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Effect of Chemical Parameters on Structure-Function Relationships of Cheese

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EFFECT OF CHEMICAL PARAMETERS ON STRUCTURE-FUNCTION RELATIONSHIPS OF CHEESE

by

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ABSTRACT

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The effect of chemical parameters on cheese structure and functionality was studied by modifying the calcium, salt content, and pH of cheese. Cheese blocks were high-pressure injected from zero to five times with water, solutions of different salts, or an acid solution 14 d after manufacture. Successive injections were performed 24 h apart. After 40-42 d of refrigerated storage, cheese structure was studied by using scanning electron microscopy and digital image analysis, and cheese functionality was characterized by texture profile analysis and melting test.

Increased salt content of cheese (2.7 versus 0.1%) caused the protein matrix to become more hydrated and to expand ($P < 0.1$), though the occurrence of syneresis resulted in decreased moisture content of cheese ($P < 0.05$). Salt injection increased cheese hardness and the initial rate of cheese flow, but it decreased cheese cohesiveness ($P < 0.05$).

Increased calcium content (1.8 versus 0.3%) and decreased pH of cheese (4.7

versus 5.3) caused contraction of the protein matrix ($P < 0.05$) and release of serum. Thus, the matrix became less hydrated, and the moisture content and weight of cheese decreased ($P < 0.05$). Calcium injection decreased the pH and melting of cheese, but it increased cheese hardness ($P < 0.05$). Acid injection promoted calcium solubilization and decreased calcium content of cheese ($P < 0.05$). Above pH 5.0 (5.0-5.3), acid injection decreased cheese hardness and increased the initial rate of cheese flow ($P < 0.05$). Below pH 5.0 (5.0-4.7), acid injection decreased cheese cohesiveness, and the initial rate and extent of cheese flow ($P < 0.05$).

In conclusion, modifying the chemical composition of cheese alters protein interactions, resulting in cheese with different structural and functional properties. Increased salt content of cheese (up to 2.7%) impairs protein-to-protein interactions, and its effect is most significant when salt content increases from 0 to 0.5%. Below 5.0 (5.0-4.7), the effect of pH predominates over calcium content, and decreased cheese pH promotes protein-to-protein interactions. Increased calcium content of cheese (up to 1.8%) also promotes protein-to-protein interactions, and the content of protein-bound calcium may be the major factor controlling the functionality of most cheeses.

(187 pages)

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LIST OF ABREVIATIONS

ANOVA = analysis of variance

AOAC = Association of Official Analytical Chemists

CCP = colloidal calcium phosphate

CV = coefficient of variation

df = degrees of freedom

EDTA = ethylenediaminotetraacetate

FDA = Food and Drug Administration

GLM = general linear model

LSD = least significant difference

MS = mean square

NS = nonsignificant

R^2 = coefficient of determination

SAS = Statistical Analysis System

SEM = standard error of the mean

TCA = trichloroacetic acid

TPA = texture profile analysis

USDA = United States Department of Agriculture

GLOSSARY

ADHESIVENESS = the tendency of cheese to stick to the probe during a two-cycle compression test. Determined as the negative load area between compression cycles.

COHESIVENESS = the tendency of cheese to stick together during a two-cycle compression test. Determined as the ratio of the positive load area during the second compression cycle to that during the first compression cycle.

HARDNESS = the peak force during the first compression cycle.

MELTING = the softening and flow of cheese upon heating. Determined as the length of cheese flow within a tube immersed in hot oil (Chapter 2), or as the decrease in height when heated cheese is subjected to a constant load (Chapters 3 and 4).

TWO-BITE COMPRESSION = compression test with two successive cycles of compression.

CHAPTER 1

GENERAL INTRODUCTION

Cheese production in the US grew steadily between 1997 and 2000, slightly decreasing in 2001. On average, during the 5-year period, total cheese production grew at an annual rate of 2.2%, reaching a total production of 8.13 billion pounds in 2001 (USDA, 2002). During that period, Cheddar and Mozzarella cheese production grew at an average annual rate of 2.1% and 0.6%, respectively, reaching a total production of 2.82 and 2.63 billion pounds in 2001. This increase in production relates to the growth in cheese sales, as new consumer trends increasingly favor the use of cheese as an ingredient in a variety of dishes. As a result, cheese manufactures face the challenge of being able to consistently make cheese that meets customer specifications.

In particular, customers require cheese to have consistent and tailored functionality. Among other functional properties, the cheese is expected to be shredded and melt to the desired extent. A correct knowledge of the effect of calcium, moisture, salt, and pH on cheese functionality would allow the cheese industry to set appropriate product standards, and would facilitate the successful design of new products for specific markets. Thus, by succeeding in meeting present and future customer demands, the dairy industry would be able to keep and increase actual levels of cheese production and market sales while increasing the demand of milk from dairy farmers.

The application of different cheese-making procedures determines differences in the chemical composition and structure of cheese, which results in cheese with different functional attributes. Thus, chemical parameters such as calcium content and pH

significantly affect cheese functionality, and studies aimed at understanding their effects and mechanisms of action have been performed. However, researchers have faced difficulties when conducting these studies. Traditionally, the chemical composition of cheese has been altered by modifying the cheese-making procedure, but because of interactions between chemical parameters, such as calcium content and pH, it has been difficult to alter the chemical composition of cheese in a controlled manner. As a result, researchers are still not certain about the independent effect of calcium or that of pH on cheese functionality, and whether one of them predominates, and under which conditions.

The present research was thus intended to gain understanding about the effect of calcium, moisture, salt content, and pH on cheese functionality. A high-pressure injection system was used to modify cheese composition by injecting solutions into cheese blocks, and chemical, structural, and functional properties of cheese were analyzed. To study the effect of calcium, cheese with low calcium content was made by using a direct-acid cheese-making procedure. For determining the effect of salt, unsalted cheese (Muenster prior to brining) with normal calcium content and pH was used, and to study the effect of pH, cheese with normal calcium, salt content, and pH (Cheddar) was used. As a result, increased knowledge of cheese functionality and how to control it is now available. This should benefit the research community in further advancing our understanding of the relationship between chemical and functional attributes of cheese, and increase the ability of the dairy industry to better direct cheese functionality in meeting costumer needs.

LITERATURE REVIEW

Calcium

Content and distribution in milk. Bovine's milk has an average mineral content of 0.7%, including K, Cl, P, Na, Ca^{2+}, and Mg^{2+}, and other trace elements (Walstra et al., 1999a). Most of these minerals are present in soluble and insoluble forms. Soluble forms include ionic and salt forms, whereas insoluble forms include counter ions, molecular-bound ions, and salts.

The average content of calcium in bovine's milk is about 1200 mg/L (i.e., 30 mM) (Fox et al., 2000b), and milk is supersaturated with calcium phosphate, with a large part of it being undissolved (Walstra et al., 1999b; Fox et al., 2000b). Thus, calcium distributes between soluble forms, 32 to 34% (ionic and bound to citrate and/or phosphate), and insoluble or colloidal forms, 66 to 68% (colloidal calcium phosphate [**CCP**] and casein-bound calcium) (Walstra et al., 1999b; Fox et al., 2000b). Among the caseins, α_s- and β-caseins can bind considerable amounts of calcium, whereas κ-casein does not bind calcium strongly because of the lack of phosphoseryl clusters (Fox and Mulvihill, 1982). In addition, serum proteins also bind some soluble calcium (Bloomfield and Morr, 1973).

Calcium binding to micellar caseins involves interaction with negatively charged groups, such as phosphate esters and carboxylic groups (Bloomfield and Morr, 1973; van Hooydonk et al., 1986b; Baumy et al., 1989; Gaucheron et al., 1997), and according to Marshall and Green (1980), most binding occurs to sites in the interior of the casein micelle. When bound to phosphate, calcium participates in the formation of large

aggregates that associate with the casein micelles, which are referred to as CCP (Bloomfield and Morr, 1973). The simplest possible structure of CCP being $Ca_3(PO_4)_2$, though most evidence indicates $CaHPO_4 \cdot 2H_2O$ (Fox et al., 2000b).

There is a pseudo and dynamic equilibrium between the soluble forms of calcium, and between its soluble and insoluble forms (Walstra et al., 1999b), with various chemical and physical factors affecting this equilibrium. Some of the changes that occur in mineral and calcium equilibrium are reversible, however, if changes in casein micelle structure occur they may be irreversible (Brulé et al., 2000).

Among the chemical factors affecting calcium equilibrium, the addition of a calcium source to milk, such as $CaCl_2$, initially increases the amount of soluble calcium (Mohamed et al., 1988; Fox et al., 2000c; Lenoir and Remeuf, 2000), which then results in increased content of CCP (Walstra et al., 1999d; Fox et al., 2000c; Lenoir and Remeuf, 2000). In contrast, decreased pH causes dissociation of calcium from casein micelles, thus decreasing the amount of casein-bound calcium and increasing the content of soluble calcium (Dalgleish and Parker, 1980; Gaucheron et al., 1997). Lowered pH decreases the ionization state of phosphoseryl and other groups, which impairs electrostatic interactions, thus promoting the release of calcium from caseins, with 50% of calcium being released from casein micelles in suspension at pH 5.2 (Gaucheron et al., 1997). Also, decreased pH promotes dissociation of colloidal calcium, which further increases the content of soluble calcium, and at pH 5.2, 74% of colloidal calcium would be removed from caseins in milk (Brulé and Fauquant, 1981).

In addition, increased ionic strength decreases the binding of calcium to caseins in

milk and casein systems (Dalgleish and Parker, 1980; Parker and Dalgleish, 1981; Gaucheron et al., 1997). In contrast, heating of milk, and the corresponding increase in temperature promotes increased binding of calcium to caseins (Dalgleish and Parker, 1980; Brulé and Fauquant, 1981; Parker and Dalgleish, 1981), precipitation of calcium phosphate (Pouliot et al., 1989), and an increase in the amount of CCP that largely associates with caseins in the casein micelles (Walstra et al., 1999b), which decreases the amount of soluble calcium.

Role in casein micelles. Calcium is an important component of casein micelles, and in particular, CCP is recognized for its role in helping to maintain the integrity of casein micelles (Holt, 1983; Fox et al., 2000b). According to Brulé et al. (2000), the binding of calcium and calcium salts to caseins promotes casein aggregation, which then allows for the formation of casein micelles. As a result, the integrity of casein micelles becomes dependent on calcium, and the removal of calcium causes preferential dissociation of β- and κ-casein from casein micelles (Bloomfield and Morr, 1973). A micellar framework then remains, but if a critical amount of calcium is removed, then the micellar framework also dissociates. In contrast, the use of calcium salts helps restore the micellar casein (Robinson and Wilbey, 1998a), and adding calcium increases the micellar size after prolonged cold storage of milk (24 to 48 h at 4 to 5°C) during which the content of soluble casein increases.

Effect on milk coagulation and curd properties. Calcium content affects the rennet coagulation of milk, and a critical level of calcium, and more precisely, a critical level of ionic calcium is probably needed to initiate micelle aggregation (van Hooydonk

et al., 1986b) and to allow the casein micelles to coagulate (Fox and Nash, 1979; Fox, 1993).

Calcium addition lowers the pH of casein systems (Bringe and Kinsella, 1993) and that of milk by promoting exchange of Ca^{2+} for H^{+} (Satia and Raadsveld, 1969; Jen and Ashworth, 1970; van Hooydonk et al., 1986b; Lenoir and Remeuf, 2000). Also, increased binding of calcium and calcium-containing complexes (van Hooydonk et al., 1986b) neutralizes negative charges in casein molecules (Green and Marshall, 1977) by reacting with phosphoserine residues and/or carboxylic acid groups (Dalgleish, 1983). As a result, the rate of enzymatic reaction increases, with an overall increase in enzyme activity (Bringe and Kinsella, 1986; van Hooydonk et al., 1986b) due to increased affinity of rennet for casein micelles (Green and Marshall, 1977).

The binding of calcium to casein molecules, and the consequent neutralization of negative charges, also results in increased protein-to-protein interactions. Charge neutralization decreases electrostatic repulsion between proteins, and interactions involving hydrophobic regions of proteins are promoted, which increases the aggregation of renneted casein micelles (Green and Marshall, 1977; Dalgleish, 1983; Bringe and Kinsella, 1986; van Hooydonk et al., 1986b; Walstra, 1993; Gaucheron et al., 1997; Fox et al., 2000c). However, rather than simply reducing the surface charge of renneted casein micelles, calcium also seems to have a specific function, most certainly calcium bridging, in promoting aggregation between renneted casein micelles (Dalgleish, 1983; Walstra et al., 1999c).

As a result of increased enzymatic action and/or aggregation of casein micelles,

adding calcium reduces the rennet coagulation or gelation time of milk (Green and Marshall, 1977; McMahon et al., 1984; McMahon and Brown, 1984; van Hooydonk et al., 1986b; Lucey and Fox, 1993; Robinson and Wilbey, 1998b; Fox et al., 2000c), and may increase gel strength if added in low concentration, less than 10 mM (Jen and Ashworth, 1970; McMahon and Brown, 1984; Mohamed et al., 1988; Lucey and Fox, 1993; Wolfschoon-Pombo, 1997; Walstra et al., 1999d). This would then lead to a firmer curd (McMahon et al., 1984; van Hooydonk et al., 1986b; Kosikowski and Mistry, 1997b; Fox et al., 2000c) and increased curd syneresis (van Dijk and Walstra, 1986; Mohamed et al., 1988; Pearse and Mackinlay, 1989; Walstra, 1993; Solorza and Bell, 1998b; Walstra et al., 1999d; Fox et al., 2000d).

At high levels of added calcium, however, increased ionic strength interferes with the proper interaction of chymosin with substrate, and calcium would impair specific ionic interactions between para-casein micelles (Bringe and Kinsella, 1986). Also, high levels of calcium would increase the positive charge on caseins, thus making them less prone to aggregation (Fox et al., 2000c), causing swelling of the protein matrix, and inducing suppression of syneresis (Pearse and Mackinlay, 1989; Fox et al., 2000d). Decreased interaction between caseins may even inhibit gelation by causing the gel network to be less extensive (McMahon et al., 1984), and if gelation occurs, a less extensive protein network may lead to a softer cheese curd (Fox et al., 2000c) and increased curdiness in fresh cheese (McMahon et al., 1984).

The ability of calcium to promote interaction between caseins, and thus to help maintain the integrity of casein micelles determines its usefulness as an additive in

improving the coagulation properties of milk (Walstra, 1993). Thus, addition of $CaCl_2$ to milk (up to 0.02%) is a common practice in the commercial production of cheese (Fox et al., 2000a), and calcium chloride is commonly added to milk in some regions and in certain seasons to compensate for the deficient coagulating properties of milk (Lenoir et al., 2000). Also, adding calcium to milk can improve the coagulation properties of overheated milk (Dalgleish, 1993; Kosikowski and Mistry, 1997a; Walstra et al., 1999c), and of milk that has been stored at cold temperatures for prolonged periods of time (Ramet et al., 1981; Lenoir et al., 2000). In contrast, the addition of chelating agents, such as citrate, oxalate, or EDTA, reduces the level of ionic calcium and colloidal calcium phosphate in milk, which impairs interactions between casein micelles and increases the heat stability of milk (Mohammad and Fox, 1983).

Effect on cheese composition. The effect of pH on the calcium content of casein micelles is regularly exploited during cheese making to control the content of calcium in cheese. Thus, during cheese making, acidification of milk (Shehata et al., 1967; Keller et al., 1974; Lucey and Fox, 1993), decreased pH of milk at setting, lower rate of acid production (Lucey and Fox, 1993; Fox et al., 2000d), and decreased pH of whey at draining (Lawrence et al., 1983; Kiely et al., 1992; Yun et al., 1995; Fox et al., 2000d) promote decreased calcium content of cheese, and they are considered the critical factors determining the mineral content of cheese, including calcium and phosphorous (Lucey and Fox, 1993). In contrast, and as mentioned before, adding a calcium source to milk increases the amount of soluble and colloidal calcium, which may then result in cheese with increased calcium content (Satia and Raadsveld, 1969; Solorza and Bell, 1998b).

Adding calcium to milk prior to cheese making can result in cheese with increased moisture content, which is thought to result from the over-riding effect of decreased curd pH at the time of whey drawing (Cheng et al., 1997). In contrast, when added to brine solution, increased calcium concentration decreases the moisture content and weight of cheese (Geurts et al., 1972).

Effect on cheese texture and structure. The content of calcium in cheese affects the extent and degree of protein aggregation, thus determining the basic structure and texture of cheese (Lawrence et al., 1983). A high level of protein aggregation and larger protein aggregates are observed in cheeses with higher calcium content (higher cheese pH) compared to cheeses with lower calcium content (lower cheese pH [Hall and Creamer, 1972; Lawrence et al., 1987]). Retention of very high levels of calcium thus produces a hard unyielding curd that renders a cheese with harsh body (Robinson and Wilbey, 1998a). In contrast, removal of CCP increases the ability of casein micelles to absorb water, which would promote swelling of casein submicelles causing the cheese to be softer (Lawrence et al., 1983). These differences in protein aggregation determine the contrasting structure and texture of cheeses such as Swiss and Cheddar (Hall and Creamer, 1972).

Effect on cheese functionality and yield. The ability of calcium to promote interactions between proteins and its role in maintaining the structure of casein micelles results in calcium being a major factor affecting cheese functionality. Thus, during cheese making, the concentration of calcium in the curd and the proportion of soluble calcium affect the plasticizing properties of Mozzarella curd (Fox et al., 2000d). Higher levels of

calcium cause Mozzarella cheese curd to be less cohesive and elastic, which results in curd with poor stretching (Cheng et al., 1997). According to Fox et al. (2000d), successful plasticization is achieved at relatively low levels of calcium (27 mg/g of protein) and increased level of soluble calcium (40%). Under these conditions, there is increased hydration of paracasein, and the curd becomes smoother after plasticization.

In Mozzarella cheese making, good stretching is normally achieved by lowering the pH to about 5.3, and ideally, pH 5.15 (Fox et al., 2000d). Thus, the stretching characteristics of natural cheese curd depend on the content of colloidal calcium phosphate, and according to Lawrence et al. (1987), conditions are usually optimal when 75% of the CCP has been removed. This removal of calcium also allows for increased emulsification of fat by caseins, which decreases oiling off during pizza baking (McMahon et al., 1993). In contrast, in direct-acid Mozzarella cheese manufacture, successful plasticizing is achieved at a higher pH (i.e., 5.6) due to increased solubilization of CCP, which allows for the paracasein to become more hydrated (Fox et al., 2000d). The successful stretching and performance of direct-acid Mozzarella cheese with a pH of 5.6 is thus evidence of the greater importance of demineralization compared to pH per se in affecting functional properties of cheese (Kindstedt, 1991; McMahon et al., 1993).

Calcium content also affects rheological, textural, and melting properties of cheese. Increased calcium content increases cheese moduli, increasing both the storage and loss modulus (Solorza and Bell, 1995, 1998a). Thus, the cheese becomes more solid like and has a more viscous behavior. Also, increased calcium content of cheese increases cheese firmness (Lawrence et al., 1993), and the cheese becomes more brittle (Satia and

Raadsveld, 1969). According to Solorza and Bell (1988a), the effect of calcium on rheological properties of cheese is mediated via the protein network, and acts independently. Calcium seems to promote linking of material to the gel network (Solorza and Bell, 1998a), and to increase the extent of cross-linking (Solorza and Bell, 1995).

In addition, calcium content affects the melting of cheese (McMahon and Oberg, 1998), with increased calcium content normally decreasing cheese melting (Olson and Bogenrief, 1997; Paulson et al., 1998a). In contrast, increased melting is observed in cheese made by direct acidification of milk due to extensive solubilization of calcium (Keller et al., 1974), and in outer regions of cheese blocks exposed to concentrated brine, which promotes exchange of calcium by sodium (Kindstedt et al., 1992). Decreased calcium content would result in a more hydrated protein matrix, with decreased interaction between proteins and increased protein-to-water interactions (McMahon and Oberg, 1998). This then allows the proteins to flow more easily, and cheese meltability increases.

There is also some evidence that adding calcium chloride to milk during the early stages of cheese making increases cheese yield (Wolfschoon-Pombo, 1997; Walstra et al., 1999d), and Solorza and Bell (1998b) observed increased recovery of fat and protein when calcium was added to milk.

Water

Content and distribution in milk. Water is an important and major component of foods, comprising 75 to 95% of many foods (Labuza and Lewicki, 1978). Based on quantity, water is the principal component of milk, and in average, it constitutes 87% of

the total weight of bovine's milk, with approximately 90% of water found in the milk serum, and the remaining 10% present as a component of casein micelles (Walstra et al., 1999a). Native milk proteins, caseins and whey proteins, have a strong ability to interact and bind water (Morr, 1989), and thus casein micelles are highly hydrated (Fox and Mulvihill, 1982).

According to Geurts et al. (1974b), four types of interaction between protein and water can be identified: 1) chemisorption, that is water interacting with polar side groups, which is unavailable as a solvent, 2) ice-structured, water forming ice-like structures surrounding hydrophobic groups, which is available for some reactions but not as a solvent, 3) imbibition or capillary, that is water held mechanically and sometimes by surface forces, which is available for reactions and as a solvent, and 4) steric exclusion, water layer between large molecules.

The ability of water to interact with proteins and other molecules is thus manifested in the variety of mechanisms by which they can interact, which in turn gives rise to different states of water in foods. However, there is no consensus on how to classify water fractions in food, and authors differ in the use of terms and classification of water fractions.

In foods, water has been described to exist in three basic states: tightly bound, also considered structural, held by adsorption in multilayers or hydrodynamic hydration, and bulk water, which is indistinguishable from free water (Harwalkar and Brown, 1989). Also, water in foods has been classified as either free water or restricted activity water (Morr, 1989). Thus, free or bulk water has normal vapor pressure, which among other

things is available for microbial growth and chemical reactions, and functions as a solvent. In contrast, restricted activity water, which includes monolayer, hydrogen bonded, and clathrate water, is not available as a solvent or for chemical reactions. In addition, imbibed water is physically entrapped but with similar properties to free water (Morr, 1989); the water imbibing power of foods being called either water-holding capacity or water-binding capacity (Labuza and Lewicki, 1978).

Casein hydration. Reported values on casein hydration vary widely, from 0.3 to 3.5 and 4.0 g of water/g of protein (Geurts et al., 1974b; Lelièvre and Creamer, 1978; Fox and Mulvihill, 1982; Morr, 1989), depending on the analytical method used and on whether caseins or casein micelles were considered. According to Fox and Mulvihill (1982), values of approximately 2 g of water/g of protein are more frequently reported. In addition, the binding of water to caseins is greater in milk and casein suspensions than in cheese (Geurts et al., 1974b).

Water binds to molecules because of its ability to interact with ions and ionic groups, as well as its ability to form hydrogen bonds with different food molecules through interaction with chemical groups such as hydroxyl and amide groups (Kinsella and Fox, 1986). As a result, the amount of water bound to caseins would be dependent on the physicochemical state of the proteins, which is a function of several factors including pH, temperature, protein concentration, and ionic conditions (Geurts et al., 1974b; Morr, 1989; Kneifel et al., 1991).

Even though the physicochemical state of milk proteins is expected to affect their hydration, the amount of water bound to casein seems to be unaffected by rennet

treatment, coagulation, or acid precipitation (Geurts et al., 1974b; Ruegg et al., 1974; Lelièvre and Creamer, 1978). However, at water activity values between 0.6 and 0.95, Ruegg et al. (1974) observed increased binding of water by native casein as compared to rennet-treated casein. Similarly, Creamer (1985) observed decreased solvation of casein pellets in rennet-treated skim milk. The effect of rennet on casein hydration is related to its proteolytic activity on κ-casein, and Ruegg et al. (1974) estimated that the loss of the hydrophilic glycomacropeptide from κ-casein in rennet-treated casein decreased water binding by 10%.

If the physicochemical state of proteins affects casein hydration, pH should then affect the amount of water that is bound to caseins. In this regard, Rüegg and Blanc (1976) observed pH to significantly affect the binding of water by caseins and casein micelles. Casein hydration was the lowest at pH 4.6, and in particular at high water activity (greater than 0.95), which resulted from promoted interaction between proteins and decreased protein-solvent interactions as the caseins approached their isoelectric point. Also, the addition of NaOH and increased pH of casein solutions increased protein hydration, which according to the authors would be related to the binding of Na^+ to caseins. Thus, it seems that Na^+ could have an independent effect on casein hydration, which is supported by the results of studies in which the addition of NaCl resulted in increased solvation of caseins in solution (Creamer, 1985; Curme et al., 1990). In contrast to the effect of NaCl in promoting casein hydration, addition of $CaCl_2$ has been observed to decrease casein solvation (Geurts et al., 1972; Creamer, 1985).

Regarding the effect of pH on the hydration of casein micelles, Rüegg and Blanc

(1976) observed no effect of pH at low water activity values, but minimum hydration was observed between pH 6.5 and 7.0 at high water activity (in particular when greater than 0.9). Thus, the behavior of caseins differs from that of casein micelles, and increased hydration of casein micelles seems to occur under conditions unfavorable from optimal hydration of caseins (as pH approaches 5.0, and below). It follows then, that during cheese making, acidification would decrease protein hydration as the caseins approach their isoelectric point, but casein micelle hydration would increase, most certainly as a result of increased mineral solubilization.

In addition to the effect of rennet, acidification, and addition of salts, temperature also affects casein hydration. Thus, Farrell et al. (1989) observed increased temperature (from 2 to 30°C) to decrease casein micelle hydration. According to the authors, this resulted from promoted hydrophobic interactions that increased protein-to-protein interactions and caused the casein micelles to become more compact.

Syneresis and moisture content of cheese. When held in a protein structure, water could be basically classified into two types: bound to molecules, which is no longer available as a solvent, and trapped in the protein matrix (Kneifel et al., 1991). In milk gels, about 90% of the water is mechanically enclosed within the casein network, and most of the remaining water is mechanically enclosed within casein particles that form the network (van Vliet and Walstra, 1994). Thus, protein hydration only concerns with a small amount of water, less than 0.5 g/g of protein.

During cheese making, the moisture content of cheese can be initially controlled by adjusting the casein/fat ratio (Lelièvre, 1983), and by the addition of

exopolysaccharide producing cultures (Perry et al., 1997, 1998) and fat replacers (McMahon et al., 1996). Thus, decreasing the casein/fat ratio allows for increasing the moisture content in the non-fat substance of cheese, and addition of exopolysaccharide producing cultures and fat replacers can increase the moisture content of cheese. Fat allows for moisture to be retained in the cheese (McMahon and Oberg, 1998), partly because fat decreases the flux of water due to increased tortuosity. Thus, cheeses with lower fat content loss moisture more readily (Geurts et al., 1974a). However, the major factor affecting the moisture content of cheese curd is the production of acid by lactic acid bacteria (Whitehead and Harkness, 1954).

In addition, moisture content decreases with decreased size of cheese curd, increased cooking temperature, and increased dry stirring. Also, decreased size of cheese block (Whitehead and Harkness, 1954; Geurts et al., 1980), increased duration of brining, a more concentrated brine (Geurts et al., 1974a, 1980), and drying during storage (Marcos, 1993) promote further decrease in moisture content of cheese. At very high salt concentration, shrinking of the protein matrix could further promote the outward migration of water (Geurts et al., 1974a). Other factors, such as pH and temperature have less effect on the amount of moisture lost during the brining of cheese (Geurts et al., 1980).

The amount of water bound to caseins is affected by the physicochemical state of the proteins. In contrast, the amount of trapped water is affected by different network structures that contain the water (Kneifel et al., 1991), and if protein interactions and network structures are extensively disrupted syneresis increases significantly

(Marchesseau and Cuq, 1995). Thus, in milk gels, the main cause of syneresis is the alteration of protein interactions and the concomitant rearrangement of the protein network (Walstra et al., 1985; van Vliet and Walstra, 1994; Marchesseau and Cuq, 1995). This includes the breaking of protein strands followed by the formation of new interactions between casein particles (Walstra et al., 1985).

According to classical theories of polymer science, a decrease in the net charge of the polymer, a decrease in solvent-polymer interaction, and/or an increase in the number of crosslinkages in the network would promote contraction of the gel matrix and syneresis (Lelièvre and Creamer, 1978). Thus, when structural rearrangements lead to an increase in the number of bonds, the network would contract, the water-holding capacity of the gel decrease, and syneresis increase (Walstra et al., 1985). However, as protein rearrangements occur causing gel contraction and syneresis, geometrical constrains would limit further rearrangements of the protein network and further syneresis.

The effect of pH, temperature, and salt content, on curd syneresis and moisture content of cheese seems to result then from their effect on the extent and nature of protein interactions, which would manifest in different structural arrangements of the protein matrix. Similarly, the release of serum during storage of process cheese would also result from structural readjustments of the protein network that result in decreased water-holding capacity of cheese (Marchesseau and Cuq, 1995).

Rearrangements of the protein matrix during storage may, however, result in increased water-holding capacity of cheese (Ramkumar et al., 1997; McMahon et al., 1999). In their study, McMahon et al. (1999) observed the amount of expressible serum

to decrease during the storage of part-skim and reduced-fat Mozzarella cheese, with little serum being expressed on d 14, and none on d 21. It appears that during storage, the protein matrix absorbs serum, thus becoming more hydrated and expanding. The increase in hydration of the protein matrix was not however related to an increase in the amount of protein-bound water, but to an increase in the amount of entrapped water.

Physical and chemical factors not only affect the extent but also the rate of syneresis. Syneresis rate is determined by a pressure gradient exerted on the protein network and the resistance of flow through the network (Walstra et al., 1985). Thus, application of external pressure, decreased pH, and increased temperature increase the rate of syneresis of rennet-induced curd, partly by causing shrinkage of the casein particles. Other factors, such as geometrical constrains, the composition of the gel, amount of rennet added, the addition of salts, and milk pre-treatment may also affect the rate of syneresis.

During ripening, biochemical processes, glycolysis, proteolysis, and lipolysis, increase the concentration of low molecular weight solutes in the water phase of cheese, which results in increased proportion of bound water, and less water available for microbial growth and chemical reactions (Marcos, 1993). These biochemical processes are promoted by increased storage temperature, which also promotes distribution of moisture within cheese blocks, with moisture transferring from high temperature to low temperature areas of the block (Reinbold and Ernstrom, 1988). Thus, during the first months of storage, the cheese would normally have increased water holding capacity, a more uniform distribution of moisture throughout the block, and decreased water activity.

Effect on cheese functionality. Water is the most important diluent of food solids (Ross, 1997), and it affects several attributes of foods, including shelf life, organoleptic properties, microbial growth, enzyme activity, and chemical reactions (Labuza and Lewicki, 1978; Ross, 1997). In addition, water affects physical properties of foods (Labuza and Lewicki, 1978; Ross, 1997), and specially, mechanical strength, elasticity, plasticity, and flow of foods (Labuza and Lewicki, 1978). In milk products, water acts as a plasticizer of non-fat solids, thus also affecting the physical state and molecular mobility of solids (Ross, 1997).

In particular, moisture content affects the hardness, cohesiveness, and melting of cheese. Increased moisture content causes the cheese to become softer, more cohesive and elastic, and less crumbly (Creamer and Olson, 1982; Lawrence et al., 1987; Tunick et al., 1991; McMahon et al., 1993; Guinee et al., 2000). In addition, increased moisture content promotes flowability or melting of cheese (Tunick et al., 1991; McMahon et al., 1993, 1999; Olson and Bogenrief, 1997; Perry et al., 1997; McMahon and Oberg, 1998; Guinee et al., 2000).

Increased hydration of the protein matrix results in decreased protein-to-protein interactions and increased protein-to-water interactions (McMahon et al., 1999). Thus, water would act as a lubricant or plasticizer between proteins (Tunick et al., 1991), which favors the flow of proteins and melting of cheese (Tunick et al., 1991; McMahon et al., 1999). Therefore, the functionality of low-fat cheese can be improved by increasing its moisture content so that the moisture/protein ratio is similar or even higher than that in full-fat cheese (McMahon and Oberg, 1998), and increased moisture content and a more

hydrated protein matrix can render reduced-fat cheese with similar textural and melting properties as full-fat cheese (Tunick et al., 1991).

Salt

Content and distribution in milk. Sodium and chloride occur naturally in milk, and bovine's milk has an average concentration of 48 and 110 mg/l, respectively (Walstra et al., 1999b). Whereas sodium distributes between colloidal and soluble forms, chloride is only present in solution (Holt, 1997; Walstra et al., 1999b). Thus, even though most of sodium is present in solution (95%), a portion of it associates with casein micelles as counter ions (Walstra et al., 1999b). Also, sodium chloride is not totally ionized in solution, and a fraction of it is present in the form of soluble salt.

Effect on casein and milk properties. When salt is added to milk or casein systems, calcium and phosphate dissociate from within casein micelles and into solution (Casiraghi and Lucisano, 1991; Gatti and Pires, 1995; Aoki et al., 1999; Gaucheron et al., 2000). Thus, the content of CCP decreases, which may cause micellar dissociation and render the casein micelles with a loosen structure (Aoki et al., 1999). In particular, at low levels (20 mM), adding salt to milk promotes dissociation of κ-casein (Fox and Nash, 1979). Displacement of calcium from casein micelles as a result of salt addition may then cause increased hydration or solvation of caseins in milk (Creamer, 1985).

Increased dissociation of κ-casein from casein micelles would be expected to make the casein micelles more prone to aggregation. However, when salt is added to milk, the rennet coagulation time increases (Fox and Nash, 1979; Goddard and Augustin, 1995). According to Fox and Nash (1979), this is an indication that some other change,

such as CCP solubilization, outweighs the effect of salt in promoting κ-casein dissociation, and coagulation is impaired. However, Goddard and Augustin (1995) observed no effect of salt addition on the concentration of ionic calcium. They proposed that salt may alter hydrophobic interactions and hydration forces, which would promote conformational changes in the proteins rendering them less prone to aggregation. In their study, decreased interaction between proteins resulted also in weaker gels.

In heated milk, the dissociation of micellar κ-casein complexed with whey proteins is affected by ionic strength (Singh and Fox, 1987). Increasing the salt concentration would decrease electrostatic repulsions between caseins by shielding negatively charged groups, which may prevent κ-casein dissociation. Charge neutralization and decreased repulsion may then promote interaction between casein micelles. This would agree with the observation that adding low levels of NaCl (20 mM) decreases the heat stability of milk (Fox and Nash, 1979; Aoki et al., 1999).

Effect on cheese composition. In addition to its role on casein micelles in milk, it is suggested that adding salt would promote calcium solubilization from paracasein in casein pellets and cheese (Creamer, 1985; Kindstedt et al., 1992), thus displacing calcium from the protein matrix and into the serum. Thus, salting would result in cheese with decreased calcium content. However, Paulson et al. (1998b) and Schroeder et al. (1988) observed that when salt was added to cheese calcium content remained the same. Decreased calcium content of cheese upon brine salting has been reported, but only when the brine solution contained low calcium content (e.g., 0.1%) or no calcium at all (Geurts et al., 1972). In contrast, no calcium was lost from the cheese when enough calcium was

added to the brine (0.6%).

In addition, calcium solubilization from caseins in cheese would decrease protein-to-protein interactions, and the protein matrix could become more hydrated. However, salting of cheese, and in particular brine salting, normally promotes syneresis and decreased moisture content of cheese (Geurts et al., 1972, 1974a; O'Connor, 1974; Thakur et al., 1975; Schroeder et al., 1988; Kindstedt et al., 1992; Guinee and Fox, 1993; Kelly et al., 1996; Kristiansen et al., 1999; Mistry and Kasperson, 1998; Prasad and Alvarez, 1999), which may be an indication of decreased hydration of the protein matrix.

During brining, less salt is normally absorbed than moisture is lost, and thus the moisture content and weight of cheese decreases (Geurts et al., 1972, 1974a). This process of salt uptake and outward migration of moisture of cheese in brine is described as a mutual impeded diffusion process, and it is referred as pseudo diffusion (Geurts et al., 1974a). In addition, at very high salt concentration (20% wt/v), brining may cause shrinkage of the cheese matrix (Geurts et al., 1972), which promotes the release of serum, thus further decreasing the moisture content of cheese (Geurts et al., 1980).

Increased salt content has also resulted in cheese with no decreased moisture content (Cervantes et al., 1983; Paulson et al., 1998b). Thus, the method of salting affects the moisture content of cheese, with dry salting resulting in cheese with higher moisture content compared to brine salting or a mix of dry and brine salting (Paulson et al., 1998b; Guinee et al., 2000).

In brine-salted cheese, there is normally an inverse gradient of moisture and salt content from the surface to the interior of the cheese. Thus, there is higher salt and lower

moisture content at the surface compared to the interior of brine-salted cheese. However, in Mozzarella cheese with soft-surface defect, both salt and moisture content are higher at the surface (Kindstedt et al., 1996). This is thought to be related to a thermal gradient within the cheese, which operates as a driving force promoting the migration of water from the interior, which is warmer, to the surface of the cheese, which is cooler. The occurrence of such thermal gradient is favored by the brining of Mozzarella cheese in cold brine (4°C compared to higher temperatures, 12 to 20°C).

Adding salt to cheese also affects cheese composition by influencing microbial activity (Thakur et al., 1975; Turner and Thomas, 1980; Thomas and Pearce, 1981; Schroeder et al., 1988; Guinee and Fox, 1993). Adding low levels of salt to milk (1.5%) may promote starter activity, but higher levels (2.5%) have the opposite effect (Irvine and Price, 1961), and lactose utilization by starter bacteria may be totally inhibited at a salt concentration of 5% (Turner and Thomas, 1980). As a result of inhibited starter activity, cheese with high salt content (e.g., 6% salt in moisture) has increased level of residual lactose (Turner and Thomas, 1980; Thomas and Pearce, 1981), which results in cheese with higher pH (O'Connor, 1974; Turner and Thomas, 1980; Thomas and Pearce, 1981; Kelly et al., 1996; Kristiansen et al., 1999). Also, non-starter bacteria may then metabolize available lactose increasing the content of D-lactic acid in cheese (Turner and Thomas, 1980).

In addition, salt content may also affect cheese proteolysis by affecting microbial and enzyme activity, with high salt levels decreasing the rate and/or extent of proteolysis (Fox and Walley, 1971; Thakur et al., 1975; Thomas and Pearce, 1981; Schroeder et al.,

1988; Kelly et al., 1996; Mistry and Kasperson, 1998). This would result from the bacteriostatic effect of salt and decreased moisture content of salted cheese (Thakur et al., 1975). In particular, increased salt content decreases the content of water-soluble (Thakur et al., 1975; Kelly et al., 1996) and acid-soluble (Kristiansen et al., 1999) nitrogen in cheese, and the hydrolysis of α_{s-1}- and β-casein (Thomas and Pearce, 1981; Kelly et al., 1996).

In Cheddar cheese, increased salt content inhibits β-casein hydrolysis most certainly as a result of decreased chymosin action (Kelly et al., 1996). It is possible that promoted aggregation of β-casein at high ionic strength makes it difficult for the enzyme to access cleavage sites, and therefore the amount of intact casein increases at higher salt content of cheese. However, decreased β-casein hydrolysis with increased salt content of Danbo-type cheese has been linked to decreased plasmin activity (Kristiansen et al., 1999). Thus, differences in cheese composition, salting method, and/or ripening seem to affect the activity of chymosin and plasmin differentially.

Effect on cheese functionality. The direct and indirect effects of salt on chemical composition affect functional properties of cheese and may alter cheese structure. Among other effects, salt content affects textural properties of cheese. Thus, increased salt content increases the hardness or firmness of cheese, decreases cheese cohesiveness (Cervantes et al., 1983; Schroeder et al., 1988; Banks et al., 1993; Prasad and Alvarez, 1999), and at high salt content the cheese becomes more crumbly (Banks et al., 1993).

In addition, salt content affects cheese melting, even though results differ between studies. Thus, increased salt content decreased the melting of Mozzarella cheese (Olson,

1982), but increased the melting of nonfat Mozzarella cheese (Paulson et al., 1998b). Salt affects the hydration of the protein matrix in cheese, with increased salt content leading to a more hydrated matrix (McMahon and Oberg, 1998). Thus, increased salt content would impair interactions between proteins and promote protein-to-water interactions, which helps to explain the observed increased melting of salted cheese.

The salt content of cheese may also affect cheese structure. Increased salt content of cheese would promote solubilization of caseins (Guo and Kindstedt, 1995; Guo et al., 1997), causing the protein matrix to become more hydrated and to swell (Guo and Kindstedt, 1995; Guo et al., 1997; Kindstedt and Guo, 1998; Paulson et al., 1998b). Swelling of the cheese matrix has been observed when the salt concentration of brine was below 20% (wt/v) and no calcium was added to the brine (Geurts et al., 1972). Under these conditions, salt seemed to increase protein solubility, which resulted in cheese with a weaker body. However, the opposite would be expected for cheese in brine with very high salt concentration. Thus, when the salt concentration in the brine was above 20% (wt/v), salting caused shrinkage of cheese, up to 30% at the highest concentrations.

The influence of salt on cheese quality is ultimately manifested by its effect on the grading and overall acceptability of cheese by consumers. In general, consumers favor cheeses with intermediate salt content, 1.2 to 1.8% (O'Connor, 1974; Lindsay et al., 1982; Kelly et al., 1996). Lower salt content, and no salting in particular, leads to cheese with higher moisture content, increased proteolysis, softer and more open body, increased bitterness, and decreased flavor (Thakur et al., 1975; Banks et al., 1993). In addition, consumers show preference for cheese salted with sodium chloride compared to

potassium chloride, and increased content of potassium chloride leads to bitterness (Lindsay et al., 1982).

pH

pH and buffering properties of milk. The pH of bovine's milk is normally about 6.7, and ranges from 6.6 to 6.8 (Walstra et al., 1999a). In addition, milk contains a variety of acidic and basic groups that result in milk having buffering action over a wide range of pH values (Singh et al., 1997), with a maximum in buffering capacity during acid titration at pH 5.1 (Lucey et al., 1993b). The buffering of milk is primarily determined by phosphates, soluble and colloidal, and proteins present in milk (Singh et al., 1997; Walstra et al., 1999a). Both pH and buffering capacity depend on temperature (Singh et al., 1997; Walstra et al., 1999a) and compositional variation of milk (Singh et al., 1997).

Moderate heating, such as pasteurization, promotes losses of CO_2 and precipitation of calcium phosphate with concomitant release of H^+ (Singh et al., 1997), which decreases the pH of milk (Singh et al., 1997; Walstra et al., 1999a). Heating of milk above 100°C increases the buffering peak at pH 5.0, probably due to heat-induced precipitation of calcium and phosphate and the concomitant increase in the concentration of CCP (Lucey et al., 1993a). Furthermore, heating milk at 120°C shifts the buffering peak to a lower pH, from 5.0 to 4.4, which may result from a change in structure and/or composition of CCP. In addition, freezing, dilution, and concentration may also affect the pH and buffering properties of milk (Singh et al., 1997).

During cheese manufacture, milk pH decreases either as a result of lactic acid production by starter bacteria and/or as a result of acid addition. However, the rate at

which the pH of milk decreases is affected by the buffering properties of milk (Lucey et al., 1993b). During acidification, solubilization of CCP results in the formation of phosphate ions, which combine with H^+ resulting in buffering, and maximum buffering capacity of milk has been observed at pH 5.1 (Lucey et al., 1993b). However, when milk was titrated from acidic to basic conditions, low buffering was observed at pH 5.1, and maximum buffering capacity occurred at pH 6.3. According to Lucey et al. (1993b), during back-titration, CCP has already been solubilized, and maximum buffering occurs when $Ca_3(PO_4)^2$ forms at pH 6.3, with the release of H^+ from HPO_4^{2-} and $H_2PO_4^{1-}$.

Effect on caseins and milk salts. In contrast to heating, lowering the pH of milk or casein suspensions causes solubilization of minerals, mainly calcium and phosphate from caseins (Keller et al., 1974; van Hooydonk et al., 1986a; Visser et al., 1986; Dalgleish and Law, 1989; Kiely et al., 1992; McMahon et al., 1993; Lucey et al., 1996; Le Graët and Gaucheron, 1999), which results in increased content of soluble minerals, and calcium in particular (Fox and Ernstrom, 1969; Kindstedt et al., 2001). This acid-induced solubilization of minerals is independent of milk temperature in the range of 4 to 30°C (Dalgleish and Law, 1989).

At low temperature, acidification, and the concomitant solubilization of calcium and phosphate, normally causes caseins to dissociate from the casein micelles (Roefs et al., 1985; van Hooydonk et al., 1986a; Dalgleish and Law, 1988; McMahon et al., 1993). However, the total amount and proportion of caseins dissociated varies with pH and temperature. Thus, acidification at low temperature (4°C) favors increased dissociation of caseins and of β-casein in particular, and a peak of dissociation is observed at pH 5.1

(Dalgleish and Law, 1988). In contrast, the effect of pH on casein dissociation is minimized at high temperature (30°C). In general, even though significant amounts of α_s-casein dissociate from casein micelles, β-casein is the largest single component of dissociated caseins (Dalgleish and Law, 1988).

Acidification of milk, and the concomitant solubilization of minerals and casein dissociation, leads to decreased interaction between proteins at pH 5.4 or 5.3, which allows for increased solvation and solubility of caseins (Roefs et al., 1985; van Hooydonk et al., 1986a). However, at pH 5.2, increased protein-to-protein interactions promote aggregation, which results in increased structural heterogeneity of milk gels with areas of concentrated caseins and areas devoid of aggregates (Visser et al., 1986). Further lowering of pH, especially below 5.0, would then promote protein-to-protein interactions as the caseins approach their isoelectric point and electrostatic repulsions are minimized (Visser et al., 1986; van Vliet and Walstra, 1994; Marchesseau et al., 1997). Thus, the isoelectric precipitation of caseins causes contraction of protein aggregates during the formation of milk gels (Visser et al., 1986), which would oppose further dissociation of caseins from casein micelles (Dalgleish and Law, 1988).

Effect on cheese composition. The effect of pH on caseins and milk salts leads to alterations in the chemical composition of cheese. Different chemical parameters of cheese are thus affected by pH and the concomitant dissociation of minerals, mainly calcium and phosphate. Among other parameters, pH affects the calcium, moisture, rennet, and plasmin content of cheese, as well as enzyme and microbial activity.

The pH of cheese is determined by the amount of acid produced and by the

buffering capacity of the curd, mainly caseins and phosphate (Lucey and Fox, 1993). In particular, the pH of Cheddar cheese is basically determined by the curd acidity at salting and the buffering capacity of the curd (Lawrence and Gilles, 1982). However, salt addition significantly affects the pH of cheese throughout storage.

As a result of decreased pH, calcium and other minerals are solubilized from casein micelles and into the serum (Lawrence et al., 1983; Ramkumar et al., 1997; Kindstedt and Guo, 1998), and the content of soluble calcium in cheese increases (Kindstedt et al., 2001; Watkinson et al., 2001). In addition, decreased pH and increased mineral solubilization normally results in cheese with decreased calcium content (Keller et al., 1974; Anis and Ladkani, 1988; Kiely et al., 1992; Yun et al., 1995).

In general, decreased pH results in cheese with reduced moisture content (Taneya et al., 1992; Ramkumar et al., 1997; Watkinson et al., 2001). However, lowered pH of casein gels, in the range of 4.7 to 4.1, has resulted in decreased syneresis (Lucey et al., 1997). Similarly, in process cheese, Geurts et al. (1974a) observed decreased pH (e.g., 4.7 compared to 5.7) to lower the loss of moisture relative to the gain in salt content of cheese. The authors proposed that at low pH the protein matrix becomes more rigid and it tends to resist shrinkage. Thus, less moisture is expelled from within the cheese.

The pH of cheese also affects plasmin and rennet retention in cheese. Thus, the pH at draining determines the proportion of plasmin in cheese (Lawrence et al., 1987), with decreased plasmin retention at lower pH values (Roefs et al., 1985). In contrast, decreased pH increases the retention of calf rennet in cheese, but it has no effect on the retention of microbial rennet (Creamer et al., 1985). Increased retention and activity of

rennet may then lead to increased hydrolysis of α_{s-1}-casein, whereas increased plasmin activity promotes β-casein hydrolysis. Thus, by influencing the retention and activity of proteolytic enzymes, and microbial activity, pH affects the extent and pattern of proteolysis. Lowered pH, e.g., from 6.2 to 5.2, decreases the extent of proteolysis, which is manifested in decreased levels of water- and acid-soluble nitrogen in cheese (Creamer et al., 1988; Watkinson et al., 2001). This would result from decreased microbial and enzymatic activity (Creamer et al., 1988). However, decreased pH of direct-acid, low-moisture Mozzarella cheese (5.6 compared to 5.9) resulted in increased level of primary proteolysis, i.e. pH 4.6-soluble nitrogen (Feeney et al., 2002).

When lowering the pH of process cheese from 6.2 to 5.2, Watkinson et al. (2001) observed lower cheese pH to significantly decreased the extent of β-casein degradation at 87 d. The authors proposed that lowered cheese pH decreased the activity of plasmin, which may then result in decreased breakdown of β-casein and lower level of non-protein nitrogen. In contrast to these observations and suggestion, Feeney et al. (2002) observed increased β-casein degradation at lower cheese pH, 5.6 compared to 5.9, probably due to increased plasmin activity.

There is also evidence that pH affects the retention of total solids and fat in the cheese. Thus, increased acidification during manufacture of direct-acid Mozzarella cheese, and decreased pH, from 5.6 to 5.0, has resulted in decreased retention of solids and decreased cheese yield (Anis and Ladkani, 1988).

Changes in pH after the cheese is salted and made into a block will depend upon the availability of residual lactose and the ability of starter bacteria to continue

metabolizing lactose to lactic acid. This, in turn, is influenced by the salt-in-moisture content of cheese (Lawrence and Gilles, 1982). Salting at higher levels, e.g., 6.5% compared to 3.5% (salt in moisture), causes the cheese to have increased content of residual lactose. However, due to decreased microbial activity, cheese pH usually remains unchanged and at a higher value. In contrast, if the content of salt in the moisture phase is lower than 4%, microbial activity is favored, and the initial pH of cheese usually decreases.

Effect on cheese texture and structure. The pH of cheese is one of the two primary determinants of cheese texture, an important attribute of cheese because it is the first to be judged by consumers (Lawrence et al., 1987). This effect of pH on cheese texture is particularly significant because of its influence on calcium and phosphate solubilization, which results in changes in the protein network.

In cheeses of higher pH (5.2), larger protein aggregates are present in the protein matrix compared to cheeses of lower pH (e.g., 5.0 [Hall and Creamer, 1972; Lawrence et al., 1987]), and they bind together forming long chains (Lawrence et al., 1987). Lowering of pH would then increase calcium solubilization and decrease electrostatic repulsions between proteins as the caseins approach their isoelectric point, and at pH 5.0, the protein matrix has a less well-defined structure (Taneya et al., 1992) with smaller protein aggregates (Hall and Creamer, 1972; Lawrence et al., 1987). Below pH 5.0, protein-to-protein interactions significantly increase (van Vliet and Walstra, 1994), and cheese is characterized by having protein aggregates of even smaller size (Hall and Creamer, 1972; Lawrence et al., 1987). Then, at pH 4.8, and as a result of extensive mineral

solubilization and dissociation of caseins, protein aggregates seem to loss their identity (Lawrence et al., 1987).

Effect on cheese functionality. Among other functional properties, pH affects the stretching of cheese curd. Thus, decreased pH improves the stretching of curd (Guinee et al., 2002), and optimum stretching and maximum cohesion is normally achieved at pH 5.2, when 75% of CCP has been solubilized (Lawrence et al., 1987). At this pH, chains of casein aggregates are still present in the protein matrix, and they contribute structural integrity to the matrix. However, at pH 5.2, bonds between the proteins are weaker because of mineral solubilization, which would allow for the rearrangement of protein interactions to proceed at a higher rate compared to both higher and lower pH (van Vliet and Walstra, 1994), and in turn facilitate the expression of moisture from cheese curd. However, Ramkumar et al. (1997) observed that decreased quantity of serum could be expressed from cheese with lower pH, 5.2 compared to 5.6. In contrast, further lowering of pH to or below 4.8 causes the cheese to loose cohesion and stretchability because of extensive structural disruption of the protein matrix (Lawrence et al., 1987).

Even though decreased pH, in the range of pH 5.8 to 5.2, increases the strength of skim-milk gels (Goddard and Augustin, 1995), lower pH normally decreases cheese firmness. Thus, cheese with lower pH normally has less of a solid-like behavior (Ramkumar et al., 1998), becomes less firm and more crumbly, and hardness decreases (Creamer and Olson, 1982; Marchesseau et al., 1997; Paulson et al., 1998a; Ramkumar et al., 1998; Watkinson et al., 2001; Guinee et al., 2002). Marchesseau et al. (1997) suggested that decreased structural uniformity of cheese with lower pH would not allow

for even distribution of stress, which then results in cheese of lower pH (5.2 compared to 5.6) having decreased firmness.

The effect of pH on rheological and fracture properties of cheese seems to result mainly from changes in calcium-mediated protein interactions as a result of calcium solubilization (Watkinson et al., 2001). Thus, small differences in pH, e.g., 5.2 compared to 5.1, that result in cheese with similar calcium content have no effect on hardness or other textural properties of cheese (Yun et al., 1993, 1995).

The pH of cheese may also affect cheese melting (McMahon and Oberg, 1998). Thus, decreased pH of direct-acid Mozzarella cheese, from 5.9 to 5.0, increases cheese flowability and melting (Anis and Ladkani, 1988; Guinee et al., 2002). In contrast, Kindstedt et al. (2001) observed that low-moisture, part-skim Mozzarella lost the ability to flow and melt when pH was lower than 5.0. Thus, changes in pH seem to exert different effects on cheese melting depending upon whether they occur above or below pH 5.0. In addition, when the change in pH is small, e.g. 5.2 compared to 5.1, and/or calcium content remains basically unchanged, decreased pH seems to have no effect on cheese melting (Yun et al., 1993, 1995; Paulson et al., 1998a).

OBJECTIVES

The overall objective of this research project was to determine how changes in the chemical composition of cheese, by means of injecting concentrated ionic solutions or water into it, affect cheese structure and functionality. In particular, our specific research objectives were:

1. To determine the effect of calcium on cheese structure, and to relate changes in structure to changes in cheese functionality.
2. To determine the effect of water on cheese structure, and to relate changes in structure to changes in cheese functionality.
3. To determine the effect of salt on cheese structure, and to relate changes in structure to changes in cheese functionality.
4. To determine the effect of pH on cheese structure, and to relate changes in structure to changes in cheese functionality.

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CHAPTER 2

EFFECT OF CALCIUM AND WATER INJECTION ON STRUCTURE-FUNCTION RELATIONSHIPS OF CHEESE*

ABSTRACT

Our objectives were to determine the effect of calcium and water injection on cheese structure and to relate changes in structure to changes in functional properties of cheese. Cheese with fat and moisture content similar to that of low-moisture part-skim Mozzarella was made according to a direct-acid, stirred/pressed-curd procedure. The cheese was then cut into blocks that were high-pressure injected from one to five times, with either water or a 40% calcium chloride solution. Successive injections were performed 24 h apart. After 42 d of refrigerated storage, cheese microstructure and functionality were analyzed. When injected three or more times, water tended to increase cheese weight. The control, uninjected cheese, had the typical structure of a stirred/pressed-curd cheese: protein matrix interspersed with areas that originally contained fat and/or serum. Injecting water increased the area of cheese matrix occupied by protein, but it did not affect textural properties or melting of cheese. In contrast, when calcium was injected, a decrease in cheese weight was observed that was manifested through syneresis. The moisture content and pH of the cheese decreased as well. Calcium injection also decreased the area of cheese matrix occupied by protein. Cheese hardness increased, and cohesiveness and melting of cheese decreased upon calcium injection.

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We concluded that adding calcium to cheese alters how the proteins interact, which is manifest as changes in cheese microstructure. Such changes in cheese structure provide an understanding of changes in functional attributes of the cheese.

INTRODUCTION

Calcium content affects the rennet coagulation of milk. Calcium addition lowers the pH of milk by promoting exchange of Ca^{2+} for H^{+} (Satia and Raadsveld, 1969; Jen and Ashworth, 1970), and neutralizes negative charges in casein molecules (Green and Marshall, 1977) by reacting with phosphoserine residues and/or carboxylic acid groups of casein molecules (Dalgleish, 1983). As a result, the affinity of rennet for casein micelles is enhanced and the rate of enzymatic reaction increases (Green and Marshall, 1977). Also, charge neutralization facilitates protein-to-protein interactions between casein molecules, which increase the aggregation of renneted casein micelles (Green and Marshall, 1977; Dalgleish, 1983). As a result of increased enzymatic action and aggregation of casein micelles, adding calcium to milk reduces the rennet coagulation time (Green and Marshall, 1977), and may increase gel strength if added in low concentration (Jen and Ashworth, 1970).

During cheese making, the pH at draining determines the retention of minerals, mainly calcium and phosphorous, in the cheese curd (Lawrence et al., 1983). The content of calcium then affects the extent and degree of protein aggregation determining the basic structure and texture of cheese (Lawrence et al., 1983). In cheeses with higher calcium content (higher cheese pH), a high level of protein aggregation and bigger protein

aggregates are observed compared to cheeses with lower calcium content (lower cheese pH [Hall and Creamer, 1972]). These differences in protein aggregation determine the contrasting structure and texture of cheeses such as Swiss and Cheddar.

Calcium content also affects cheese functionality. According to Paulson et al. (1998a), increased calcium content resulted in decreased melting of nonfat Mozzarella cheese. Also, for any given pH value, there is a tendency for Cheddar cheese to become firmer as the calcium content of cheese increases (Lawrence et al., 1993).

The effect of calcium on cheese making parameters and cheese composition has been usually studied by adding a calcium source, such as calcium chloride, to milk, during the early stages of cheese making (Satia and Raadsveld, 1969; Jen and Ashworth, 1970; Dalgleish, 1983; Solorza and Bell, 1998). However, changes in the calcium content of cheese are interdependent with changes in curd pH. According to Lawrence et al. (1993), the difficulty in conducting experiments on the chemical composition of cheese is that the variables of interest, especially pH and calcium content of cheese, are interdependent and influenced by a variety of parameters during cheese making. This makes it difficult to segregate the effect of pH per se from the effect of pH-induced changes (Lucey and Fox, 1993). One way to overcome this difficulty is to change the calcium content of cheese by adding calcium after the cheese is manufactured. In this way, both the cheese-making procedure and initial chemical composition of the cheese can be kept the same. Calcium could be added to the cheese after manufacture by injecting a concentrated solution at high pressure. High-pressure injection has been successfully used to inject fluids into cheese (Lee et al., 1978; Olson, 1979) and meat

(Hendricks and Hansen, 1991).

Few studies have characterized changes in cheese structure as a result of changes in the chemical composition of cheese (Kiely et al., 1992; Tunick et al., 1993; Paulson et al., 1998b), and none has yet determined the effect of calcium content on cheese structure. Thus, limited data is available for determining relationships between the structure and functionality of Mozzarella cheese, on the basis of the chemical composition of cheese.

OBJECTIVES

The objectives of the present research were to determine the effect of calcium and water injection on the structure of a cheese with similar fat and moisture content to that of low-moisture part-skim Mozzarella, and to relate changes in structure to changes in functional properties of the cheese.

MATERIALS AND METHODS

Cheese Making

Nine-kilogram blocks of cheese were made according to a direct-acid, stirred/pressed-curd procedure. Pasteurized, full-fat, non-homogenized milk at 5°C was acidified by adding citric acid (1 g per kilogram of milk) and sufficient acetic acid solution (10% wt/wt) to lower the pH of milk to 5.4. The milk was then warmed to 33°C and 0.1 ml of double strength rennet (Chymax, Rhodia, Madison, WI) per kilogram of milk was added. After 15 min, the curd was cut, allowed to heal for 5 min, and cooked

with stirring for 30 min at 33°C. The whey was then drained and the cheese curd dry-salted (2% wt/wt). After salting, the curd was placed into a 9-kg cheese mold and pressed overnight. The cheese was then trimmed and cut into 0.3- to 0.4-kg blocks that were vacuum-packaged and stored for 10 d at 4°C, before the injection was performed.

Cheese Injection

A 2-stage homogenizer (Crepaco, Model 3DDL-3535, Chicago, IL) served as the pump for injection and pressure on the homogenizer valve was set at 1400 psi. The burst duration was controlled via a solenoid-operated valve on the outlet line and set to 1 s. Solutions flowed through a sapphire nozzle (0.02 cm in diameter) and into the cheese. The cheese was high-pressure injected with either distilled water or a 40% (wt/wt) calcium chloride ($CaCl_2.2H_2O$) solution (4.2 M). Injections were performed using a 1 x 1-cm injection pattern applied to one side of the cheese block (5 x 9 cm) and then to the opposite side. In average, a total number of 64 injection sites were used for each block of cheese. The cheese block was then blotted with paper towels to remove extraneous fluid and vacuum-packaged. Successive injections were performed 24 h apart so that a series of cheeses was obtained that had been injected from one to five times. The cheeses were then stored at 4°C for 42 d to allow time for complete distribution of ions and moisture so that chemical equilibrium could be reached (see Tables B.1 and B.2, and Figure B.1 in Appendix B).

Chemical Composition

Fat content was determined using a modified Babcock method (Richardson,

1985), moisture content by using the vacuum oven AOAC method 926.08 (1990), and calcium and sodium by inductively coupled plasma-atomic emission spectroscopy (US Environmental Protection Agency, 1992). A pH meter (model IQ240, IQ Scientific Instruments, Inc., San Diego, CA), with a stainless steel probe (model PH06-SS, IQ Scientific Instruments, Inc., San Diego, CA), was used for determining cheese pH, which was measured by taking a cheese sample from the cheese block and inserting the pH probe.

Scanning Electron Microscopy

The control, uninjected cheese, and cheese that had been injected five times with water or calcium solution were selected for examining cheese microstructure. Cheese samples (approximately 1 x 1 x 10 mm) were fixed in 2% glutaraldehyde solution for 1 h at room temperature (22°C). The glutaraldehyde solution was then changed, and the samples stored in new solution for 2 d at 4°C. After refrigerated storage, the samples were processed according to McManus et al. (1993). Samples were frozen in liquefied Freon 22 (-159°C) (Mallinckrodt Inc., Paris, KY), transferred to liquid nitrogen, cryofractured perpendicular to their long axis, and thawed in 2% glutaraldehyde solution. They were then dehydrated in a graded ethanol series followed by fat extraction with Freon 113 (Mallinckrodt Inc., Paris, KY). After overnight storage in Freon 113 at 4°C, the samples were rehydrated by reversing the graded ethanol series, and washed with a 0.1 M sodium cacodylate buffer (Electron Microscopy Sciences, Fort Washington, PA), pH 7.2. The samples were then post-fixed for 2 h with a solution containing 1% OsO_4 (Electron Microscopy Sciences, Fort Washington, PA) and 1.5% $K_4Fe(CN)_6 \cdot 3H_2O$

(Fisher Scientific Co., Fair Lawn, NJ). This solution was replaced by a 2% tannic acid (Mallinckrodt Inc., Paris, KY) solution in cacodylate buffer, and the samples were left for 3 h at room temperature. The tannic acid solution was then replaced with the mentioned solution of osmium tetroxide and potassium ferrocyanate, and samples left for 4 h. This solution was later replaced with an aqueous solution of 1% hydroquinone (Mallinckrodt Inc., Paris, KY), and samples were left overnight. After the heavy metal impregnation, the samples were washed with distilled water, dehydrated in a graded ethanol series, and critical-point dried in a critical-point drier (Model 1200; Polaron, Waterford, England) with CO_2. Samples were viewed in a field emission scanning electron microscope (Model S-4000T FESEM, Hitachi Scientific Instruments, Mountain View, CA) operated at 3 kV. Images from each sample, at 1500 X magnification, from two fields were recorded on Kodak TMX 120 film, and digitally using Spectrum 2.0 software (The Dindima Group Pty. Ltd. Ringwoood, Victoria. Australia). Fields were randomly selected from areas of the sample that exhibited planes of fracture of good quality.

Image Analysis

Digital images, with pixels in the gray scale 0 to 255 (from black to white) were uploaded into Adobe Photoshop® 4.0. The images were then converted from their gray-scale values to binary images in which gray pixels were converted to either white or black pixels by applying the threshold function. In the original digital images, dark pixels corresponded to areas of the micrograph occupied by fat/serum pockets, while light pixels corresponded to areas occupied by protein matrix. Then, when thresholding, pixels having a gray value lower than the threshold level were converted to black pixels,

while those having a gray value higher than the threshold level were converted to white pixels. However, because of the occurrence of superficial pockets and areas of protein matrix with pronounced fracture angles, some micrographs had under- and overestimated values of area occupied by fat/serum pockets when a single threshold level for all images was selected. To overcome this problem, a threshold level of either 60 or 70 was selected for each micrograph depending on the individual image characteristics. Thus, a more precise differentiation between dark and light areas was obtained, as determined by visually matching the original micrographs with their corresponding binary images. The proportions of dark and light pixels, and the areas occupied by them were then determined by applying the histogram function. Thus, the area of cheese matrix occupied by fat/serum pockets (dark areas) and protein matrix (light areas) was determined.

Cheese Functionality

Cheese weight was recorded before injection and after 42 d of storage at 4°C. Also, after refrigerated storage, cheese functionalities, melting and Texture Profile Analysis (**TPA**) were analyzed. Melting was performed according to a modified tube test (Bogenrief and Olson, 1995). Duplicate cheese plugs, 15 g in weight, were cut from the cheese and placed into glass tubes, which were sealed with rubber stoppers and immersed in mineral oil (90°C). The distance the cheese melted was then measured at 10 min. TPA was performed using a two-bite compression test run on a texture profile analyzer (Model 5542, Instron Corp., Canton, MA). The compression factor was 75% and the crosshead speed was set at 20 mm/min. Samples, 20 mm long by 16 mm in diameter, were taken from the cheese immediately after removal from the refrigerator, and tested at

approximately 5°C. Hardness and cohesiveness were determined by analyzing the data according to Bourne (1978).

Experimental Design and Statistical Analysis

The experiment was conducted in triplicate as a randomized block design with cheese-making day as the block factor. Treatments were calcium and water injection, along with a control, uninjected cheese. Treatment levels were one to five according to the number of injections performed on the cheese block. Two cheese samples were analyzed for each variable except weight. For scanning electron microscopy, two cheese samples from one replication were analyzed. Thus, each sample was considered as a replicate for analysis. Statistical analysis, ANOVA, was performed using SAS® (1991). Individual comparisons, Contrast or LSD, between calcium injected, water injected and control, uninjected cheeses were also performed. Significance was declared at $P \leq 0.05$ (for summary of statistical analysis see Table A.1 in Appendix A).

RESULTS

Cheese Composition

The moisture and fat content of the control, uninjected cheese (Table 2.1) was in agreement with the US specifications for low-moisture part-skim Mozzarella cheese. The calcium content was as expected, according to the cheese-making procedure, whereas the sodium content was higher than expected (Table 2.1). Using the direct-acid cheese-making procedure resulted in a cheese that had less than half the amount of calcium normally present in low-moisture part-skim Mozzarella cheese, 0.3% compared

TABLE 2.1. Cheese composition

Variable	Mean	SEM
Fat (%)	21.3	0.3
Moisture (%)	50.2	1.4
pH	5.46	0.12
Calcium (%)	0.28	0.01
Sodium (%)	0.95	0.10

to 0.7% (Kosikowski and Mistry, 1997). Similar lower calcium contents have been reported when making nonfat Mozzarella cheese with direct acidification of milk (Paulson et al., 1998a, 1998b).

Water and calcium injection affected the weight of cheese blocks (Table 2.2). Even though it was not significant, water injection tended to increase cheese weight. Part of the injected water was contained within the cheese block, and after five injections the weight of the cheese block had increased by 5% (Figure 2.1), with no serum released from the cheese during storage. In contrast, calcium injection significantly decreased the weight of cheese (Figure 2.1), and considerable serum was observed in the cheese package during storage (including overnight storage between successive injections). After each injection, the cheese had been blotted so the serum in the package was serum expelled from the cheese rather than residual injectant not incorporated into the cheese block.

Calcium content was unaffected by water injection (Table 2.2), and it remained at 0.3% (Figure 2.2). In contrast, calcium injection significantly increased the calcium content of cheese (Table 2.2). After five injections, the calcium content of the cheese was

TABLE 2.2. Statistical results for the effect of adding calcium chloride (40% w/w) and water on chemical and functional properties of Mozzarella cheese after 42 d of storage at 4°C

Source	Model[1]	Contrast		
Variable		Uninjected v Calcium	Uninjected v Water	Calcium v Water
		P	*P*	*P*
Calcium	< 0.001	< 0.001	NS[2]	< 0.001
Moisture	< 0.001	< 0.001	NS	< 0.001
pH	< 0.001	< 0.001	NS	< 0.001
Weight	< 0.001	< 0.001	NS	< 0.001
Cohesiveness	< 0.01	< 0.001	NS	< 0.001
Hardness	< 0.05	< 0.01	NS	< 0.001
Melting	< 0.001	< 0.001	NS	< 0.001

[1] $Y_{jkl} = \mu + T_j + B_k + e_{jk} + d_{jkl}$, where Y is the variable of interest, μ is the overall mean, T is the treatment effect, B is the block effect, e is the error term, and d is the subsample effect.
[2]NS: not significant, i.e., $P > 0.05$

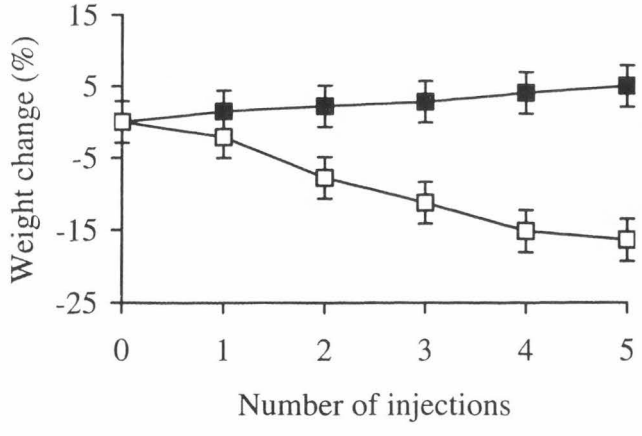

Figure 2.1. Weight of Mozzarella cheese blocks injected with either a calcium chloride solution (40% w/w) (□) or water (■) and stored for 42 d at 4°C. Successive injections performed 24 h apart. Error bar = 0.5 x LSD

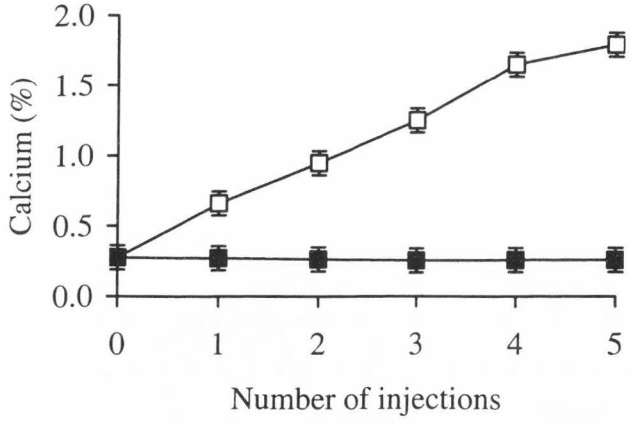

Figure 2.2. Calcium content of Mozzarella cheese injected with either a calcium chloride solution (40% w/w) (□) or water (■) and stored for 42 d at 4°C. Successive injections performed 24 h apart. Error bar = 0.5 x LSD

1.8%, compared to 0.3% in the control, uninjected cheese (Figure 2.2). Each calcium injection increased the calcium content of cheese another 0.3%.

Even though water injection had no significant effect on the moisture content and pH of cheese ($P > 0.05$), injecting calcium significantly affected both (Table 2.2). Thus, the moisture content of cheese significantly decreased with calcium injection (Table 2.2), from an initial 50% down to 37% after five injections (Figure 2.3). This decrease in moisture content was associated with syneresis observed between successive calcium injections and during subsequent storage of the cheese. Also, a significant decrease in cheese pH occurred upon calcium injection (Table 2.2), from 5.5 to 4.6 after five injections (Figure 2.4).

Cheese Microstructure

The control, uninjected cheese had a structure typical of a stirred/pressed-curd cheese, with protein matrix interspersed with areas that originally contained fat and/or

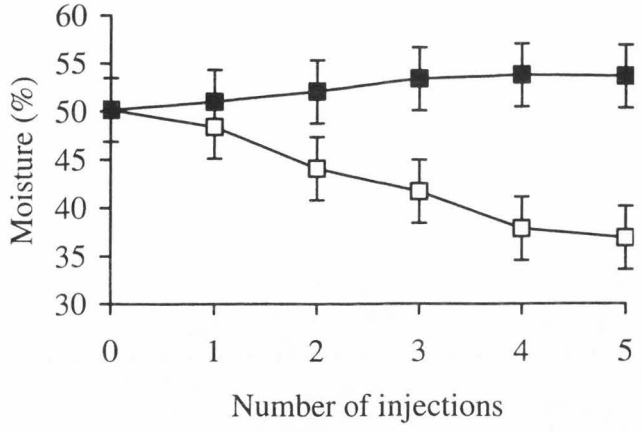

Figure 2.3. Moisture content of Mozzarella cheese injected with either a calcium chloride solution (40% w/w) (□) or water (■) and stored for 42 d at 4°C. Successive injections performed 24 h apart. Error bar = 0.5 x LSD

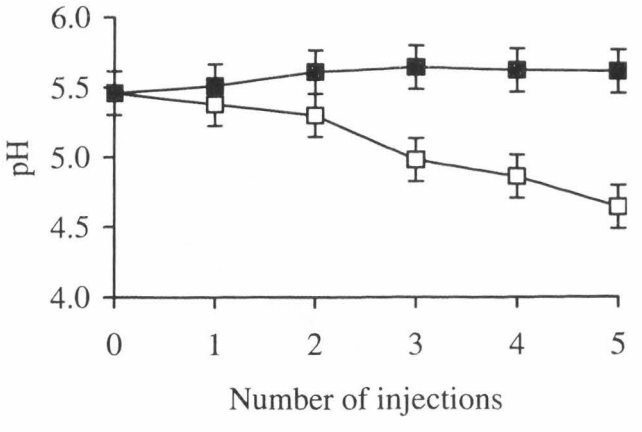

Figure 2.4. Mozzarella cheese pH after injection with either a calcium chloride solution (40% w/w) (□) or water (■) and storage for 42 d at 4°C. Successive injections performed 24 h apart. Error bar = 0.5 x LSD

serum (Figure 2.5A). In contrast, the water injected cheese had fewer void spaces distributed throughout the protein matrix (Figure 2.5B). The structure of calcium-injected cheese was similar to that of the control cheese, although the void spaces appeared larger (Figure 2.5C). The binary images obtained when the threshold function was applied to the micrographs are shown in Figure 2.6. In these images fat/serum pockets (black) were clearly differentiated from the protein matrix (white). For the control cheese (Figure 2.6A), the protein matrix occupied 81% of the cheese matrix, with fat/serum pockets occupying the remaining 19%. Injecting water significantly increased the area of cheese matrix occupied by protein (Figure 2.6B). After five injections, the protein matrix occupied 92% of the cheese matrix area ($P < 0.05$). In contrast, injecting calcium significantly decreased the area of cheese matrix occupied by protein, from 81% to 69% ($P < 0.05$) (Figure 2.6C). Thus, when compared to the water-injected cheese, the protein matrix in calcium-injected cheese occupied 25% less of the micrograph area ($P < 0.05$).

Cheese Functionality

Water injection had no effect on textural and melting properties of cheese. In contrast, injecting calcium into cheese affected both (Table 2.2). Thus, calcium injection significantly increased cheese hardness up until four injections (Figure 2.7), and significantly decreased both cheese cohesiveness and extent of melting (Figures 2.8 and 2.9, respectively). There was a large decrease in cheese cohesiveness and extent of melting after the first two calcium injections with further injections causing little decrease.

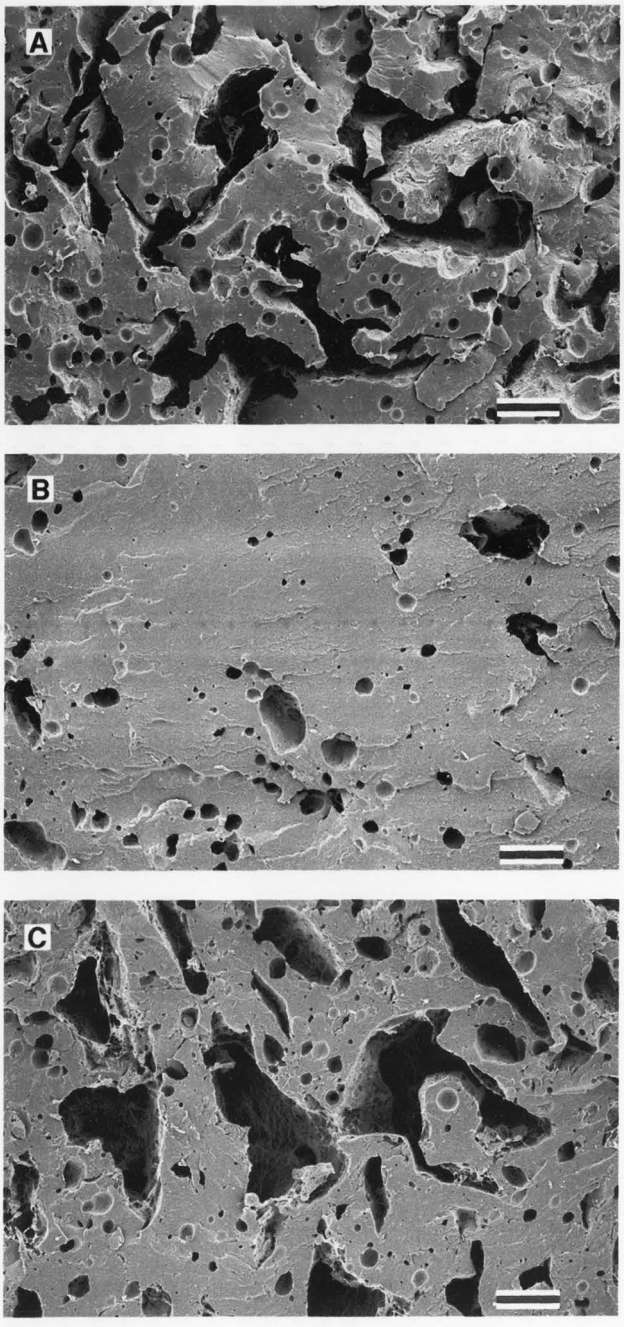

Figure 2.5. Scanning electron micrographs of Mozzarella cheese after 42 d of storage at 4°C. A: uninjected cheese; B: water-injected cheese (5 injections); C: calcium-injected cheese (5 injections). Bar = 10 μm

Figure 2.6. Binary image of scanning electron micrographs 2.5A, 2.5B, and 2.5C, after thresholding. Bar = 10 μm

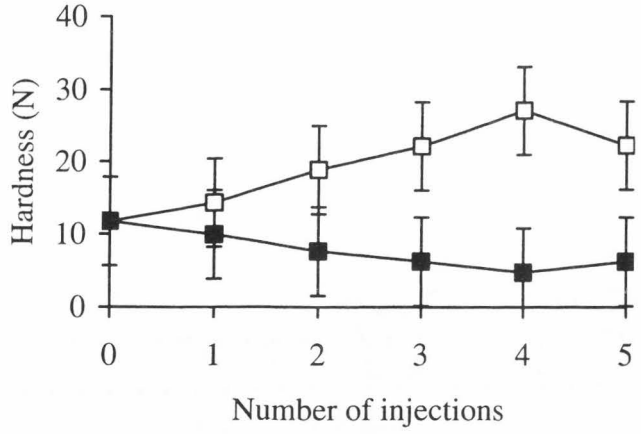

Figure 2.7. Hardness of Mozzarella cheese injected with either a calcium chloride solution (40% w/w) (□) or water (■) and stored for 42 d at 4°C. Successive injections performed 24 h apart. Error bar = 0.5 x LSD

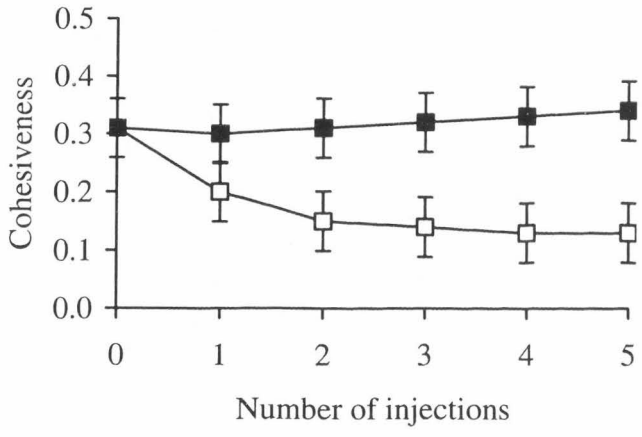

Figure 2.8. Cohesiveness of Mozzarella cheese injected with either a calcium chloride solution (40% w/w) (□) or water (■) and stored for 42 d at 4°C. Successive injections performed 24 h apart. Error bar = 0.5 x LSD

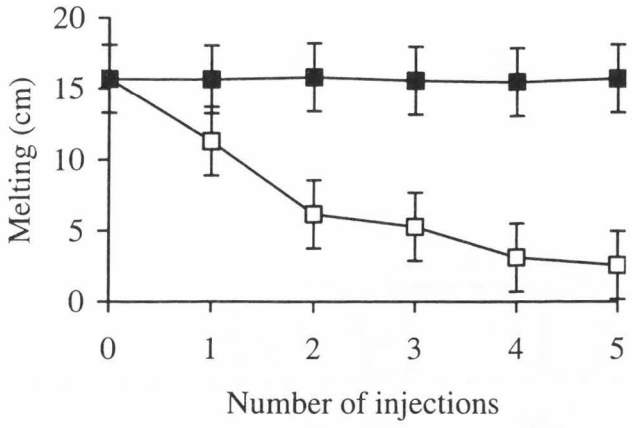

Figure 2.9. Melting of Mozzarella cheese injected with either a calcium chloride solution (40% w/w) (□) or water (■) and stored for 42 d at 4°C. Successive injections performed 24 h apart. Error bar = 0.5 x LSD

DISCUSSION

Chemical Composition

Acidification of milk or casein micelle suspensions causes dissociation of calcium and other minerals such as phosphorous and magnesium from the micelle and into the serum (Keller et al., 1974; Dalgleish and Law, 1989; Le Graët and Gaucheron, 1999). Consequently, cheese made by direct acidification of milk normally has lower calcium content (Shehata et al., 1967; Keller et al., 1974; Paulson et al., 1998a, 1998b) than cheese made by traditional procedures in which starter cultures are used to lower the pH of milk during cheese making. Thus, the lower calcium content of the uninjected cheese resulted from calcium solubilization brought about by the acidification of milk to pH 5.4 prior to renneting. Also, fat retention may decrease when making cheese by direct acidification methods (Keller et al., 1974) as it was observed in the present experiment.

The syneresis that occurred after calcium injection indicates that adding calcium

promoted protein-to-protein interactions within the cheese matrix, which resulted in decreased hydration of caseins and decreased capacity of the cheese to hold water present in pockets throughout the matrix. Calcium binds to casein molecules such as α_{s1}-casein (Dalgleish and Parker, 1980) and β-casein (Parker and Dalgleish, 1981; Baumy et al., 1989), thus participating in the formation and aggregation of casein micelles in milk (Dalgleish and Parker, 1980; Gaucheron et al., 1997). Divalent cations such as calcium react with negatively charged groups in casein molecules including phosphoserine residues (Baumy et al., 1989; Gaucheron et al., 1997) and possibly also carboxyl, phenolic, sulfhydryl, and imidazole groups (Gaucheron et al., 1997). As calcium binds to casein molecules and negative charges are neutralized, electrostatic repulsion between proteins decreases and protein-to-protein interactions involving hydrophobic regions are promoted (Gaucheron et al., 1997). Consequently, adding calcium to milk facilitates micelle aggregation (Green and Marshall, 1977; Dalgleish, 1983), and increases gel strength when added in levels lower that 10 mM (Jen and Ashworth, 1970). Similarly, calcium injected into cheese seems to promote interactions between proteins within the cheese matrix, which then resulted in contraction of the protein matrix.

The binding of calcium decreases the hydration of casein micelles (Green, 1982), whereas solubilization of micellar calcium can promote hydration of the paracasein in cheese (Kindstedt and Guo, 1997). Accordingly, calcium injection decreased protein solvation in the cheese. As protein-to-protein interactions increased, there was a concomitant contraction of the protein matrix and release of water from the matrix. As water molecules are released from the protein matrix they would initially accumulate in

pockets distributed throughout the matrix. This can be described as microsyneresis, in which increased strength and number of interactions between proteins cause a contraction of the protein matrix and serum accumulates in the pockets distributed throughout the matrix. Expulsion of serum from the cheese block (macrosyneresis) would not be observed until the changes in the protein matrix become substantial enough to exert an actual shrinkage of the cheese block. Consequently, the loss of serum from within the cheese block caused decreased moisture content and weight of cheese.

In addition to its effect on moisture content, calcium injection also decreased cheese pH. When calcium is added to milk, calcium binding to casein promotes the release of protons that lowers the pH of milk (Satia and Raadsveld, 1969; Jen and Ashworth, 1970). Similarly, injecting calcium into cheese seems to promote an exchange of protons from caseins that decreases the pH of cheese.

Cheese Microstructure

Lowering the calcium content of cheese by means of decreasing cheese pH can lead to decreased fusion of paracasein particles (Kiely et al., 1992) and increased hydration of the protein matrix (Kindstedt and Guo, 1997). As a result, the protein matrix swells (Kindstedt and Guo, 1997), and a more continuous three-dimensional network is observed in the cheese (Kiely et al., 1992). However, water injection promoted swelling of the protein matrix even though the calcium content of the cheese was unaffected. Soluble calcium was not determined, but changes in its concentration are thought to help explain the rearrangement of the protein matrix observed upon water injection. In contrast, calcium injection caused a contraction of the protein matrix. Adding calcium

promoted interactions between proteins, which would increase the fusion of paracasein particles and decrease the hydration of the protein matrix. Thus, the protein matrix contracted, and serum was released from within the matrix.

Cheese Functionality

As calcium content of the cheese increased, serum was released from within the cheese matrix and the moisture content of cheese decreased. This would in turn decrease cheese cohesiveness. Tunick et al. (1991) reported such a decrease in cheese cohesiveness as the moisture content of low- (23% average) and high-fat (46% average) Mozzarella cheese decreased from 57% to 52%, and from 52% to 47%, respectively. Also, the lower pH of the cheese in calcium-injected cheese possibly affected cheese cohesiveness. A progressive dissociation of casein micelles into smaller aggregates occurs as the pH of cheese curd decreases (Hall and Creamer, 1972; de Jong, 1978; Roefs et al., 1985), and below pH 4.8 casein aggregates loose their identity, and cohesion is lost (Lawrence et al., 1987). Thus, decreased moisture content and pH would promote decreased cohesiveness of cheese upon calcium injection.

In addition to its effect on cheese cohesiveness, calcium injection increased cheese hardness. Calcium binding to the protein matrix increases the rigidity of the cheese (Barbano, 1999). Thus, as calcium injection promoted protein-to-protein interactions, possibly through calcium bridging and charge neutralization, serum was expelled from within the protein matrix and the cheese became firmer. Accordingly, in Cheddar cheese, for any given pH value there is a tendency for the cheese to become firmer as the calcium content of the cheese increases (Lawrence et al., 1993).

Calcium injection also affected cheese meltability, and in agreement with Paulson et al. (1998a), increased calcium content led to decreased melting of cheese. In their study, Paulson et al. (1998a) observed that increased calcium content of nonfat Mozzarella cheese, from 0.3% to 0.6%, resulted in decreased melting. They also found cheese pH to have less effect than calcium content in affecting cheese functionality. In fact, pH in the range of 5.8 to 5.3 had no effect on either the structure (data not published) or meltability of cheese when the calcium content of the cheese was kept the same (0.6%). Thus, even though decreased cheese pH upon calcium injection possibly affected cheese structure and functionality, we think it played a secondary role compared to calcium content.

CONCLUSIONS

Increasing the calcium content of cheese alters how the proteins in the cheese matrix interact. It appears that calcium promotes protein-to-protein interactions, probably through calcium bridging and charge neutralization. Such increased and stronger interactions between proteins cause contraction of the protein matrix and expulsion of serum from the matrix. As the protein matrix becomes less hydrated, and protein-to-protein interactions are promoted, more energy must be applied to overcome these interactions and allow the proteins to flow when heated. Thus, cheese hardness increases and cohesiveness and meltability decrease when the calcium content of the cheese increases.

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CHAPTER 3

EFFECT OF SALT ON STRUCTURE-FUNCTION RELATIONSHIPS OF CHEESE*

ABSTRACT

Our objective was to determine the effect of salt on structural and functional properties of cheese. Unsalted, Muenster cheese was obtained on 1 d, vacuum-packaged, and stored for 10 d at 4°C. The cheese was then cut into blocks that were vacuum-packaged. After 4 d of storage at 4°C, cheese blocks were high-pressure injected one, three, or five times, with a 20% (wt/wt) sodium chloride solution. Successive injections were performed 24 h apart. After 40 d of storage at 4°C, cheese blocks were analyzed for chemical, structural, and functional attributes. Injecting sodium chloride increased the salt content of cheese, from 0.1% in the control, uninjected cheese to 2.7% after five injections. At the highest levels, salt injection promoted syneresis, and after five injections the moisture content of cheese decreased from 41% to 38%. However, the increased salt content caused a net weight gain. Cheese pH, soluble nitrogen, and total and soluble calcium content were unaffected. Cheese injected five times had a 4% increased area of cheese occupied by protein matrix compared to uninjected cheese. Hardness, adhesiveness, and initial rate of cheese flow increased, and cohesiveness decreased upon salt injection. However, the final extent of cheese flow, or melting was unaffected. We concluded that adding salt to cheese alters protein interactions, such that

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the protein matrix becomes more hydrated and expands. However, increasing the salt content of cheese did not cause an exchange of calcium with sodium. Therefore, calcium-mediated protein interactions remain a major factor controlling cheese functionality.

INTRODUCTION

When considering the available data regarding the effect of salt content on the chemical composition and functional properties of cheese, it is observed that there is no data reporting on some possible effects of salt (e.g., soluble calcium content), that some results seem to contradict each other (e.g., cheese melting), and that there is not enough information available for determining structure-function relationships of cheese. Following, some of these issues, for which there is not enough information available or that seem to be contradictory, are introduced and the objectives of our study presented.

Adding salt to milk or casein systems promotes dissociation of calcium and phosphate from within casein micelles and into solution (Casiraghi and Lucisano, 1991; Gatti and Pires, 1995; Gaucheron et al., 2000). It has been suggested that salt addition would promote calcium solubilization from paracasein in casein pellets and cheese (Creamer, 1985; Kindstedt et al., 1992), thus displacing calcium from the protein matrix and into the serum. Paulson et al. (1998) and Schroeder et al. (1988) observed that when salt was added to cheese calcium content remained the same. However, soluble calcium was not determined. Therefore, whether adding salt to cheese would cause mobilization of calcium from caseins and into solution remains uncertain.

Displacement of calcium from casein micelles by adding salt to milk may cause

increased hydration or solvation of caseins (Creamer, 1985). However, in cheese, salt addition normally promotes syneresis and decreases the moisture content of cheese (Kindstedt et al., 1992; Guinee and Fox, 1993; Mistry and Kasperson, 1998; Schroeder et al., 1988). Thus, in contrast to results in milk systems, adding salt to cheese seems to cause decreased hydration of caseins. However, increased salt content has also resulted in no decrease in the moisture content of cheese (Cervantes et al., 1983; Paulson et al., 1998). Therefore, increasing the salt content of cheese may not cause moisture losses of cheese, and whether caseins become less hydrated upon salting of cheese is not clear.

Adding salt to cheese also affects cheese composition by influencing microbial activity (Thomas and Pearce, 1981; Schroeder et al., 1988; Guinee and Fox, 1993). Even though adding low levels of salt to milk may promote starter activity, higher levels have the opposite effect (Irvine and Price, 1961). In addition, salt content may also affect cheese proteolysis by affecting microbial and enzyme activity, with high salt levels decreasing the rate and/or extent of proteolysis (Fox and Walley, 1971; Schroeder et al., 1988; Mistry and Kasperson, 1998). Thus, salt content may affect both cheese pH and proteolysis, which could in turn affect cheese functionality.

Salt content affects cheese functionality either directly or indirectly by mediating other changes in cheese composition. Increased salt content causes increased hardness and decreased cohesiveness of cheese (Cervantes et al., 1983; Schroeder et al., 1988). However, results that seem to be contradictory have been reported regarding the effect of salt content on cheese melting. Thus, increased salt content decreased the melting of Mozzarella cheese (Olson, 1982), but increased the melting of nonfat Mozzarella cheese

(Paulson et al., 1998). Additional work may prove helpful to better understand how salt content affects cheese melting.

The salt content of cheese may also affect cheese structure. Increased salt content of cheese would promote solubilization of caseins (Guo and Kindstedt, 1995; Guo et al., 1997), causing the protein matrix to become more hydrated and to swell (Guo and Kindstedt, 1995; Guo et al., 1997; Paulson et al., 1998). However, very limited data (Paulson et al., 1998) is available for determining relationships between cheese structure and functionality as affected by salt content of cheese. Determining structure-function relationships of cheese would help better understand the effect of salt on cheese functionality.

OBJECTIVES

The objectives of the present research were to determine the effect of salt on cheese structure and to relate changes in structure to changes in functional properties of the cheese.

MATERIALS AND METHODS

Cheese

Three 3-kg loaves of unsalted, Muenster cheese were obtained from a cheese-production facility on the day of manufacture, vacuum-packaged, and stored for 10 d at 4°C. The loaves were then cut into 0.5- to 0.6-kg blocks that were vacuum-packaged and stored for an additional 4 d at 4°C prior to injection.

Cheese Injection

A 2-stage homogenizer (Crepaco, Model 3DDL-3535, Chicago, IL) served as the pump for injection. It had an outlet line that went through a solenoid-operated valve and to an injection head, which had 13 nozzles aligned 1 cm apart from one another. Adjusting the homogenizer valves allowed for changing the pressure of injection, which was set at 17 MPa. The burst duration was controlled via the solenoid-operated valve and set to 1 s. Injected solution flowed through sapphire nozzles (0.02-cm internal diameter) and into the cheese. Cheese blocks were accurately weighted and then high-pressure injected one, three, or five times, with a 20% (wt/wt) sodium chloride solution (3.8 M). Successive injections were performed 24 h apart and according to an injection pattern of 1 x 1 cm applied to two opposite sides of the cheese block. During injection, only a portion of dispensed solution is effectively retained in the cheese block (visual observation). Therefore, after injection, cheese blocks were blotted with paper towels to remove extraneous fluid and cheese weight recorded. The cheese was then vacuum-packaged and stored for an additional 40 d at 4°C prior to analysis (see Tables B.1 and B.2, and Figure B.1 in Appendix B).

Chemical Composition

Fat content was determined using a modified Babcock method (Richardson, 1985), moisture content by using the vacuum oven AOAC method 926.08 (1990), and sodium chloride according to AOAC method 971.19 (model 926 salt analyzer; Corning, Medfield, MA) (1990). Total and soluble calcium was determined by inductively coupled plasma-atomic emission spectroscopy (US Environmental Protection Agency, 1992). To

determine soluble calcium, cheese samples (5 g) were blended with 50 g of water using a hand-held, high-speed homogenizer, and transferred to a beaker. The blending container was then rinsed with water (150 g), and the water transferred to the beaker. After standing for 20 min, the solution was filtered through Whatman # 42 filter paper. The filtrate was then analyzed for calcium content. A pH meter (model IQ240, IQ Scientific Instruments, Inc., San Diego, CA), with a stainless steel probe (model PH06-SS, IQ Scientific Instruments, Inc., San Diego, CA), was used to determine cheese pH, which was measured by taking a sample from the cheese block and inserting the pH probe into it. Proteolysis was determined by measuring non-protein nitrogen. Cheese samples (1.5 g) were blended with 30 ml of trichloroacetic acid solution (12% wt/wt) using a hand-held, high-speed homogenizer, and transferred to a beaker. The blending container was then rinsed with 20 ml of trichloroacetic acid solution, and the solution transferred to the beaker. After standing for 20 min, the solution was filtered through Whatman # 42 filter paper, and nitrogen content in the filtrate measured by Kjeldahl method.

Scanning Electron Microscopy

Cheese samples (approximately 1 x 1 x 10 mm) were taken and fixed in fresh 2% glutaraldehyde solution and stored at 4°C. After refrigerated storage, the samples were processed according to McManus et al. (1993). Samples were frozen in liquefied Freon 22 (-159°C) (Mallinckrodt Inc., Paris, KY), transferred to liquid nitrogen, cryofractured perpendicular to their long axis, and thawed in 2% glutaraldehyde. They were then dehydrated in a graded ethanol series followed by fat extraction with Freon 113 (Mallinckrodt Inc., Paris, KY). After overnight storage in Freon 113 at 4°C, the samples

were rehydrated, by reversing the graded ethanol series, and washed with a 0.1 M sodium cacodylate buffer (Electron Microscopy Sciences, Fort Washington, PA), pH 7.2. The samples were then post-fixed for 2 h with a solution containing 1% OsO_4 (Electron Microscopy Sciences, Fort Washington, PA) and 1.5% $K_4Fe(CN)_6 \cdot 3H_2O$ (Fisher Scientific Co., Fair Lawn, NJ). This solution was replaced by a 2% tannic acid (Mallinckrodt Inc., Paris, KY) solution in cacodylate buffer, and the samples were left for 3 h at 20°C. The tannic acid solution was then replaced with the mentioned solution of osmium tetroxide and potassium ferrocyanate, and samples left for 4 h. This solution was later replaced with an aqueous solution of 1% hydroquinone (Mallinckrodt Inc., Paris, KY), and samples left overnight. After post-fixing, the samples were washed with distilled water, dehydrated in a graded ethanol series, and air-dried. Samples were then coated with a gold-iridium mix using a sputter coater (model 108, Kurt J. Lesker, PA). After coating, samples were viewed in a field emission scanning electron microscope operated at 3 kV. Images from each sample, at 1500 X magnification, from three fields were recorded on Kodak TMX 120 film, and digitally using Spectrum 2.0 software (The Dindima Group Pty. Ltd., Ringwoood, Victoria, Australia). Fields were randomly selected from areas of the sample that exhibited good quality planes of fracture.

Image Analysis

Digital images, with pixels in the gray scale 0 to 255 (from black to white) were uploaded into Adobe Photoshop® 4.0. The images were then converted from their gray-scale values to binary images in which gray pixels were converted to either white or black pixels by applying the Threshold function. In the original digital images, dark

pixels corresponded to areas of the micrograph occupied by pockets that originally, mainly contained fat and/or serum, while light pixels corresponded to areas occupied by protein matrix. Then, when thresholding, pixels having a gray value lower than the threshold level were converted to black pixels, while those having a gray value higher than the threshold level were converted to white pixels. A threshold level of 95 allowed making a precise differentiation between dark and light areas as determined by visually matching the original and binary images. The proportions of black and white pixels, and the areas occupied by them were then determined by applying the Histogram function. Thus, the area of cheese matrix occupied by fat/serum pockets (dark areas) and protein matrix (light areas) was determined.

Cheese Functionality

After 40 d of storage at 4°C, cheese was removed from its packaging, dried with paper towels, and re-weighted. Melting was performed using the UW Meltmeter (University of Wisconsin-Madison, WI). Duplicate cheese samples, 3 cm in diameter and 0.7 cm in height, were tested at 60°C with the height of cheese recorded every 0.2 s for 40 s. Initial rate of cheese flow was defined as the rate (mm/s) at which cheese height decreased during the first 2 s of the test. Also, the final extent of cheese flow (decrease in height) at 40 s was determined. Texture profile analysis was performed using a two-bite compression test run on a texture profile analyzer (Model 5542, Instron Corp., Canton, MA). The compression factor was 75% and the crosshead speed was set at 20 mm/min. Samples, 20 mm long by 16 mm in diameter, were taken from the cheese immediately after removal from the refrigerator, and tested at approximately 5°C. Hardness,

cohesiveness, and adhesiveness were determined by analyzing the data according to Bourne (1978).

Experimental Design and Statistical Analysis

The experiment was conducted in triplicate as a completely randomized design. Three treatments, corresponding to number of injections one, three, or five, along with a control, uninjected cheese, were considered in the experiment. Two cheese samples were analyzed for each variable except weight, soluble and total calcium, and soluble nitrogen, and their mean considered for analysis of variance. For scanning electron microscopy, three cheese samples from one replication were analyzed. Thus, each sample was considered as a replicate for analysis. Statistical analysis (GLM and LSD) was performed using SAS® (1999) (for summary of statistical analysis see Table A.2 in Appendix A).

RESULTS

Cheese Composition

The moisture content of cheese was in compliance with the standard of identity for Muenster cheese, lower than 46%, however the fat content as a percent of solids was lower than required, 49% compared to 50% in dry basis (FDA, 1991) (Table 3.1). Calcium and sodium chloride content were as expected.

In accordance to previous results (Appendix B), injecting a concentrated solution of sodium chloride significantly increased the salt content of cheese (Table 3.2). Each injection increased the salt content of cheese by 0.5% in average, and after five injections the salt content increased from 0.1% (uninjected cheese) to 2.7% (Figure 3.1).

TABLE 3.1. Cheese composition of unsalted cheese

Variable	Mean	CV
Fat (%)	29.0	1.0
Moisture (%)	40.8	1.6
pH	5.45	0.2
Calcium (%)	0.7	7.8
Salt (%)	0.08	25

TABLE 3.2. Statistical results for the effect of adding sodium chloride on chemical and functional properties of unsalted Muenster cheese after 40 d of storage at 4°C

Source	Model[1]
Variable	
	P
Salt	0.0001
Moisture	0.0454
Weight	0.0002
Hardness	0.0008
Adhesiveness	0.0364
Cohesiveness	0.0003
Flow rate	0.0001

[1] $Y_j = \mu + T_j + e_j$, where Y is the variable of interest, μ is the overall mean, T is the treatment effect, and e is the error term

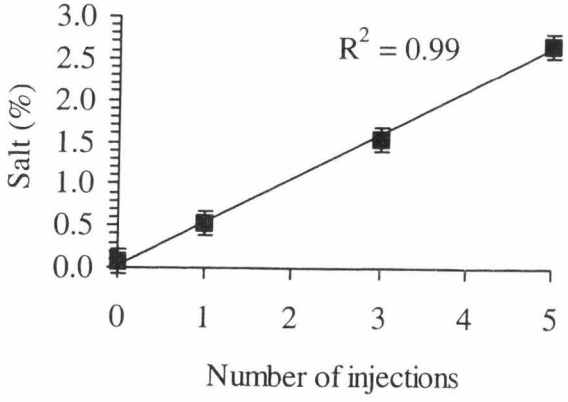

Figure 3.1. Salt content of unsalted Muenster cheese injected with a sodium chloride solution (20% wt/wt) and stored for 40 d at 4°C. Successive injections performed 24 h apart. Error bar = LSD/2

Salt injection promoted syneresis, and after refrigerated storage, drops of serum were observed inside the package of cheese blocks injected three and five times. After each injection the cheese had been blotted, so the serum in the package was serum expelled from the cheese rather than residual injectant not incorporated into the cheese block. Thus, the moisture content of cheese significantly decreased after five injections (Table 3.2), from 41% in the control cheese to 38% (Figure 3.2). Accordingly, adding salt normally promotes syneresis and decreases the moisture content of cheese (Schroeder et al., 1988; Kindstedt et al., 1992; Guinee and Fox, 1993; Mistry and Kasperson, 1998). However, even though injecting salt at the highest levels caused moisture losses, the increased salt content resulted in a significant increase in cheese weight (Table 3.2), with a net weight gain of 1.9% after five injections (Figure 3.3). In contrast, in brine-salted cheese the weight of cheese blocks normally decreases upon salting (Geurts et al., 1972; Guinee and Fox, 1993).

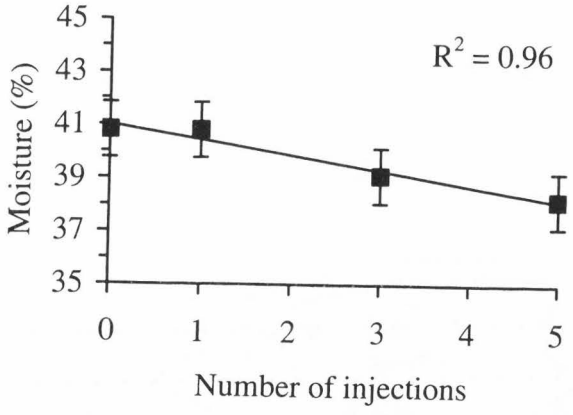

Figure 3.2. Moisture content of unsalted Muenster cheese injected with a sodium chloride solution (20% wt/wt) and stored for 40 d at 4°C. Successive injections performed 24 h apart. Error bar = LSD/2

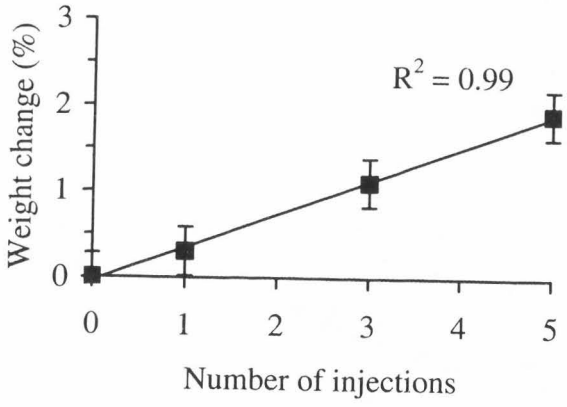

Figure 3.3. Weight change of unsalted Muenster cheese injected with a sodium chloride solution (20% wt/wt) and stored for 40 d at 4°C. Successive injections performed 24 h apart. Error bar = LSD/2

In agreement with previous studies (Cervantes et al., 1983; Kindstedt et al., 1992; Guo et al., 1997), and in contrast to the results of Thomas and Pearce (1981), increased salt content of cheese did not affect cheese pH, and the pH of cheese remained at 5.5. Also, the increased salt content had no effect on the content of soluble nitrogen, an indicator of extent of proteolysis. Similarly, Kindstedt et al. (1992) observed no differences in proteolysis between sections of low-moisture and low-moisture part-skim Mozzarella cheese with high- and low-salt content (2 to 3% compared to 0.4%). As previously reported (Schroeder et al., 1988; Paulson et al., 1998), salt content had no effect on total calcium content of cheese. In addition, soluble calcium was unaffected by adding salt to cheese and remained at approximately 50% of total calcium.

Cheese Microstructure

The control, uninjected cheese had a structure typical of a stirred/pressed-curd cheese, with protein matrix interspersed with areas that originally contained fat and/or serum (Figure 3.4A). The structure of salt-injected cheese looked similar to that of the control cheese, with fat/serum pockets ranging in size between 1 and 11 μm in diameter or length observed throughout the cheese matrix (Figure 3.4B). Applying the Threshold function of the software allowed obtaining binary images of the original micrographs (Figure 3.5). In these images, fat/serum pockets (black areas) were clearly differentiated from the protein matrix (white areas). For the control cheese, the protein matrix occupied 84% of the cheese matrix, with fat/serum pockets occupying the remaining 16% (Figure 3.5A). Although only significant at $P < 0.1$, cheese injected five times had a 4% increased area of cheese occupied by protein matrix compared to the uninjected cheese

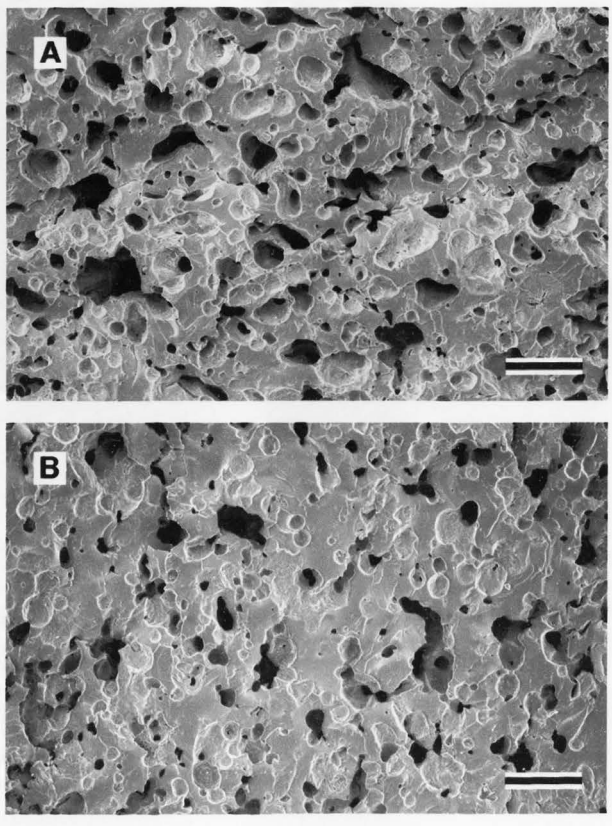

Figure 3.4. Scanning electron micrographs of Muenster cheese after 40 d of storage at 4°C. A: unsalted cheese (uninjected); B: salt-injected cheese (5 injections). Bar = 10 μm

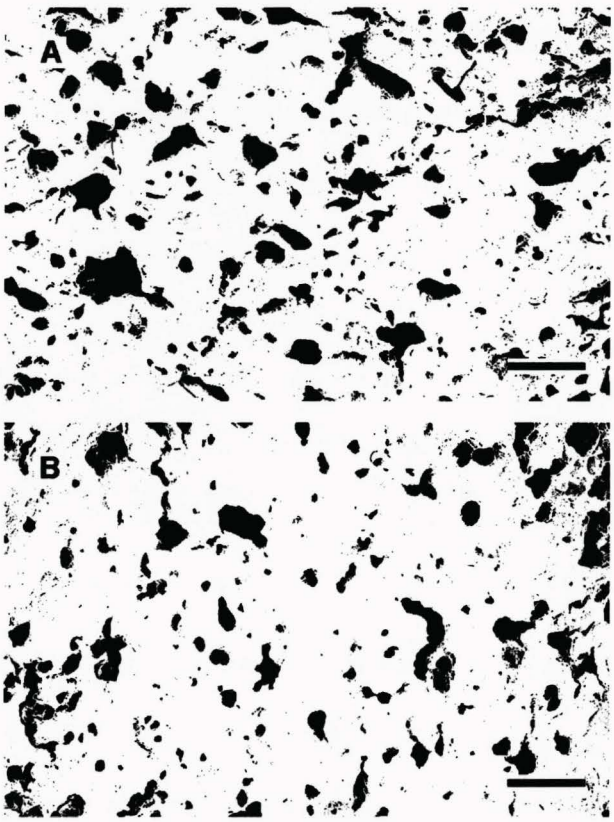

Figure 3.5. Binary image of scanning electron micrographs 3.4A and 3.4B after thresholding. Bar = 10 μm

(Figure 3.5B). Thus, after five injections the protein matrix occupied 88% of the cheese matrix, with fat/serum pockets occupying the remaining 12%. This is in agreement with the results of Paulson et al. (1998), who observed salted nonfat Mozzarella to have a more homogeneous cheese matrix with increased area occupied by protein matrix when compared to unsalted cheese.

Cheese Functionality

Salt injection significantly affected the hardness of cheese (Table 3.2). In agreement with previous studies (Cervantes et al., 1983; Schroeder et al., 1988; Mistry and Kasperson, 1998), increased salt content caused increased hardness of cheese, but no further increase was observed after three injections (Figure 3.6). During the analysis, cheese blocks injected five times partially collapsed, losing structural integrity when compression approached 70%. Cheese adhesiveness and cohesiveness were also significantly affected by salt injection (Table 3.2). Injected cheese had increased adhesiveness, but there was no further difference after one injection (Figure 3.7). Also, in agreement with Cervantes et al. (1983) and Schroeder et al. (1988), increased salt content of cheese decreased cheese cohesiveness (Figure 3.8).

Injecting salt significantly affected the initial rate of cheese flow (Table 3.2). Even though salt injection increased the initial rate of flowing, no further increase was observed after three injections, and cheese injected five times had decreased flow rate compared to cheese injected once (Figure 3.9). In previous studies, increased salt content decreased the melting of young Mozzarella cheese (Olson, 1982), and increased the melting of nonfat Mozzarella cheese (Paulson et al., 1998). In contrast, in the present

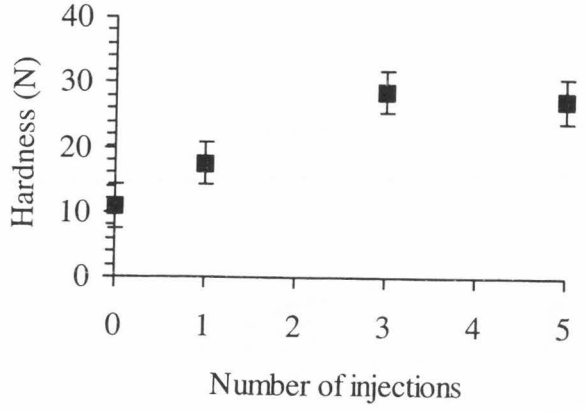

Figure 3.6. Hardness of unsalted Muenster cheese injected with a sodium chloride solution (20% wt/wt) and stored for 40 d at 4°C. Successive injections performed 24 h apart. Error bar = LSD/2

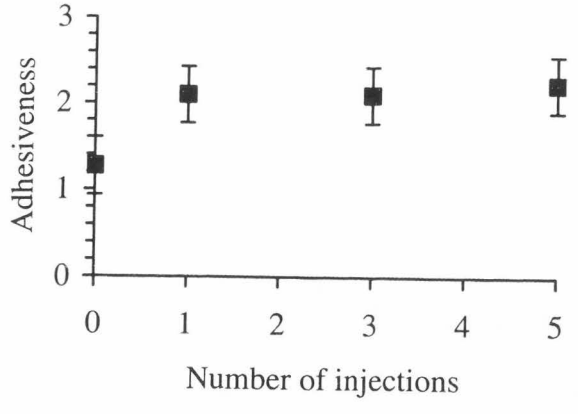

Figure 3.7. Adhesiveness of unsalted Muenster cheese injected with a sodium chloride solution (20% wt/wt) and stored for 40 d at 4°C. Successive injections performed 24 h apart. Error bar = LSD/2

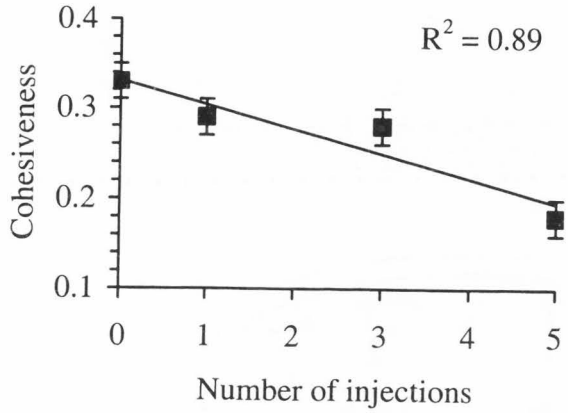

Figure 3.8. Cohesiveness of unsalted Muenster cheese injected with a sodium chloride solution (20% wt/wt) and stored for 40 d at 4°C. Successive injections performed 24 h apart. Error bar = LSD/2

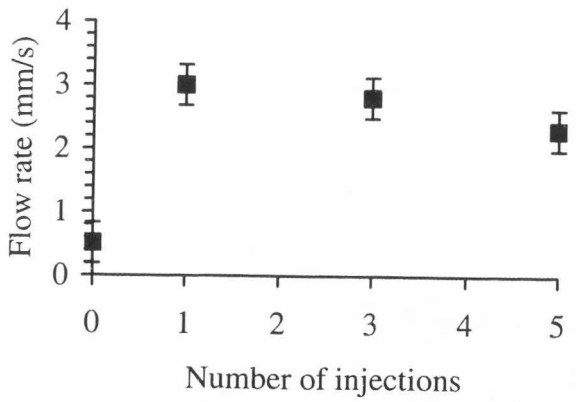

Figure 3.9. Initial rate of flow of unsalted Muenster cheese injected with a sodium chloride solution (20% wt/wt) and stored for 40 d at 4°C. Successive injections performed 24 h apart. Error bar = LSD/2

experiment the final extent of cheese flow, or melting, was unaffected by the increased salt content of cheese.

DISCUSSION

Chemical Composition

Moisture retention. In previous work (Chapter 2), injection of a calcium solution into cheese induced contraction of the protein matrix with concomitant release of serum and loss of moisture, which resulted in a less hydrated protein matrix. Thus, in the present experiment, the occurrence of syneresis and moisture losses of cheese after three injections was indication of a possibly less hydrated protein matrix. But determining whether salting of cheese by injecting a concentrated salt solution caused decreased hydration of the protein matrix requires further considerations.

When salt is added to an aqueous solution, the volume occupied by that solution increases. In cheese, serum represents an aqueous solution, and injection of a concentrated brine solution increased the salt content of cheese. Considering that salt mostly remains in the water phase of cheese, salt injection resulted in increased salt concentration in the serum. Then, as a result of increased salt concentration, the volume of serum would increase, and the magnitude of this change could be estimated based upon the increase of salt-in-water concentration in the cheese. However, the increase in volume is not strictly equivalent to the increase in salt content. In particular, at a salt-in-water concentration of 6.9% (that achieved after five injections), the volume occupied by cheese serum would increase by 2% compared to the 0.2% salt-in-water content of the

uninjected cheese. However, the volume of pockets in cheese for allocating serum and the water-holding capacity of the protein matrix is limited. Considering water to be uniformly distributed throughout the cheese matrix, and that the cheese remains with no deformation or increase in its total volume, if the protein matrix expands, serum must be displaced from within pockets.

Assuming the data on micrograph area corresponds with the volume of cheese occupied by protein matrix or serum/fat pockets, the volume of cheese occupied by protein matrix increased by 4%. Thus, upon addition of salt the protein matrix became more hydrated. However, the protein matrix has limited water-holding capacity, and while there was some migration of serum into the protein matrix there was also migration of excess water out of the cheese. This was observed as syneresis and the cheese had a net loss of moisture (1.6 g per 100 g of cheese after five injections).

In general, when cheese is brined, there is a decrease in the moisture content of cheese. However, when cheese was brined, so as to produce a final salt content of 1.1% and 1.8%, there was no difference in the moisture content of cheese (Cervantes et al., 1983). In some cases, salting may even result in cheese with higher moisture content than unsalted cheese. Paulson et al. (1998) made nonfat Mozzarella with final salt contents of 0.1 to 2.2%, and observed no decrease in the moisture content of cheese. The only difference in moisture content was between unsalted cheese and cheese that was dry-salted before cooking and stretching, the latter having increased moisture content. However, the cheese was made according to a direct-acid cheese-making procedure that promoted solubilization of calcium from casein and into solution. As a result, the cheese

had low calcium content, 0.4%, which would decrease interactions between proteins, enhancing the effect of salt in promoting protein-to-water interactions. Thus, the water-holding capacity of cheese curd increased when salt was added, and syneresis was inhibited resulting in cheese with increased moisture content. In addition, increasing the salt content of cheese above about 0.5%, by using hot brine solution during cooking/stretching of the curd, had no further influence on moisture content. Therefore, whether salting decreases or increases the moisture content of cheese depends on the amount of salt added, method of salting (e.g., dry-salting, brining, or injection of salt solution), and probably on the chemical composition of cheese (e.g., low or high calcium content).

pH and proteolysis. Salting of cheese can influence cheese pH through its effect on microbial activity (Thomas and Pearce, 1981; Guinee and Fox, 1993). Adding low levels of salt to milk (up to 1.5%) may promote starter activity but higher levels (2.5% and above) have the opposite effect (Irvine and Price, 1961). In cheese, depressed microbial activity at high salt levels (above 6% salt-in-water) leads to increased level of residual lactose and higher cheese pH (Thomas and Pearce, 1981). However, the level of salt at which starter activity decreases depends on bacterial specie and strain, and moisture content of cheese (Guinee and Fox, 1993). In the present experiment, salt was not injected into cheese until 14 d after manufacture. Generally, there is no residual lactose in cheese after two weeks of ripening (McSweeney and Fox, 1993), so that injecting salt would not cause a change in pH by altering microbial usage of sugar and amount of acid produced in cheese during further storage.

The pH of cheese during long-term storage typically increases as a result of proteolysis, by formation of NH_3 (Fox et al., 1993). Salt content may influence cheese proteolysis by affecting microbial and enzyme activity, with high salt levels decreasing the rate and/or extent of proteolysis (Fox and Walley, 1971; Schroeder et al., 1988; Mistry and Kasperson, 1998). However, in the present experiment there were no differences in proteolysis (measured as TCA-soluble nitrogen) based on salt content of cheese. In agreement with our results, Kindstedt et al. (1992) also observed no differences in proteolysis between sections of Mozzarella cheese with high- and low-salt content.

Soluble calcium. It has been proposed that adding salt promotes calcium solubilization from the paracasein matrix of rennet-treated casein pellets and cheese (Creamer, 1985; Kindstedt et al., 1992). Calcium can be lost from cheese during brining if the brine solution contains low calcium content (e.g., 0.1%) or no calcium at all. However, this is not related to increasing the salt content of cheese because when enough calcium is added to the brine (0.6% calcium content) there is no loss of calcium from the cheese (Geurts et al., 1972). In the present experiment the content of total and soluble calcium was unaffected by the increased salt content of cheese. Thus, solubilization of calcium in cheese is independent of salt content.

Cheese Microstructure

It has been proposed that sodium chloride in the serum phase of Mozzarella cheese would promote solubilization of caseins and increased protein-to-water interactions. Thus, the protein matrix becomes more hydrated and swells, occupying an

increased area of cheese matrix, becoming more continuous and homogeneous in appearance (Guo and Kindstedt, 1995; Guo et al., 1997; Paulson et al., 1998). Our results support this role of salt increasing the hydration of proteins by altering protein interactions. This occurs by increased salt content impairing interactions between proteins and promoting protein-to-water interactions. Thus, a partial relaxation of the protein matrix would occur that would allow water from within pockets to migrate into the protein matrix. As a result, the protein matrix became more hydrated and swelled, occupying increased area of cheese matrix.

Cheese Functionality

Hardness. Increasing the salt content of cheese increases cheese hardness, but the cheese becomes more brittle (Olson, 1982; Cervantes et al., 1983; Mistry and Kasperson, 1998). According to Cervantes et al. (1983), salt affects cheese hardness by promoting interactions between proteins. In the present experiment, cheese hardness increased after one injection of salt, but after further injections (i.e., with salt content greater than 0.5%) the effect of salt on cheese hardness was confounded with decreased moisture content of cheese. However, it is unclear why interactions between proteins that affect hardness could increase when our analysis of microstructural data suggests impaired protein-to-protein interactions and increased protein-to-water interactions when the salt content of cheese is increased.

Cheese can be considered as a gel that results from the effective interaction of proteins to form aggregates leading to the initial formation of protein strands, and then of a matrix that entraps serum, fat, and bacteria. However, proteins in cheese not only

interact among themselves, but also with water, fat, and salts; the nature and extent of these interactions depending on the ionic environment of cheese and processing conditions. Adding sodium chloride to protein suspensions or cheese increases the ionic strength of the system. In general, increasing the ionic strength of water causes salting-in or increased solubility of proteins. It seems that a similar phenomenon happens in cheese because the protein matrix swelled, suggesting increased protein-to-water interactions in salted cheese. This was also observed by Paulson et al. (1998). Such an increased hydration would increase the thickness of strands that make up the internal structure of the protein matrix of cheese. Swelling of the strands in the protein matrix may then compensate for decreased interactions between proteins, and the protein matrix would have increased capacity to withstand deformation during compression, so that cheese hardness increases.

At high ionic strength the solubility of proteins frequently decreases and proteins come out of solution. In this experiment, after five injections the protein matrix appeared more hydrated compared to unsalted cheese, indicating that "salting-out" of proteins in cheese requires a salt-in-water content greater than 7%.

Cohesiveness and adhesiveness. According to Cervantes et al. (1983), salt content affects cheese cohesiveness independently of other variables by modifying interactions with other cheese constituents. In our experiment, the effect of salt decreasing cheese cohesiveness was confounded with decreased moisture content of cheese, which may also affect cheese cohesiveness (Tunick et al., 1991). Increasing ionic strength by injecting salt can affect protein interactions at more than one level. For

example, more extensive short-range interactions, such as those involving increased hydration and thickness of strands in the protein matrix, could contribute to increased hardness of cheese. In contrast, weaker and/or decreased long-range interactions, and water loss from pockets throughout the cheese may lead to a less elastic cheese matrix, resulting in decreased cheese cohesiveness. In addition to changes in cohesiveness, anything that changes the ability of the proteins to interact with water or other proteins can also influence cheese adhesiveness. Injecting salt increased cheese adhesiveness, which may be a result of increased ability of proteins to interact with water and other non-protein elements. However, increasing the salt content of cheese from 0.5% to 2.7% did not cause any further increase in adhesiveness.

Melting. According to Olson (1982), higher salt content (2% compared to 1%) decreases the melting of young Mozzarella cheese. In contrast, Paulson et al. (1998) reported increased melting when the salt content of nonfat Mozzarella cheese increased from 0.14% to 0.4%, with further increases in salt content, up to 2.2%, having no effect on cheese melting. Also, salting increased the melting of cheese on d 1, but had no effect on the melting of cheese on d 24. In the present experiment, salt content had no effect on the final extent of cheese flow, or melting. The different results between these studies can be better understood when differences in the chemical composition and age of the cheeses are considered.

Olson (1982) studied cheese with higher fat and calcium content than Paulson et al. (1998) did, but both reported on young cheese. At the level of salt reported by Olson (1982) to decrease cheese melting, Paulson et al. (1998) observed no effect on cheese

melting. Adding salt to cheese increases the ionic strength, and in cheese with relatively high calcium content (0.7% for Mozzarella made by standard procedures), such in the study of Olson (1982), this may lead to increased interactions between proteins that decrease cheese melting. However, in the study of Paulson et al. (1998), similar increase in salt content led to lower ionic strength because the cheese had low calcium content (0.4%). As a result, interactions between proteins did not increase significantly, and cheese melting was unaffected. In addition, at lower salt levels than those reported by Olson (1982), when the salt content of cheese increased from 0.14% to 0.4%, Paulson et al. (1998) observed increased cheese melting. Thus, a relatively small increase in the salt content of unsalted cheese with low calcium content seems to promote protein-to-water interactions. As a result, interactions between proteins are impaired and protein hydration increases, which results in increased melting of young cheese.

In the present experiment the cheese also had higher fat and calcium content compared to the cheese used by Paulson et al. (1998). However, the results agree for cheeses more than 24 d old, and over a wide range of salt content (from 0.1% to 2.2%), with increased salt content having no effect on cheese melting. When these observations are compared to those reported by Olson (1982) and Paulson et al. (1998) in young cheese, it seems that changes associated with the aging of cheese inhibited the effect of salt content on cheese melting regardless of the difference in fat and calcium content of cheese.

Even though salt injection had no effect on the final extent of cheese flow, it increased the initial rate of cheese flow. After one injection of salt, there was an initial

increased flow rate, but no increase was observed after further injections. This suggests that increased hydration of the protein matrix favored protein-to-water interactions. Therefore, by impairing interactions between proteins and promoting protein-to-water interactions, salt injection caused partial relaxation of the protein matrix that initially favored the flowing of cheese, but that had no significant effect on the final extent of flow of a full-fat cheese whose calcium content of 0.7% remained the same.

In summary, the effect of salt on cheese melting varies according to cheese age (e.g., few days compared to several weeks old), the content of salt (e.g., 0.5% compared to 2.0%), and probably with cheese composition (e.g., calcium content: 0.4% compared to 0.7%). From our observations in this experiment and previous studies (Chapter 2; Paulson et al., 1998), salt content affected cheese functionality to a lower extent than calcium content did, and we agree with Lawrence et al. (1983) that calcium is a primary determinant of cheese functionality. In addition, calcium content appears to limit the extent to which changes in salt content can affect cheese structure, and may determine whether adding salt increases or decreases the melting of young cheese. Discussing the effect of chemical composition on cheese functionality by considering first the effect of a changing ionic environment on protein interactions, protein aggregates, and cheese structure helps to better understand and integrate observations.

CONCLUSIONS

Adding salt increases the ionic strength in cheese, which promotes increased solvation of proteins, thus altering protein interactions. Such increased protein-to-water

interactions cause partial relaxation of the protein matrix, which becomes more hydrated and swells. The influence of salt on cheese functionality is most prevalent in the range of 0% to 0.5%, in which case adding salt increases cheese hardness, adhesiveness, and the initial rate of cheese flow. At salt contents above 0.5%, salt appears to further increase hardness and decrease cohesiveness of cheese. Increasing the salt content of cheese did not, however, affect cheese melting, although this may not be the case for young cheese. In addition, increased salt content did not cause an exchange of calcium with sodium, and soluble calcium remained constant. Therefore, calcium-mediated protein interactions remain a major factor controlling the functionality of cheese.

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CHAPTER 4

EFFECT OF pH ON STRUCTURE-FUNCTION RELATIONSHIPS OF CHEESE

ABSTRACT

Our objective was to determine the effect of pH on chemical, structural, and functional properties of cheese, and to relate changes in structure to changes in cheese functionality. Cheddar cheese was obtained from a cheese-production facility and stored at 4°C. Ten days after manufacture, the cheese was cut into blocks that were vacuum-packaged and stored for 4 d at 4°C. Cheese blocks were then high-pressure injected one, three, or five times, with a 20% (wt/wt) glucono-δ-lactone solution. Successive injections were performed 24 h apart. Cheese blocks were then analyzed after 40 d of storage at 4°C. Acid injection decreased cheese pH, from 5.3 in the uninjected cheese to 4.7 after five injections. Decreased pH increased the content of soluble calcium and slightly decreased the total calcium content of cheese. At the highest level, injection of acid promoted syneresis. Thus, after five injections the moisture content of cheese decreased from 34% to 31%, which resulted in decreased cheese weight. Injecting acid also decreased cheese hardness and cohesiveness, and the cheese became more crumbly. The initial rate of cheese flow increased when pH decreased from 5.3 to 5.0, but it decreased when cheese pH was further lowered to 4.7. The final extent of cheese flow also decreased at pH 4.7. We concluded that lowering the pH of cheese alters protein interactions, which then affects cheese functionality. At pH greater than 5.0, calcium solubilization decreases protein-to-protein interactions. In contrast, at pH lower than 5.0,

the acid precipitation of proteins overcomes the opposing effect caused by increased calcium solubilization and decreased calcium content of cheese, and protein-to-protein interactions increase.

INTRODUCTION

When reviewing the literature on the effect of pH on cheese properties, the first realization is that considerable amount of research has been done on this topic (Keller et al., 1974; Creamer et al., 1988; Kiely et al., 1992; Marchesseau et al., 1997; Ramkumar et al., 1997, 1998; Kindstedt et al., 2001; Watkinson et al., 2001). In fact, the effect of pH has not only been studied in cheese but also in milk and casein systems. In this regard, the most significant effect of decreased pH is to promote mineral solubilization (Visser et al., 1986; van Hooydonk et al., 1986; Dalgleish and Law, 1989; Kiely et al., 1992; Le Graët and Gaucheron, 1999) and casein dissociation from casein micelles (Roefs et al., 1985; van Hooydonk et al., 1986; Dalgleish and Law, 1988) both of which alter milk properties by affecting the extent and nature of protein interactions.

Knowing how pH affects the properties of milk and casein micelles has provided a basis for understanding its effects on cheese. However, researchers have encountered a major limitation in that modifying cheese pH causes changes in other chemical parameters of cheese (Lawrence et al., 1983; Lucey and Fox, 1993). This makes it difficult to segregate the effect of pH from that of changes in total and soluble calcium content, moisture content, extent and pattern of proteolysis, and their interactions. As a result, and despite the extensive work already done, some of the fundamental questions

still remain unanswered. Thus, we are yet not certain about the independent effect of pH, or that of calcium, and which one is predominant, and under which conditions.

In trying to overcome the limitation of confounding effects, alternative methods that may allow for independently modifying the pH of cheese could be applied. Thus, Kindstedt et al. (2001) modified the pH of shredded cheese by exposing the cheese to either ammonia or acetic acid vapors. However, this requires shredding of the cheese, which limits the analysis of textural properties. An alternative to this approach is to modify the pH of cheese by high-pressure injecting concentrated acid solutions into cheese blocks, a method previously used for modifying other chemical parameters of cheese (Chapters 2 and 3). This method for modifying cheese pH allows for a more comprehensive study that includes changes in chemical composition, structure, and textural properties of cheese.

OBJECTIVES

The objectives of the present research were then to determine the effect of pH on the chemical, structural, and functional properties of cheese, and to relate changes in structure to changes in cheese functionality.

MATERIALS AND METHODS

Cheese

A 19-kg block of Cheddar cheese was obtained from a cheese-production facility and stored at 4°C. Ten days after manufacture the cheese was cut into 0.4- to 0.5-kg

blocks that were vacuum-packaged and stored for an additional 4 d at 4°C prior to injection.

Cheese Injection

Cheese was high-pressure injected one, three, or five times with a 20% (wt/wt) glucono-δ-lactone (GDL) solution as described in Chapter 3. A 2-stage homogenizer served as the pump for injection, and solution exited the system at high speed through a multi-nozzle injection head. Pressure of injection was set at 17 MPa, and the burst duration was set to 1 s. Successive injections were performed 24 h apart and according to an injection pattern of 1 x 1 cm applied to two opposite sides of the cheese block. After injection, cheese blocks were blotted with paper towels, weighed, vacuum-packaged, and then stored for an additional 40 d at 4°C prior to analysis.

Chemical Composition

Fat content was determined using a modified Babcock method (Richardson, 1985), moisture content by using the vacuum oven AOAC method 926.08 (1990), and sodium chloride according to AOAC method 971.19 (model 926 salt analyzer; Corning, Medfield, MA) (1990). Protein content was determined by measuring nitrogen content (Kjeldahl method) and multiplying by 6.38. Total and soluble calcium was determined by inductively coupled plasma-atomic emission spectroscopy (US Environmental Protection Agency, 1992). For determining soluble calcium, cheese samples (5 g) were blended with 50 g of water using a hand-held, high-speed homogenizer, and transferred to a beaker. The blending container was then rinsed with water (150 g), and the water transferred to

the beaker. After standing for 20 min, the solution was filtered through Whatman # 42 filter paper. The filtrate was then analyzed for calcium content. A pH meter (model 520A, Orion Research Inc., Boston, MA), with a glass probe (spear combo, Corning, Medfield, MA), was used for determining cheese pH, which was measured by taking a cheese sample from the cheese block and inserting the pH probe into it. Proteolysis was determined by measuring non-protein nitrogen. Cheese samples (3 g) were blended with 40 ml of trichloroacetic acid solution (12% wt/wt) using a hand-held, high-speed homogenizer. After standing for 30 min, the solution was filtered through Whatman # 42 filter paper, and nitrogen content in the filtrate measured by Kjeldahl method.

Scanning Electron Microscopy

Cheese samples (approximately 1 x 1 x 10 mm) were taken and fixed in fresh 2% glutaraldehyde solution at room temperature, and then stored at 4°C. After refrigerated storage, the samples were processed according to McManus et al. (1993), but as modified in Chapter 3. Thus, samples were frozen in liquefied Freon 22, transferred to liquid nitrogen, cryofractured perpendicular to their long axis, and thawed in 2% glutaraldehyde. They were then dehydrated in a graded ethanol series followed by fat extraction. After overnight storage, the samples were rehydrated, and washed with sodium cacodylate buffer, pH 7.2. The samples were then post-fixed with a solution containing osmium tetroxide and potassium ferrocyanate, and staining enhanced by a tannic acid solution in cacodylate buffer. After post-fixing, the samples were washed with distilled water, dehydrated in a graded ethanol series, and air-dried. Samples were then coated with a gold-iridium mix. After coating, samples were viewed in a field emission

scanning electron microscope operated at 3 kV. Images, at 1500 X magnification, from eight fields were recorded on film and digitally. Fields were randomly selected from areas of the sample that exhibited good quality planes of fracture.

Image Analysis

Digital images of electron micrographs were uploaded into Adobe Photoshop® 4.0, and brightness and contrast were adjusted so that images looked alike. Following, images with pixels in the gray scale 0 to 255 (from black to white) were analyzed as described in Chapters 2 and 3. Images were converted from their gray-scale values to binary images in which gray pixels were converted to either white or black pixels by applying the threshold function of the software. In the original digital images, dark pixels corresponded to areas of the micrograph occupied by pockets that originally, mainly contained fat and/or serum, whereas light pixels corresponded to areas occupied by protein matrix. Then, when thresholding, pixels having a gray value lower than the threshold level were converted to black pixels, while those having a gray value higher than the threshold level were converted to white pixels. A threshold level of 120 was found to provide for a precise differentiation between dark and light areas as determined by visually matching the original and binary images. The proportions of black and white pixels, and the areas occupied by them were then determined by applying the histogram function of the software. Thus, the area of cheese matrix occupied by fat/serum pockets (dark areas) and protein matrix (light areas) was determined.

Cheese Functionality

After 40 d of storage at 4°C, cheese was removed from its packaging, blotted with paper towels, and re-weighed. Melting was analyzed using the UW Meltmeter (University of Wisconsin-Madison, WI) and as described by Wang et al. (1998). Duplicate cheese samples, 3 cm in diameter and 0.7 cm in height, were tested at 60°C with the height of cheese recorded every 0.2 s for 60 s. Initial rate of flowing was defined as the rate (mm/s) at which cheese height decreased during the first 5.0 s of the test. Also, the final extent of cheese flow (cheese height) at 40.0 s was determined. Texture profile analysis was performed as described in Chapters 2 and 3. Thus, a two-bite compression test was run on an Instron 5542 (Instron Corp., Canton, MA), with a 75% compression factor and crosshead speed set at 20 mm/min. Samples, 20 mm long by 16 mm in diameter, were taken from the cheese immediately after removal from the refrigerator, and tested at approximately 5°C. Hardness, cohesiveness, and adhesiveness were determined by analyzing the data according to Bourne (1978).

Experimental Design and Statistical Analysis

The experiment was conducted in triplicate as a completely randomized design. Three treatments, corresponding to number of injections (one, three, or five) along with a control, uninjected cheese, were considered in the experiment. Two cheese samples were analyzed for all variables except cheese weight, soluble and total calcium, and soluble nitrogen content, and their mean considered for analysis of variance. For scanning electron microscopy, at least five cheese samples from one replication were analyzed, and the image of five fields was selected for analysis. Thus, each field was considered as a

replicate for analysis. Statistical analysis (GLM and LSD) was performed using SAS® (1999) (for summary of statistical analysis see Table A.3 in Appendix A).

RESULTS

When cheese is high-pressure injected, injection sites are visually observable immediately after injection, but are usually no longer visible after a few days of refrigerated storage. This was the case in previous work, when water (Chapter 2) or a sodium chloride solution (Chapter 3) was injected into cheese. In contrast, when acid was injected, injection sites were still visible after 40 d of storage. The same phenomenon was also observed when calcium was injected into cheese (Chapter 2).

In addition to observing injection sites, after 40 d of refrigerated storage, white crystals were observed on the surface of cheese blocks injected three and five times; being more abundant in cheese blocks injected five times. The crystals tended to arrange in clover-like structures with a cross-section length of up to 6 mm.

Chemical Composition

The moisture and fat content of the cheese was 34% and 30%, respectively. Calcium content was 0.8% and sodium chloride 1.7%.

Injecting a concentrated solution of glucono-δ-lactone significantly decreased cheese pH ($P < 0.01$), and after five injections cheese pH decreased from 5.3 in the control, uninjected cheese to 4.7 (Figure 4.1). In addition, acid injection promoted syneresis, and after refrigerated storage there was free serum inside the package of cheese blocks injected five times. After each injection the cheese had been blotted, so the serum

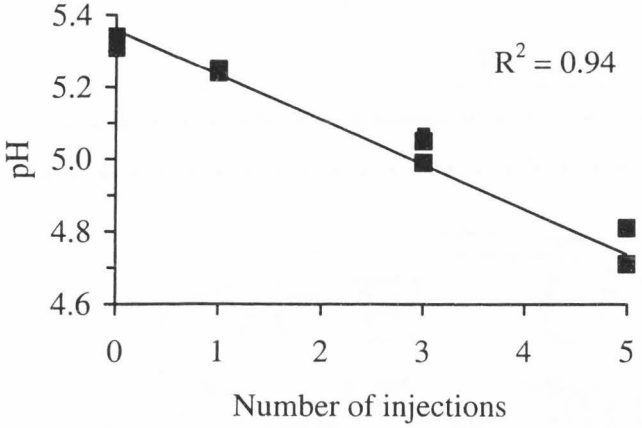

Figure 4.1. pH of Cheddar cheese injected with a glucono-δ-lactone solution (20% wt/wt) and stored for 40 d at 4°C. Successive injections performed 24 h apart. Error bar = LSD/2

in the package was serum expelled from the cheese rather than residual injectant not incorporated into the cheese block. Thus, acid injection significantly affected the moisture content of cheese ($P < 0.01$), and after five injections moisture content decreased from 34% in the control cheese to 31% (Figure 4.2). This is in agreement with previous observations in which decreased pH resulted in cheese with reduced moisture content (Taneya et al., 1992; Ramkumar et al., 1997; Watkinson et al., 2001). As a result of syneresis and moisture loss, cheese weight was significantly affected by acid injection ($P < 0.01$), and after five injections cheese weight decreased (Figure 4.3).

Decreased cheese pH promoted significant calcium solubilization ($P < 0.01$), and soluble calcium content increased from 3.5 mg/g of cheese in the uninjected cheese to 4.7 mg/g of cheese after five injections (Figure 4.4). Thus, the proportion of calcium in soluble form increased from 45% at pH 5.3 to 75% at pH 4.7. This agrees with decreased pH of cheese curd promoting calcium solubilization from casein and into the serum as

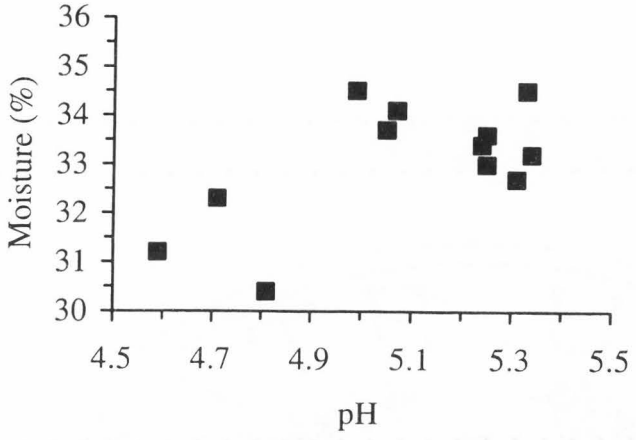

Figure 4.2. Moisture content of Cheddar cheese injected with a glucono-δ-lactone solution (20% wt/wt) and stored for 40 d at 4°C. Successive injections performed 24 h apart. Error bar = LSD/2

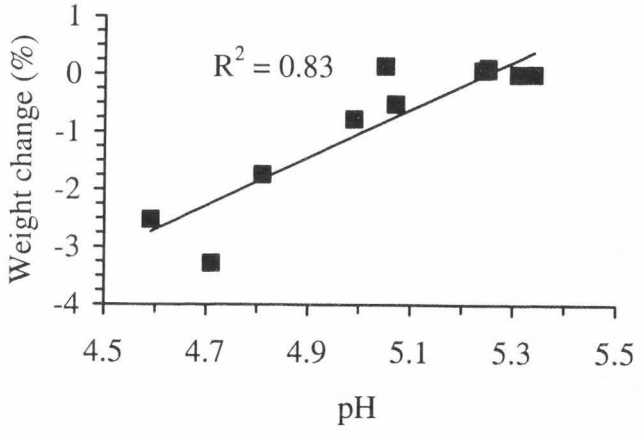

Figure 4.3. Weight of Cheddar cheese injected with a glucono-δ-lactone solution (20% wt/wt) and stored for 40 d at 4°C. Successive injections performed 24 h apart. Error bar = LSD/2

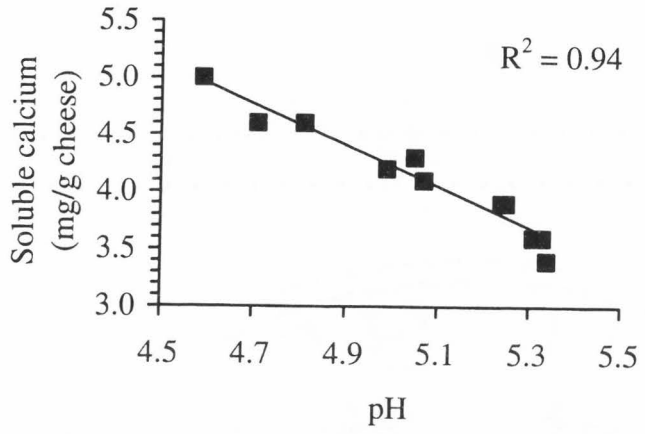

Figure 4.4. Soluble calcium content of Cheddar cheese injected with a glucono-δ-lactone solution (20% wt/wt) and stored for 40 d at 4°C. Successive injections performed 24 h apart. Error bar = LSD/2

previously reported by Lawrence et al. (1983) and Ramkumar et al. (1997). In addition, the total calcium content of cheese was significantly affected by acid injection ($P < 0.05$). Even though it remained unchanged after three injections, calcium content decreased after five injections (Figure 4.5), which is in accordance with previous observations in which cheese with lower pH had decreased calcium content (Keller et al., 1974; Kiely et al., 1992).

The content of TCA-soluble nitrogen after 40 d of storage was significantly reduced ($P < 0.01$) by injecting acid into cheese (Figure 4.6). Similarly, Watkinson et al. (2001) observed decreased acid-soluble and water-soluble nitrogen in cheese with lower pH, and Creamer et al. (1988) reported decreased content of acid-soluble amino groups in Cheddar cheese with lower pH.

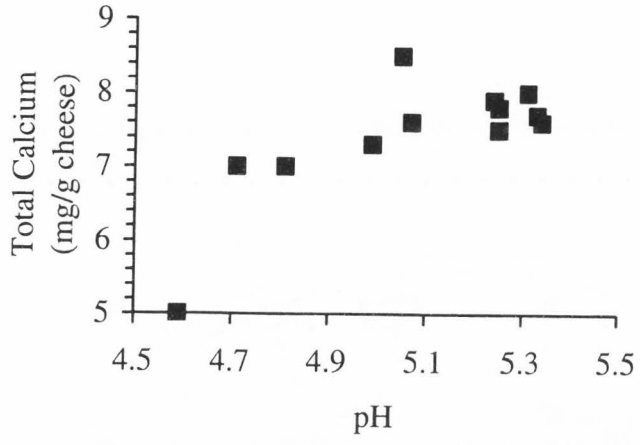

Figure 4.5. Total calcium content of Cheddar cheese injected with a glucono-δ-lactone solution (20% wt/wt) and stored for 40 d at 4°C. Successive injections performed 24 h apart. Error bar = LSD/2

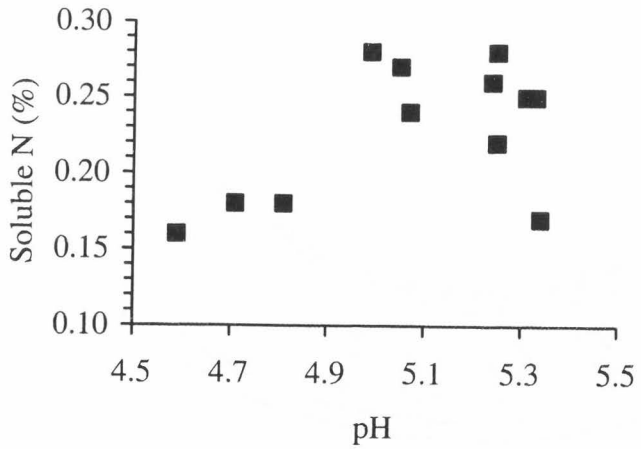

Figure 4.6. TCA-soluble nitrogen content of Cheddar cheese injected with a glucono-δ-lactone solution (20% wt/wt) and stored for 40 d at 4°C. Successive injections performed 24 h apart. Error bar = LSD/2

Cheese Microstructure

The control, uninjected cheese (pH 5.3) had a structure typical of a stirred/pressed-curd cheese, with protein matrix interspersed with areas that originally contained fat and/or serum (Figure 4.7A). The structure of acid-injected cheese (pH 4.7) looked similar to that of the control cheese (Figure 4.7B). Applying the threshold function of the software allowed for obtaining binary images of the original micrographs (Figure 4.8). In these images, fat/serum pockets (black areas) were clearly differentiated from the protein matrix (white areas). For the control cheese, the protein matrix occupied 82% of the cheese matrix, with fat/serum pockets occupying the remaining 18% (Figure 4.8A). However, after five injections with acid, the area of cheese occupied by protein matrix significantly decreased ($P < 0.05$). Thus, the protein matrix occupied 80% of the cheese matrix, with fat/serum pockets occupying the remaining 20% (Figure 4.8B).

Cheese Functionality

Acid injection significantly affected cheese hardness ($P < 0.01$), and after three injections the cheese became more crumbly and had decreased hardness (Figure 4.9). In agreement with our results, it has been reported that cheese with lower pH normally becomes less firm and more crumbly, and hardness decreases (Marchesseau et al., 1997; Paulson et al., 1998; Ramkumar et al., 1998; Watkinson et al., 2001). The cohesiveness of cheese was also significantly affected by acid injection ($P < 0.05$). Initially, cohesiveness was unaffected, but it decreased after five injections (Figure 4.10). In contrast, pH had no effect on cheese adhesiveness, which is in agreement with the results reported by Watkinson et al. (2001).

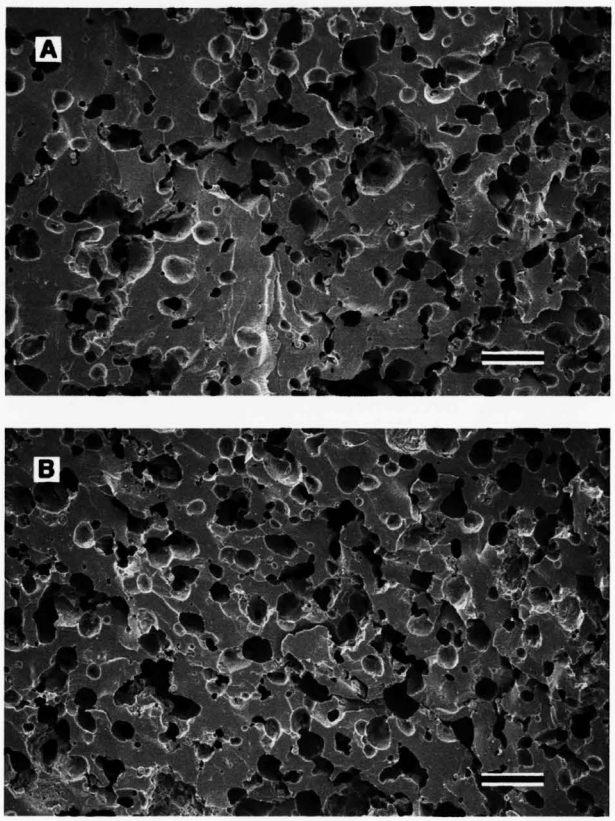

Figure 4.7. Scanning electron micrographs of Cheddar cheese after 40 d of storage at 4°C. A: uninjected cheese (pH 5.3); B: acid-injected cheese (5 injections; pH 4.7). Bar = 10 μm

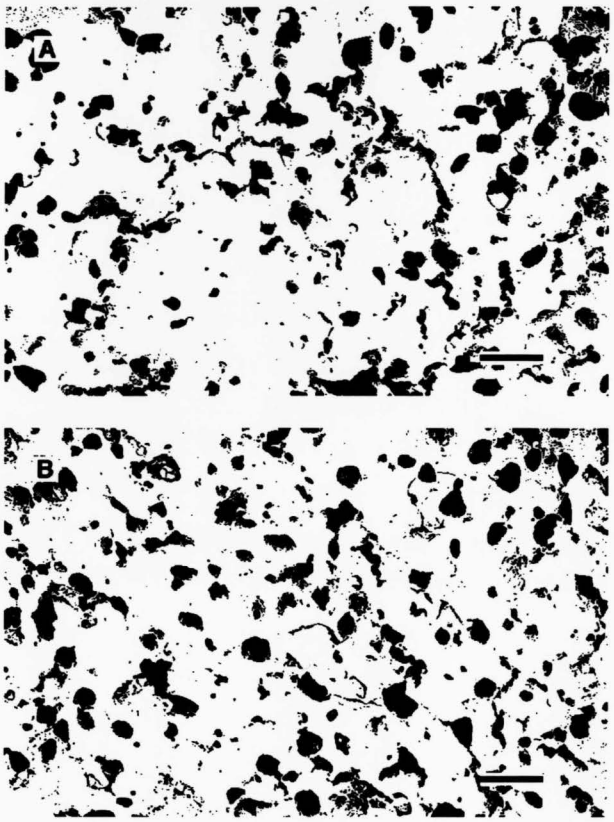

Figure 4.8. Binary image of scanning electron micrographs 4.7A and 4.7B after thresholding. Bar = 10 μm

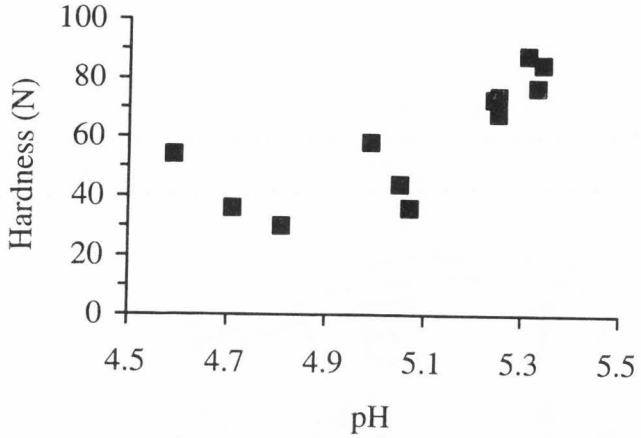

Figure 4.9. Hardness of Cheddar cheese injected with a glucono-δ-lactone solution (20% wt/wt) and stored for 40 d at 4°C. Successive injections performed 24 h apart. Error bar = LSD/2

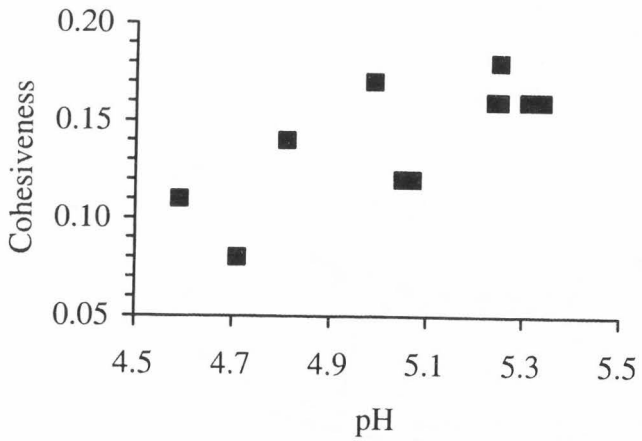

Figure 4.10. Cohesiveness of Cheddar cheese injected with a glucono-δ-lactone solution (20% wt/wt) and stored for 40 d at 4°C. Successive injections performed 24 h apart. Error bar = LSD/2

During the melting test, the initial rate of cheese flow was significantly affected by cheese pH ($P < 0.01$). Initially, decreased pH promoted the flow of cheese at a higher rate (Figure 4.11). Thus, after three injections (pH 5.0), the cheese flowed at the highest rate. However, after five injections (pH 4.7), the initial rate of cheese flow decreased. The extent of cheese flow, as determined by the height of the cheese sample after 40.0 sec, was also significantly affected by cheese pH ($P < 0.01$), and after five injections the cheese flowed to a lesser extent (Figure 4.12). In agreement with our results, Kindstedt et al. (2001) observed that low-moisture part-skim Mozzarella lost the ability to flow and melt when pH was lower than 5.0.

DISCUSSION

Chemical Composition

Moisture. In previous work, injection of a concentrated solution of either calcium or sodium chloride resulted in syneresis and moisture losses of cheese although for different reasons (Chapters 2 and 3). When the dynamics of injecting a fluid into cheese are considered, the volume of serum in the cheese initially increases upon injection. However, pockets in the cheese matrix are already full, thus unless the protein matrix expands to accommodate the extra fluid serum will be expelled from within the cheese and syneresis will occur. If the solute portion of the injected fluid remains in the cheese, then the moisture content of the cheese may actually decrease.

Injecting salt into cheese increases the hydration and water holding capacity of the protein matrix, which results in increased moisture retention and expansion of the matrix

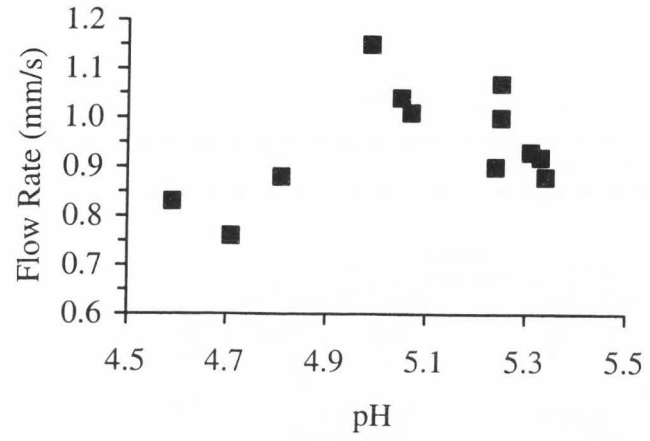

Figure 4.11. Initial flow rate of Cheddar cheese injected with a glucono-δ-lactone solution (20% wt/wt) and stored for 40 d at 4°C. Successive injections performed 24 h apart. Error bar = LSD/2

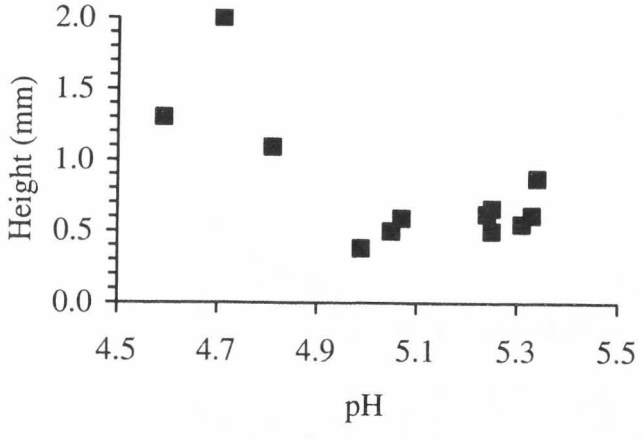

Figure 4.12. Melted cheese height (extent of flow) of Cheddar cheese injected with a glucono-δ-lactone solution (20% wt/wt) and stored for 40 d at 4°C. Successive injections performed 24 h apart. Error bar = LSD/2

(Chapter 3). However, the expansion of the protein matrix was insufficient to retain all the fluid and increased solid content, which resulted in syneresis during storage and a net reduction in the moisture content of cheese. In contrast, calcium injection promotes interaction between proteins that causes contraction of the protein matrix, decreased water-holding capacity of the matrix, release of water, and syneresis (Chapter 2). In the present experiment, lowering the pH of cheese resulted in syneresis (especially after five injections) possibly because of both increased volume of serum and contraction of the protein matrix.

At pH 5.2 there is increased CCP solubilization and decreased interactions between proteins, which allows for increased solvation of caseins (van Hooydonk et al., 1986). Thus, at pH 5.2, increased hydration of the protein matrix would be expected, leading to increased moisture content of cheese (Keller et al., 1974). In addition, at pH 5.2, the presence of weakest and/or fewer bonds between proteins allows for the rearrangement of protein interactions to proceed at a higher rate compared to both higher and lower pH (van Vliet and Walstra, 1994), which in turn would facilitate the expression of moisture from cheese curd. However, Ramkumar et al. (1997) observed that decreased quantity of serum could be expressed from cheese with lower pH, 5.2 compared to 5.6. It is possible then, that the ease with which protein interactions can rearrange at pH 5.2 may also facilitate the redistribution of moisture within the cheese matrix during early storage, thus increasing the water-holding capacity of the cheese.

Further lowering of pH, especially below 5.0, would promote protein-to-protein interactions as the caseins approach their isoelectric point and electrostatic repulsions are

minimized (Visser et al., 1986; Marchesseau et al., 1997). Thus, the ability of proteins to interact with water and the water-holding capacity of the protein matrix would decrease below pH 5.0, which then results in increased syneresis and decreased moisture content of cheese.

Calcium. Decreasing the pH of milk causes dissociation of minerals, mainly calcium and phosphorous, from colloidal calcium phosphate into soluble ions and complexes (Keller et al., 1974; van Hooydonk et al., 1986; Visser et al., 1986; Dalgleish and Law, 1989; Lucey et al., 1996; Le Graët and Gaucheron, 1999). As a result, the content of soluble calcium in cheese increases as pH is lowered (Kindstedt et al., 2001; Watkinson et al., 2001), and in the present experiment, there was an inverse linear relationship between pH and soluble calcium content (0.35% at pH 5.3 and 0.47% at pH 4.7). Thus, after three injections the amount of protein-bound calcium decreased from 17 to 14 mg/g. Lowering the pH of cheese from 5.0 to 4.7 further decreased the amount of protein-bound calcium, from 14 to 6 mg/g, and the amount of total calcium slightly decreased, presumably because of syneresis and loss of calcium in the expelled serum.

Proteolysis. In the present experiment, lowering cheese pH from 5.3 to 5.0 resulted in increased content of TCA-soluble nitrogen in cheese after 40 d of refrigerated storage. However, lowering cheese pH from 5.0 to 4.7 resulted in less proteolysis occurring during cheese storage. This decrease in proteolysis is probably because of decreased microbial and enzymatic activities at lower pH (Creamer et al., 1988). In particular, lower cheese pH decreases the activity of plasmin, which may then result in decreased breakdown of β-casein (Watkinson et al., 2001).

Cheese Microstructure

The solubilization of minerals from caseins that is brought about by decreased pH at low temperature leads first to decreased interactions between proteins, and caseins normally dissociate from the casein micelles (Roefs et al., 1985; van Hooydonk et al., 1986; Dalgleish and Law, 1988). Thus, lowering the pH of milk from 6.7 to 5.4 or 5.3 increases the solubility of caseins (Roefs et al., 1985) and leads to the presence of smaller casein aggregates (Visser et al., 1986). In cheese, at pH 5.3 or 5.2, larger aggregates are observed in the protein matrix compared to cheeses with lower pH (5.0) (Hall and Creamer, 1972; Lawrence et al., 1983, 1987). Thus, we propose a model for the protein matrix of cheese at pH 5.3 that is characterized by the presence of relatively large high-density protein aggregates, 10 to 12 nm in diameter, and by having a relatively well-defined structure in which protein strands can be identified (Figure 4.13A).

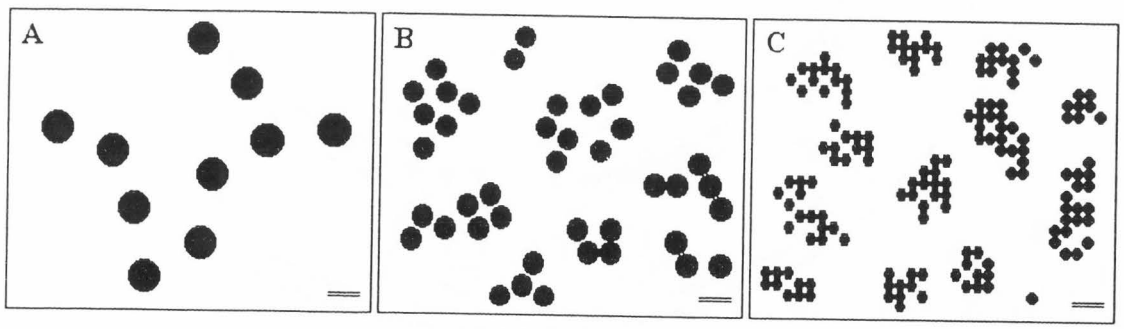

Figure 4.13. Diagram modeling the protein matrix of Cheddar cheese at pH 5.3 (uninjected): A; at pH 5.0 (three injections): B; and at pH 4.7 (five injections): C. Dark circles represent high-dense protein aggregates. Bar = 10 nm

Further lowering of pH, below 5.2, increases calcium solubilization and decreases electrostatic repulsions between proteins as the caseins approach their isoelectric point. As a result, casein aggregates in milk concentrate in fields, thus increasing the heterogeneity of the system (Visser et al., 1986), and at pH 5.0 the protein matrix of cheese has a less well-defined structure (Hall and Creamer, 1972; Taneya et al., 1992) with smaller protein aggregates (Hall and Creamer, 1972; Lawrence et al., 1983, 1987). This is represented in our model by a protein matrix that has smaller aggregates, 6 to 7 nm in diameter, which locate closer to one another, resulting in shorter protein strands and a less well-defined structure (Figure 4.13B).

Interactions between proteins significantly increase below pH 5.0 (van Vliet and Walstra, 1994), which results in the contraction of protein aggregates during the formation of milk gels (Visser et al., 1986), and cheese is characterized by having protein aggregates of even smaller size (Hall and Creamer, 1972; Lawrence et al., 1983, 1987). Thus, at pH 4.7 the protein matrix in our model is characterized by the presence of high-density aggregates 2 to 4 nm in diameter that tend to cluster, making protein strands no longer visible, and decreasing the structural uniformity of the matrix (Figure 4.13C).

In agreement with the previous description, injecting acid into cheese caused a decrease in pH that after three injections (pH 5.0) impaired protein-to-protein interactions due to calcium solubilization, but that significantly increased interactions between proteins after five injections (pH 4.7) because of decreased electrostatic repulsion. Thus, at pH 4.7, the acid precipitation of the caseins overcame the opposing effect of calcium

solubilization, and a net increase in protein-to-protein interactions caused contraction of the protein matrix.

Cheese Functionality

Hardness. Lowering the pH of cheese normally results in cheese with decreased hardness (Paulson et al., 1998; Watkinson et al., 2001). Thus, the cheese has less of a solid-like behavior (Ramkumar et al., 1998) and it may become more elastic (Keller et al., 1974). In contrast, Creamer et al. (1988) observed no significant correlation between the pH and hardness of Cheddar cheese (pH range of 5.3 to 4.9). However, when comparing cheeses with similar calcium content (0.91% and 0.95%) but different pH (4.9 and 5.1), cheese with lower pH had decreased hardness. Also, in their study, modifying cheese pH resulted in changes in other chemical parameters of cheese. Thus, the effect of pH on cheese hardness may depend on the range of pH values considered, and it may be confounded by changes in other chemical parameters of the cheese.

Watkinson et al. (2001) observed process cheese with lower pH, from 6.2 to 5.2, to have decreased firmness and to become more crumbly. In their study, lower pH was accompanied by decreased moisture and increased soluble calcium content of cheese, and by changes in the pattern and extent of proteolysis in cheese. They suggested, however, that the effect of pH on rheological and fracture properties of cheese mainly resulted from changes in calcium-mediated protein interactions as a result of calcium solubilization. In addition, increased structural uniformity of the matrix has been observed to increase cheese firmness (Rayan et al., 1980), and Marchesseau et al. (1997) observed process cheese to have a less homogeneous and dense protein network at pH 5.2 compared to pH

5.7. Thus, they suggested that decreased structural uniformity could not allow for even distribution of stress, which would then result in cheese of lower pH (5.2) having decreased firmness.

Our results are in agreement with previous observations in which lowering the pH of cheese resulted in decreased hardness. In particular, we think that lowering the pH of cheese from 5.3 to 5.0 affects cheese hardness by affecting calcium-mediated protein interactions through changes in the distribution of calcium between its soluble and insoluble forms, which is in agreement with the proposition of Watkinson et al. (2001). Thus, after three injections, decreased pH caused solubilization of calcium from casein aggregates that decreased interactions between proteins and that probably facilitated structural rearrangements in the protein matrix. Decreased protein-to-protein interactions resulted then in the weakening of the protein matrix that led to decreased hardness of cheese; an effect that can be better understood with insight from polymer and materials science.

From a material science's point of view, cheese could be considered a composite material in which two main structural components are recognized: protein and fat. Protein is the polymeric material that makes up the structure of the matrix, whereas fat participates either as a filler (non-homogenized milk) or as a copolymer (homogenized milk). The strength of a composite material depends on its composition, the properties of the polymeric and filler material, and on the nature and extent of their interactions or cross-linking (Calvert, 1997). Also, orientation of the polymeric material and structural regularity increases the strength and toughness of the material. In the present experiment,

after three injections with acid solution, the gross composition of cheese was unchanged, and no major changes in the state of fat would be expected. Therefore, changes in the state of the polymeric material, protein, and its interactions would account for changes in textural properties of cheese.

The strength of a material can be enhanced by increasing the molecular weight of the polymeric constituent, the chain length, the extent of cross-linking, and the orientation or structural regularity of the material; all of which improve the transfer of load between polymeric units (Calvert, 1997). Calcium promotes protein interactions (Chapter 2), probably through calcium bridging and charge neutralization. Accordingly, the solubilization of calcium from casein would decrease the extent of interaction or cross-linking between the polymeric units, protein aggregates. This would then impair the transfer of stress, thus decreasing the hardness of cheese. As proposed in our model, it is possible that decreased interaction between proteins resulted also in decreased size of protein aggregates and/or decreased length of protein strands in the matrix (Figure 4.13B), which would further decrease the hardness of cheese. In addition, decreased interaction between proteins could also influence the structural regularity of the protein matrix, which could in turn affect cheese hardness as previously suggested by Marchesseau et al. (1997).

In contrast to the observed decreased hardness of cheese when pH decreased from 5.3 to 5.0, further lowering of pH, from 5.0 to 4.7, had no effect on cheese hardness. Even though the cheese had decreased calcium and moisture content, no further decrease in cheese hardness was observed after five injections (pH 4.7). It is possible that as the

caseins approached their isoelectric point, increased protein-to-protein interactions compensated for the decreased calcium and moisture content of cheese that would make the cheese less hard and more crumbly. Applying the same principles from materials science, the acid precipitation of proteins could lead to increased interaction between neighboring protein aggregates, which now locate closer to one another (Figure 4.13C). This would then facilitate the transfer of stress between these polymeric units, thus promoting increased hardness to an extent that possibly compensated for the opposing effect of decreased calcium and moisture content of cheese.

Cohesiveness. In accordance with previous work (Chapters 2 and 3), altered protein interactions affected cheese cohesiveness. However, cheese cohesiveness significantly decreased only after five injections, when the cheese had decreased moisture and calcium content. Both, decreased moisture and increased calcium content have been associated with decreased cohesiveness of cheese (Chapter 3), and moisture content may per se affect cheese cohesiveness (Tunick et al., 1991). Thus, the effect of pH on cohesiveness was confounded with decreased calcium and moisture content of cheese. As previously proposed (Chapter 3), it is possible that decreased long-range protein interactions caused the cheese to become less cohesive and elastic, and more crumbly. Even though interaction between proteins was favored at pH 4.7, they probably involved neighboring aggregates and did not extend considerably throughout the matrix (Figure 4.13C). This would be in agreement with the observed decreased hardness of cheese, as less extended, short-range protein interactions would result in decreased transfer of stress between polymeric units.

Flow. The effect of pH on cheese flow was also related to altered protein interactions. When pH decreased from 5.3 to 5.0, calcium was solubilized from caseins and the amount of protein-bound calcium decreased. This resulted in cheese with increased initial rate of flow. Calcium is a strong promoter of protein-to-protein interactions, and its solubilization would decrease interactions between proteins, thus facilitating the initial flow of cheese. However, after three injections, and even though calcium had been solubilized, the total calcium content remained the same, and decreased pH had no effect on the final extent of cheese flow. Therefore, the change in calcium distribution between its insoluble and soluble forms, and the decreased amount of protein-bound calcium observed after three injections did not alter protein interactions to such an extent that would affect the final extent of cheese flow. Similarly, Paulson et al. (1998) observed no effect of pH, in the range of 5.8 to 5.3, on the melting of nonfat Mozzarella cheese whose calcium content remained unchanged.

In contrast, lowering pH from 5.0 to 4.7 decreased both the initial rate and the final extent of cheese flow. At pH 4.7, the cheese had increased content of soluble calcium, and decreased amount of protein-bound and total calcium content, which would decrease protein-to-protein interactions. However, after five injections, the decrease in cheese flow and the contraction of the protein matrix were indications of increased interaction between proteins. As the pH of cheese decreases from 5.0 to 4.7, caseins approach their isoelectric point and electrostatic interactions decrease, which would favor protein-to-protein interactions. Thus, below pH 5.0, the acid precipitation of caseins overcame the opposing effect of calcium solubilization and decreased calcium content,

resulting in a net increase in protein-to-protein interactions that significantly impaired the flow of cheese.

CONCLUSIONS

We concluded that lowering the pH of cheese by injecting an acid solution alters protein interactions, which then affects cheese functionality. Decreased pH not only promotes calcium solubilization and decreased calcium content of cheese, which impair interactions between proteins, but it also leads to isoelectric precipitation of caseins, which favors interactions between proteins. At low levels of acid injection, calcium solubilization is the predominant factor, and interactions between proteins decrease. Thus, the content of protein-bound calcium would direct cheese functionality when the pH of cheese is above 5.0. In contrast, at high levels, acid injection promotes protein-to-protein interactions as the caseins approach their isoelectric point. Thus, at pH values below 5.0, the acid precipitation of caseins overcomes the opposing effect caused by increased calcium solubilization and decreased calcium content of cheese, and there is a net increase of protein-to-protein interactions.

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CHAPTER 5

GENERAL SUMMARY AND CONCLUSIONS

A series of experiments were designed and conducted to determine the effect of calcium, moisture, salt content, and pH on cheese functionality. The experiments were successful in allowing us to achieve our objectives, and increased information and improved understanding of cheese functionality and how to control it is now available. The following conclusions summarize the major findings of this research:

1. Increasing the calcium content of cheese from 0.3 to 1.8% promoted protein-to-protein interactions, probably because of charge neutralization and calcium bridging, which resulted in contraction and decreased water-holding capacity of the protein matrix. Thus, syneresis was observed after each injection, and it continued during cheese storage leading to moisture losses and decreased weight of cheese blocks. In addition, as a result of increased protein-to-protein interactions, cheese hardness increased and cheese melting decreased.
2. Increasing the moisture (from 50 to 54%) or salt (from 0.1 to 2.7%) content of cheese resulted in a more hydrated and expanded protein matrix with impaired protein-to-protein interactions. Thus, the water-holding capacity of the matrix increased. However, if the matrix is unable to accommodate all the injected fluid, syneresis will occur during cheese storage. In addition, when salt is added, the solute fraction of the injected fluid was preferentially retained within the cheese, which resulted in syneresis during storage and decreased moisture content of cheese. Increased salt content (0.5-2.7% compared to 0.1%) increased cheese

hardness and the initial rate of cheese flow. The most significant effect of salt on cheese texture and melting was observed when its content increased from 0 to 0.5%.

3. Decreasing the pH of cheese altered protein interactions. However, the effect of decreased cheese pH differed depending on whether pH was above or below 5.0. Above pH 5.0 (5.0 to 5.3), lower cheese pH impaired protein-to-protein interactions via increased calcium solubilization and decreased content of protein-bound calcium. This resulted in cheese with decreased hardness and increased initial rate of flow. In contrast, below pH 5.0 (5.0 to 4.7), as the caseins approached their isoelectric point, and despite the increased solubilization of calcium, decreased cheese pH promoted protein-to-protein interactions. Thus, below pH 5.0, the protein matrix contracted and the water-holding capacity of the matrix decreased. As a result, syneresis and moisture losses occurred, and the initial rate and final extent of cheese flow decreased.

In summary, when the results of this research are considered together, the content of protein-bound calcium seems to be the major factor controlling the functionality of most cheeses. In addition, the use of a high-pressure injection system allows for the effective modification of the chemical composition of cheese. Thus, solutions of different ionic species, at different concentrations, and with different pH can be injected into cheese for research or product development studies.

As previously indicated by some researchers, the independent modification of chemical parameters of cheese is difficult. However, it is in fact necessary to understand

the interaction between these chemical parameters, such as pH and calcium content, because they determine cheese functionality. Also, the influence of chemical composition on cheese functionality can be better understood when considering the effect of a modified ionic environment on protein interactions. Accordingly, changes in chemical composition can be basically classified into two groups: those that favor and those that impair protein-to-protein interactions.

Future research on cheese functionality could greatly benefit by having input from other areas of science such as polymer and materials science. This would certainly improve the ability of researchers to better understand the effect of chemical parameters on cheese functionality. In addition, detailed characterization of cheese ultrastructure by means of electron microscopy and digital image analysis may allow for modeling structure-function relationships of cheese.

APPENDICES

APPENDIX A

SUMMARY OF DATA FROM STATISTICAL ANALYSIS

TABLE A.1. Summary of statistical data (GLM and contrasts analysis) for the effect of calcium and water injection on structure-function relationships of cheese

Variable	df	MS	F value	Pr > F
Source				
Weight				
Model	12	157.10	13.93	< 0.001
Error	19	11.27		
Contrast				
Control v Water	1	26.49	2.35	> 0.05
Control v Calcium	1	276.75	24.54	< 0.001
Water v Calcium	1	1355.08	120.15	< 0.001
Calcium				
Model	43	0.51	48.96	< 0.01
Error	22	0.01		
Contrast				
Control v Water	1	0.00	0.10	> 0.05
Control v Calcium	1	4.86	469.09	< 0.001
Water v Calcium	1	15.02	1449.45	< 0.001
Moisture				
Model	43	74.30	4.95	< 0.01
Error	22	15.01		
Contrast				
Control v Water	1	33.69	2.24	> 0.05
Control v Calcium	1	351.40	23.40	< 0.001
Water v Calcium	1	1808.08	120.42	< 0.001
pH				
Model	43	0.20	5.84	< 0.01
Error	22	0.03		
Contrast				
Control v Water	1	0.10	2.99	> 0.05
Control v Calcium	1	0.91	27.34	< 0.001
Water v Calcium	1	4.86	145.19	< 0.001
Hardness				
Model	43	107.91	2.08	< 0.05
Error	22	51.81		
Contrast				
Control v Water	1	116.07	2.24	> 0.05
Control v Calcium	1	418.77	8.08	< 0.01
Water v Calcium	1	2924.69	56.45	< 0.001
Cohesiveness				
Model	43	0.01	3.28	< 0.01
Error	22	0.00		
Contrast				
Control v Water	1	0.00	0.05	> 0.05
Control v Calcium	1	0.14	37.68	< 0.001
Water v Calcium	1	0.44	121.46	< 0.001
Melting				
Model	43	45.78	5.69	< 0.01
Error	22	8.05		
Contrast				
Control v Water	1	0.02	0.00	> 0.05
Control v Calcium	1	499.67	62.05	< 0.001
Water v Calcium	1	1482.05	184.05	< 0.001

TABLE A.2. Summary of statistical data (GLM and LSD analysis) for the effect of salt on structure-function relationships of cheese

Variable Source	**df**	**MS**	**F value**	**Pr > F**
Salt				
Model	3	4.0031	166.56	0.0001
Error	8	0.0240		
Moisture				
Model	3	5.1779	4.24	0.0454
Error	8	1.2205		
Weight				
Model	3	2.2710	25.07	0.0002
Error	8	0.0906		
Hardness				
Model	3	213.6656	16.96	0.0008
Error	8	12.5964		
Adhesiveness				
Model	3	0.5897	4.66	0.0364
Error	8	0.1267		
Cohesiveness				
Model	3	0.0117	21.56	0.0003
Error	8	0.0005		
Flow Rate				
Model	3	3.8885	33.81	0.0001
Error	8	0.1150		

TABLE A.3. Summary of statistical data (GLM and LSD analysis) for the effect of pH on structure-function relationships of cheese

Variable Source	df	MS	F value	Pr > F
pH				
Model	3	0.2324	65.76	0.0001
Error	8	0.0035		
Moisture				
Model	3	4.4189	8.72	0.0067
Error	8	0.5067		
Weight				
Model	3	4.5004	21.99	0.0003
Error	8	0.2046		
Soluble Calcium				
Model	3	0.7719	40.28	0.0001
Error	8	0.0192		
Total Calcium				
Model	3	1.5431	3.41	0.0734
Error	8	0.4525		
Soluble Nitrogen				
Model	3	0.0047	4.49	0.0074
Error	8	0.0010		
Hardness				
Model	3	1273.1031	14.94	0.0012
Error	8	85.2353		
Cohesiveness				
Model	3	0.0020	4.24	0.0455
Error	8	0.0005		
Flow Rate				
Model	3	0.0328	7.69	0.0096
Error	8	0.0043		
Extent of Flow				
Model	3	0.5939	8.67	0.0068
Error	8	0.0685		

APPENDIX B

PRELIMINARY WORK ON CALCIUM AND SALT DISTRIBUTION

CALCIUM AND SALT DISTRIBUTION IN INJECTED CHEESE

ABSTRACT

Our objective was to determine whether calcium and salt in injected cheese would be evenly distributed over 1-cm distance from the injection site in 30 d. Salted and unsalted cheese was made according to direct-acid, stirred-pressed curd procedures. After manufacture, cheeses were cut into blocks that were vacuum-packaged, and stored for 3 wk at 4°C. After storage, salted cheese was high-pressure injected with a 40% (wt/wt) calcium chloride solution, and unsalted cheese with a 23% (wt/wt) sodium chloride solution. Injection was performed once, in a single and centered row in the top of the cheese block, with injection sites 1 cm apart. After injection, cheese blocks were blotted with paper towels to remove extraneous fluid, and then vacuum-packaged and stored at 4°C. After storage, cheese blocks were sectioned in half, perpendicular to the injection path, and the bottom portion discarded. Three 0.5-cm thick slices, to both sides of the injection line were then cut and analyzed for calcium or salt content. Calcium-injected cheese, analyzed 4 wk after injection, had increased calcium content compared to uninjected cheese, and had even distribution of calcium over 1 cm from the injection line. Salt-injected cheese was analyzed 6 wk after injection and had increased salt content compared to uninjected cheese. Also, injected cheese had even distribution of salt over 1.5 cm from the injection line. We concluded that injecting a concentrated solution of calcium or salt allows for increasing their content in the cheese, and that injecting these solutions using a 1 x 1-cm injection pattern allows for even distribution of ions in 30 d.

INTRODUCTION

Calcium is normally added to milk in the early stages of cheese making to improve coagulation properties. Adding calcium increases the affinity of rennet for casein, which increases the rate of enzymatic reaction (Green and Marshall, 1977), and then facilitates the aggregation of renneted casein micelles (Green and Marshall, 1977; Dalgleish, 1983). As a result, adding calcium to milk reduces the rennet coagulation time (Green and Marshall, 1977), and may increase gel strength if added in low concentration (Jen and Ashworth, 1970). During cheese making, the pH at draining determines the retention of calcium in cheese curd, which then affects the extent of protein aggregation, thus determining the basic structure and texture of cheese (Lawrence et al., 1983). In addition, calcium content affects cheese functionality. Increased calcium content increases cheese hardness or firmness (Lawrence et al., 1993), and decreases the melting of cheese (Paulson et al., 1998). Calcium content may also affect cheese structure by promoting protein-to-protein interactions.

To study the effect of calcium on chemical and functional properties of cheese, a calcium source, such as calcium chloride, has been normally added to milk during the early stages of cheese making (Jen and Ashworth, 1970; Dalgleish, 1983). However, changes in the calcium content of cheese during cheese making are interdependent with changes in curd pH, which makes it difficult to segregate the effect of pH per se from the effect of pH-induced changes (Lucey and Fox, 1993). Therefore, a method to independently modify the pH or calcium content of cheese would be useful to better understand their effect on cheese structure and functionality.

High-pressure injection has been successfully used to inject fluids into cheese (Lee et al., 1978; Olson, 1979) and meat (Hendricks and Hansen, 1991). By injecting ionic solutions after manufacture, the cheese-making procedure and initial chemical composition are kept the same, which may then allow for modifying the chemical composition of cheese by altering one variable at a time. However, injection of ionic solutions to alter chemical, structural, and functional attributes of cheese requires knowledge of the time needed to obtain uniform distribution of ions in the cheese before analyses are performed. Therefore, it was our objective to determine whether even distribution of calcium in cheese, over 1-cm distance from injection site, could be obtained in 30 d.

During manufacture, salt is normally added to cheese either in dry form, onto cheese curd or on the surface of cheese blocks, or by immersion of cheese blocks into brine. In either case, salt affects chemical and functional properties of cheese, and is thought to affect cheese structure. Salt affects cheese composition by promoting syneresis, which decreases the moisture content of cheese (Sutherland, 1974; Schroeder et al., 1998), and by influencing microbial activity (Olson, 1982; Cervantes et al., 1983; Schroeder et al., 1988; Guinee and Fox, 1993). High salt levels decrease starter activity (Irvine and Price, 1961), thus increasing residual lactose and cheese pH. In addition, salt content may also affect cheese proteolysis by affecting microbial and enzyme activity, with high salt levels decreasing the rate and/or extent of proteolysis (Olson, 1982; Cervantes et al., 1983; Schroeder et al., 1988). As a result, according to Sutherland (1974), the salt-in-moisture content would be the most important factor affecting the

ripening of Cheddar cheese, determining the degree of maturity and flavor quality of cheese (Sutherland, 1977).

Salt content also affects cheese functionality. Increased salt content increases the hardness and decreases the cohesiveness of cheese (Cervantes et al., 1983; Schroeder et al., 1988), and may influence cheese melting (Olson, 1982; Paulson et al., 1998). In addition, salt directly contributes to cheese flavor (Guinee and Fox, 1993). However, significant variation in salt content within and between cheese blocks has been reported (Sutherland, 1977; Morris et al., 1985), and uniform distribution of salt throughout the cheese is rarely achieved. Thus, variation in chemical composition within the same block of cheese causes portions of the cheese block to have different functional attributes, which decreases the overall quality of cheese, and in particular if intended for direct consumption. High-pressure injection of a concentrated salt solution into cheese or cheese curd may allow for attaining uniform distribution of salt in cheese in a relative short period of time. Therefore, our objective was to determine whether even distribution of salt in cheese, over 1-cm distance from injection site, could be obtained in 30 d.

MATERIALS AND METHODS

Cheese Making

Calcium distribution. Cheese, 9-kg block, was made according to a direct-acid, stirred/pressed curd procedure. Pasteurized, full fat, non-homogenized milk was acidified at 5 °C by adding citric acid (1 g per kilogram of milk), and acetic acid solution (5% wt/wt) to lower the pH of milk to 5.8. The milk was then warmed to 33 °C, and rennet

(Chymax, Rhodia, Madison, WI) added. After 15 min, the curd was cut and then allowed to heal for 5 min. Following 5 min of stirring, half the whey was drained, and glucono-δ-lactone added to further acidify the cheese curd to pH 5.2. The temperature was then raised to 42 °C and cheese curd cooked with stirring for 45 min. The remaining whey was then drained, and the cheese curd dry-salted (2% wt/wt). After salting, the curd was placed into a 9-kg mold and pressed overnight. The cheese was then trimmed and cut into 0.4- to 0.6-kg blocks (5 x 6 x 10 cm) that were vacuum-packaged and stored for 3 wk at 4 °C prior to injection.

Salt distribution. Cheese, 9-kg block, was made according to a direct-acid, stirred/pressed curd procedure. Pasteurized, skim, non-homogenized milk was preacidified at 5 °C by adding citric acid (1 g per kilogram of milk), and acetic acid solution (5% wt/wt) to lower the pH of milk to 5.8. The milk was then warmed to 27 °C, and rennet (Chymax, Rhodia, Madison, WI) added. After 15 min, the curd was cut and then allowed to heal for 5 min. Following 5 min of stirring, half the whey was drained, and glucono-δ-lactone added to further acidify the cheese curd to pH 5.0. Temperature was then raised to 42 °C and cheese curd cooked with stirring for 90 min. The remaining whey was then drained, and cheese curd left to drain for 90 min. After draining, cheese curd was placed into a 9-kg mold and pressed overnight. The cheese was then trimmed and cut into 0.3- to 0.4-kg blocks (5 x 6 x 8 cm) that were vacuum-packaged and stored for 3 wk at 4 °C prior to injection.

Cheese Injection

A 2-stage homogenizer (Crepaco, Model 3DDL-3535, Chicago, IL) served as the pump for injection and pressure on the homogenizer valve was set at 10 MPa. The burst duration was controlled via a solenoid-operated valve on the outlet line and set to 1.5 s. Solutions flowed through a sapphire nozzle (0.02-cm internal diameter) and into the cheese. The cheese was high-pressure injected with either a 40% (wt/wt) calcium chloride ($CaCl_2.2H_2O$) solution (4.2 M), or a 23% (wt/wt) sodium chloride solution (4.4 M). Injection was performed once, in a single and centered row in the top of the cheese block, with injection sites 1 cm apart (Figure B.1). After injection, the cheese block was blotted with paper towels to remove extraneous fluid and vacuum-packaged. Cheese blocks injected with calcium were then stored at 4°C for 4 wk, and those injected with salt were stored at 4°C for 6 wk to allow time for distribution of ions and moisture in the cheese prior to analysis.

Chemical Composition

Fat content was determined using a modified Babcock method (Richardson, 1985), moisture content by using the vacuum oven AOAC method 926.08 (1990), calcium by inductively coupled plasma-atomic emission spectroscopy (US Environmental Protection Agency, 1992), and sodium chloride according to AOAC method 971.19 (model 926 salt analyzer; Corning, Medfield, MA) (1990). A pH meter (model IQ240, IQ Scientific Instruments, Inc., San Diego, CA), with a stainless steel probe (model PH06-SS, IQ Scientific Instruments, Inc., San Diego, CA), was used for determining cheese pH, which was measured by taking a cheese sample from the cheese

block and inserting the pH probe. For calcium and salt content determination, injected cheese blocks were first sectioned in half, perpendicular to the injection path, and the bottom portion discarded. During injection, injectant solution entered the cheese only 3 to 4 cm deep. As a result, only the top portion of the cheese was effectively injected and therefore retained for analysis. Three 0.5-cm thick slices, to both sides of the injection line were then cut and analyzed for calcium or salt content (Figure B.1).

Experimental Design and Statistical Analysis

The experiment was conducted in triplicate and the data analyzed according to a repeated measures designs with slice as a pseudo-main factor. Statistical analysis (contrasts) was performed using SAS® (1999).

RESULTS

Cheese Composition

The results for the chemical composition of the control, uninjected cheeses are presented in Table B.1. The calcium content of salted cheese was as expected and lower than what is normal for a cheese such as Mozzarella, 0.3% compared to 0.7% (Kosikowski and Mistry, 1997). Even though cheese curd was acidified during cheese making to pH 5.2 for salted cheese, and to pH 5.0 for unsalted cheese, pH increased during cheese storage to 5.7 and 5.5, respectively.

Calcium Distribution

Injecting calcium significantly increased the calcium content of cheese ($P < 0.01$),

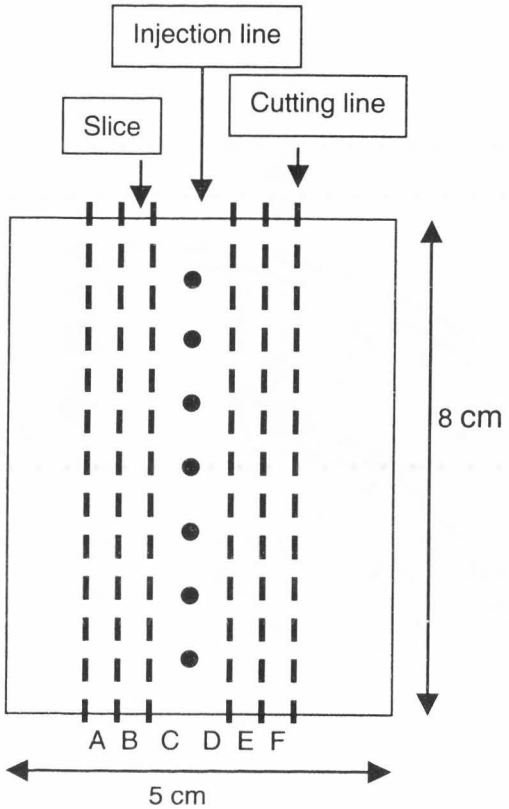

Figure B.1. Diagram of cheese block viewed from the top showing injection sites (●) and slices (A to F) cut for analysis of calcium or salt content

TABLE B.1. Chemical composition of uninjected cheese

	Calcium distribution		Salt distribution	
Variable	Mean	CV	Mean	CV
Fat (%)	25	0.0	22	1.6
Moisture (%)	45	1.4	42	1.0
pH	5.7	0.5	5.5	0.5
Calcium (%)	0.33	0.0	*	
Salt (%)	*		0.16	0.0

* Content not determined

from 0.33% in the control, uninjected cheese to 0.43%, in average for the three slices analyzed (Table B.2). In addition, after 4 wk of storage the cheese had even distribution of calcium over 1-cm distance from the injection line.

Salt Distribution

Salt injection significantly increased the salt content of cheese ($P < 0.01$), from 0.16% in the control, uninjected cheese to 0.25%, in average for the three slices analyzed (Table B.2). Also, after 6 wk of storage, injected cheese had even distribution of salt over 1.5-cm distance from the injection line.

DISCUSSION

Chemical Composition

Cheese made by direct acidification of milk normally has lower calcium content than cheese made by traditional procedures in which starter cultures are used to lower the

TABLE B.2. Calcium and salt content of injected cheese

Variable	Distance from injection line (cm)		
	0-0.5	0.5-1	1-1.5
Calcium content (%)[1]	0.45[a]	0.44[a]	0.41[b]
Salt content (%)[2]	0.25[a]	0.25[a]	0.24[a]

[1] Calcium-injected cheese; [2] Salt-injected cheese
[a,b] Means with different superscripts differ at $P < 0.05$

pH of milk during cheese making (Keller et al., 1974; Paulson et al., 1998). Acidification of milk or casein micelle suspensions causes dissociation of calcium from the micelle and into the serum (Keller et al., 1974; Dalgleish and Law, 1989). Thus, the lower calcium content of the uninjected salted cheese resulted from calcium solubilization brought about initially by the acidification of milk to pH 5.8 prior to renneting, and then by the acidification of cheese curd to pH 5.2. Similarly, and even though it was not determined, the unsalted cheese most certainly had low calcium content as well. However, even though acidification of milk and cheese curd during cheese making caused extensive mineral solubilization, cheese pH increased after manufacture.

Calcium Distribution

Distribution of ions in injected cheese would result from deflection and diffusion processes. First, upon injection, deflection of the injectant solution as it enters the cheese would provide initial dispersion of ions throughout the cheese matrix. To enter the cheese, the injectant solution has to overcome opposing forces, or resistance from cheese components, especially protein. However, due to variation in chemical composition and

curd kneading the level of resistance to the incoming injectant varies throughout the cheese. Thus, as the injectant enters the cheese it follows a path through areas of lower resistance. Then, increased localized ion concentrations generate gradients in the cheese that operate as a driving force for ions to diffuse in relative short distances.

Diffusion of ions in cheese is impaired by tortuosity and friction due to the presence of fat and protein, and by the viscosity of the aqueous phase of cheese (Geurts et al., 1974). Thus, the process is normally recognized as pseudo diffusion, and diffusion coefficients determined in cheese would be lower that those in pure water. Also, calcium ions interact with the protein fraction of cheese, possibly via electrostatic interactions and/or calcium bridging (Baumy et al., 1989; Gaucheron et al., 1997), thus further restricting the ability of ions to diffuse. In spite of these limitations, calcium ions distributed through 1.5-cm distance from the injection line, and the cheese had even distribution of calcium over 1-cm distance after 4 wk of storage. Therefore, high-pressure injection of a concentrated calcium solution according to 1 x 1-cm pattern would allow a uniform increase in the calcium content of cheese in 30 d.

Salt Distribution

Similar considerations to those mentioned for injection and distribution of calcium apply to injection and distribution of salt. Thus, salt distribution results from both deflection of injectant solution and diffusion of ions throughout the cheese matrix. Also, the diffusion of sodium chloride in cheese can be described as an impeded process, and is referred to as pseudo diffusion (Geurts et al., 1974; Sutherland, 1977). In brine-salted (Geurts et al., 1974; Guinee and Fox, 1983) and dry-salted cheese (Guinee and

Fox, 1983) the pseudo diffusion coefficient of salt was 0.2 cm^2/d, which is lower than that of salt in pure water, 1.0 cm^2/d at 12.5°C. According to the authors, this is explained by the presence of fat and protein, and the viscosity of the aqueous phase that significantly impair the movement of sodium and chloride ions throughout the cheese matrix. In a model system, Wiles and Baldwin (1996) reported a diffusion coefficient for salt in water of 0.1 cm^2/d, which properly fit experimental data. To the list of factors explaining the lower diffusion coefficient of salt in cheese compared to salt in pure water, the authors added the low moisture content of cheese, 35%, and the occurrence of differences in salt content over long distances, 5 to 10 cm, throughout the cheese block. Also, interaction of salt with the protein fraction of cheese further decreases the diffusion of ions (Payne and Morison, 1999). In spite of these limitations, the cheese had even distribution of salt through 1.5-cm distance from the injection line after 6 wk of storage. Assuming that diffusion proceeded at a constant rate, high-pressure injection of a concentrated salt solution according to 1 x 1-cm pattern would allow uniform increase in the salt content of cheese in 30 d.

It seems however, that uniform salt distribution could be obtained in a few days after high-pressure injection of cheese. Lee et al. (1980) reported Mozzarella cheese to reach equilibrium in salt content over a 1.5-cm thick section in 7 d, and Morris et al. (1985) found that small individual pieces of dry-salted Cheddar cheese (2 x 2 x 6 cm) attained salt equilibrium in 48 h. However, in 20-kg cheese blocks, uniform distribution of salt was not achieved after 24 wk. According to Morris et al. (1985), the presence of many boundaries with no consistent direction between curd particles may reduce the rate

of diffusion, and there is no consistent gradient of salt throughout the cheese, as in brine salting, that could promote salt diffusion. During injection, small pools with high salt concentration would be created throughout the cheese matrix. Short-range gradients in salt content of cheese would then promote diffusion of salt in short distances that could overcome the presence of curd boundaries with random direction. As a result, the cheese may reach uniform distribution of salt in a relative short period of time. Therefore, further research to determine whether high-pressure injected cheese can reach uniform salt distribution in a few days would be useful.

CONCLUSIONS

Injecting a concentrated solution of calcium or sodium chloride allows for increasing the content of calcium or salt in the cheese. Injecting these solutions according to a 1 x 1-cm injection pattern allows for even distribution of ions in the cheese to occur in 30 d. Therefore, calcium- or salt-injected cheese can be analyzed for chemical and functional attributes 30 d after injection. However, even distribution of calcium and salt in injected cheese may be achieved in a shorter period of time. Further studies for determining calcium and salt distribution during the first 30 d of injection would be useful.

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APPENDIX C

PERMISSION LETTER

January 17, 2003

Andres J. Pastorino
Research Assistant, PhD Program
Utah State University
Nutrition and Food Sciences Department
750N 1200E
Logan, UT 84322-8700

Dear Andres J. Pastorino:

I give you permission to reprint and include the following material in your dissertation for completion of your PhD in the Nutrition and Food Sciences Department at Utah State University, Spring 2003:

Article title: Effect of calcium and water injection on structure-function relationships of cheese (Journal of Dairy Science 2003 -In press-); co-authored with A. Pastorino, C. Hansen, and D. McMahon.

I enjoyed working with you on this project and wish you and your family happiness and success.

Sincerely,

Neal Ricks
Scientist
General Mills
9000 Plymouth Ave N
Minneapolis, MN 55427

CURRICULUM VITAE

Andres J. Pastorino

(December 2002)

Candidate for the Degree of Doctor of Philosophy

Dissertation: Effect of Chemical Parameters on Structure-Function Relationships of Cheese

Major Field: Nutrition and Food Sciences

EDUCATION

PhD. Nutrition and Food Sciences. 2002. Utah State University

Postgraduate. Business Administration. 1996. Catholic University of Uruguay

BS. Agricultural Engineering. 1995. University of Uruguay

AWARDS

Research Assistant of the year. 2002-2003. Nutrition and Food Sciences Dept.

Dissertation Fellowship. 2002. Utah State University

Phi Kappa Phi. 2001. Utah State University Chapter

National Dean's List. 2001.

Research Vice President's Fellowship. 1998-1999. Utah State University

Scholarship. 1996. Catholic University of Uruguay

EXPERIENCE

Research Assistant. 1998-2002
Western Dairy Center, Nutrition and Food Sciences Dept. Utah State University. Logan, Utah.

Designed and conducted experiments on the effect of chemical parameters on cheese structure and functionality. The project involved cheese making, the injection of solutions into cheese by means of using a high-pressure injection system, the characterization of cheese structure by using electron microscopy and image analysis, and the determination of textural and melting properties of cheese. In addition, methodologies were developed for digital analysis of images. Findings were presented at several state and national meetings, and four scientific papers were written (either published or to be published in peer-reviewed journal).

In addition to research work, assisted in teaching the course Food Technology and Health. This involved preparing material for lectures, giving lectures, grading written quizzes and book reports, and having office hours for assisting students.

Plant Manager. 1997
Florentino Sande S.A. Montevideo, Uruguay. Manufacturer of cookies and crackers.

Managed personnel in meeting production goals according to established plans. During that year, the company achieved an all-time production record. Evaluated personnel and recommended promotions, which were implemented.

PUBLICATIONS

Referred Articles

Pastorino, A. J., C. Hansen, and D. J. McMahon. 2003. Effect of salt on structure-function relationships of cheese. J. Dairy Sci. 86:60-69.

Pastorino, A. J., N. P. Ricks, C. Hansen, and D. J. McMahon. 2003. Effect of calcium and water injection on structure-function relationships of cheese. J. Dairy Sci. 86:105-113.

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Non Referred Articles

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Pastorino, A. J., N. P. Ricks, C. Hansen, and D. J. McMahon. 2000. Effect of water and calcium injection on structure-function attributes of Mozzarella cheese. 2000 IFT Annual Meeting. Book of Abstracts:86.

PRESENTATIONS

American Dairy Science Association. 2001.

Institute of Food Technologists. 2000 and 2002.

Utah Academy of Sciences Arts & Letters. 2000-2002.

Western Dairy Center. 2001 and 2002.

ACTIVITIES

Food Sciences Club. Utah State University

Student Health Advisory Committee. Utah State University

PROFESSIONAL AND HONOR MEMBERSHIPS

American Dairy Science Association

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