decrease in FP when CO₂ was added at 0°C. Once CO₂ was dissolved in the skim phase of a milk, the extent of pH reduction and FP depression depended on protein concentration and protein type (i.e., CN and SP).

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