Modification of the Kjeldahl noncasein nitrogen method to include bovine milk concentrates and milks from other species¹

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ABSTRACT

The objective of our research was to modify the current indirect casein method for bovine milk to enable it to be applied to bovine milk, bovine milk concentrates, and milks of other species that contain a protein concentration up to 9% (wt/wt). Our work used a series of bovine milk concentrates from about 3 to 9% protein with the same casein as a percentage of true protein to determine the amount of buffer required and pH of the noncasein nitrogen (NCN) filtrate to achieve consistent estimates of casein and casein as percent of true protein. As the concentration of protein in milk increased (either in bovine milk concentrates or in milks of other species), the amount of buffer needed for the NCN sample preparation method to achieve a filtrate pH of 4.6 increased. In the first part of the study using a series of bovine milk concentrates, it was demonstrated that the method gave more consistent predictions of casein as a percentage of true protein when the final NCN filtrate pH was between 4.5 and 4.6 at 38°C. When the amount of buffer added to the sample was not sufficient (i.e., the filtrate pH was too high), the filtrates were not clear. A polynomial equation was developed for prediction of the amount of acetic acid or sodium acetate buffer required to achieve pH 4.5 to 4.6 for milk protein concentrations from 3 to 9% protein using bovine milk and milk concentrates. When the equation developed using cow milk was applied to goat, sheep, and water buffalo milks, it correctly predicted the volume of reagents needed to achieve a final NCN filtrate pH of 4.6 at 38°C. We also verified as part of this work that the ability to measure NPN content of milk was not influenced by protein content of milk in the range

from 3 to 9% protein. The results of this study will be used as the basis for proposed changes in the official methods for measurement of the casein content of milk to expand the scope of the method so it can be used to achieve accurate results for milk concentrates and milks of other species.

Key words: casein, Kjeldahl, noncasein nitrogen, nonprotein nitrogen

INTRODUCTION

In 1938, Samuel J. Rowland published a paper (Rowland, 1938a) describing the analytical procedures for fractionation, measurement, and classification of N-containing compounds in milk. The fractions that he defined were total nitrogen (TN), noncasein nitrogen (NCN), NPN, proteose-peptone, and globulin N. The methods described by Rowland (1938a,b) were used with good results for the determination of the N distribution in a large number of samples of normal and abnormal bovine milk. The TN, NCN, and NPN are the main N fractions for the determination of N distribution in bovine milk used commonly today. The NCN method uses 2 solutions (acetic acid and sodium acetate) which, when combined, form an acetate buffer. However, Rowland (1938a,b) did not combine the 2 solutions and then add them to milk. The results of Rowland (1938a,b) indicated that it was important to lower the pH of the milk and water mixture beyond 4.6 with the addition of acetic acid, and then after a short incubation period at 38°C the acetate solution was added to bring the pH back up to approximately 4.6. The 2-step addition allows the pH to go below the isoelectric point with acetic acid addition, which provides ruggedness to the method to accommodate milks of other species with slightly lower isoelectric points of CN than bovine milk. Rowland (1938a,b) analyzed only bovine milks and used a fixed volume of the buffer solution with a 10-mL milk sample. This is reflected in the current International Dairy Federation (IDF, 2004, method 29–1) and AOAC International methods (AOAC International, 2010, methods numbers 991.20,

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991.21, and 998.05) for indirect measurement of casein N content of milk. Need exists in the global dairy industry for a modified NCN method that works bovine milks, milks of other species (e.g., sheep, goat, and water buffalo), and bovine milk concentrates. Recent work with milk protein concentrates has demonstrated that more buffering capacity of the reagents is needed (Zhang and Metzger, 2011). It is likely that milks with higher protein content will require a different amount of buffer to achieve the optimum pH for preparation of an NCN filtrate that is free of CN. The objective of our research was to modify the current indirect CN method for bovine milk to enable it to be applied to bovine milk, bovine milk concentrates, and milks of other species that contain a range of protein concentration up to 9%.

MATERIALS AND METHODS

Experimental Design and Statistical Analysis

The study was conducted in 2 phases. In phase I, 4 bovine milk protein concentrations (from about 3 to 9%) in combination with 6 different buffer concentrations at constant buffer volume addition in the NCN method were evaluated to determine the effect of buffer concentration on filtrate pH, clarity, and NCN content with milks with a wide range of protein content. All percentages reported in the current paper are weight/weight unless otherwise stated. This was replicated 3 times. In phase I, we encountered difficulties keeping the highest concentration of sodium acetate in solution during refrigerated storage, which prevented us from exploring higher buffer concentrations to achieve the target pH of the NCN filtrate. Therefore, we used a different strategy in phase II of our work. We increased the volume of addition of the low-concentration buffer that is specified in the original NCN method to progressively increase the buffering capacity used for precipitation of the CN for higher-protein samples instead of increasing buffer concentration. In phase II, 3 different volumes of buffer addition were used, one that equaled the buffering capacity of the highest buffer concentration used in phase I, and 2 volumes that exceeded the highest buffer concentration used in phase I. In phase II, the performance of the NCN method was evaluated for bovine milk and milk concentrates from about 3 to 9% protein, sheep, water buffalo, and goat milks using 3 different volumes of buffer addition.

To determine if milk protein and buffer concentration had an effect on estimated NCN content, casein as a percentage of TP (**CN%TP**), and NCN filtrate pH, an ANOVA using the Proc GLM procedures of SAS (SAS version 8.02, SAS Institute Inc., Cary, NC)

was conducted using a split-plot model. Milk protein concentration and replicate were category variables in the whole plot, whereas buffer concentration was used as a continuous variable with linear, quadratic, and cubic terms for buffer concentration and their interaction with whole plot terms as the subplot. The milk by replicate interaction was used as the error term to test the significance of terms in the whole plot whereas the model error term was used to test for significance of all the other terms in the model. Distortion of the ANOVA by multicollinearity in the model was minimized by mean centering the buffer concentration using a mathematical transformation (Glantz and Slinker, 2001). Buffer concentration was transformed as follows: $\text{buffer concentration} = \text{buffer concentration} - [(\text{highest buffer concentration} - \text{lowest buffer concentration})/2]$. This mathematical transformation made the data set orthogonal with respect to buffer concentration.

Current Status of the NCN Method for CN Determination

The official methods for measurement of NCN in both IDF and AOAC are based on the principles of the Rowland (1938a,b) method for fractionation of the N-containing compounds of milk and the collaborative study of the NCN method by Lynch et al. (1998). The current method uses a 10-mL milk test portion at $38 \pm 1^\circ\text{C}$ that is diluted with 75 mL of water at $38 \pm 1^\circ\text{C}$. To the diluted milk, 1 ± 0.02 mL of 10% acetic acid was added to lower the pH below 4.6; then, the mixture was swirled and held for 10 min at $38 \pm 1^\circ\text{C}$. After 10 min, 1 ± 0.02 mL of 1 N sodium acetate solution is added to bring the pH back up to about 4.6. Lynch et al. (1998) discussed the use of a correction factor in the calculation of the casein result to take into account the volume occupied in the milk by the CN and fat removed from the NCN filtrate. In the present study, we measured the fat content of the milks by ether extraction (AOAC International, 2010; method 989.05) and calculated the applicable correction factor for each sample.

It was assumed that buffer capacity was sufficient with these two 1-mL additions for bovine milks of all protein levels; the principle goes back to the original work by Rowland (1938a,b). Prior to the collaborative study conducted by Lynch et al. (1998), ruggedness testing was not done to determine the range of protein over which the buffer concentration specified in the method was adequate because the NCN method was a long-standing official method and the primary purpose of the collaborative study was to develop the new direct CN method that would produce results that would agree with the more traditional indirect method of CN determination on bovine milk. Recent interest

in the application of the direct and indirect CN determination methods (Lynch et al., 1998) to milks from other species and bovine milk concentrates that contain higher protein content has raised a question about the accuracy of the NCN method when used on milks with higher protein contents. Nelson and Barbano (2005) recognized that higher protein concentration in micro-filtration retentates caused cloudy NCN filtrates when a 10-mL sample size was used and found 1 mL of the acetic acid and sodium acetate solutions specified in the method were not sufficient for high-protein milk concentrates. They reduced the sample size of the milk protein concentrate used to deliver about the same amount of protein in a test portion as there would have been in 10 mL of average bovine milk. In the present study, we will determine the buffering capacity needed to produce a filtrate pH of 4.6 and achieve accurate NCN tests for milks from 3 to 9% protein using a 10-mL test portion of milk or milk concentrates.

Milks Used in the Study

Phase I: Bovine Milk Protein Concentrates (About 3 to 9%). Raw whole bovine milk was pasteurized with a plate heat exchanger (Model 080-S, AGC Engineering, Manassas, VA) at 72°C and a holding time of 16 s. The pasteurized milk was cooled to 50°C and run through a centrifugal cream separator (model 619, DeLaval, Poughkeepsie, NY) to produce skim milk with a fat content of about 0.07%. The raw milk was pasteurized to reduce bacteria count and reduce bacterial growth that could occur during subsequent UF processing at 50°C. Minimum pasteurization time and temperature were used to minimize heat denaturation of whey proteins and binding to CN micelles (Lynch et al., 1998). The pasteurization was expected to cause a small increase in CN as a percentage of true protein, but that would be consistent in milk across all protein levels. The 50°C skim milk was fractionated using a pilot-scale UF system equipped with a polyethersulfone spiral wound membrane (model 3838, Gea Niro Inc., Hudson, WI; nominal separation cutoff: 10,000 Da, surface area: 6.8 m²) at 50°C. The inlet pressure was 276 kPa and the retentate outlet pressure was 103 kPa with no back pressure on the permeate side. Immediately before UF processing, the spiral-wound UF membrane system was given a short cleaning cycle, as described elsewhere (Evans et al., 2009). Approximately 320 kg of skim milk was processed with the UF system in batch recirculation mode to a concentration factor of 3× (about 9% true protein). After processing, the UF system was cleaned as described elsewhere (Evans et al., 2009). Approximately 2 L of UF permeate (about 0.03% true protein) and 2 L of UF retentate (about

9% true protein) were collected. The 3× UF retentate was diluted with UF permeate to obtain a series of 4 milks that contained approximately 3, 5, 7, and 9% protein. Because all 4 milks originated from the same milk source within each replicate of our experiment, the CN as a percentage of true protein in all 4 milks should be very similar within a replicate. The preparation of milk concentrates was replicated 3 times in different weeks starting with a different batch of raw whole milk.

Phase II: Bovine Milk Concentrates, Sheep, Water Buffalo, and Goat Milks. Bovine milk formulations were made up in the same manner as described above for phase I. Raw sheep, water buffalo, and goat milks were collected at farms in New York State. The sheep were of the East Friesian breed. The water buffalo were predominantly Riverine water buffalo. The goats were Toggenburg, Nubian, and Alpine breeds. The milks were collected at each farm (about 1 L per farm or group of animals), transported to the laboratory on ice, and analyzed within 1 or 2 d of arrival at the laboratory. The 4 sheep milks used in the study were collected from 2 different farms in different weeks. The goat milks were from 4 different farms collected on the same day. The 4 water buffalo milks were from 1 farm and each milk represented a blend from 4 different water buffalo collected on the same day. The water buffalo in different groups were at different stages of lactation. All of the samples for NCN analysis within a species were prepared on the same day and analyzed as a single batch.

Buffers Used in the Study

Preliminary Evaluation of Buffer Concentrations. Preliminary work was conducted to determine the range of buffer concentrations that would be needed to provide enough buffering capacity to analyze bovine milk concentrates with protein content up to about 9% protein using the NCN method to measure casein (TN – NCN). Six buffering capacities were achieved by using 1 mL each of the following pairs of solutions: 1.75 (10%) and 1 mol/L, 2.98 (17%) and 1.7 mol/L, 4.20 (24%) and 2.4 mol/L, 5.43 (31%) and 3.1 mol/L, 6.65 (38%) and 3.8 mol/L, and 7.88 (45%) and 4.5 mol/L of acetic acid and sodium acetate solutions, respectively. These were added to the milk plus water mixtures with increasing protein concentrations. A control test was also done to determine the pH of water when 1 mL of each of these solutions was added.

Phase I: Buffer Concentrations. Based on the results of the preliminary work, it was determined that 6 buffer concentrations would be used for phase I of the experiment ranging from buffer concentration of 1.75 (10% vol/vol), 2.98 (17% vol/vol), 4.20 (24% vol/vol),

5.43 (31% vol/vol), 6.65 (38% vol/vol), and 7.88 mol/L (45% vol/vol) for the acetic acid solution and 1.00, 1.70, 2.40, 3.10, 3.80, and 4.50 mol/L for the sodium acetate solution. After 3 replicates with milks of 3 to 9% protein were completed in phase I using the 6 buffer concentrations it was observed that, even with the highest buffer concentration, we were not consistently able to attain an NCN filtrate pH lower than 4.6 (reported below) for the highest protein concentration (9%). It was impractical to use concentrations of sodium acetate higher than 4.5 mol/L because of crystallization when the reagent was stored in the refrigerator. Once the sodium acetate solution crystallized, it was very difficult to redissolve. Therefore, we decided to conduct phase II of the study using increasing volumes of addition of the 10% acetic acid and 1 *N* sodium acetate solutions specified in AOAC International (2010; 998.05; 33.2.64) and IDF (2004; 29:2004) NCN method instead of increasing acetic acid and sodium acetate concentrations with a 1-mL addition.

Preliminary Evaluation of Buffer Volumes.

To determine the buffer volumes needed to attain the buffering capacity necessary to achieve an NCN filtrate pH of 4.6 for bovine milks with protein concentrations up to 9%, we chose 8 different volumes each (1, 1.7, 2.4, 3.1, 3.8, 4.5, 5.2, and 5.9 mL) of 1.75 mol/L (10% vol/vol) of glacial acetic acid and 1.0 mol/L of sodium acetate to be added to the 4 milk protein concentrations.

Phase II: Buffer Volumes. Based on the results of phase I and the preliminary evaluation of buffer volumes, we chose 4.5, 5.5, and 6.5 mL each of 1.75 mol/L (10% vol/vol) of acetic acid and 1.0 mol/L of sodium acetate for phase II of the study to achieve a higher buffering capacity in the method than we achieved in phase I. The 4.5-mL addition of acetic acid solution and sodium acetate solution was equivalent to the 1-mL addition of the highest buffer concentration used in phase I. There were modifications made to the AOAC International (2010) NCN method for the volume portion of the study. Reduced amounts of water were used to compensate for the larger volume of the acetic acid and sodium acetate solutions added. The water volume added for buffer volume 1 (4.5 mL of buffer) was 68 mL of water, volume 2 (5.5 mL of buffer) was 66 mL of water, and volume 3 (6.5 mL of buffer) was 64 mL of water. Class A serological pipets were used to deliver the amount of acetic acid and sodium acetate and a graduated cylinder was used to measure the volume of water added. The blank volumes used for our study were: buffer volume 1 used 2.25 mL for each reagent and 45.5 mL of water, buffer volume 2 used 2.75 mL for each reagent and 44.5 mL of water, and buffer volume 3 used 3.25 mL of each reagent and 43.5 mL of water.

Measurement of N Fractions

Total, NCN, and NPN. The TN fraction was determined by macro-Kjeldahl (IDF, 2014; 20–1:2014; AOAC International, 2010; method 991.20), as described by Barbano et al. (1991). The NPN was measured using (AOAC International, 2010; method 991.21), as described by Barbano et al. (1991). The 0.01 *N* HCl used for the titration of NPN was made by dilution from the 0.1 *N* HCl used to titrate the TN fraction. Digestions and distillations were conducted using a traditional Labconco Kjeldahl apparatus (model# 21178, Labconco, Kansas City, MO) with adjustable heaters for individual flasks. The digestion flasks had a total capacity of 500 mL and a 50-mL class A buret was used after distillation for titrations. Reagents used in our study were nitrogen-free 95 to 98% H₂SO₄ (A300, Certified ACS, Fisher Scientific, Fairlawn, NJ), nitrogen-free CuSO₄·5H₂O (C493, Certified ACS, Fisher Scientific) as 0.05 g/mL of H₂O solution, nitrogen-free K₂SO₄ (Alfie Packers, Omaha, NE), 50% NaOH solution (wt/wt) and nitrate-free (SS410, Certified ACS, Fisher Scientific), methyl red (M296-free acid, color index number 13020, Certified ACS, Fisher Scientific) and bromocresol green (B383, Certified ACS, Fisher Scientific) indicator solution (0.2% wt/vol methyl red and 0.2% wt/vol bromocresol green), 4% aqueous H₃BO₄ (A73, Certified ACS, Fisher Scientific) with 6 mL of indicator solution added per liter of 4% boric acid, and HCl standard solution 0.1000 *N* (SA54–20, Certified 0.0995 to 0.1005 *N*, Fisher Scientific). Quality control and evaluation of the Kjeldahl method performance was done as described by Lynch and Barbano (1999).

Measurement of pH of NCN Filtrates. To determine if the filtrates from the NCN method achieved a pH of 4.6 (isoelectric point of CN) or lower in both phase I and II, we collected all filtrates, removed the 50-mL portion that was added to a Kjeldahl flask for NCN analysis, and the remainder of each filtrate was poured into a 90-mL snap-lid plastic vial (Capitol Plastic Products, Fultonville, NY). Filtrates were observed for cloudiness or particulate matter. Vials containing filtrate were placed into an agitating water bath at 38 ± 1°C. The pH was measured using a glass AccuTupH⁺ probe (model 13–620–185, Fisher Scientific) and an Accumet 925 pH meter (Fisher Scientific) and was calibrated with a pH 7 and 4 buffer held in a water bath at 38 ± 1°C using the buffer manufacturers' temperature corrections for buffer pH at 38°C. The pH electrode was immersed in 3 *M* KCl solution at 38°C between readings to maintain the electrode at the same temperature as the samples to be measured. This provided more rapid electrode response and more stable readings.

Table 1. Effect of increasing buffer concentration on final pH

Buffer number	Buffer [mol/L (% vol/vol)]		pH	
	Acetic acid	Sodium acetate	Acetic acid and water	Acetic acid, sodium acetate and water
1	1.75 (10%)	1.00	3.28	4.49
2	2.98 (17%)	1.70	3.15	4.48
3	4.20 (24%)	2.40	3.05	4.48
4	5.43 (31%)	3.10	2.99	4.47
5	6.65 (38%)	3.80	2.95	4.47
6	7.88 (45%)	4.50	2.92	4.48

Measurement of Residual CN in NCN Filtrates. Proteins in the NCN filtrate were identified by reducing SDS-PAGE. A microfluidic chip-based electrophoresis system (Experion Automated Electrophoresis Station, Bio-Rad Laboratories Inc., Hercules, CA) and protein testing kit (Experion Pro260 Analysis Kit, Bio-Rad Laboratories Inc.) were used to determine the relative quantities of α_{s1} -CN, β -CN, α_{s2} + κ -CN, α -LA, β -LG, BSA, and lactoferrin. Between 85 and 90% of the total peak area in each electropherogram was accounted for by the sum of these 7 protein fractions. All reagents were supplied by Bio-Rad Laboratories Inc. and included in the Pro260 Analysis Kit, unless otherwise noted.

The Pro260 gel and fluorescent gel stain were prepared according to the manufacturer's instructions. The reducing buffer was prepared by combining 30 μ L of the Pro260 kit sample buffer with 1 μ L of β -mercaptoethanol. The buffer contains lithium dodecyl sulfate, which binds to the proteins. The fluorescent gel stain, in turn, binds to the lithium dodecyl sulfate, fluoresces in proportion to the amount of protein present, and is detected by the Experion system. Each sample was diluted with deionized water to a true protein concentration of 0.09%, based on Kjeldahl data, then 4 μ L of the diluted sample and 2 μ L of the reducing buffer were combined in a 0.25-mL snap-cap microcentrifuge tube (Fisher Scientific). In addition, a ladder sample containing known marker proteins was prepared in the same manner by combining 4 μ L of the Pro260 kit ladder and 2 μ L of reducing buffer. The sample buffer and ladder buffer mixtures were centrifuged briefly at $2,000 \times g$ for 30 s at 25°C (Mini Centrifuge, Bio-Rad Laboratories Inc.) to collect the liquid in the bottom of the tubes, and then tubes were placed in a boiling water bath for 5 min. The condensates in the tubes were then centrifuged again ($2,000 \times g$ for 30 s at 25°C) and the samples and ladder were further diluted by adding 84 μ L of deionized water to each tube. Each microfluidic chip, analogous to a gel slab, was loaded with a prepared ladder, 9 prepared samples, and a prepared skim milk sample to run as a check for consistency of

analysis from chip to chip. Each sample was analyzed in duplicate from a common tube. The area under the peak corresponding to each protein fraction was calculated using Experion Software (Version 3.20, Bio-Rad Laboratories Inc.) by manually assigning peaks and using a straight baseline integration approach.

RESULTS AND DISCUSSION

Preliminary Data: pH Produced by Different Buffer Concentrations

The addition of acetic acid to water produced a pH of less than 4.6 (Table 1). Upon the addition of sodium acetate, the pH of the water plus acetic acid mixture increased to approximately pH 4.48 (Table 1). If these same reagents were added to a milk plus water mixture, we would expect that initially the pH of the diluted milk would be much lower than pH 4.6 and then after the sodium acetate addition the pH would increase to approximately pH 4.6. As expected, despite the wide range of buffer concentrations, the final pH of the buffer was about the same regardless of buffer strength (Table 1).

Phase I: Different Buffer Concentrations

pH Data. At the lowest buffer concentration (i.e., the concentration currently used in the NCN method), the pH of the NCN filtrates at all protein concentrations was higher than pH 4.6 (Table 2). This indicates that the current method for bovine milk may not have sufficient buffering capacity to achieve a filtrate pH of 4.6 at 40°C for bovine milks at normal protein levels (i.e., P1 in Table 2). Increasing milk protein concentration resulted in an observed increase in NCN filtrate pH ($P < 0.05$) within each buffer concentration (Table 2), as demonstrated by the significant effect of term for milk in the ANOVA (Table 3). Within each protein concentration, increasing buffer concentration resulted in an observed decrease in the NCN filtrate pH ($P < 0.05$, Table 3). The pH of the filtrate with buffer 1 increased

Table 2. Average noncasein nitrogen (NCN) filtrate pH ($n = 3$) in phase I using a fixed 1-mL addition of reagents at 6 different buffer concentrations and 4 different bovine milk true protein concentrations (P1 = 2.91%, P2 = 4.84%, P3 = 6.77% and P4 = 8.66%)

Buffer number	Buffer, acetic acid (mol/L)	Buffer, sodium acetate (mol/L)	P1 pH	P2 pH	P3 pH	P4 pH
1	1.75	1.00	4.79	4.91	5.06	5.17
2	2.98	1.70	4.64	4.72	4.82	4.89
3	4.20	2.40	4.59	4.65	4.72	4.77
4	5.43	3.10	4.56	4.61	4.66	4.70
5	6.65	3.80	4.52	4.57	4.61	4.67
6	7.88	4.50	4.50	4.54	4.57	4.63

by 0.38 pH units with increasing protein concentration, whereas the pH of filtrates with buffer concentration 6 only increased by 0.13 pH units, which is supported by the significant effect of the interaction of buffer by milk in the ANOVA data presented in Table 3.

Kjeldahl Data. As expected, because of the way the milk concentrates were produced, there was a difference ($P < 0.01$) in both CP and true protein across the 4 milks (Table 4). The UF process used to produce a milk protein concentrate with about 9% protein and the dilution of that concentrate to formulate milks with progressively decreasing protein content should have maintained a constant NPN concentration in all of the milks of different protein content. A constant NPN concentration in all the milks was expected (Table 4) because the primary compound in the NPN fraction of milk is urea (Walstra and Jenness, 1984; Webb et al. 1974), and urea should easily pass through a 10-kDa molecular weight cut-off UF membrane. However, a progressive increase ($P < 0.05$) in NPN concentration from P1 to P4 was observed (Table 4). Why was NPN higher with higher protein concentration? Two possible explanations exist. (1) Urea is only 50 to 70% of the NPN in milk (Walstra and Jenness, 1984; Webb et al., 1974) and one or more minor NPN compounds other than urea that are soluble in 12% TCA may not have

passed through the UF membrane or attached to something larger (e.g., milk fat globule membrane) during the UF process. Or, (2) an error was present in the NPN method.

The calculation of NPN concentration was as follows: $[1.4007 \times (V_s - V_b) \times N] / \{ (W_f \times W_m) / [W_t - (W_m \times 0.065)] \}$, where V_s and V_b = milliliters of titrant used for sample and blank, respectively; N = normality of HCL solution; W_f = weight (g) of 20 mL of filtrate; W_m = weight (g) of milk; and W_t = weight (g) of milk plus 40 mL of 15% TCA solution. Factor 0.065 in the denominator assumes that milk contains about 3.5% fat and 3.0% true protein (i.e., $0.035 + 0.030$). Factor may need to be adjusted if liquid dairy products of different composition are analyzed (i.e., concentrated or fractionated skim, whole milk products, and so on). The possibility that protein concentration influenced the ability of the NPN method to correctly measure the NPN content was something that we addressed in the current study and is discussed below.

Did the observed increase in NPN concentration in milks with increasing protein concentration reported in Table 4 occur because the calculation in the NPN method to compensate for the increasing volume of the precipitate produced by the TCA was not correct? To answer this question, the 4 milks from replicate 3 of

Table 3. Type III sum of squares and probability values (in parentheses) for ANOVA to determine the effect of milk, replicate (rep), and buffer on noncasein nitrogen (NCN), CN, casein as a percent of total protein (CN%TP), and pH on NCN filtrates from phase I

Source	df	NCN	Casein	CN%TP	pH
Milk	3	5.283 (<0.01)	91.590 (<0.01)	8.401 (<0.01)	0.126 (<0.01)
Rep	2	0.049 (<0.01)	0.635 (<0.01)	0.269 (0.078)	0.016 (<0.01)
Milk $\times$ rep	6	0.019 (<0.01)	0.462 (<0.01)	1.542 (0.0004)	0.044 (<0.01)
Buffer	1	0.007 (<0.01)	0.007 (<0.01)	1.199 (<0.01)	0.059 (<0.01)
Buffer $\times$ milk	3	0.006 (<0.01)	0.006 (<0.01)	2.003 (<0.01)	0.005 (<0.01)
Buffer $\times$ rep	2	0.001 (0.032)	0.001 (0.0324)	NS	0.0006 (0.037)
Buffer $\times$ buffer	1	0.004 (<0.01)	0.004 (<0.01)	0.212 (0.045)	0.002 (<0.01)
Buffer $\times$ buffer $\times$ rep	2	0.018 (0.075)	0.002 (0.0075)	0.419 (0.021)	NS
Buffer $\times$ buffer $\times$ milk	3	0.030 (<0.01)	0.030 (<0.01)	8.468 (<0.01)	0.011 (<0.01)
Buffer $\times$ buffer $\times$ buffer	1	0.007 (<0.01)	0.007 (<0.01)	0.753 (0.0003)	0.023 (<0.01)
Buffer $\times$ buffer $\times$ buffer $\times$ milk	3	0.009 (<0.01)	0.009 (<0.01)	2.693 (<0.01)	0.0009 (0.017)
Buffer $\times$ buffer $\times$ buffer $\times$ rep	2	NS	NS	NS	0.0007 (0.022)
Buffer $\times$ buffer $\times$ buffer $\times$ buffer	1	NS	NS	0.240 (0.034)	0.001 (0.0002)

Table 4. Mean (n = 3) percentage total nitrogen (TN, ×6.38), TP (true protein), and NPN for milk concentrates used in phase I

Replicate	CP ¹				TP ¹				NPN			
	P1	P2	P3	P4	P1	P2	P3	P4	P1	P2	P3	P4
1	3.219	5.198	7.243	9.294	3.031	5.003	7.047	9.088	0.187	0.195	0.196	0.207
2	3.024	4.905	6.716	8.509	2.849	4.720	6.526	8.311	0.175	0.185	0.191	0.197
3	3.099	5.029	6.966	8.856	2.914	4.836	6.771	8.655	0.186	0.193	0.195	0.201
Mean	3.114 ^d	5.044 ^c	6.975 ^b	8.886 ^a	2.932 ^d	4.853 ^c	6.781 ^b	8.685 ^a	0.183 ^d	0.191 ^c	0.194 ^b	0.202 ^a

^{a-d}Mean values within TN, within TP, and within NPN with different letters are significantly different from each other ($P < 0.01$).

¹CP = crude protein (total nitrogen × 6.38), TP = (TN – NPN) × 6.38.

phase I (Table 4) were each analyzed for NPN content with and without any added urea. Three different and progressively increasing levels of added urea were created (Sigma Aldrich, St. Louis, MO; Lot #38H0022, minimum 99.5% purity), as shown in Table 5. The measured NPN concentrations were calculated using the equations described above and the residual differences between the measured and added urea, expressed as a percent ($N \times 6.38$) are shown in Figure 1. The measured amount of added NPN was in good agreement with the known amount of urea added at all protein concentrations from about 3 to 9% true protein. The overall recovery of the added urea was 99.88% and a change in the recovery of added urea was not detected ($P > 0.05$) with increasing true protein concentration [i.e., the slopes of the lines for U1, U2, and U3 in Figure 1 were not different ($P > 0.05$) from zero]. Therefore, we concluded that the increase in NPN content of the milks shown in Table 4 was most likely due to a minor 12% TCA-soluble NPN compound that could not pass freely through the 10-kDa UF membrane and no effect of increasing protein concentration on the measurement of milk NPN was detectable.

Both NCN and casein concentration increased ($P < 0.05\%$) with increasing protein concentration as expected, because UF was used to produce the series of milks (Table 6 and Figure 2.). The expected behavior of the CN%TP among the 4 milk protein concentrations is a novel aspect of the design of our study and should provide a unique advantage in the evaluation of how well modifications of the NCN method worked. Our goal was to make the method work well over a wider range of protein concentrations that are encountered when bovine milk concentrates and milks of different species are analyzed. The expected CN%TP in the 4 milks of very different protein concentrations were expected to be identical within replicates in our study because the UF process concentrates both the CN and whey protein and does not allow protein to pass through the UF membrane into the permeate. This allowed us to analyze each of the 4 milks using a range of different buffer strengths (6 in phase I) in our study and observe both the pH of the NCN filtrates (Table 2) and the measured CN%TP reported in Table 6. When the buffering capacity was not sufficient to achieve a final pH of the NCN filtrate of 4.6 or a little lower, then we expected that the observed CN%TP would be more variable and in general lower when the pH of the NCN filtrate was significantly higher than pH 4.6. As protein content increased from milk P1 to P4, the pH of the filtrates increased (e.g., pH of filtrates for milks P3 and P4 at the lowest buffer concentrations as seen in Table 2). The filtrates with the highest pH, P3 and P4 with buffer 1 (Table 2), were cloudy and the rate of

Table 5. The NPN concentrations of milks with 4 different true protein contents (P1 = 2.91%, P2 = 4.84%, P3 = 6.77%, and P4 = 8.66% true protein) with 3 levels of added urea expressed as percent $\text{NPN} \times 6.38$ for the actual amount of urea added, the measured amount of added urea detected by the Kjeldahl method, and the measured amount of added urea minus the amount of urea added

Urea level added	Urea added (%N $\times$ 6.38)				Added urea measured (%N $\times$ 6.38)				Measured urea minus added urea (%N $\times$ 6.38)			
	P1	P2	P3	P4	P1	P2	P3	P4	P1	P2	P3	P4
0	0	0	0	0	0	0	0	0	0	0	0	0
1	0.0261	0.0258	0.0258	0.0263	0.0260	0.0232	0.0293	0.0226	0	-0.0027	0.0035	-0.0037
2	0.0966	0.0956	0.0963	0.0953	0.1006	0.0994	0.0939	0.1010	-0.0001	0.0038	-0.0024	0.0058
3	0.1541	0.1536	0.1512	0.1547	0.1551	0.1501	0.1507	0.1542	0.0009	-0.0027	-0.0005	-0.0005

passage of filtrate through the filter was much slower when the filtrate pH was too high. This is a key visual indicator for the analyst that something is going wrong in the method. At the same time, the CN%TP for milks P3 and P4 were lower ($P < 0.05$) than at higher buffer concentrations (Table 6). This is supported by the fact that in the ANOVA (Table 3), an effect was noted of the linear interaction of buffer concentration by milk protein concentration and a quadratic and cubic interaction of buffer by milk protein concentration were also observed. All these effects had a probability of $P < 0.01$ and, based on their type III sum of squares these terms, explained most of the variation in the data (Table 3).

This complex but systematic behavior of CN%TP among the 4 milk concentrations as a function of increasing buffer concentration, and its relationship to the observed pH of the filtrates can be best seen in Figure 2. In general, it appeared that when the buffering capacity was much too low and pH of the NCN filtrate was much too high, then the CN%TP for that milk was low (i.e., lines for P4 in Figure 2). As buffer concentration increased, the estimate of CN%TP increased for P4. As the filtrate pH arrives at 4.6 and a little lower, the CN%TP increased. This behavior was present within each milk protein concentration, but the behavior was progressively out of phase from left to right in the graph (Figure 2) as protein concentration increased. In reality, all 4 of the milks regardless of protein concentration should have had the same CN%TP within replicate in our study because of the way the milks were manufactured using UF. It can be observed in Figure 2 that, as the buffer concentration increased, the CN%TP measured for all 4 milks became similar and less variable at the $3.8\times$ and $4.5\times$ buffer concentrations [i.e., $3.8\times$ and $4.5\times$ buffers = 6.65 (38% vol/vol) and 7.88 mol/L (45% vol/vol) for the acetic acid solution and 3.80 and 4.50 mol/L for the sodium acetate solutions, respectively]. This is a key indicator that the method was more robust for milks of all protein concentrations if a higher buffer concentration was used and the pH of the NCN filtrate was pH 4.6 or slightly lower.

The advantage of using the mixture of acetic acid and sodium acetate in combination is that they form a buffer as shown in Table 1. The analyst will not dramatically overshoot the target pH on the low side in the final filtrate. If an analyst was just using an acid by itself instead of buffer, a risk of making the final NCN filtrate pH too low and causing a negative effect on the results exists, as noted by Zhang and Metzger (2011). As can be seen from the NCN filtrate pH data in Table 2 and on the graph in Figure 2, we did not achieve the filtrate pH of 4.6 on the milk with the highest protein content (P4). In addition, the average protein content

Figure 1. Residual plot of differences between amount of urea measured and amount of urea added (expressed as $\%N \times 6.38$) to 4 milks with different true protein concentrations. U1, U2, and U3 were 3 targeted levels of urea added ($\%N \times 6.38$ was 0.025, 0.095, and 0.15, respectively) to each of 4 milks with different true protein levels (P1 = 2.91%, P2 = 4.84%, P3 = 6.77%, and P4 = 8.66% true protein).

for the 3 replicates of the P4 milk was 8.66% protein, which was lower than our goal of 9%. Therefore, a slightly higher buffer concentration would be required to achieve our goal of the method being adequate for milk concentrates with protein contents up to 9%. Using a higher buffer concentration would appear to be the recommended action, but a technical difficulty occurred with the solubility of the sodium acetate at higher concentrations. Therefore, at this point we decided to change our strategy in the method development and evaluated the use of increasing the volume of reagent added using the lowest buffer concentration instead of increasing buffer concentrations. The approach of using different volumes of buffer addition to increase the effective total buffering capacity in the analysis was investigated in phase II of our study.

Phase II: Different Buffer Volumes

pH Data. A preliminary evaluation of volumes of buffer required to achieve pH 4.6 in the NCN filtrates with various milk protein contents, using the 4 milks from replicate 2 and 8 different volumes of buffer [1.75 (10%) and 1 mol/L of acetic acid and sodium acetate solutions, respectively], was conducted and the results are reported in Table 7. For the milk with 8.31% protein, it took just 5.2 mL of the buffer solutions to achieve a pH slightly lower (Table 7, pH 4.59) than pH 4.6. Given that the protein content of the P4 sample was less than the target value of 9%, we decided to use the acetic acid and sodium acetate solution volumes of 4.5, 5.5, and 6.5 mL in phase II to ensure that enough buffering capacity was present for the bovine milk concentrate with 9% protein. The 4.5-mL solution addition would be the equivalent of the highest buffer concentration

used in phase I of the study. The NCN filtrate pH data and the protein content of the 4 milks used in phase II are shown in Table 7. It took about 5.5 mL of each of the 2 solutions to achieve an NCN filtrate pH lower than 4.6 in the P4 milk that contained 8.66% protein.

Residual CN in NCN Filtrates. Milks from 3 individual cows were analyzed for pH, nitrogen fractions, and individual protein types present in the NCN filtrates using 2 different acetic acid and sodium acetate volumes to produce NCN filtrates with pH higher and lower than the target pH of 4.6. The 3 milks differed in protein content (Table 8). As expected, the 3 milks prepared with 1 mL of reagents had a mean NCN filtrate pH of 4.79 and those prepared with 5 mL of reagents had a mean NCN filtrate pH of 4.48 (Table 8). The NCN was higher (0.69 vs. 0.73%) and the casein (TN – NCN) was lower (2.50 vs. 2.46%) when the pH of the NCN filtrate was lower. It is apparent from the data in Table 8 that there is probably always a trace of some type of CN in the NCN filtrate, even at pH 4.6. However, the data in Table 8 demonstrate that the type of CN present changes as a function of pH above and below pH 4.6. At pH higher than 4.6, the NCN filtrates (pH 4.79) were visibly cloudy and the electrophoresis data indicated both β -CN and α_{s1} -CN in those 3 filtrates. The NCN filtrates at a pH of 4.48 contained no detectable β -CN or α_{s1} -CN, but did contain a higher level of κ - + α_{s2} -CN than the NCN filtrates that had a mean pH of 4.79.

Kjeldahl Data. The milks used for the phase II volume experiment were the same milks used for replicate 3 of phase I; therefore, the CP, true protein, and NPN values for these milks are given in Table 4. The NCN and CN contents of these 4 milks when using 3 different buffer volumes are shown in Table 9. The NCN and CN

Figure 2. Casein as percentage of true protein (CN%TP) and non-casein nitrogen (NCN) filtrate pH at the 6 different buffer concentrations used in phase I. Buffer concentrations were: 1.75 (10% vol/vol), 2.98 (17% vol/vol), 4.20 (24% vol/vol), 5.43 (31% vol/vol), 6.65 (38% vol/vol), and 7.88 mol/L (45% vol/vol) for the acetic acid solution and 1.00, 1.70, 2.40, 3.10, 3.80, and 4.50 mol/L for the sodium acetate solution, corresponding to buffer concentration factor 1 $\times$, 1.7 $\times$, 2.4 $\times$, 3.1 $\times$, 3.8 $\times$, and 4.5 $\times$, respectively on the x-axis. Solid symbols are data for CN%TP for the 4 protein levels (P1 = 2.85%, P2 = 4.72%, P3 = 6.53%, and P4 = 8.31%) and open symbols are data for the 4 protein levels (P1 = 2.85%, P2 = 4.72%, P3 = 6.53%, and P4 = 8.31%).

contents increased with increasing protein content, as expected (Table 9). The CN%TP was very consistent among the 3 buffer volumes, and the P2, P3, and P4 milks all had very similar CN%TP. The mean CN%TP for the P1 sample was a bit lower than P2, P3, and P4. The Kjeldahl analyses for all the P1 milk at the 3 buffer volumes were run together as a batch, and it appears that something caused a slight shift of the results low for the all the P1 samples. Overall, using an increasing volume of the low concentration of acetic acid and sodium acetate solutions seems to be a more practical approach for the routine NCN method than using a constant volume of the solutions at higher concentration. It appears that a modification of the NCN method to use either 4.5, 5.5, or 6.5 mL of the acetic acid solution on all milks might provide a more rugged method that could be applied to a wide range of milks from various species of animals and to milk concentrates. In the next sections, we applied the 3 highest buffer volumes to milks of other species to evaluate the performance of the method, particularly with respect to its ability to achieve the correct pH of the NCN filtrate.

Goat Milk

The CP, true protein, and NPN content of 4 different goat milks are shown in Table 10. These were goat

Table 6. Average ($n = 3$) noncasein nitrogen (NCN), CN, and casein as a percentage of true protein (CN%TP) in phase I using a fixed 1-mL addition of reagents at 6 different buffer concentrations and 4 different (P1 = 2.93%, P2 = 4.85%, P3 = 6.78%, and P4 = 8.68%) bovine milk true protein concentrations for phase I

Buffer concentration ¹	NCN				CN				CN%TP			
	P1	P2	P3	P4	P1	P2	P3	P4	P1	P2	P3	P4
1	0.717	1.156	1.604	2.071	2.352	3.887	5.371	6.815	80.248	80.111	79.227	78.470
2	0.789	1.161	1.540	1.950	2.325	3.883	5.435	6.937	79.306	80.008	80.161	79.868
3	0.815	1.192	1.538	1.927	2.299	3.852	5.437	6.959	78.427	79.373	80.178	80.126
4	0.796	1.209	1.581	1.936	2.318	3.835	5.394	6.921	79.071	79.027	79.554	80.027
5	0.790	1.195	1.608	1.963	2.324	3.849	5.367	6.923	79.274	79.316	79.144	79.708
6	0.788	1.186	1.606	1.963	2.326	3.858	5.369	6.924	79.335	79.505	79.185	79.718
Mean	0.790 ^d	1.183 ^c	1.579 ^b	1.968 ^a	2.324 ^d	3.861 ^c	5.396 ^b	6.918 ^a	79.277 ^b	79.557 ^a	79.575 ^a	79.653 ^a

^{a-d}Means for NCN, CN, and CN%TP within the same row that do not share a common superscript are different ($P < 0.05$).

¹Buffers 1 to 6 (1-mL addition of each solution): (1) 1.75 mol/L (10%) and 1 mol/L; (2) 2.98 mol/L (17%) and 1.7 mol/L; (3) 4.20 mol/L (24%) and 2.4 mol/L; (4) 5.43 mol/L (31%) and 3.1 mol/L; (5) 6.65 mol/L (38%) and 3.8 mol/L; (6) 7.88 mol/L (45%) and 4.5 mol/L of acetic acid and sodium acetate solutions, respectively.

milks from 4 different farms. The goat milks had true protein contents that were generally higher than typical Holstein milks, but the proteins were not exceedingly high and were within the range of protein contents seen from some breeds of dairy cattle (e.g., Jersey). The NPN content of the goat milks (Table 10) was higher than typically seen for cow milk. Park et al. (2007) reported that goat and human milks have higher NPN content than cow milk (Table 4). The pH of the NCN filtrates for goat milks were all lower than pH 4.6, which is consistent with the protein level based on the data for cow milk (Table 11). All of the solution volumes used (i.e., 4.5, 5.5, and 6.5 mL) provided more than enough buffering capacity to achieve consistent estimates of CN%TP regardless of buffer volume (Table 12).

Sheep Milk

The CP, true protein, and NPN content of 4 different sheep milks are shown in Table 10. These were sheep milks from 2 different farms with milk collected from each farm on 2 different days in different weeks. Milks 1 and 4 are from the same farm. The sheep milks had true protein contents that were higher than typical Holstein milks and higher than goat milks. The NPN content of the sheep milks was higher than typically seen for cow milk and there was a large difference in the NPN content of the milk from the 2 different farms. The farm with the lower NPN was a very large farm (milking 400 sheep) that used a TMR feeding system with an optimization of the nutrient composition of the ration. The other farm producing sheep milk had an unusually

high NPN content relative to cow milk and relative to the other sheep farm. Milks 2 and 3 were produced by a small farm that was less intensively managed. When NPN content of ruminant milks vary, it is normally due to differences in urea concentration in the milks. Taylor (1922) reported that changes in milk NPN content of goat milk were highly correlated with variation in blood urea content. This is generally true for all ruminants. Milk urea content varies in proportion to blood urea content. Taylor (1922) also indicated that concentration of fat, protein, and ash varied inversely with milk volume produced, whereas lactose concentration varied directly with milk volume. Taylor found no correlation of milk NPN content with the volume of milk produced. Morgante (2004) identified 2 causes of high NPN content in sheep milk: (1) when lactation sheep are fed large amounts of concentrates, or NPN, and too much protein or nitrogen exists in the diet for the amount of fermentable carbohydrate to form the carbon framework for protein synthesis by the rumen microflora; and (2) when milk-producing sheep are grazing on spring pasture that is rich in NPN or a high level of easily degradable proteins. The milk samples used in our study were collected in the fall. High milk NPN content is correlated with decreased fertility and increased incidence of reproductive problems in ruminants (Morgante, 2004).

The pH of the NCN filtrates for sheep milks (Table 11) were all lower than pH 4.6, even though the protein contents of the sheep milk were higher than both cow and goat milks. The 4.5 mL of buffer and higher buffer volumes were all sufficient for the protein level in the

Table 7. Eight preliminary buffer volume treatments [1.75 (10%) and 1 mol/L of acetic acid and sodium acetate solutions, respectively] used to determine the buffer volumes required to reach pH 4.6 for 4 different milk protein concentrations using the milks from replicate 2 of phase I for 10 mL milk sample, and average NCN filtrate pH ($n = 3$) in phase I of replicate 3 using 3 different buffer volumes (4.5 mL, 5.5 mL and 6.5 mL) and 4 different bovine milk true protein concentrations¹

Acetic acid solution	Sodium acetate solution (mL)	P1 pH	P2 pH	P3 pH	P4 pH
Buffer volume (mL)					
1	1	4.78	4.96	5.06	5.17
1.7	1.7	4.65	4.76	4.82	4.89
2.4	2.4	4.56	4.64	4.70	4.75
3.1	3.1	4.53	4.60	4.65	4.69
3.8	3.8	4.50	4.56	4.60	4.64
4.5	4.5	4.48	4.54	4.57	4.61
5.2	5.2	4.47	4.52	4.54	4.59
5.9	5.9	4.47	4.50	4.52	4.56
NCN filtrate pH					
4.5	4.5	4.51	4.54	4.59	4.62
5.5	5.5	4.48	4.51	4.55	4.58
6.5	6.5	4.47	4.50	4.53	4.55

¹Total protein for buffer volume = 2.85, 4.72, 6.53, and 8.31% for P1, P2, P3, and P4, respectively. Total protein for NCN filtrate pH = 2.91, 4.84, 6.77, and 8.66% for P1, P2, P3, and P4, respectively.

Table 8. Noncasein N (NCN) filtrate pH, nitrogen fractions expressed on a protein basis (% $\times 6.38$), and individual protein types and distribution (relative %) in NCN filtrates for milk from 3 different cows when acetic acid and sodium acetate solutions [1.75 (10%) and 1 mol/L, respectively] were added at 1 and 5 mL in the preparation of the NCN filtrate

Cow number	Reagent (mL)	Filtrate pH	TN ¹	NCN ¹	NPN ¹	TP	CN ¹	CN%TP ¹	α -LA	β -LG	β -CN	α_{s1} CN	$\kappa + \alpha_{s2}$ CN	BSA	LF ¹
1	1	4.82	3.53	0.79	0.15	3.38	2.73	80.88	31.81	57.29	2.03	0.69	2.19	4.81	1.80
2	1	4.76	3.08	0.59	0.15	2.92	2.49	85.07	30.26	62.42	0.95	0.44	2.48	1.72	1.72
3	1	4.79	2.97	0.69	0.16	2.81	2.28	81.00	25.87	67.80	1.33	0.37	2.58	1.03	1.03
1	5	4.49	3.53	0.83	0.15	3.38	2.70	79.81	30.14	58.61	ND ²	ND	6.68	3.46	1.20
2	5	4.48	3.08	0.62	0.15	2.92	2.45	83.97	25.41	59.76	ND	ND	10.25	3.72	0.87
3	5	4.48	2.97	0.73	0.16	2.81	2.24	79.76	23.34	67.43	ND	ND	6.69	1.59	0.95
Mean	1	4.79	3.19	0.69	0.15	3.04	2.50	82.32	29.11	62.50	1.44	0.50	2.42	2.52	1.52
Mean	5	4.48	3.19	0.73	0.15	3.04	2.46	81.18	26.26	61.93	ND	ND	7.87	2.92	1.01

¹TN = total nitrogen $\times 6.38$; NCN = noncasein nitrogen $\times 6.38$; NPN = nonprotein nitrogen $\times 6.38$; TP = true protein = TN – NPN; CN = TN – NCN; CN%TP = casein as a percentage of true protein; and LF = lactoferrin.

²ND = not detected.

Table 9. Average ($n = 3$) noncasein nitrogen (NCN; %, $\times 6.38$), CN, and casein as a percentage of true protein (CN%TP) in milks from phase I replicate 3 using 3 different buffer [1.75 (10%) and 1 mol/L of acetic acid and sodium acetate solutions, respectively] volumes (4.5, 5.5, and 6.5 mL), and 4 different (P1 = 2.91%, P2 = 4.84%, P3 = 6.77%, and P4 = 8.66%) bovine milk true protein concentrations

Buffer volume (mL)	NCN (%)				CN (%)				CN%TP			
	P1	P2	P3	P4	P1	P2	P3	P4	P1	P2	P3	P4
4.5	0.809	1.182	1.592	1.964	2.290	3.848	5.374	6.892	78.610	79.567	79.365	79.631
5.5	0.800	1.169	1.568	1.953	2.300	3.860	5.398	6.903	78.934	79.820	79.724	79.753
6.5	0.804	1.167	1.560	1.929	2.295	3.863	5.406	6.927	78.770	79.876	79.850	80.034
Mean	0.804	1.172	1.573	1.948	2.295	3.857	5.393	6.907	78.771	79.755	79.646	79.806

Table 10. Mean (% $\times 6.38$) protein for CP, true protein (TP), and NPN for goat, sheep, and water buffalo milks

Test method	Goat				Sheep				Water buffalo			
	Milk 1	Milk 2	Milk 3	Milk 4	Milk 1	Milk 2	Milk 3	Milk 4	Milk 1	Milk 2	Milk 3	Milk 4
CP	3.949	3.936	4.253	3.613	5.448	5.838	5.943	5.530	5.135	5.117	5.819	6.410
TP	3.699	3.689	4.021	3.357	5.243	5.508	5.614	5.283	4.883	4.838	5.587	6.155
NPN	0.250	0.247	0.232	0.256	0.205	0.330	0.329	0.247	0.253	0.278	0.233	0.255

Table 11. Mean noncasein nitrogen (NCN) filtrate pH for goat, sheep, and water buffalo milks as function of volume (mL) of added acetic acid [1.75 mol/L (10%)] and sodium acetate at (1 mol/L)

Buffer volume	Goat				Sheep				Water buffalo			
	Milk 1	Milk 2	Milk 3	Milk 4	Milk 1	Milk 2	Milk 3	Milk 4	Milk 1	Milk 2	Milk 3	Milk 4
4.5	4.51	4.52	4.54	4.51	4.51	4.53	4.53	4.56	4.59	4.58	4.58	4.56
5.5	4.49	4.50	4.51	4.49	4.50	4.51	4.51	4.53	4.55	4.54	4.54	4.53
6.5	4.48	4.49	4.49	4.48	4.49	4.50	4.49	4.51	4.52	4.51	4.51	4.51

Table 12. Goat milk: mean percentages for noncasein nitrogen (NCN; %, $\times 6.38$), CN, and casein as percentage of true protein (CN%TP) as function of volume (mL) of added acetic acid [1.75 mol/L (10%)] and sodium acetate (1 mol/L)

Buffer volume	NCN				CN				CN%TP			
	Milk 1	Milk 2	Milk 3	Milk 4	Milk 1	Milk 2	Milk 3	Milk 4	Milk 1	Milk 2	Milk 3	Milk 4
4.5	1.009	1.042	1.109	0.868	2.941	2.894	3.144	2.745	79.498	78.427	78.186	81.770
5.5	1.016	1.054	1.113	0.875	2.928	2.864	3.138	2.731	79.136	77.640	78.043	81.354
6.5	1.022	1.072	1.115	0.882	2.931	2.868	3.138	2.724	79.234	77.748	78.028	81.141

Table 13. Sheep milk mean percentages for noncasein nitrogen (NCN; %, $\times 6.38$), CN, and casein as percentage of true protein (CN%TP) as function of volume (mL) of added acetic acid [1.75 mol/L (10%)] and sodium acetate (1 mol/L)

Buffer volume	NCN				CN				CN%TP			
	Milk 1	Milk 2	Milk 3	Milk 4	Milk 1	Milk 2	Milk 3	Milk 4	Milk 1	Milk 2	Milk 3	Milk 4
4.5	1.341	1.380	1.406	1.328	4.107	4.458	4.541	4.202	78.321	80.939	80.827	79.548
5.5	1.333	1.376	1.398	1.315	4.110	4.462	4.544	4.215	78.377	81.014	80.955	79.795
6.5	1.338	1.393	1.409	1.333	4.090	4.445	4.534	4.196	77.997	80.702	80.765	79.440

sheep milks used in our study. All of the buffer volumes provided more than enough buffering capacity to achieve consistent estimates of CN%TP with the same milk (Table 13).

Water Buffalo Milk

The CP, true protein, and NPN content of 4 different water buffalo milks are shown in Table 10. These were water buffalo milks from 4 different groups of water buffalo on the same farm. The water buffalo milks had true protein contents that were generally higher than typical Holstein milks and higher than goat milks. The NPN content of the water buffalo milks was higher than typically seen for cow milk. The pH of the NCN filtrates for water buffalo milks were all lower than pH 4.6 and even though the protein content of the water buffalo milks were higher than both cow and goat milks. The 4.5 mL of buffer and higher buffer volumes were all sufficient to achieve a pH lower than 4.6 (Table 11) for the protein level in the water buffalo milks in the current study. All of the buffer volumes provided more than enough buffering capacity to achieve consistent estimates of CN%TP regardless of buffer volume (Table 14).

Relationship Between Milk Protein Concentration and NCN Filtrate pH

The data from the series of milk concentrates and the observed milk pH values from the phase I and II sections of the current study were combined to determine the relationship between the protein content of a milk sample and the volume of the low-concentration buffer solution required to achieve an NCN filtrate pH of 4.6 for bovine milk and milk concentrates. A graph and the polynomial regression equations for the relationship between mL of buffer [1.75 (10%) and 1 mol/L of acetic acid and sodium acetate solutions, respectively] and the pH of the NCN filtrate for the P1, P2, P3, and P4 milks are shown in Figure 3. The 4 equations from Figure 3 were used to calculate the milliliters of buffer solution required to produce an NCN filtrate with a pH of 4.6 for each protein content. Those volumes were plotted as a function of the true protein content of the milk, as shown in Figure 4. From this graph a cubic polynomial prediction equation was developed that can be used to calculate the volume of buffer solutions needed for any true protein content of milks and milk concentrates in the range of 3 to 9% protein. The equation is $y = 0.0345x^3 - 0.4984x^2 + 2.6296x - 2.0314$, where x is the true protein content of the milk content and y is the volume of buffer solutions required to produce a filtrate pH of 4.6. This equation was developed using the data collected for bovine milk. Can this equation be applied

Buffer volume	NCN				CN				CN%TP			
	Milk 1		Milk 2		Milk 3		Milk 4		Milk 1		Milk 2	
	Milk 1	Milk 2	Milk 3	Milk 4	Milk 1	Milk 2	Milk 3	Milk 4	Milk 1	Milk 2	Milk 3	Milk 4
4.5	1.041	1.129	1.125	1.290	4.094	3.988	4.695	5.120	83.847	82.413	84.035	83.190
5.5	1.040	1.135	1.111	1.295	4.096	3.981	4.709	5.116	83.879	82.285	84.286	83.113
6.5	1.039	1.109	1.092	1.277	4.096	4.008	4.727	5.133	83.889	82.842	84.616	83.397

Figure 3. The pH of noncasein nitrogen (NCN) filtrate as a function of 4 different bovine milk protein concentrations (P1, P2, P3, and P4). Regression equations were, P1: $y = -0.0004x^3 + 0.0582x^2 - 0.2944x + 5.0145$; P2: $y = -0.0055x^3 + 0.0793x^2 - 0.3969x + 5.2206$; P3: $y = -0.0072x^3 + 0.1047x^2 - 0.525x + 5.4705$; and P4: $y = -0.0093x^3 + 0.1309x^2 - 0.6283x + 5.6548$, where y = filtrate pH and x = milliliters of each single strength buffer ingredient (i.e., acetic acid and sodium acetate solution), with R^2 values of 0.989, 0.988, 0.992, and 0.909, respectively. The single strength buffer was 1.75 mol/L (10% vol/vol) for the acetic acid solution and 1.00 mol/L for the sodium acetate solution.

to milks of different species to predict the amount of buffer required to achieve pH 4.6 in the NCN filtrate?

To answer this question, a group of goat, sheep, and water buffalo milks were collected. The true protein content of each milk was determined as previously described by infrared milk analysis (Kaylegian et al., 2006a, b). The protein test result of each milk was in-

Figure 4. The volume of single strength acetic acid and sodium acetate solutions [1.75 mol/L (10% vol/vol) for the acetic acid solution and 1.00 mol/L for the sodium acetate solution] needed to achieve a pH of 4.6 as a function of true protein content of milk for a 10-mL milk sample, where x = true protein (%).

serted in the third-degree polynomial equation given above to calculate the volume of acetic acid and sodium acetate needed to achieve an NCN filtrate with a pH 4.6 at 40°C. The calculated volume and a volume 0.5-mL larger and smaller were used to prepare 3 NCN filtrates from each milk. The pH of each NCN filtrate was determined as described above and the results are shown in Table 15. The mean pH achieved at the predicted levels of acetic acid and sodium acetate addition averaged for the group of milks of other species was 4.594

Table 15. The protein content of milk and the measured pH of the noncasein nitrogen (NCN) filtrates prepared from those goat, sheep, and water buffalo milks when the volume of acetic acid and sodium acetate solutions [1.75 (10%) and 1 mol/L, respectively] expected to produce a pH of 4.6 was calculated using the polynomial equation derived from cow milks and added at the predicted volume and at ± 0.5 mL to a 10-mL milk sample

Milk source	Amount of buffer added (pH)			Milk true protein (%)
	Predicted volume - 0.5 mL	Predicted volume ¹	Predicted volume + 0.5 mL	
Goat 1	4.644	4.597	4.545	2.96
Goat 2	4.603	4.559	4.501	2.78
Goat 3	4.609	4.561	4.521	2.77
Water buffalo 1	4.656	4.623	4.590	4.78
Water buffalo 2	4.644	4.601	4.573	4.72
Water buffalo 3	4.658	4.604	4.571	4.46
Water buffalo 4	4.639	4.603	4.572	4.57
Water buffalo 5	4.631	4.601	4.569	4.57
Sheep 1	4.634	4.596	4.579	4.51
Sheep 2	4.636	4.610	4.575	4.69
Sheep 3	4.621	4.599	4.558	4.57
Mean	4.634	4.596	4.559	4.12

¹Predicted volume of acetic acid and sodium acetate solutions to using the equation where $y = 0.0345x^3 - 0.4984x^2 + 2.6296x - 2.0314$, where x is the percent true protein content of the milk content and y is the volume in milliliters of acetic acid and sodium acetate solutions required to produce a filtrate pH of 4.6.

for the level of addition designed to achieve an NCN filtrate pH of 4.6. The equation for prediction of the amount of acetic acid and sodium acetate solution that was developed using bovine milk and milk concentrates worked well when applied to milks from other species.

CONCLUSIONS

As the concentration of protein in milk increased (either in bovine milk concentrates or in milks of other species), the amount of buffer needed in the NCN sample preparation method to achieve a filtrate pH of 4.6 increased. In the first part of the study using a series of bovine milk concentrates, we demonstrated that the method gave more consistent predictions of CN as a percentage of true protein when the NCN filtrate pH was between 4.5 and 4.6. When the amount of buffer added to the sample was not sufficient (i.e., the filtrate pH was too high), the filtrates were not clear. A polynomial equation was developed for prediction of the amount of acetic acid and sodium acetate buffer required to achieve the target NCN filtrate pH for milk protein concentrations from 3 to 9% protein using bovine milk and milk concentrates. When this equation developed using cow milk was applied to goat, sheep, and water buffalo milks, it correctly predicted the volume of reagents needed to achieve an NCN filtrate pH of 4.6. We also verified as part of this work that the ability to measure NPN content of milk was not influenced by protein content of milk in the range from 3 to 9% protein. The results of this study will be used as the basis for proposed changes in the official methods for measurement of the CN content of milk to expand the scope of the method so it can be used to achieve accurate results for milk concentrates and milks of other species.

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