Influence of Calcium and Phosphorus, Lactose, and Salt-to-Moisture Ratio on Cheddar Cheese Quality: pH Buffering Properties of Cheese

P. Upreti,* P. Bühlmann,† and L. E. Metzger*¹

*MN-SD Dairy Foods Research Center, Department of Food Science and Nutrition, University of Minnesota, St. Paul 55108

†Department of Chemistry, University of Minnesota, Minneapolis 55455

ABSTRACT

The pH buffering capacity of cheese is an important determinant of cheese pH. However, the effects of different constituents of cheese on its pH buffering capacity have not been fully clarified. The objective of this study was to characterize the chemical species and chemical equilibria that are responsible for the pH buffering properties of cheese. Eight cheeses with 2 levels of Ca and P (0.67 and 0.47% vs. 0.53 and 0.39%, respectively), residual lactose (2.4 vs. 0.78%), and salt-to-moisture ratio (6.4 vs. 4.8%) were manufactured. The pH-titration curves for these cheeses were obtained by titrating cheese:water (1:39 wt/wt) dispersions with 1 N HCl, and backtitrating with 1 N NaOH. To understand the role of different chemical equilibria and the respective chemical species in controlling the pH of cheese, pH buffering was modeled mathematically. The 36 chemical species that were found to be relevant for modeling can be classified as cations (Na^+ , Ca^{2+} , Mg^{2+}), anions (phosphate, citrate, lactate), protein-bound amino acids with a side-chain pK_a in the range of 3 to 9 (glutamate, histidine, serine phosphate, aspartate), metal ion complexes (phosphate, citrate, and lactate complexes of Na^+ , Ca^{2+} , and Mg^{2+}), and calcium phosphate precipitates. A set of 36 corresponding equations was solved to give the concentrations of all chemical species as a function of pH, allowing the prediction of buffering curves. Changes in the calculated species concentrations allowed the identification of the chemical species and chemical equilibria that dominate the pH buffering properties of cheese in different pH ranges. The model indicates that pH buffering in the pH range from 4.5 to 5.5 is predominantly due to a precipitate of Ca and phosphate, and the protonation equilibrium involving the side chains of protein-bound glutamate. In the literature, the precipitate is often referred to as amorphous colloidal calcium phosphate. A comparison of experimental data and model predictions shows that the buff-

ering properties of the precipitate can be explained, assuming that it consists of hydroxyapatite [$\text{Ca}_5(\text{OH})(\text{PO}_4)_3$] or $\text{Ca}_3(\text{PO}_4)_2$. The pH buffering in the region from pH 3.5 to 4.5 is due to protonation of side-chain carboxylates of protein-bound glutamate, aspartate, and lactate, in order of decreasing significance. In addition, pH buffering between pH 5 to 8 in the backtitration results from the reprecipitation of calcium and phosphate either as CaHPO_4 or $\text{Ca}_4\text{H}(\text{PO}_4)_3$.

Key words: Cheddar cheese, pH buffering, calcium, phosphate

INTRODUCTION

The influence of pH on the flavor, texture, and melting properties of cheese has been well recognized, and the pH buffering capacity of cheese is an important determinant of cheese pH. Hence, it is important to control the pH buffering properties of cheese to obtain desired cheese characteristics. The contribution of different constituents of milk to the pH buffering properties of cheese and other dairy products has been a subject of study for several decades (Whittier, 1929, 1933; Wiley, 1935a,b; Lucey et al., 1993), and has been recently reviewed by Salaün et al. (2005). However, there still lacks consensus on what factors govern the pH buffering properties of cheese and should be controlled during cheese manufacturing.

In general, the constituents that contribute to the pH buffering capacity of cheese can be broadly divided into 2 categories: 1) proteins, and 2) weak acids, bases, and their complexes with metal cations (Salaün et al., 2005). Proteins and free amino acids are major contributors to pH buffering in any biological system, including cheese or milk. The pH buffering capacity of proteins results from amino acids with basic and acidic side chains, and interactions of cations with functional groups in these side chains affect the pH buffering capacity (Salaün et al., 2005). The presence of several different amino acids, their sequence, spatial orientation, and the environment in which they occur in proteins complicate the quantitative contribution of proteins to the pH buffering capacity of cheese (Salaün et

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¹Corresponding author: lmetzger@umn.edu

al., 2005). To add to the complexity, some ionizable groups in native proteins are inaccessible to protonation or deprotonation but become accessible after denaturation caused by major changes in pH (Singh et al., 1997).

In addition to proteins, nonprotein substances such as weak acids, bases, and their complexes with metal cations contribute to buffering in cheese. The most important species are phosphate, citrate, lactate, carbonate, acetate, and propionate. Relevant metal ions are Ca^{2+} and Mg^{2+} . The ability of the acids to become deprotonated at pH values determined by their H^+ dissociation constants (pK_a) directly contributes to pH buffering. The presence of ions that alter the ionic strength of the medium indirectly influences pH buffering, because it shifts the apparent pK_a values of these acids. Moreover, many weak acids can form complexes with metal ions (e.g., Ca^{2+} , Mg^{2+}), further modifying the pH buffering capacity of cheese.

In addition, the formation of precipitates such as Ca^{2+} or Mg^{2+} phosphate, Ca^{2+} or Mg^{2+} citrate, and Ca^{2+} or Mg^{2+} lactate occurs in cheeses. Acids that are produced during cheese ripening due to fermentation of residual sugars influence the solubilization of such precipitates and indirectly affect pH buffering (Hassan et al., 2004). Past research showed the presence of a precipitate (referred to as colloidal calcium phosphate, **CCP**) within the casein micelles of cheese. Although the composition of CCP is uncertain, the importance of CCP in pH buffering in cheese is well recognized (Lucey et al., 1993; Lucey and Fox, 1993). Colloidal calcium phosphate was proposed to be composed of CaHPO_4 (Van Slyke and Bosworth, 1915), amorphous $\text{Ca}_3(\text{PO}_4)_2$ (Ling, 1936; Schmidt, 1980), a mixture of CaHPO_4 and $\text{Ca}_3(\text{PO}_4)_2$ (Porcher and Chevallier, 1923), $3\text{Ca}_3(\text{PO}_4)_2 \cdot \text{CaH} \cdot \text{citrate}$ (Pyne and McGann, 1960), or $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ (Holt et al., 1989). Although observations with x-ray absorption spectroscopy (Holt and Hukins, 1991) indicated the presence of brushite (CaHPO_4), ^{31}P solid-state nuclear magnetic resonance spectra (Bak et al., 2001) suggested the presence of hydroxyapatite [$\text{Ca}_5(\text{OH})(\text{PO}_4)_3$] in casein micelles.

Indeed, the quantitative discussion of calcium phosphate precipitation in biological systems is not easy. The formation of thermodynamically less stable precipitates is often favored by the kinetics of nucleation (Lu and Leng, 2005). Hence, the type of precipitate formed at a certain pH and given concentrations of Ca and phosphate often cannot be predicted correctly from solubility products alone. Also, nucleation rates are influenced by the presence of other ions (e.g., citrate) and proteins (Schmidt, 1980), and the interconversion of thermodynamically less stable precipitates into thermodynamically more stable ones is often observed. The

latter is particularly true for the amorphous precipitates that are often formed initially. Therefore, the identification of the component(s) of CCP in cheese is a challenging problem. In view of the buffering properties of cheese, it is important to note that the contribution of different forms of CCP to the pH buffering capacity of cheese would not be identical. For example, because of the different degree of protonation, a $\text{Ca}_3(\text{PO}_4)_2$ precipitate would provide higher pH buffering capacity than CaHPO_4 with an equal amount of phosphorus.

Variations in the pH buffering capacity of cheese can arise due to several factors. For instance, the variation in mineral and protein content of cheese can result from the varying composition of milk, different pretreatments of milk before cheese making (heating, acidification), modifications in the cheese-making process (set or drain pH, rate and extent of acidification), salting, and the cheese ripening conditions (Lucey and Fox, 1993; Salaün et al., 2005). Hence, it is important to identify the key species that control the pH buffering capacity of cheese and, consequently, control their concentrations.

This study was carried out to specifically identify the chemical species and the mechanisms involved in pH buffering of cheese in different pH regions. To investigate this, cheeses with different levels of total Ca and P were manufactured. The cheeses also differed in their residual lactose and the salt-to-moisture ratio (**S/M**), which led to differences in their contents of lactate and other water-soluble organic acids (Upreti et al., 2006). All cheeses were compared in view of differences in titration curves and pH buffering properties. The chemical species that caused pH buffering of cheese-water dispersions were interpreted assuming that these dispersions are in a thermodynamic quasi-equilibrium. This means that the conditions where calcium phosphate precipitation occurs are not necessarily defined by thermodynamics and can be kinetically determined. For example, it is well known that phosphate and calcium form precipitates that can subsequently slowly convert to thermodynamically more stable solids. The quasi-equilibrium model accounts for this by including only equilibria for precipitates that are directly formed from solution species. A similar, albeit much less comprehensive, approach was used by Whittier (1933) to predict the pH buffering action of calcium phosphate in milk.

MATERIALS AND METHODS

Cheese Manufacturing

The manufactured Cheddar cheeses differed in 3 factors (Ca and P, residual lactose, and S/M) at 2 levels, giving a total of 8 different treatments. The treatments

Table 1. Average chemical composition of cheeses expressed as percentage by weight of cheese (mean of 3 replicates)

	Treatments ¹							
	HHH	HHL	HLH	HLL	LHH	LHL	LLH	LLL
Moisture	32.07 ^a	33.80 ^{bc}	33.07 ^{ab}	35.21 ^{de}	34.08 ^{bcd}	35.94 ^e	34.39 ^{cd}	37.57 ^f
Fat	35.93 ^a	34.95 ^{ab}	35.66 ^{ab}	34.75 ^{abc}	34.48 ^{bcd}	33.62 ^{cd}	34.67 ^{abc}	33.32 ^d
Protein	26.40 ^a	25.55 ^{abc}	26.02 ^{ab}	25.29 ^{bcd}	25.15 ^{cd}	24.77 ^{cd}	25.29 ^{bcd}	24.46 ^d
Salt	2.04 ^{bc}	1.68 ^d	2.14 ^{ab}	1.73 ^{cd}	2.28 ^{ab}	1.63 ^d	2.47 ^a	1.75 ^{cd}
S/M	6.37 ^a	4.98 ^b	6.48 ^a	4.92 ^b	6.71 ^a	4.53 ^b	7.17 ^a	4.65 ^b
Lactose (DL)	1.52 ^a	1.35 ^c	0.32 ^{de}	0.11 ^e	1.64 ^{ab}	1.41 ^{bc}	0.49 ^d	0.27 ^e
Total Ca	0.69 ^a	0.68 ^a	0.67 ^a	0.66 ^a	0.55 ^b	0.54 ^b	0.55 ^b	0.51 ^b
Total P	0.48 ^a	0.48 ^a	0.48 ^a	0.47 ^a	0.42 ^b	0.42 ^b	0.41 ^b	0.40 ^b

^{a-f}Means in a row with common superscript do not differ ($P \geq 0.05$).

¹Treatments: high Ca and P-high lactose-high S/M (HHH); high Ca and P-high lactose-low S/M (HHL); high Ca and P-low lactose-high S/M (HLH); high Ca and P-low lactose-low S/M (HLL); low Ca and P-high lactose-high S/M (LHH); low Ca and P-high lactose-low S/M (LHL); low Ca and P-low lactose-high S/M (LLH); and low Ca and P-low lactose-low S/M (LLL).

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Titration Curves

The pH-titration curves for the cheeses were measured on the day following their manufacturing. The pH titrations were performed similarly as described by Lucey et al. (1993). Titrations were done in duplicate for each sample. Cheese-water dispersions were prepared by homogenizing 3 g of cheese with 17 g of water using a high-shear mixer-homogenizer (model 17105, Omni International, Waterbury, CT) at a setting of 4 for 1 min. To each cheese dispersion, 100 mL of water was then added to obtain a final cheese-water dispersion with a dilution factor of 1:39 (wt/wt). The cheese-water dispersions used in this study were more diluted than in previously published studies (Ollikainen, 1990; Lucey et al., 1993; Hassan et al., 2004), in which dilutions of 1:4 to 1:10 (wt/wt) were used. This large dilution factor was chosen to prevent activity coefficients from changing significantly throughout the titrations. Activity coefficients for a complex medium like a concentrated cheese dispersion are unknown and can only be projected. At the rather high dilution used in this study, the activity coefficients are close to unity and—due to the negligible dilution by the titrant—constant

throughout the titration. Consequently, the activities of all ions were assumed equal to their concentrations.

The cheese-water dispersions were titrated at room temperature with 1 N HCl to a pH of 3.0, and then backtitrated with 1 N NaOH to a pH of 9.0. The titrant was added using a 711 Liquino dispenser (Metrohm Ltd., Herisau, Switzerland) at a rate of 0.05 mL/min, unless otherwise noted. Throughout the titrations, the samples were stirred to ensure proper sample mixing. The pH was measured potentiometrically every 5 s using a double-junction sleeve-type Ag/AgCl reference electrode (Mettler Toledo, Wilmington, MA), a pH half-cell glass electrode (Inlab, Mettler Toledo), and an EMF 16 potentiometer (Lawson Laboratories Inc., Malvern, PA).

Calculation of pH Buffering Indices

Changes in pH resulting from the addition of titrant were used to calculate pH buffering indices (dB/dpH) as follows:

$$\text{dB/dpH} =$$

$$\frac{\text{Volume of titrant added (L)} \times \text{Normality of titrant (N)}}{\text{Change in pH}}.$$

This equation does not take into account the dilution of the sample with titrant, as was suggested by Van Slyke (1922). A volume correction for these rather diluted samples would have had only a small effect on the resulting buffering curves. Moreover, a volume correction for a highly pH-buffered system is questionable because the pH in a buffered system hardly depends on the sample volume. A 5-point Savitzky-Golay filter was used to improve the signal-to-noise ratio.

Table 2. List of association constants, complexation constants, and solubility products utilized for modeling (Brintzinger, 1965; Martell and Smith, 1975; Kotrlý, and Šůcha, 1985; Lu and Leng, 2005)

Association constants		Complexation constants ¹		Solubility products	
Phosphate	$10^{-2.12}$, $10^{-7.12}$, $10^{-12.67}$	Ca phosphate MHL/M.HL MH ₂ L/M.H ₂ L	457.09 25.7	Ca ₃ (PO ₄) ₂	2.02×10^{-33}
Citrate	$10^{-3.14}$, $10^{-4.77}$, $10^{-6.39}$	Ca citrate ML/M.L MHL/M.HL MH ₂ L/M.H ₂ L	$10^{3.55}$ $10^{2.13}$ 10	CaHPO ₄	2.63×10^{-7}
Lactate	$10^{-3.08}$	Ca lactate	17.37	Ca ₄ H(PO ₄) ₃	2.0×10^{-49}
Histidine ²	$10^{-6.04}$	Mg phosphate MHL/M.HL MH ₂ L/M.H ₂ L	$10^{2.81}$ $10^{1.66}$	Ca ₅ (OH)(PO ₄) ₃	2.34×10^{-59}
Phosphoserine ²	$10^{-5.67}$	Mg citrate ML/M.L MHL/M.HL	$10^{3.45}$ $10^{1.81}$	Mg ₃ (PO ₄) ₂	6.31×10^{-26}
Aspartic acid ²	$10^{-3.86}$	Ca complex of Asp ³	3.16	MgHPO ₄	1.51×10^{-6}
Glutamic acid ²	$10^{-4.31}$	Mg complex of Asp ³	3.47	Tricalcium citrate	4.79×10^{-16}
		Ca complex of Glu ⁴	3.24	Trimagnesium citrate	1.26×10^{-13}
		Mg complex of Glu ⁴	3.39		
		Ca complex of SerP ⁵	30.90		
		Mg complex of SerP ⁵	37.15		

¹M is the concentration of metal ion, and L is the concentration of ligand.

²Side chain.

³Estimated from corresponding value for propanoic acid (ML/M.L).

⁴Estimated from corresponding value for butanoic acid (ML/M.L).

⁵Estimated from corresponding value for monophosphate ester of methanol (ML/M.L).

Mathematical Modeling of pH Buffering Curves

All chemical species (ions, complexes, precipitates, amino acids) that could contribute to pH buffering of cheese were considered in a preliminary evaluation. The equilibrium constants at 25°C of the reactions into which these chemical species are involved were adopted from the literature (see Table 2; Brintzinger, 1965; Martell and Smith, 1975; Kotrlý and Šůcha, 1985; Lu and Leng, 2005). Table 3 shows the total concentrations of the components of the cheese curds for which modeling is discussed in this paper. The majority of Na in cheese is introduced as NaCl. Therefore, the Na content of cheese was estimated from the chloride concentration as determined by Mohr's titration. The total concentration of Ca and phosphate was measured using atomic and UV-visible spectroscopy, respectively. The organic phosphate content was measured by precipitation of the protein fraction of cheese with 12% (wt/wt) TCA, followed by the measurement of phosphate in the precipitate by UV-visible spectroscopy. Serine phosphate was assumed to represent all organic phosphate. The inorganic phosphate content was calculated by the difference between total and organic phosphate. The con-

centration of lactic and citric acids was determined using HPLC. The concentrations of Mg, histidine, aspartic acid, and glutamic acid were estimated based on literature data (USDA, 2005). Using these values, a semi-quantitative prescreening was performed to identify species that did not significantly contribute to buffering (see Results and Discussion). These calculations were performed using program code written in Mathematica5 (Wolfram Research, Inc., Champaign, IL).

Fully quantitative modeling was performed for all the species that were shown to be significant by the prescreening. A set of equations representing the chemical equilibria and mass and charge balances was considered for every pH value of interest. A set of equations excluding the formation of a precipitate was solved initially for every pH value to give a first set of concentrations for all chemical species. For any pH at which no precipitate formed at equilibrium, this first set represented physically meaningful, correct concentrations. However, if for a given pH, a precipitate is expected for equilibrium conditions, this manifested itself by negative concentrations in the first obtained set of concentrations. Whenever such evidently wrong concentra-

Table 3. Molar concentration of chemical species in cheese:water dispersions (1:39 wt/wt) used as input variables for mathematical modeling of pH buffering curves¹

Chemical species	Treatments ² (molar concentration)	
	HLH at d 1	LHL at d 1
Total Ca	0.0043	0.0034
Total Na	0.0082	0.0067
Total Mg	0.0003	0.0003
Total lactic acid	0.0028	0.0028
Total citric acid	0.0002	0.0002
Total phosphate	0.0039	0.0029
Serine phosphate ³	0.0015	0.0015
Inorganic phosphate ⁴	0.0024	0.0014
Histidine (total, not protein-bound ⁵)	0.0015, 0.00001	0.0014, 0.00001
Aspartic acid (total, not protein-bound ⁵)	0.0031, 0.00003	0.0029, 0.00003
Glutamic acid (total, not protein-bound ⁵)	0.0108, 0.0002	0.0102, 0.0002

¹From (USDA, 2005; Upreti and Metzger, 2006; Upreti et al., 2006).

²Treatments: HLH = high Ca and P, low lactose, high salt-in-moisture; LHL = low Ca and P, high lactose, and low salt-in-moisture.

³Assumed to represent all organic phosphate.

⁴Calculated as total phosphate – organic phosphate.

⁵Concentration of free amino acids at 6 mo of ripening (adapted from Shakeel-Ur-Rehman et al., 2004).

tions were obtained for a specific pH in the first calculation, the program automatically expanded the original set of equations to account for the precipitation of hydroxyapatite (unless noted otherwise, see Results and Discussion), and the expanded set of equations was solved to give a second set of (without exception positive and meaningful) concentrations for all chemical species.

RESULTS AND DISCUSSION

Experimental Titration and pH Buffering Curves

The pH buffering capacities of cheeses were determined from pH titration curves of cheese dispersions as measured with strong acids or bases (Ollikainen, 1990; Lucey and Fox, 1993; Lucey et al., 1993; Hassan et al., 2004). Figure 1 shows the experimental titration curve and pH buffering curve of a cheese prepared with the HLH treatment, as measured on the day following its manufacture. The cheese–water dispersion (dilution 1:39 wt/wt) was first brought from its initial pH (~5.8) to a pH of 3 by addition of 1 *N* HCl, and then backtitrated to pH 9 by addition of 1 *N* NaOH.

The regions in the titration curve (Figure 1a) that show no or only small slopes represent ranges of high pH buffering. If the derivative of a pH titration curve is plotted, minor changes in buffering capacity become more readily recognizable (Van Slyke, 1922). For example, Figure 1b shows regions of high pH buffering as peaks in the pH buffering curve. Ideally, backtitration yields sections of a titration and a buffering curve that overlaps with the corresponding sections obtained in the initial titration. Evidently, this is not true for the

titration and buffering curves shown in Figure 1. Differences between the forward and back titration sections (Figure 1b) indicate reactions that are irreversible on the timescale of the titration, as will be discussed below. Similar titration and pH buffering curves were obtained for all 8 differently prepared cheese curds in our study. The most noticeable differences between the buffering curves were observed for the pH buffering peak at pH 5.1. The peak height for cheeses with high Ca and P (HHH, HHL, HLH, HLL) were higher than for cheeses with low Ca and P (LHH, LHL, LLH, LLL). This shows that Ca²⁺ and phosphates have a significant role in pH buffering in cheese in this pH region [see below “pH buffering between pH 4.5 and 5.5 (Region 1)”].

Identification of pH Buffering Regions in Cheese

The pH buffering curves typically show 5 distinct regions of interest, and are labeled 1 to 5 in Figure 1b. Differences in absolute values for peak maxima have been observed by different researchers (Salaün et al., 2005), and are related to differences in the cheese composition, differences in cheese dilution factors, and the rate of addition of acid/base of different concentrations.

In our experiments, the cheese–water dispersions were titrated from their initial pH of typically ~5.8. Strong pH buffering was observed from the starting pH down to pH 4.5, with a peak maximum at pH 5.1 (region 1). The continued addition of acid to the cheese dispersion resulted in a shoulder at about pH 4.0 (region 2), followed by a steep increase in the pH buffering capacity at lower pH (region 3). The shoulder around pH 4.0 is apparent in the pH buffering curves for other cheese

Figure 1. Typical titration a) and buffering b) curves of a 1:39 cheese:water dispersion (shown: HLH treatment, high Ca and P, low lactose, high salt-in-moisture); dB/dpH = pH buffering index. (1 = buffering peak between pH 6 and pH 4 during forward titration; 2 = buffering shoulder at pH 4 during titration; 3 = steep buffering at pH 3 during forward titration; 4 = buffering peak at pH 6 during back titration; 5 = buffering peak above pH 9 during back titration).

samples as well (see Figure 2, LHL). After reaching a pH of 3.5 or lower, the cheese–water dispersions were backtitrated using 1 N NaOH. No pH buffering peak at pH ~5.1 was observed, but pH buffering with a peak maximum at pH 6.0 was detected (region 4) in the back-titration. Continued addition of base showed a region of increased pH buffering at pH 9.0 and higher.

Although similar pH buffering in cheese was observed by others, there does not seem to be consensus about the chemical species and mechanisms that contribute to pH buffering in different pH regions. To understand which chemical species and chemical equilibria contribute to pH buffering in different pH regions, mathematical modeling of the pH buffering curves was pursued. Owing to the marked differences in experimental pH buffering curves for the HLH and LHL treat-

ments, buffering curves for these treatments are used here for comparison of experimental results to the theoretical curves predicted by the mathematical model.

Mathematical Modeling

To understand quantitatively the extent to which each chemical species contributes to pH buffering in cheese, it was assumed that during a titration all the chemical species that contribute to pH buffering in cheese were at equilibrium. Seventy chemical species and precipitates were identified as possibly influencing pH buffering in the pH range of 3 to 9. They can be broadly classified as follows: cations (Na^+ , Ca^{2+} , Mg^{2+}); anions (Cl^- , OH^- , and, in various states of protonation, phosphate, citrate, lactate, formate, acetate, propionate, and butyrate); complexes (Na^+ , Ca^{2+} , and Mg^{2+} phosphates; Na^+ , Ca^{2+} , and Mg^{2+} citrates; Ca^{2+} lactate); precipitates (Ca^{2+} and Mg^{2+} phosphates, Ca^{2+} and Mg^{2+} lactates, Ca^{2+} and Mg^{2+} citrates, Ca^{2+} and Mg^{2+} chloride, Ca^{2+} and Mg^{2+} hydroxide); free and protein-bound amino acids; and Ca^{2+} and Mg^{2+} complexes of free and bound amino acids.

Recognizing the large number of variables and equations that would be needed to consider all these species and precipitates, a prescreening of the chemical species was performed using criteria based on total concentration, complexation constants, and solubility products. Total concentrations of Ca, Na, lactic acid, citric acid, phosphate, and serine-phosphate were measured analytically (Upreti and Metzger, 2006; Upreti et al., 2006), and the Mg, histidine, aspartic acid, and glutamic acid content was estimated using published values (USDA, 2005). For different cheese curds, total concentrations representing respective curds were used as input variables for modeling (Table 3). Because the pH buffering capacity is related to the concentration of chemical species, the species that are present in very small amounts have negligible contributions to the pH buffering capacity of cheese. For instance, free amino acids have a concentration that is less than 1% of the concentration of protein-bound amino acids (Shakeel-Ur-Rehman et al., 2004) and, therefore, contribute to pH buffering much less than do protein-bound amino acids. Hence, free amino acids were not considered for the quantitative evaluation (Table 3). In addition, it was assumed that only the side chains of proteins are relevant for protonation and complex formation equilibria, whereas the terminal ammonium and carboxylate groups of proteins occur only in small concentrations and have a negligible effect on pH buffering. Therefore, only amino acids with side chains containing a functional group with a pKa in the range of 3 to 9 were considered. To assess the complexation of metal cations and the side-

Figure 2. Experimental (left) vs. predicted (right) buffering curves for a) HLH (high Ca and P, low lactose, high salt-in-moisture), and b) LHL (low Ca and P, high lactose, low salt-in-moisture) cheeses. Predicted changes in concentration of different chemical species with change in pH are shown in panel c; GluH, AspH, SerPH, and HisH represent the protonated side chain groups of glutamate (Glu), aspartate (Asp), serine phosphate (SerP), and histidine (His), respectively; LacH = protonated lactate (Lac), and HAP = hydroxyapatite.

chain groups of aspartate, glutamate, serine phosphate, and histidine, the respective complex formation constants for propionic acid, butanoic acid, monomethyl phosphate, and imidazole were used.

Metal ion complexes were disregarded when an estimate of their concentration, based on the complex formation constant and the total concentration of the involved species, showed that the true complex concentra-

tion would be negligibly small. For example, the formation of a monosodium citrate complex was ignored because its concentration is low when estimated based on the total citrate concentration. Evidently, the true complex concentration of monosodium citrate complex is even lower than this rough estimate because only a fraction of the total citrate is in its fully deprotonated state, and the formation of complexes between citrate and Ca^{2+} and Mg^{2+} further lowers the concentration of free citrate. Similarly, contributions from Na phosphates, Mg lactate, and from soluble or insoluble aggregates between chlorides and hydroxides of Ca^{2+} , Mg^{2+} , and Na^+ were found to be negligible. Also, the concentrations of Ca^{2+} and Mg^{2+} complexes of free amino acids and side chains of histidine and aspartic acid were found to be too low to be relevant. Finally, although it is possible in more concentrated solutions to obtain simultaneous precipitates of, for example, CaHPO_4 and $\text{Ca}_3(\text{PO}_4)_2$ (Whittier, 1933), it was found that for the total phosphate and Ca^{2+} concentrations of the cheese-water dispersions investigated in this study, CaHPO_4 precipitate formation under thermodynamic equilibrium is not expected. In addition, owing to the experimental evidence that the precipitate in cheese or CCP is hydroxyapatite [$\text{Ca}_5(\text{OH})(\text{PO}_4)_3$; Bak et al., 2001], hydroxyapatite was considered for the mathematical modeling unless mentioned otherwise.

This elimination process provided a shorter list of 36 relevant species falling into the following categories: cations (Na^+ , Ca^{2+} , Mg^{2+}); anions (phosphates, citrates, lactates, in various states of protonation); complexes (Na^+ , Ca^{2+} , and Mg^{2+} complexes of phosphates, citrates, lactate, and side-chains of protein-bound amino acids as indicated in Table 2); precipitate of hydroxyapatite [$\text{Ca}_5(\text{OH})(\text{PO}_4)_3$]; and protein-bound glutamate, histidine, serine phosphate, and aspartate side chains.

The equilibrium reactions and mass balances corresponding to these chemical species were identified, and the set of 36 equations was solved for a range of pH values between 3 and 9 to give the pH dependence of the concentration of all 36 species (Figure 2c) and, thereof, titration and pH buffering curves (Figure 2a,b).

Qualitative Comparison of Experimental vs. Predicted pH Buffering Curves

As shown in Figure 2, the predicted pH buffering curves exhibit the pH buffering regions 1, 2, and 3, which are similar to the experimental curves. However, a peak corresponding to peak 4 in the experimental data is missing in the predicted curves. A qualitative comparison of the pH buffering regions of 1, 2, 3, and 4, and the quantitative contributions of different chemical

species to pH buffering in these regions are described below.

pH Buffering Between pH 4.5 and 5.5 (Region 1).

As can be seen in Figure 2a, the predicted curve for HLH shows a pH buffering maximum at pH ~5.1, which matches our experimental results. A similar peak is missing in the experimental data for LHL (Figure 2b) because the starting pH of that cheese dispersion was already below 5.1. Nonetheless, it is worth noticing that the peak area of pH buffering peak 1 in the calculated curve is smaller in LHL compared with HLH. Whittier (1929) attributed a similar peak maximum at pH 5.2 to caseins (micelles), and Lucey and Fox (1993) suggested that pH buffering in this region is due to CCP. Wiley (1935b) proposed that a peak maximum at pH 5 in the pH buffering curve of milk corresponded to Ca, phosphate, citrate, and caseins. To identify what factors primarily influence pH buffering in this region, the pH dependence of the concentration of different chemical species was predicted in this study with the mathematical model (Figure 2c). It was found that pH 6.0 marks the beginning of solubilization of hydroxyapatite, which is complete when pH 5.0 is reached. This explains why the pH buffering peak at pH 5.1 was not observed for the LHL treatment, in which the initial pH of the cheese dispersion was already below 5.1 when the titration began (Figure 2b). Because the buffering curves for all 8 tested cheese curds show (data not shown), and as confirmed by the model calculations, the pH of cheese dispersions must be above 5.0 to observe a pH buffering peak due to solubilization of hydroxyapatite. Because solubilization of hydroxyapatite leads to the formation of phosphate ions that can be protonated, it indirectly affects pH buffering (Lucey et al., 1993; Lucey and Fox, 1993). To quantitatively illustrate the contribution of this precipitate to the pH buffering capacity, the mathematical model was modified to remove the contribution of precipitate from the pH buffering curve. Figure 3 shows a pH buffering curve for a cheese-water dispersion as predicted from all 36 species (solid line), and if the contribution of hydroxyapatite was removed (line with open triangles). The latter curve predicts a remarkable decrease in pH buffering at pH 4.5 to 5.5. This clearly shows that the pH buffering peak in this region is dominated by the solubilization of a calcium phosphate precipitate. The calculations show that in cheeses prepared by all 8 different treatments, there was always an excess of free Ca^{2+} relative to free phosphate; that is, the amount of the calcium phosphate precipitate is limited by the concentration of inorganic phosphate present. Thus, the pH buffering capacity of a cheese curd in this buffering region is higher if the curd contains more phosphate.

Figure 3. Predicted buffering curves after subtraction of contributions from hydroxyapatite (Δ) only; and hydroxyapatite and glutamate ($\circ$). The original buffering curve is also shown (solid curve).

Surprisingly, although the intensity of the pH buffering peak diminished when the contribution of hydroxyapatite was removed (see Figure 3), there was still significant residual pH buffering in this region. The species concentrations shown in Figure 2c suggest an important role of the side chain of protein-bound glutamate. Indeed, the removal of the contribution of glutamate from the predicted buffering curves illustrates the significance of this species for pH buffering in this region (Figure 3). For a cheese prepared with the HLH treatment, the contributions of hydroxyapatite and the side chains of protein-bound glutamate to pH buffering in the region from pH 4.5 to 5.5 are 37 and 41%, respectively.

A comparison of the experimental and predicted pH buffering curves in Figure 2 shows that the theoretical curves overestimate the pH buffering capacity of the cheese–water dispersions. For example, the peak height for pH buffering peak 1 in the predicted curve is ~ 0.0017 , whereas the experimental curve shows a peak height of ~ 0.0006 . To assess if these overestimates were due to the use of equilibrium constants unsuitable for such dispersions, pH buffering curves were calculated using K_{sp} values for hydroxyapatite that were either 10^3 smaller or larger than the K_{sp} value indicated in Table 2. The results showed significant shifts in the position of the peak maximum (for $K_{sp} = 2.34 \times 10^{-62}$, the pH maximum was at 4.7, and for $K_{sp} = 2.34 \times 10^{-56}$ at 5.7), as opposed to a decrease in peak height (data not shown). Hence, the small experimentally observed peak maxima cannot be explained by inaccuracies in the value of K_{sp} . However, if a calcium phosphate precipitate solubilizes slowly, or if a protein does not unfold quickly upon addition of acid during a titration, these species will not fully contribute to pH buffering capacity

Figure 4. Effect of speed of titration on pH. Arrows indicate when the addition of acid was stopped.

as observed within the timescale of the titration. Therefore, it was expected that titrating a cheese–water dispersion down to a pH of ~ 5.0 and stopping the addition of acid at that point would be followed by a gradual increase in pH. This was indeed confirmed experimentally when cheese dispersions were titrated with 3 different rates of acid addition. In each case, the addition of acid was stopped when the pH reached ~ 5 . As Figure 4 shows, the pH of each mixture was found to increase gradually after the addition of acid was stopped, as expected. Higher initial rates of acid addition led to larger subsequent pH drifts. Analogous results were observed by Wiley (1935b), who found that when milk was titrated quickly, pH buffering maxima occurred at pH 5. However, if milk was allowed to stand for 2 h after the addition of hydrochloric acid, and pH measurements were made thereafter, maximum pH buffering occurred at pH 5.5.

In the present study, all pH titration curves, other than those shown in Figure 4, were performed by addition of titrant at a rate of 0.05 mL/min. For this rate, Figure 4 shows that there is an increase in ~ 0.3 pH units upon complete solubilization of the calcium phosphate precipitate and unfolding of proteins. Hence, if given enough time for equilibration, the amount of titrant added at a rate of 0.05 mL/min to decrease the pH to 5.0 was, under equilibrium conditions, only sufficient to lower the pH to 5.3. To assess the accuracy of the mathematical model, we determined the amount of titrant predicted by the model that was necessary to reach the pH obtained in the experiment, after allowing for pH drift. This value was then compared with the actually added amount of titrant. As the data in Table 4 show, the model predicts the buffering capacity of the cheese–water dispersion within $\pm 13\%$. The residual error probably results from experimental inaccuracies

Table 4. Comparison of predicted vs. actual volume of titrant needed to cause same change in pH

Flow rate (mL/min)	Predicted volume (mL)	Actual volume (mL)	% error ¹
0.10	0.26	0.30	-13
0.05	0.35	0.33	+6
0.03	0.45	0.40	+13

¹% error = [(Predicted - Actual) ÷ Actual] × 100.

as well as oversimplifying assumptions in the model. However, given the complexity of this chemical system, the error seems to be rather small.

pH Buffering Between pH 3.5 and 4.5 (Region 2). The calculated curves shown in Figure 2 exhibit a shoulder at pH ~4.0, which is also apparent in our experimental data, and will be referred here as region 2. Whittier (1929) attributed the steep rise of the curve at lower pH values (<5.0) to lactate. Lucey et al. (1993) proposed a peak in the buffering curve at pH 4 due to lactate (pKa 3.8), whereas Tomasula et al. (1999) suggested the pH buffering peak at pH 4.1 to be related to aspartic acid (pKa 4.1) and glutamic acid (pKa 4.6). Walstra and Jenness (1984) suggested an additional contribution of citrate salts to pH buffering in this region. Ollikainen (1990) concluded that pH buffering in the pH range of 2 to 5 was due to carboxyl groups of amino acids (pKa 2.1 to 2.7), total lactic acid (pKa 3.8), and acetic and propanoic acids (pKa 4.8 to 4.9). In a relatively recent study, Famelart et al. (2002) observed a maximum pH buffering capacity of a ripened Emmental cheese at pH 4.3 to 4.6, and attributed it to the presence of phosphate, lactate, propionate, acetate, and acid residues of proteins, peptides, and amino acids.

Our quantitative model shows that lactate and the side chains of aspartate and glutamate dominate pH buffering in this region (Figure 2c). To establish the relative importance of these 3 species for buffering in this pH region, the contribution of these 3 species was individually subtracted from the buffer curve predicted for all 36 species (Figure 5). The removal of glutamate showed a much more significant decrease in pH buffering in the pH region below pH 4.5 than aspartate and lactate. For the HLH cheese, buffering in the region from pH 4.5 to 3.5 is due to glutamate, aspartate, and lactate, with relative contributions of 48, 15, and 6%, respectively. Protonation of water molecules contributes 26% to the buffering index. Because of its higher concentration (see Figure 2c), glutamate contributes to buffering much more than aspartate and lactate.

pH Buffering Below pH 3.5 (Region 3). As the pH falls below 3.5, there appears to be a sudden increase in the pH buffering capacity (region 3). This is apparent

Figure 5. Predicted buffering curves after individual subtraction of lactate (●), aspartate (△), and glutamate (○). The original buffering curve is also shown (solid curve).

in both the predicted and experimental curves. The model illustrates that the increased apparent buffering capacity represents the protons required for the protonation of water at this low pH (see Figure 6). Due to the rather high dilution of the cheese–water dispersions, this effect is relatively large in this study. Although a contribution of lactate to buffering in this region might be suspected (Figure 2c), it does not affect the predicted curves significantly. This finding contrasts with conclusions drawn in previous studies of somewhat less diluted samples, where an increase in pH buffering at pH ~2 was explained by pH buffering due to carboxyl groups of amino acids (pKa 2.1 to 2.7) or various protein residues (Ollikainen, 1990; Lucey et al., 1993).

Figure 6. Demonstration of influence of autoprotolysis of water at pH ~3.0 (○). Predicted buffering curve after subtraction of serine phosphate and histidine (△). The original buffering curve is also shown (solid curve).

Figure 7. Theoretical buffering curves as predicted for the formation of different calcium phosphate precipitates.

Backtitration: pH Buffering Between pH 5 and 8 (Region 4). In the backtitration, the experimental results show a pH buffering peak between pH 5 and 8 (peak maximum at pH 6). Similar peaks were observed by Wiley (1935a), who attributed this buffering maximum to calcium phosphate precipitation. Similar conclusions were drawn by Lucey and Fox (1993), who attributed the strong pH buffering effect in the pH range from 6 to 7 to the formation of $\text{Ca}_3(\text{PO}_4)_2$. They concluded that $\text{Ca}_3(\text{PO}_4)_2$ precipitation leads to the release of H^+ (from HPO_4^{2-} and H_2PO_4^-), which can combine with the OH^- titrant, causing pH buffering. Salaün et al. (2005) attributed pH buffering at pH 6.0 to organic (phosphoserine) and inorganic phosphates. However, no such peak was predicted by our theoretical model, and in the same pH range, only weak buffering by protein-bound glutamate, serine phosphate, and histidine, and hydroxyapatite precipitate was predicted (see Figure 2c). To quantitatively illustrate the relative importance of each of the above-mentioned species in pH buffering in this pH region, the contributions of these species were subtracted from the curve as predicted for all 36 species (Figure 6). About 27% of buffering in the pH region from 5 to 8 was attributed to serine phosphate and histidine. The model predicts that another 47% is due to hydroxyapatite precipitate, and 19% due to protein-bound glutamate residues.

Evidently, the model prediction in this pH region does not match the experimental data well. This is probably

explained by the types of precipitates present at different stages of our titrations. Acid was added to the cheese–water dispersions, which solubilized the calcium phosphate precipitates. In the backtitration with base, reprecipitation occurred. Given the complex chemistry of calcium phosphate precipitates, it appears likely that a different precipitate was formed in the backtitration than the CCP that was initially present in the cheese–water dispersion before acid was added. Therefore, program code was written to predict pH buffering curves as they would be observed upon precipitation of 4 different calcium phosphate precipitates, namely $\text{Ca}_3(\text{PO}_4)_2$, brushite (CaHPO_4), octacalcium phosphate [$\text{Ca}_4\text{H}(\text{PO}_4)_3$], or hydroxyapatite [$\text{Ca}_5(\text{OH})(\text{PO}_4)_3$]. Figure 7 shows that the formation of different precipitates would result in very different pH buffering curves. It appears that the pH buffering peak with a peak maximum at pH 6.0, formed in the backtitration, can be explained by the formation of CaHPO_4 or octacalcium phosphate [$\text{Ca}_4\text{H}(\text{PO}_4)_3$] but not by $\text{Ca}_3(\text{PO}_4)_2$ or hydroxyapatite.

Figure 7 is also interesting in view of peak 1, which is observed at pH 5.1 when the cheese dispersions are titrated initially with 1 N HCl. As mentioned above, the precipitate that dissolves at this pH is commonly referred as CCP. Figure 7 shows that both the theoretically predicted peak maxima for $\text{Ca}_3(\text{PO}_4)_2$ and hydroxyapatite correspond well with the experimental values. These conclusions are consistent with research

suggesting the presence of $\text{Ca}_3(\text{PO}_4)_2$ (Ling, 1936; Schmidt, 1980) or hydroxyapatite (Bak et al., 2001), but seem to contradict other conclusions suggesting CCP contains brushite (CaHPO_4 ; Holt et al., 1989; Holt and Hukins, 1991) or octacalcium phosphate [$\text{Ca}_8\text{H}(\text{PO}_4)_6$].

CONCLUSIONS

There is major overlap between the contributions from different chemical species to pH buffering of cheese in different pH regions. The mathematical model described here allows the quantitation of the relative contributions from different species. It shows that the effect of Mg and citrate to pH buffering in cheese is minor, owing to their relatively low concentrations. Both CCP and glutamate contribute significantly to pH buffering between pH 4.5 to 5.5, which should be interesting to cheese makers because of the effect of these species on the final pH of cheeses. In addition, the contribution of lactic acid to buffering at pH < 5.0 confirms the role of lactic acid in determining the pH of cheese. Hence, the control of Ca, P, and lactic acid (as determined by residual lactose and S/M) is crucial for controlling cheese pH, and hence, cheese quality.

It is important to recognize that the concentration of many of the 36 species considered by this mathematical model cannot be readily determined experimentally. Any analytical method involving a separation would fail to do so because any separation would affect the large number of interrelated chemical equilibria into which the 36 chemical species are involved. Also, a spectroscopic quantitation of all these species would be extremely complicated to perform, and chemical sensors for many of these species are not available yet. Therefore, a mathematical model is uniquely suited to illustrate how individual species influence pH buffering in different pH regions. Such a model could also be used to predict how cheese pH could be changed by adjusting one or more of the constituent chemical species. This has practical implications for process cheese manufacturers who can use different levels and types of emulsifying salts to adjust cheese pH. However, additional studies will have to be conducted to test the ability of this model to predict properties of cheese in more concentrated and even undiluted forms.

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