

Utah State University

DigitalCommons@USU

---

Annual Reports

Western Dairy Center

---

2009

## 2008 Annual Report

Various Authors

Follow this and additional works at: [https://digitalcommons.usu.edu/wdc\\_reports](https://digitalcommons.usu.edu/wdc_reports)



Part of the [Dairy Science Commons](#)

---

### Recommended Citation

Authors, Various, "2008 Annual Report" (2009). *Annual Reports*. Paper 12.

[https://digitalcommons.usu.edu/wdc\\_reports/12](https://digitalcommons.usu.edu/wdc_reports/12)

This Report is brought to you for free and open access by the Western Dairy Center at DigitalCommons@USU. It has been accepted for inclusion in Annual Reports by an authorized administrator of DigitalCommons@USU. For more information, please contact [digitalcommons@usu.edu](mailto:digitalcommons@usu.edu).



## **INTRODUCTION**

The Western Dairy Center is now in its 22<sup>nd</sup> year as part of the National Dairy Foods Research Center program supported by the US dairy industry. There have been many changes through that time since the driving forces in the Center's dairy research program included Gary Richardson, William Sandine, Floyd Bolyfelt and Rodney Brown with a number of younger researchers filling out the ranks. Along with C. A. Ironstrom who had even earlier organized the Dairy Research Advisory Board here at Utah State University, all these professors have retired or moved on to other endeavors. However, the one thing constant through the years has been the desire for performing innovative research that is relevant and useful beyond the borders of the universities.

Over the same time period, there have been changes in the industry we serve, especially here in the western states where milk production has increased so dramatically and set the standards for large-scale dairy food manufacturing. With critical support from Dairy Management Inc., the Western Dairy Center is being re-tooled to be the research and technology resource needed for today's dairy industry. As well as our focus on conducting dairy foods research that makes a difference, we recognize the diversity of needs within the region that includes the growing interest in artisan cheesemaking, a need for ongoing technical training in the dairy industry, the building up of dairy food expertise in the western region, and education of students who will be our future innovators.

Pictures

Don from last year

Carl from last year



## CONTENTS

Introduction .....	1
--------------------	---

### RESEARCH PROJECTS AND PAPERS

#### *Low-Fat Cheese Research Projects*

Low Fat Cheese Platform Study - Objective 3 .....	7
Effect of Fat Removal on Cheese Microenvironment and Starter Culture Metabolism in Cheddar Cheese.....	11
Innovative Approaches for Improving Low Fat Mozzarella Cheese .....	17
Low Fat Cheese Texture Enhancement Portfolio .....	25
Low Fat Natural Cheese Strategic Platform Study - Objective 1 .....	29
Improve the Flavor of Low Fat Cheese by Adding Innovative Cultures and/or Flavoring Systems.....	39
Investigating the Filled Gel Model for the Role of Fat in Cheese .....	43

#### *Value-Added Research Projects*

Understanding How Salt Level Influences Perception of Cheese Flavor .....	57
Partitioning of Omega-3 Fatty Acids Between Cheddar Cheese Curd and Whey .....	61
Vitamin D Fortification of Cheddar Cheese.....	65
Development Cheddar Cheese Containing Probiotics.....	73

#### *Milk and Whey Research Projects*

Synthesis, Characterization, and Bioactivity of Lactose Lauryl Esters .....	83
Development of Descriptive Taste Panel for Dairy Application .....	99
Iron-Binding by Milk Mineral .....	109
Effect of Milk Fat Globular Membrane (MFGM) in Providing Protection Against Gastrointestinal Stress.....	119

#### *Papers*

Acid Coagulation of Milk .....	123
Influence of Brine Concentration and Temperature on Composition, Microstructure, and Yield of Feta Cheese .....	137
Fortification of Reduced-Fat Cheddar Cheese with $\omega$ -3 Fatty Acids .....	151
Harddening of High Protein Nutrition Bars and Sugar/Polyol-Protein Phase Separation .....	163
Production of High Protein Cheddar Cheese with an Improved Extrusion-Modified Texture .....	179
Effect of High Intensity Ultrasound (HIU) of Functional Properties of Whey Proteins .....	187
Antioxidant Mechanism of Milk Mineral .....	201
Myoglobin Oxidation in a Model System as Affected by Nonheme Iron and Iron Chelating Agents .....	207

***LOW-FAT CHEESE RESEARCH  
PROJECTS***

# Low-Fat Cheese Platform Study - Objective 3

**JEFF BROADBENT:** Utah State University

Objective 3: How do the changes in cheese composition that occur when making cheese with lower fat levels influence what happens to the bacteria in cheese.

There were two components to this objective:

- 3.1: Measure the starter and nonstarter lactic acid bacterial populations in the cheeses and how they change during 9-mon storage of the cheese
- 3.2: Quantification of organic acids, sugars, salt by chloride content, and moisture in all cheeses at each time point

## BACKGROUND

There is a lack of information and scientific understanding related to the flavor defects that are prominent in lowfat cheese. A key to understanding why flavor development in low fat cheese is so different to that which occurs in full fat cheese (for which there is a large knowledge base accessible by scientists and the cheese industry) is to determine the differences in microbial populations and microbial activities between cheeses with different fat levels. This project addresses the research target of better understanding the effects of micro-environment and make-procedures of lowfat cheese on the microbiology and consequent flavor chemistry and flavor sensory properties of such cheese (Scientific Recommendations for Product Innovation Research of the Low Fat Cheese Expert Panel Meeting held on March 30<sup>th</sup> and 31<sup>st</sup>, 2006).

Experimental Approach:

Under the strategic platform project, the following cheeses were made with the same culture, standardized make procedures, and standardized compositions, to investigate the effect of fat content on flavor, texture, microbiology:

- Cheese A. Full fat cheddar made by a stirred curd method
- Cheese B. Full fat cheddar with the same proximate composition as Cheese A but made using stirred curd method with a water curd washing step.
- Cheese C. 50% reduced fat cheddar made using stirred curd method with washing step
- Cheese D. Low fat cheddar made using stirred curd method with washing step plus use of preacidification of milk prior to renneting.

The USU component of Objective 3 was directed exclusively at Subobjective 3.1: Measure the starter and nonstarter lactic acid bacterial populations in the cheeses and how they change during 9-mon storage of the cheese.

Cheeses made at USU include duplicate vats of cheese A (made Oct. 3, Nov. 15), B (Oct. 4 and Nov. 14), C (Oct. 12 and Nov. 21) and cheese D (Oct. 10 and Nov. 1); UW cheese include duplicate samples of cheese A (made Feb. 2), B (Nov. 15) and cheese D (Oct. 10 and Oct. 31); Cal-Poly products include cheese A (Nov. 21 and Feb. 6), B (Nov. 21 and Feb. 6), C (Oct. 26 and Nov. 15), and D (Oct. 26 and Nov. 14).

All cheeses were analyzed at USU for starter and NSLAB populations on wk 2, wk 6, 3 mo, 6 mo and 9 mo. Lactococcal starter bacteria were enumerated on Elliker's agar incubated at 30°C, and NSLAB populations were enumerated on Rogosa agar incubated at 37°C. In addition, cheese extracts were plated on M17 agar and incubated at <5°C to determine the presence of adventitious streptococci, and on MRS agar incubated at 10, 30 and 45°C to collect DNA from the total microbiological population for denaturing gradient gel electrophoresis (DGGE). As cheeses were sampled for starter and NSLAB, total DNA was also extracted from the cheese. The DNA collected from all samples was frozen at -80°C until DGGE was performed.

For DGGE, the variable V3 region of the 16S rDNA in cell pellets or DNA extracted from cheese was amplified by PCR using primers that are complementary to conserved regions of the eubacterial 16S rRNA gene. The fragments are then separated in a Denaturing Gradient Gel Electrophoresis System (CBS Scientific Company, Solna Beach, CA, USA) using an 8% (wt/vol) acrylamide gel (in 1X TAE: 40mM Tris acetate [pH 7.4], 20 mM

sodium acetate, 1mM Na<sub>2</sub>-EDTA) with a chemical gradient from 25 to 60% denaturant. After electrophoresis, the gels were stained with SYBR Gold solution (Molecular Probes Inc., Carlsbad, CA, USA) then photographed using a UV transilluminator). Bands of interest were excised and placed in separate tubes, and then DNA was eluted from the gel using the procedure outlined by Koo and Jaykus (2000). DNA eluted from the excised DGGE bands was purified, cloned into *E. coli*, and then sequenced. Sequences were compared against the nucleotide database to determine the closest phylogenetic relative (usually a specific species) from which each band was derived.

In addition to microbiological sampling and DGGE, exploratory microarray experiments were also performed to evaluate the utility of a "phylochip" designed at the Lawrence Berkeley National laboratory for rapid identification of bacteria in environmental samples. For this work, the complete 16S rDNA gene was amplified from pooled DNA samples collected from 2 wk- and 6 mo-old type B and D cheeses made at Utah State. The DNA was sent to Dr. Gary Anderson at LBNL, who performed the hybridizations and data normalization.

## PROJECT OUTCOMES

The microbiological counts showed several interesting trends: 1) initial NSLAB levels were generally much lower in A&B cheese versus cheese D in product made at all 3 sites; and 2) starter viability declined at a more rapid rate in A&B cheese versus C&D in product made at all 3 sites; and 3) microbiological profiles of cheese made in UT or CA were more similar, regardless of cheese type, than they were to WI cheese. NSLAB levels in low-fat cheese ("D") exceed 10<sup>6</sup> by 6 wks in all samples, but few type A or B cheeses even reach that level by 6 mo. NSLAB levels in the 50% reduced fat (type "C") cheese were intermediate to those seen in full- versus low-fat cheeses.

Another observation was that populations of starter bacteria remained stable, and sometimes increased, in type C and D cheeses out to 3 mo before showing any decline. In contrast, starter numbers in type A and B cheeses generally declined by at least 2 orders of magnitude by 3 mo. DGGE of all Utah, Wisconsin, and California cheeses showed the NSLAB fraction of all cheeses includes *Lactobacillus curvatus*, but several other species of bacteria, including *Lactobacillus helveticus*, *Lactobacillus casei*, *Lb. coryniformis*, *Lactobacillus plantarum*, *Streptococcus thermophilus*, *Bacillus cereus/thuringiensis* and *Bacillus licheniformis* were also detected in different cheeses. Finally, results from the phylochip experiments indicated this microarray cannot discriminate microbes to the species level as needed by our project.

### Other Analyses Related to This Project

Redox potential (Eh) of USU cheeses was measured over time at USU in Carl Brodersen's laboratory using specially designed Ag/AgCl oxidation-reduction probes that had very small platinum wire tips for easy insertion into the cheese blocks (ORP-146C, Lazar Research Laboratories, Los Angeles, CA). Holes were bored 3 inches into the cheese blocks with a small trier, then the probe is firmly seated into the cheese, and the hole around the probes are sealed with wax. Redox (Eh) values were recorded from a pH/mV meter each week. The Eh is calculated by adding the measured potential, E<sub>Ag</sub>, and the reference potential for a Ag/AgCl probe at 4°C, E<sub>R</sub>. The resulting equation is: Eh= E<sub>Ag</sub> + E<sub>R</sub>, where E<sub>R</sub> = 220 mV at 4°C. The accuracy of the probes was checked at six-week intervals using a freshly calibrated redox probe that is inserted into a separate part of the block and allowed to undergo overnight equilibration before Eh is measured. If results suggested the original probe has lost activity, then that probe was removed and the newly calibrated probe left in the cheese.

**Subobjective 3.2.** Quantification of organic acids, sugars, salt by chloride content, and moisture in all cheeses at each time point, was performed in Dr. James Steele's laboratory at the University of Wisconsin. These are attributes important to microbial physiology and it is critical that these analyses be done in the same way so that the values can be compared across all of the trials.

Blocks of cheese were sampled as received, frozen and stored and processed in batches. Organic acids (such as D/L lactate, acetate, formate, citrate, succinate etc) and carbohydrates (lactose, galactose) present in the cheese aqueous phases were determined by HPLC as described by Zeppa et al. (2001) and modified by Upreti et al. (2006). Detection of organic acids was accomplished with an Hitachi L-4500A diode-array detector connected in series with a pulsed-amphoteric detector (Cai et al. 2005). The isomers of lactic acid were quantified separately using enzymatic detection kits from R-Biopharm, Inc. (Southmarshall, MI).

Measurement of TCA/PTA soluble nitrogens, enzymes, and potential NSLAB substrates was performed only on the UW manufactured cheeses. Nucleic acids, serine-phosphate free and bound, and glycoproteins concentrations will be quantified via methods recently developed by the Steele laboratory. Starter autolysis (general AP activity), other

starter enzymes (X-prolyl dipeptidyl aminopeptidase, post-prolyl endopeptidase activity on N-acetyl beta-CN (f203-209)-pNA) and various other starter metabolites (such as ethanol, acetaldehyde, etc.) were determined. Enzyme activity analyses were performed on fresh cheese sample while samples will be frozen and batched for the other tests.

## References

- Christensen, J.E., M.E. Johnson, and J.L. Steele. 1995. Production of Cheddar cheese using a *Lactococcus lactis* ssp. *cremoris* SK11 derivative with enhanced aminopeptidase activity. *Int. Dairy J.* 5:367-379.
- Koo, K., and L. Jaykus. 2000. Method for avoiding PCR-inhibiting contaminants when eluting DNA from polyacrylamide gels. *BioTechniques* 29:694-696.
- Upuri, P., L.L. McKay and L.E. Metzger. 2006. Influence of calcium and phosphorus, lactose, and salt-to-moisture ratio on cheddar cheese quality: Changes in residual sugars and water-soluble organic acids during ripening. *J. Dairy Sci.* 89:429-443.
- Zepp, G., L. Conterno, and V. Gerbi. 2001. Determination of organic acids, sugars, diacetyl, and acetoin in cheese by high performance liquid chromatography. *J. Agric. Food Chem.* 49:2722-2726.

## RESULTS AND DISCUSSION

Under the strategic platform project, the following cheeses were all made with the same culture, standardized makeprocedures, and standardized compositions, to investigate the effect of fat content on flavor, texture, microbiology:

- Cheese A. Full fat cheddar made by a stirred curd method
- Cheese B. Full fat cheddar with the same proximate composition as Cheese A but made using stirred curd method with a water curd washing step.
- Cheese C. 50% reduced fat cheddar made using stirred curd method with washing step
- Cheese D. Low fat cheddar made using stirred curd method with washing step plus use of preacidification of milk prior to renneting.

Our research is directed exclusively at Objective 3.1: Measure the starter and nonstarter lactic acid bacterial populations in the cheeses and how they change during 9-mon storage of the cheese.

Cheeses made at USU include duplicate vats of cheese A (made Oct. 3, Nov. 15), B (Oct. 4 and Nov. 14), C (Oct. 12 and Nov. 21) and cheese D (Oct. 10 and Nov. 1); UW cheese include duplicate samples of cheese A (made Feb. 2), B (Nov. 15) and cheese D (Oct. 10 and Oct. 31); Cal-Poly products include cheese A (Nov. 21 and Feb. 6), B (Nov. 21 and Feb. 6), C (Oct. 26 and Nov. 15), and D (Oct. 26 and Nov. 14).

Microbiological sampling for starters and NSLAB in A, B, C, and D cheeses manufactured at each facility at scheduled sampling intervals (2wk, 6 wk, 3, 6, 9 mo) is complete. As cheeses were sampled, we also collected DNA from wells from plates incubated at 10, 30, or 45°C for later DGGE analysis, and we also extracted DNA samples from these cheeses for DGGE.

As was reported previously, microbiological counts showed several interesting trends: 1) initial NSLAB levels are generally much lower in A&B cheese versus cheese D in product made at all 3 sites; and 2) starter viability appears to decline at a more rapid rate in A&B cheese versus C&D in product made at all 3 sites; and 3) microbiological profiles of cheese made in UT or CA were more similar, regardless of cheese type, than they were to WI cheese. NSLAB levels in low-fat cheese ("D") exceed  $10^6$  by 6 wks in all samples, but few type A or B cheeses even reach that level by 6 mo. NSLAB levels in the 50% reduced fat (type "C") cheese were intermediate to those seen in full- versus low-fat cheeses.

Another observation is that populations of starter bacteria appear to remain stable, and sometimes increased, in type C and D cheeses out to 3 mo before showing any decline. In contrast, starter numbers in type A and B cheese generally declined by at least 2 orders of magnitude by 3 mo. Of course, raw numbers reveal nothing about the different types of NSLAB that may be present in each cheese, and we have performed DGGE studies to address that issue. At present, DGGE of all Utah, Wisconsin, and California cheeses has been completed, but sequencing of DGGE fragments is still underway. Results to date suggest the NSLAB fraction of all cheeses includes *Lactococcus curvatus*, but several other species of bacteria, including *Lactobacillus helveticus*, *Lactobacillus casei*, *Streptococcus thermophilus*, *Bacillus cereus/thuringiensis* and *Bacillus licheniformis* have also been detected in different cheeses. Finally, results from the phylochip experiments indicate that this microarray cannot discriminate microbes to the species level as needed by our project.

# Effect of Fat Removal on Cheese Microenvironment and Starter Culture Metabolism in Cheddar Cheese

**JEFF BROADBENT:** Utah State University

**JIM STEELE:** University of Wisconsin-Madison

**ROBERT WARD:** Utah State University

Overall Objective: To determine how the cheese microenvironment created by fat reduction and concomitant changes in make procedure affects the physiology and metabolism of *Lactococcus lactis* starter bacteria. To address this goal, we will perform the following objective:

Objective 1: Develop and utilize a model system to investigate the impact of different cheese microenvironments on the physiology of commercial *L. lactis* starter bacteria.

## SUMMARY

Flavor development in bacterial-ripened cheese (e.g., Cheddar) is primary due to the action of lactic acid bacteria (LAB) and enzymes in the ripening curd. Knowledge of the mechanisms by which LAB affect cheese flavor has facilitated industry efforts to accelerate or intensify flavor development in many traditional cheese varieties. Unfortunately, empirical efforts to extend this information into low-fat cheese systems have not proved successful, and low-fat products continue to suffer from low intensity of desirable flavors and/or from pronounced off-flavor defects.

From a purely technological perspective, many of the flavor limitations in low-fat cheese might be overcome through the addition of dairy flavors or enzymes. However, these technologies add significant costs to the product, and low-fat cheeses already cost more to make than their full fat equivalents. Because consumers may not be willing to buy low-fat products if they cost a lot more than full fat cheese, solving flavor problems in low-fat cheese through dairy flavors or enzyme technologies is currently an unattractive option for the cheese industry. A more practical solution to flavor can likely be found through combining flavors or enzymes with more effective culture systems. Compared to dairy flavors or enzyme addition, culture technology is an inexpensive means to secure flavor development, and one that should therefore be further explored and optimized for industry to offset the price concerns associated with low-fat cheese products.

Flavor problems in low-fat products are most likely explained by a scenario wherein starter physiology itself (and thus overall metabolism) is altered by differences in the physico-chemical environment in ways that affect the production of flavor- and aroma-active metabolites. If this hypothesis is correct, efforts to develop effective culture systems for low-fat cheese will require more specific knowledge of how the cheese physico-chemical environment affects starter cell physiology. The goal of this study is to determine how *Lactococcus lactis* metabolism is affected by fat reduction and its concomitant changes in cheese make procedure impart on the cheese microenvironment (e.g., S/M ratio, lactate content, pH, etc.). Specific knowledge of the nature of these perturbations will provide basic information needed by industry to develop strains, through mutagenesis or other methods, that enhance flavor development in low-fat and nonfat Cheddar cheese.

## BACKGROUND

Transformation of bland-flavored curd into delicious mature cheese is a complex and dynamic process whose intricacies are scripted by the milk type and composition, the cultures and enzymes present or added to the cheese milk, and the manufacturing and ripening conditions used. Many cheeses need to be stored at low temperature for months or even years before they attain characteristic flavor and body attributes. During this time, termed the curing or ripening period, microorganisms and enzymes in the cheese matrix act on milk constituents in a manner that is

partly dictated by the curd microenvironment (e.g., cheese pH,  $a_w$ , salt content,  $E_p$ , temperature, etc.) and which ultimately gives the desired product (Fox et al., 1993).

Types of LAB that occur in internally bacterial-ripened cheeses such as Cheddar, Dutch, Swiss, and Italian varieties, include deliberately added strains (e.g., starters and adjunct cultures) and adventitious species (primarily nonstarter LAB or NSLAB) that enter cheese through milk or processing equipment. Modern sanitation and Good Manufacturing Practices help minimize initial numbers of NSLAB in cheese, yet these organisms invariably appear and grow to high numbers during ripening (Peterson and Marshall, 1990). In Cheddar cheese, numbers of *Lactococcus lactis* starter bacteria frequently exceed  $10^9$  colony-forming units (cfu) per gram when ripening begins. As maturation proceeds, the harsh cheese ripening environment (little or no residual lactose, pH 5.0 to 5.3, 4-6% salt in moisture, 5-13°C) gradually takes its toll and starter viability declines. A fraction of the dying starter cells undergo autolysis, which releases intracellular enzymes and cellular components (e.g., sugars and nucleic acids) into the cheese matrix (Fryer, 1969). At the same time, NSLAB populations (whose initial numbers are typically less than  $10^2$  cfu/g in cheese made under good sanitary conditions with high quality pasteurized milk) begin to grow and eventually plateau at cell densities of  $10^7$ - $10^8$  cfu/gram after 3-9 mo of aging (Peterson and Marshall, 1990). Microbiological studies have shown NSLAB populations in bacterial-ripened cheeses may be quite diverse, but are usually dominated by facultatively fermentative species of lactobacilli or, far less frequently, by pediococci (Fryer, 1969; Beresford et al., 2001; Broadbent et al., 2003; Broome et al., 1990a; Crow et al., 2001; Sherwood, 1939). Depending on the species that is used (and whether or not the particular strain can grow in ripening cheese), populations of adjunct bacteria may mirror the trend for starter or NSLAB fractions.

Though a link between LAB activity and cheese flavor attributes was postulated more than 100 years ago (see Fryer, 1969), the variation and complexity that exists in cheese microbiota and enzyme content confounded early efforts to establish a causal role for these bacteria in flavor development. This limitation was overcome in the late-1950's, when sensory studies of aseptically manufactured Cheddar cheese showed that starter-free, gluconolactone-acidified cheese failed to develop Cheddar flavor, while cheese made with *L. lactis* starter bacteria developed characteristic, balanced flavor (Law et al., 1976; Reiter et al., 1967). The same investigations also showed NSLAB could modify basic flavor notes and accelerate flavor development. More recently, use of *Lactobacillus* spp. NSLAB isolates as adjunct cultures for Cheddar cheese manufacture has indicated these bacteria may influence flavor in at least 3 ways: they may intensify (i.e., accelerate) typical flavor development, impart atypical (but desirable) flavor notes, or promote off-flavor development (Fryer, 1969; Crow et al., 2001; Sherwood, 1939; Broome et al., 1990b; Lynch et al., 1999; McSweeney et al., 1994; Swearingen et al., 2001). In addition, NSLAB have also been associated with cheese quality defects such as open body (via gas production) and formation of calcium lactate crystals (Fryer, 1969; Johnson et al., 1990; Khalid et al., 1990).

Given the causal role of LAB in flavor development, efforts to define the biochemical basis for flavor changes in cheese have logically focused on the microbiology and physiology of species found in cheese (for recent reviews see Beresford et al., 2001; El Soda et al., 2000; Fox and Wallace, 1997; Marilley and Casey, 2004; Rattray and Fox, 1999). Those efforts have identified many of the most important biochemical and chemical processes in maturation, and have shown starter, adjunct, and NSLAB have an intimate role in most of those processes including lactose fermentation, conversion of milk proteins (primarily caseins) into peptides and free amino acids, catabolism of amino acids into volatile aroma compounds, lipase/esterase activity, and citrate catabolism.

Knowledge of the mechanisms by which LAB affect cheese flavor has facilitated industry efforts to promote flavor development in many traditional cheese varieties, but empirical efforts to extend this information into low-fat cheese systems have not proved successful. As a result, low-fat or nonfat bacterial-ripened cheeses continue to suffer from low intensity of desirable flavor or pronounced off-flavors. From a purely technological perspective, many of the flavor limitations in low-fat cheese might be overcome through the addition of dairy flavors or enzymes. The costs associated with this technology are estimated to be 1-5 cents per pound, however, and would be incurred on top of the already premium cost to make low-fat cheese. As a result, exclusive use of flavors or enzyme technologies to solve the flavor problems in low-fat products is not an attractive option at this time. Industry concerns about product cost is supported by a recent consumer study that found there is little support for low-fat cheese product, if their cost is substantially higher than that of full fat cheese.

A more cost-effective solution to the flavor problems in low-fat cheese can likely be found through combining flavors or enzymes with culture systems that deliver better flavor in these products. Compared to dairy flavors or enzyme addition, culture technology is an inexpensive means to secure flavor development, and one that should be further explored and optimized for industry to offset the price concerns associated with low-fat cheese products.

These deficiencies in low-fat cheese flavor are most likely explained by one of two scenarios: 1) starter physiology (and thus metabolic end-product profile) is the same in all cheeses, but sensory perception of those metabolites is altered by differences in the physico-chemical environment (e.g., fat, moisture, or salt in moisture contents); or 2) starter physiology itself (and thus overall metabolism) is altered by differences in the physico-chemical environment in ways that affect the production of flavor- and aroma-active metabolites. A third possibility, of course, is that each of these scenarios has a role in the atypical flavor profile of low- and nonfat cheese systems.

Though little information is available on the differences in flavor- or aroma-active metabolites in full versus reduced fat cheese, work by Milo and Reineccius (1997) noted important differences in volatile components from full fat versus 40% fat reduced Cheddar cheese, and suggested that these differences might be due to the higher water content in the reduced fat cheese. More recently, Carunchia Whetstone et al. (2006) investigated sensory properties and volatile chemical profiles in full- and 50 % reduced fat Cheddar manufactured by a novel fat removal process after aging is complete. Those authors discovered that the great majority of volatile aroma-active compounds remained in the aqueous-containing cheese matrix versus the removed fat fraction and, more interestingly, found that cheeses with either fat level had nearly identical flavor profiles. Given these findings, and the central role of lactic acid bacteria in flavor development, it is our hypothesis that the second scenario provided above (i.e., starter physiology and metabolism is altered by perturbations in the cheese microenvironment) has the greatest effect on flavor development in low- or nonfat cheese. If this hypothesis is correct, efforts to develop effective culture systems for low-fat cheese will require more specific knowledge of how the cheese physico-chemical environment affects starter cell physiology. Thus, the goal of this study is to determine how *Lactococcus lactis* metabolism is affected by fat reduction and its concomitant changes in cheese make procedure impart on the cheese microenvironment (e.g., S/M ratio, lactate content, pH, etc.). This knowledge is expected to generate basic information industry needs to develop starter strains, through mutagenesis or other methods, which enhance flavor development in low-fat and nonfat Cheddar cheese. To attain the goals of this project, we will complete the following experiments:

**Objective 1:** Develop and utilize a model system to investigate the impact of different cheese microenvironments on physiology of commercial *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* starter bacteria.

These experiments will utilize cells incubated in a Cheddar Cheese Extract (CCE) medium using laboratory-scale (1 to 2 L) bioreactors. The CCE medium for these experiments will be prepared from 6 wk-old low fat Cheddar cheese made at the Western Dairy Center. To determine the impact of cheese microenvironment on starter volatiles production and transcriptional profile, we will use 6 strains of *L. lactis* starter bacteria that include strains recommended by industry suppliers for aged full fat or low-fat cheese production.

Our first experiments will evaluate differences in production of volatile compounds by each of the lactococcal strains in CCE designed to mimic low-fat or full-fat cheese microenvironments (Table 1) at 10°C, except that redox will not be adjusted. Those experiments will be followed by a series of independent experiments to investigate the effects of salt-in-moisture (3.7 vs 4.75%), temperature (5 vs 10°C), redox (+340 vs -200), and D-lactate (500 vs 700 ppm). Once these data sets are complete, we will evaluate the effect of cell “conditioning” and exposure to gluconic acid on production of volatile compounds in low-fat CCE. “Conditioned” cells will be prepared by incubating strains in full-fat CCE for at least 5 sequential transfers before inoculating them into low-fat CCE.

The influence of cheese environment on volatiles production by the starter will be performed under the supervision of Dr. Robert Ward using a solid phase microextraction GC-MS approach essentially as described by Lee et al. (2007). Once volatiles analyses are complete, Drs. Broadbent, Ward and Steele will individually review the volatiles data obtained from these experiments and prioritize the lactococcal RNA samples that should be included in the transcriptome component of this work. Priority for these selections will be based on pair-wise comparisons that we feel will provide the greatest insight to effect of microenvironment on cell physiology.

It is our expectation that the combined results from volatiles profiling and transcriptional studies will generate critical knowledge needed to understand how starter physiology is altered by perturbations in the cheese microenvironment. The molecular information derived from this work should allow us to build a “metabolic fingerprint” for flavor production in low-fat and full-fat cheese that may reveal new strategies to develop starter strains, through mutagenesis or other methods, that produce more intense and desirable flavor notes in low-fat Cheddar cheese.



**Table 1.** Cheese environmental conditions to be used in the study.

Component	Low-fat model	Full-fat model <sup>a</sup>	Full-fat control <sup>a</sup>
Salt-in-moisture (%) <sup>b</sup>	3.7%	4.75	4.75
Lactate <sup>b</sup>	5500 ppm L-lactate 700 ppm D-lactate	5500 ppm L-lactate 500 ppm D-lactate	5500 ppm L-lactate 500 ppm D-lactate
Temperature	5, 10°C	5, 10°C	5, 10°C
Redox <sup>c</sup>	+340, -200, none	+340, -200, none	+340, -200, none
pH <sup>d</sup>	5.1	5.1	5.1

<sup>a</sup>Full-fat model and control cheese will be prepared from CCE collected from washed curd low-fat and full-fat Cheddar, respectively.

<sup>b</sup>Reflects typical S/M (at press) and lactate contents (at 3 mo) of washed curd low-fat and full fat cheese made and analyzed under the 2006 DMI collaborative low-fat platform project. Because some chloride ion may be lost during CCE preparation, CaCl<sub>2</sub> may be added to balance the sodium and chloride content of the CCE.

<sup>c</sup>Stable oxidative or reducing conditions (Eh = 340 ± 30 mV or -200 ± 30 mV, respectively) will be generated as described by Kieronczyk et al. (2006) with addition of 24 mmol potassium ferricyanide [K<sub>3</sub>Fe(CN)<sub>6</sub>] or 30 mmol dithiothreitol (DTT) to the medium.

<sup>d</sup>Initial pH; adjusted after lactate addition.

## PROGRESS TO DATE

The bioreactor studies on volatiles and gene expression that will be performed in this project will utilize cells incubated in Cheddar cheese extract (CCE) formulated to mimic different cheese microenvironments. To obtain uniform results, we needed to prepare a large batch of CCE powder that can meet all the needs of the project. Thus, more than 500 lbs of full and low-fat Cheddar cheeses were manufactured at the USU dairy plant and aged 6 weeks (the time at which HPLC analysis confirmed residual lactose and galactose levels had fallen below quantifiable levels). After 6 wks, the samples were shredded and frozen in preparation for lyophilization and subsequent processing into CCE powder. All of the cheeses have been aged, shredded, frozen and lyophilized once. Because the 2<sup>nd</sup> round of extraction and lyophilization would have taken more than one year, we worked with the Western Dairy Center staff to develop a novel, high-throughput system for generating the final CCE aqueous extract using our pilot plant UF capabilities. Preliminary studies confirmed we can concentrate our powdered cheese very rapidly using this method, and that the product supports growth of nonstarter lactic acid bacteria (NSLAB). Importantly, this process is also amenable to using frozen shredded cheese as the input, which will allow us to eliminate the need for initial lyophilization in future CCE preparations. Using this method, we have concentrated all of the lyophilized cheese we prepared for the study into approximately 70L of CCE extract. That extract is being rapidly lyophilized, and we anticipate having all the material in place to start fermentations in the next quarter using the computer-controlled, lab-scale, twin bioreactor system we acquired for this project.

We have also collected and characterized the lactococci that will be used in this study, which include *L. lactis* LL 605, *L. lactis* LL 145, *L. lactis* LL 071, *L. lactis* LL 052, *L. lactis* LL 074, and *L. lactis* LL 011. The cultures were grown in M-17 lactose broth for 24 hours at 30°C, then streaked for single colony isolation on M-17 lactose agar. Individual colonies were inoculated into M-17 lactose broth, grown, and then samples were frozen at -80°C until needed. In addition, total DNA was isolated from each strain and their identity as *Lactococcus lactis* was confirmed by PCR and sequencing of the 16S rRNA gene. The uniqueness of each strain was also determined by plasmid DNA profiles; each strain was found to possess a unique plasmids content whose molecules ranged in size from ~2,000 bp to 15,000 bp.

To prepare for headspace volatiles analysis via GCMS that will be performed in CCE, trial runs were conducted using locally obtained cheddar cheese in order to characterize baseline data for full fat cheese. Two solid phase microextraction fibers (SPME) fibers were tested in these trials, Car/PDMS and PDMS/DVB, and analytes were separated on an RTX-Wax column. The most comprehensive results were obtained with the PDMS/DVB SPME

fiber that produced a chromatograph of 79 individual peaks. Peak identification will be performed using the NIST-EPA MS library.

In similar fashion, we established methods for the microarray work that will also be performed with CCE-grown lactococci by customizing a software program called "r" that will be used to analyze gene expression studies. Preliminary analysis of the software using microarray data collected from another project has allowed us to become familiar with its nuances. We have also determined the vendor for the lactococcal microarrays that will be used in the project. In summary, we anticipate that all materials will be in place to start GCMS and microarray studies in the next quarter.

## References

- Beresford, T. P., N. A. Fitzsimons, N. L. Brennan, and T. M. Cogan. Recent advances in cheese microbiology. *Int. Dairy J.* 11:259-274.
- Broadbent, J. R., B. McKernan, C. J. Oberg, K. Houck, and M. E. Johnson. 2003. Influence of adjunct use and cheese microenvironment on nonstarter lactic acid bacteria populations in Cheddar-type cheese. *J. Dairy Sci.* 86:2773-2782.
- Broome, M. C., Krause, D. A., and Hickey, M. W. 1990a. The isolation and characterization of lactobacilli from Cheddar cheese. *Aust. J. Dairy Technol.* 45:60-66.
- Broome, M. C., D. A. Krause, and M. W. Hickey. 1990b. The use of non-starter lactobacilli in Cheddar cheese manufacture. *Aust. J. Dairy Technol.* 45:67-73
- Carunchia Whetstine, M.E., M.A. Drake, B.K. Nelson, and D. Barbano. 2006. Flavor profiles of full-fat and reduced-fat cheese and cheese fat made from aged Cheddar with the fat removed using a novel process. *J. Dairy Sci.* 89:505-517.
- Crow, V., Curry, B., and Hayes, M. 2001. The ecology of non-starter lactic acid bacteria (NSLAB) and their use as adjuncts in New Zealand Cheddar. *Int. Dairy J.* 11:275-283.
- El Soda, M., S. A. Madkor, and P. S. Tong. 2000. Adjunct cultures: recent developments and potential significance to the cheese industry. *J. Dairy Sci.* 83, 609-619.
- Fox, P. F., Law, J., McSweeney, P. L. H., and Wallace, J. 1993. Biochemistry of cheese ripening. In *Cheese: Chemistry, Physics and Microbiology*. (Ed. P. F. Fox). 2nd Edition, Volume 1, Chapman and Hall, London, pp. 389-438.
- Fox, P. F., and J. M. Wallace. 1997. Formation of flavor compounds in cheese. *Adv. Appl. Microbiol.* 45:17-85.
- Fryer, T. F. 1969. Microflora of Cheddar cheese and its influence on cheese flavour. *Dairy Sci. Abstr.* 31:471-490.
- Guinee, T.P., and P.F. Fox. 1993. Salt in cheese: physical, chemical, and biological aspects. In *Cheese: Chemistry, Physics and Microbiology*; Fox, P. F., Ed.; Chapman and Hall, London, England, Vol. 1, 2nd ed., pp. 257-302.
- Johnson, M. E., Riesterer, B. A., and Olsen, N. F. 1990. Influence of nonstarter bacteria on calcium lactate crystallization on the surface of Cheddar cheese. *J. Dairy Sci.* 73:1145-1149.
- Kieronczyk, A. R. Cachon, G. Feron, and M. Yvon. 2006. Addition of oxidizing or reducing agents to the reaction medium influences amino acid conversion to aroma compounds by *Lactococcus lactis*. *J. Appl. Microbiol.* 101:1114-1122.
- Khalid, N. M., and E.H. Marth. 1990. Lactobacilli-their enzymes and role in ripening and spoilage of cheese: a review. *J. Dairy Sci.* 73:2669-2684.
- Law, B. A., M. Castanon, and M. E. Sharpe. 1976. The effect of non-starter bacteria on the chemical composition and the flavour in Cheddar cheese. *J. Dairy Res.* 43:117-125.
- Lee, W.-J., D.S. Banavara, J.E. Hughes, J.K. Christianen, J.L. Steele, J.R. Broadbent, and S.A. Rankin. 2007. Role of cystathionine  $\beta$ -lyase in catabolism of amino acids to sulfur volatiles by genetic variants of *Lactobacillus helveticus* CNRZ32. *Appl. Environ. Microbiol.* 73:3034-3039.
- Lynch, C. M., D. D. Muir, J. M. Banks, P. L. H. McSweeney, and P. F. Fox. 1999. Influence of adjunct cultures of *Lactobacillus paracasei* ssp. *paracasei* or *Lactobacillus plantarum* on Cheddar cheese ripening. *J. Dairy Sci.* 82:1618-1628.
- Marilley, L., and M.G. Casey. 2004. Flavours of cheese products: metabolic pathways, analytical tools and identification of producing strains. *Int. J. Food Microbiol.* 90, 139-159.
- Marshall, R. T. 1993. Standard methods for the examination of dairy products, 16th ed. American Public Health Association, Washington, DC.

- McSweeney, P. L. H., E. M. Walsh, P. F. Fox, T. M. Cogan, F. D. Durain, and M. Castelo-Gonzalez. 1994. A procedure for the manufacture of Cheddar cheese under controlled bacteriological conditions and the effect of adjunct lactobacilli on cheese quality. *Irish J. Agricul. Food Res.* 33:183-192.
- Milo, C; Reineccius, G.A. 1997. *J. Agric Chem.* 45:3590-3594.
- Peterson, S. D., and R. T. Marshall. 1990. Nonstarter lactobacilli in Cheddar cheese: a review. *J. Dairy Sci.* 73:1395-1410.
- Ratray, F. P., and P. F. Fox. 1999. Aspects of enzymology and biochemical properties of *Brevibacterium linens* relevant to cheese ripening: A review. *J. Dairy Sci.* 82:891-909.
- Reiter, B.; Fryer, T. F.; Pickering, A.; Chapman, H. R.; Lawrence, R. C.; Sharpe, M. E. *J. Dairy Res.* 1967, 34, 257-272.
- Sherwood, I. R. 1939. The bacterial flora of New Zealand Cheddar cheese. *J. Dairy Res.* 10:449-454.
- Smeianov, V. V., P. Wechter, J. R. Broadbent, J. E. Hughes, B. Rodriguez, T. K. Christensen, Y. Ardo, and J. L. Steele. 2007. Comparative high-density microarray analysis of gene expression during growth of *Lactobacillus helveticus* in milk versus rich culture medium. *Appl. Environ. Microbiol.* 73:2661-2672.
- Swearingen, P. A., D. J. O'Sullivan, and J. J. Wartheson. 2001. Isolation, characterization, and influence of native nonstarter lactic acid bacteria on Cheddar cheese quality. *J. Dairy Sci.* 84:50-59.

# Innovative Approaches for Improving Low Fat Mozzarella Cheese

DONALD J. MCMAHON: Utah State University

CRAIG OBERG: Weber State University

## ABSTRACT

Two major limitations of low fat mozzarella cheese have been addressed in this research: lack of stretch and excess charring during baking, and its lack of fibrous structure in a snacking product such as string cheese. Both of these imperfections result from the decrease in the quantity of available fat. To address the melting/charring issues, a combination of a directly acidified make procedure, along with grinding the cheese curd into small particles and mixing in an oil, then pressing the cheese, were used to provide free oil in a readily available form that is released during baking of the diced cheese. The lack of fiber in a low fat string cheese was addressed by manufacturing low fat cheese through to the pasta filata step, and then blending with the hot cheese mass, a hydrated slurry containing polysaccharides. When combined, the polysaccharide did not help form the channels between protein fibers so that when the mass was extruded it did not have the appearance and texture of string cheese.

## INTRODUCTION

For good melting properties, a low fat cheese needs to have sufficient moisture and fat to prevent the cheese from dehydrating too much during baking, especially when baked on a pizza in an Impinger-type oven using hot air. The chemistry of the protein matrix can be adjusted so that it flows and has stretch properties, but in a low fat cheese there is insufficient free oil to prevent excessive surface dehydration, which then results in charring. In a cheese containing only 6% fat, the fat globules are well entrapped within the protein matrix while in a full fat cheese (or even part skim mozzarella) some of the fat is no longer in globular form and is released from the cheese as it is heated.

In recent research at Utah State University we have observed that reduced fat cheese curd can be ground (or chopped) into small pieces and then filled into hoops and pressed. Such cheese knits together well and can be easily sliced. By using this technique, a cheese curd with fat content below the final target can be made and a small amount of fat added during grinding. The amount of fat cannot be too high or it will prevent fusion of the curd particles during pressing. The final cheese will then have tiny veins and droplets of fat distributed throughout that is not intrinsically entrapped by the protein matrix. This cheese can then be diced and when spread on a pizza (or other baking applications) there would be sufficient free oil that can be released to provide a thin coating to the cheese surface.

The maximum fat level allowable for a low fat cheese is 6% fat (max. 3 g fat per 50-g reference amount). One of the requirements to meet the AHA Heart Healthy guidelines, is that such a low fat food can contain no more than 1 g of saturated fat per 1 oz serving. And since butterfat contains 63.5% saturated fat, then such a cheese can only contain 1.57 g of fat per 28 g serving, or no more than 5.6% fat. This will be the target used in this research, and this can be fat present in the original milk or added during the grinding/blending process.

The fibrous characteristic of mozzarella cheese comes about because during the pasta filata hot water cooking/stretching of the cheese curd, the fat globules undergo a type of phase separation from the protein matrix. During this process the fat globules gather and as a result of the stretching process are formed into long column of fat globules in serum with the protein consequently forming into strands that are separated by these fat-serum channels. Then when the hot cheese is extruded the protein strands are oriented so as to parallel with each other. In the absence of fat, there is nothing to keep the protein strands separated and the cheese loses stringiness unless some other substance is added that can also interfere with protein fusion to form the two phase system of protein strands

and particle channels during the mixing-extrusion process. Polysaccharide materials that do not bind to the proteins have the potential to mimic the action of fat globules in physical blocking protein fusion and allowing protein strands to form.

The polysaccharide can be added in powdered form or as a slurry. Included in this would be starches and gums. Another source of polysaccharide is that produced by EPS+ cultures such as the capsular forming *Streptococcus thermophilus* MR1C started culture that has been used for increasing moisture content of low fat mozzarella cheese and was observed in cheese to have a large polysaccharide capsule around the bacterial cells which form into chains (Perry et al., 1997,1998). This can be grown in a milk media and would have the advantage of being included in the ingredient list as part of the cultures.

## MATERIALS and METHODS

### Objective 1

**Cheesemaking.** Pizza cheese will be made using the direct acidification method developed by McMahon and Oberg (2000) for making fat-free pizza cheese that has enhanced melting properties. The cheese will be made at fat levels of 1.0, 2.75 and 4.5% so that different ratios of protein matrix-entrapped fat to exogenous fat of 1.0:4.5, 2.75:2.75 and 4.5:1.0 can be compared. The cheesemaking involves acidification of the milk prior to renneting without any addition of starter culture. The curd is cut after coagulation, stirred, then the whey drained, curd stirred until the proper moisture level in the curd is reached, and the curd salted.

The curd is then divided into four portions:

- a. cheese packed into a 10-lb hoop and pressed (Control 1)
- b. cheese ground into pieces of mean size 2 mm, then packed into a 10-lb hoop and pressed (Control 2)
- c. cheese ground into pieces of mean size 2 mm, then mixed with either 4.5, 2.75 or 1.0% (wt./wt.) nonglobular milk fat as butter oil, packed into a 10-lb hoop and pressed (Treatment 1)
- d. cheese ground into pieces of mean size 2 mm, then mixed with 5.6, 3.4, or 1.25% (wt./wt.) of globular milkfat in the form of 80% cream, packed into a 10-lb hoop and pressed (Treatment 2)

**Chemical Analysis.** Measure pH by glass electrode, salt by chloride analysis, moisture by vacuum oven, fat by Babcock, protein by combustion N, calcium and other minerals by ICP spectroscopy, water activity by headspace relative humidity.

**Storage.** Store the cheeses at 6°C for 4 months.

**Physical Testing.** Cheeses will be analyzed using a two-bite texture profile analysis with 60% compression at 2 wk, 1, 2 and 4 mo to determine extent of knitting of the curd particles. Specific parameters of interest will be hardness, elasticity, and cohesiveness.

**Structural Analysis.** Examine the cheeses at 2 wk, and 2 mo, using confocal laser microscopy to confirm the presence of fat between the curd particles as well as within the curd particle protein matrix. The cheese will be stained with Nile Red (fat stain) and fluorescein isothiocyanate (protein stain).

**Functional Analysis.** Cheese will be diced at 2 wk, 1, 2 and 4 mo, and then testing for oiling-off (Kiely et al., 1992), for stretching using a texture analyzer (Fife et al., 2002), and for its performance when cooked on a pizza (pizza, tomato pizza sauce and cheese) in an Impinger oven set at 480°F for 6 min. After cooking the pizza will be photographed to record extent of browning.

**Sensory Testing.** Determine the best cheesemaking parameters for producing a low fat pizza cheese containing 5.5% total fat, make cheese in duplicate, along with a control low fat cheese. Also determine the storage time at which melting properties for pizza-making are maintained (cheeses made using direct acidification usually do not require long aging times) and then store the cheese for 2 wk and 6 wk. At each time, conduct sensory preference testing on cheeses cooked on a pizza at 1 and 2 mo of age using an untrained panel with minimum of 70 participants.

### Objective 2

**Cheesemaking.** Low fat mozzarella cheese curd will be made from 1500 lb of 0.5% fat milk in an enclosed vat, using *Streptococcus thermophilus* starter culture. When the cheese curd reaches pH 5.3 it will be partially salted with 0.5% salt, and then formed into a homogeneous mass in cooker/stretcher using hot water containing 5% salt. The hot (130°C) cheese will then be divided into portions for addition of polysaccharides.

**Step 1 Treatments.** A variety of polysaccharides will be tested for their ability to help form fiber structure in

string cheese, and compared to a control cheese without any added ingredients. The ingredients (both dry and wet) will be added at a level of 2% dry weight of polysaccharide per wet weight of cheese, and then mixed together by kneading the curd and maintaining it at about 130°C. The hot cheese mass will then be placed in a miniature string cheese extruder and the cheese extruded through a long die with an internal diameter of about 15 mm to form the string cheese, which will then be cut into pieces and then cooled in a cold 5% brine solution.

A wide range of polysaccharides will initially be tested for their suitability to increase string formation, these will include:

- a. Starches: including modified starches including crosslinked starches, hydroxypropyl substituted starches, oxidized starches, pre-gelatinized starches.
- b. Gums: xanthan, guar, locust bean gums, carrageenan
- c. Particulated fat mimetics: cellulose gum, starch, whey protein
- d. Polysaccharide produced by EPS capsule-producing *St. thermophilus* (MR-1C) grown in milk.
- e. Polysaccharide produced by EPS capsule-producing *St. thermophilus* (MR-1C) grown in a culture media that also contains polysaccharides used for increasing yield during cheesemaking.

Store the cheese at 4°C for 1 wk and determine the fiber formation in the cheese but pulling down on the end of the cheese sample and recording the appearance of fibers/strings.

**Step 2 Treatments.** Select four ingredients from the polysaccharides tested in Step 1 above, from those that showed an increase in string formation compared to a control low fat cheese (no additives) and test over a concentration range of 0.5 to 4.0% dry weight of material per wet weight of cheese, in duplicate..

Chemical Analysis: Measure pH by glass electrode, salt by chloride analysis, moisture by microwave oven, fat by Babcock.

Storage: Store the cheeses at 4°C for 3 months.

Physical Testing: Examine the fiber formation in the cheese at 1, 6 and 12 wks, by pulling down on the end of the cheese sample and recording the length of strings of cheese that can be formed, and by photographing the cheese.

Structural Analysis: Examine the cheeses at 1, 6 and 12 wk using confocal laser microscopy to confirm the presence of the proteins in strands with the carbohydrate ingredients between the protein strands).

**Step 3.** Select the best two treatment cheeses and a control cheese (without any additive) and manufacture low fat string cheeses that are of comparable firmness to low moisture part skim mozzarella skim cheese, in triplicate.

Sensory Analysis: Perform a consumer preference study of the cheeses when they are 3 wks of age (or other time considered optimum from previous steps)..

Chemical Analysis: Measure pH by glass electrode, salt by chloride analysis, moisture by vacuum oven, fat by Babcock, protein by combustion N, calcium and other minerals by ICP spectroscopy, water activity by headspace relative humidity.

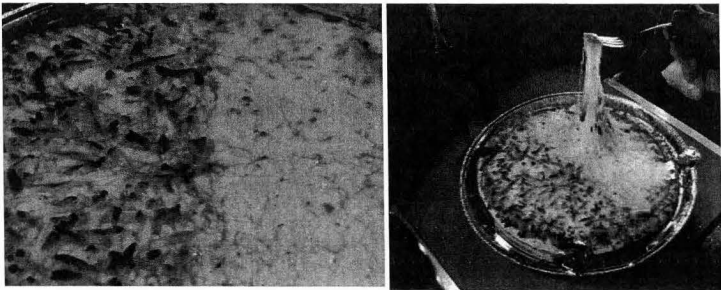
Physical Testing: Examine the fiber formation in the cheese at the same time as the sensory testing by pulling down on the end of the cheese sample and recording the length of strings of cheese that can be formed, and by photographing the cheese.

Structural Analysis: Examine the cheeses at 3 wk or age (or other chosen time) using confocal laser microscopy to confirm the presence of the proteins in strands with the carbohydrate ingredients between the protein strands).

## RESULTS

### Objective 1

A comparison of cheese performance when cooked on a pizza in an Impinger oven at 480°F for 5 min with the control cheese (left) showing characteristic charring and lack of melting of low fat cheese, and the experimental cheese (right) showing increased melting and fusion of shreds, and stretching.



Meltability was observed to improve with the milk starting with the lowest fat level, having the most free fat added as melted butter. Rep 1 and Rep 2 of cheeses of various butterfat's, grinds, and, agings have been produced. Pizzas have been made using said cheeses. Results show improvements in browning and meltability.

Proximate composition of all four cheese samples was consistent with 5.5-6.0% fat and 52-53% moisture content. The water activity for all cheese samples was 0.97. The textural analysis, free oil%, stretchability and melting profile are shown in Table 1, 2 and Figure 1 below:

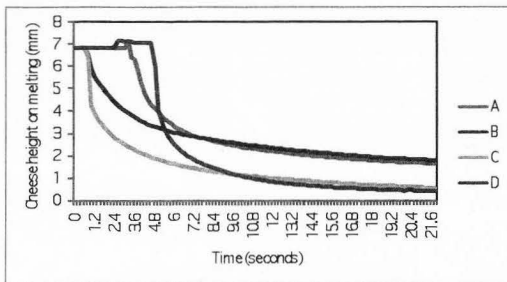
Sample Type	Hardness	Adhesiveness	Springiness	Cohesiveness	Gumminess	Chewiness
<b>2 Weeks</b>						
Control (6% fat from curd)	5681.912	-18.381	0.831	0.614 <sup>a</sup>	3663.034 <sup>A</sup>	3203.044 <sup>X</sup>
4.5%+1.5%butter	5875.010	-4.417	0.905	0.432 b	2540.520 AB	2300.360 Y
3%+3%butter	1984.685	-28.747	0.826	0.245 c	485.348 C	400.826 Z
1.5%+4.5%butter	1835.486	-0.331	0.734	0.291 c	533.343 C	391.674 Z
<b>4 Weeks</b>						
Control (6% fat from curd)	6792.125	-0.343	0.884	0.592 b	4018.280 B	3651.394 Y
4.5%+1.5%butter	12548.5	-31.312	0.897	0.708 ab	8883.315 A	7966.016 X
3%+3%butter	7166.309	-48.154	0.794	0.577 b	4133.631 B	3281.978 Y
1.5%+4.5%butter	2228.755	-2.542	0.839	0.678 ab	1511.427 C	1268.674 Z
<b>8 Weeks</b>						
Control (6% fat from curd)	5172.166	-30.839	0.836	0.707 a	3658.874 B	3058.95 Y
4.5%+1.5%butter	8036.064	-29.699	0.841	0.767 a	6166.205 A	5185.995 X
3%+3%butter	2098.451	-25.005	0.828	0.735 a	1542.265 C	1277.312 Z
1.5%+4.5%butter	1797.445	-11.247	0.805	0.698 a	1253.934 C	1009.978 Z
<b>16 Weeks</b>						
Control (6% fat from curd)	5332.134	-27.072	0.83	0.692 a	3691.029 AB	3065.065 X
4.5%+1.5%butter	5697.753	-18.612	0.844	0.765 a	4367.917 A	3678.017 X
3%+3%butter	1887.725	-23.677	0.778	0.622 a	1174.482 B	914.282 YZ
1.5%+4.5%butter	1774.814	-27.111	0.806	0.705 a	1251.088 B	1008.377 Y

**Table 1:** Textural profile analysis at 2, 4, 8, 16 weeks of storage

Different superscripts a, b, c, A, B, C, X, Y, Z within column are significantly different from each other

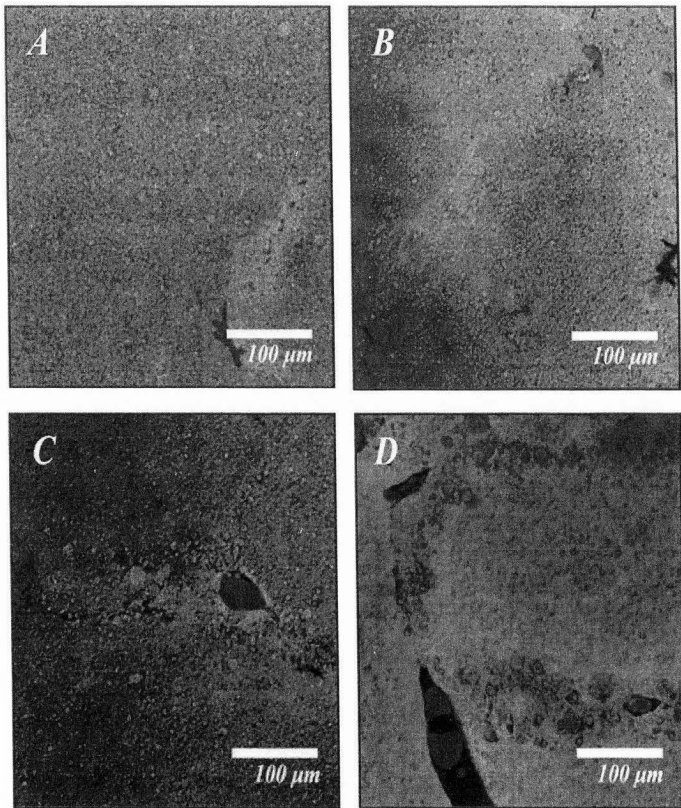
Rep 1	Sample (%Butter)	Free oil%	Stretchability (cm)	Rep 2	Sample (%Butter)	Free oil%	Stretchability (cm)
2w	0	0.30 <sup>A</sup>	6 <sup>a</sup>	2w	0	0.37 A	6a
	1.5	0.40 <sup>A</sup>	3 <sup>a</sup>		1.5	0.62 B	6a
	3	1.10 <sup>B</sup>	19 <sup>c</sup>		3	1.00 C	37b
	4.5	1.50 <sup>B,C</sup>	22 <sup>c</sup>		4.5	2.00 D	40b
4w	0	0.25 <sup>A</sup>	7 <sup>a</sup>	4w	0	0.25 A	11a
	1.5	0.37 <sup>A</sup>	9 <sup>b</sup>		1.5	0.37 A	9a
	3	1.25 <sup>B</sup>	12 <sup>b</sup>		3	0.75 B	10a
	4.5	1.25 <sup>B</sup>	18 <sup>b</sup>		4.5	1.25 C	13a
8w	0	0.25 <sup>A</sup>	6 <sup>a</sup>	8w	0	0.25 A	6a
	1.5	0.50 <sup>B</sup>	6 <sup>a</sup>		1.5	0.36 A	7a
	3	0.75 <sup>B,C</sup>	7 <sup>a</sup>		3	0.72 B	9a
	4.5	1.00 <sup>C</sup>	10 <sup>b</sup>		4.5	1.10 C	12ab
16w	0	0.20 <sup>A</sup>	10 <sup>b</sup>	16w	0	0.20 A	11a
	1.5	0.25 <sup>A</sup>	5 <sup>a</sup>		1.5	0.25 A	7a
	3	0.75 <sup>B</sup>	12 <sup>b</sup>		3	0.75 B	14ab
	4.5	0.62 <sup>B</sup>	41 <sup>c</sup>		4.5	0.62 B	38c

**Table 2:** Stretchability and %free oil for cheese samples at 2, 4, 8, and 16 weeks of storage  
Different superscript a,b,c,A,B,C within column is significantly different



**Figure 1:** Melt-profile of cheese samples (A = Control; B = 4.5% {fat from curd} + 1.5% {from added butter}; C = 3.0+3.0%; and D = 1.5+4.5%). Note, vertical displacement at being of figure is an artifact of variations in equipment start times





**Figure 2:** Laser scanning confocal microscope images of Low Fat Mozzarella Cheese (A: Control; B: 1.5% added butter; C: 3.0% added butter; and D: 4.5% added butter)

### **Objective 2**

Develop a low fat mozzarella cheese that has the same characteristics as string cheese made from cheese containing 20% fat.

Extrusion of cheese has been problematic with the cheese sticking to the extruder. Variations of temperature and pressure have been tested to improve the quality of extrusion to yield an acceptable string cheese. Modifications have not produced any improvement.

## DISCUSSION

### **Objective 1**

The reduction by comminution of cheese to small particles in the 1 to 3 mm range generates a large increase in surface area of the particles. Comminuting the cheese, releases some fat droplets at the fracture surfaces and generates free fat. For a low fat cheese, this is relatively insignificant. If the fat is reduced in the original production of the cheese curd below 6%, then additional fat can be added back to the comminuted curd so that the total becomes 6% fat in cheese. Thus the amount of fat in the cheese is greatly increased.

The free oil is present along particle boundaries within the pressed cheese. If the cheese is heated to its melting point, the free fat readily moves from the gaps to the surface of the cheese. The free oil is then available to protect the surface of the cheese from drying during forced-air convection baking, preventing charring and improving the melting of the cheese. This process generated superior meltability without raising fat levels in the cheese. The higher the added butter, the higher the meltability, but due to increased browning of cheese with 4.5% butter fat, the best combination was considered to be the cheese made with 3% butter added to curd containing 3% fat. This process is cost favorable as no extra ingredients have been added to low fat Mozzarella

### **Objective 2**

It was hoped that adding a mixture of a polysaccharide material into hot stretched low fat mozzarella cheese would create channels of polysaccharide that mimic the fat-serum channels present in higher fat cheese and cause the protein to form into strands, thus giving the cheese a string cheese appearance. There were problems in getting adequate mixing and maintaining the temperature of the cheese. A miniature string cheese formed was constructed that utilized our vertical cheese press, but cheeses with high levels of starch mixtures did not extrude properly. In future, modification in the polysaccharides with the addition of an extruder with a low friction surface, such that the lack of the fat as a lubricant, would minimize the sticking of the cheese to the extruder may yield a superior results

## Conclusions

### **Objective 1**

A cheese with enhanced melting has been developed and a provisional patent filed. Added butter fat is providing improvements in the baking of the cheese on the pizza. This is achieved by increasing moisture retention as free fat coats the outer surface, thus preventing charring. This results in an improved flowability, and stretching.

We will conduct sensory panels on low fat mozzarella cheese baked on a pizza during 2009.

### **Objective 2**

The manufacture of low fat string cheese has required numerous modification of the procedure and satisfactory results have not been produce.

## REFERENCES

- McMahon, D. J. and C. J. Oberg. 2000. Manufacture of lower-fat and fat-free pizza cheese. U.S. Patent No. 6,113,953. Issued September 5, 2000.
- Kiely, L. J.; McConnell, S. L.; Kindstedt, P. S. 1992. Melting behavior of Mozzarella/non-Mozzarella cheese blends. *Cultured Dairy Products J.* 27(2):24, 26-29.
- Fife, R. L., D. J. McMahon and C. J. Oberg. 2002. Test for measuring the stretchability of melted cheese. *J. Dairy Sci.* 85:3539-3545.
- Perry, D., D.J. McMahon and C.J. Oberg. 1998. Manufacture of low fat mozzarella cheese using exopolysaccharide-producing starter cultures. *J. Dairy Sci.* 81:563-566
- Perry, D., D.J. McMahon and C.J. Oberg. 1997. Effect of exopolysaccharide-producing cultures on moisture retention in low fat mozzarella cheese. *J. Dairy Sci.* 80:799-805.

# Low Fat Cheese Texture Enhancement Portfolio

Determine the sensory texture properties of low fat cheese made using mechanical breakup of the cheese/curd structure

DONALD J. MCMAHON: Utah State University

## ABSTRACT

Low fat cheeses will be made at three research centers using currently available mechanical size reduction techniques followed by pressing into a block. Each institution (University of Wisconsin, California State Polytechnic University, and Utah State University) will make two blocks of cheese, and send them for testing at North Carolina State University during the first quarter 2008. Methods used for making the cheese will be based on methods that are currently been used at each institution as part of other research projects. It will include use of fresh cheese curd as well as size reduction of aged cheese. Block forming will include pressing in cheese hoops as well as stuffing into casings.

After initial manufacture of the best guess cheeses, various parameters including type of cheese used, conditions of size reduction, and conditions for block forming will be investigated.

## MATERIAL AND METHODS

At each of three institutions (University of Wisconsin, Utah State University, and California Polytechnic State University) manufacture two blocks of low fat cheese using mechanical size reduction and pressing. The techniques used will depend on equipment available and previous cheesemaking experiences. These cheeses are to be made during January to February 2008 so that texture analysis can be performed for cheese one month after pressing, prior to March 31<sup>st</sup>. Proximate analysis of the cheese (moisture, fat, salt, pH, protein, calcium) was determined and cheese stored at a temperature based on past experience at each institution.

Cheeses was sent to North Carolina State University for sensory texture profiling at 1 mo and 3 mo of age (after mechanical treatment), and shared between the three institutions for comparison of texture by the PIs. A sample of each cheese will be retained for further storage and examined in promising results are obtained at 1 and 3 months.

At each of three institutions (University of Wisconsin, Utah State University, and California Polytechnic State University) manufacture two blocks of low fat cheese using mechanical size reduction and pressing. The techniques used will depend on equipment available and previous cheesemaking experiences. These cheeses are to be made during January to February 2008 so that texture analysis can be performed for cheese one month after pressing, prior to March 31<sup>st</sup>. Proximate analysis of the cheese (moisture, fat, salt, pH, protein, calcium) will be determined and cheese will be stored at a temperature based on past experience at each institution.

Cheeses will be sent to North Carolina State University for sensory texture profiling at 1 mo and 3 mo of age (after mechanical treatment), and shared between the three institutions for comparison of texture by the PIs. A sample of each cheese will be retained for further storage and examined if promising results are obtained at 1 and 3 months.

## RESULTS

### *Panelists*

The eight panelists used are members of the existing contract descriptive analysis panel in the Department of Food, Bioprocessing, and Nutrition Sciences at North Carolina State University. This panel has been trained in the

Spectrum™ method of descriptive analysis for generation of qualitative and quantitative data and has logged several hundred hours of experience with texture profiling of cheese.

### **Sensory Evaluation Method**

Descriptive analysis was conducted utilizing a 15-point product specific scale for texture. The panel members used the lexicon (Table 1) and ballot with the aid of references: Mozzarella, Muenster, sharp Cheddar and parmesan cheeses. Evaluation techniques were standardized. Samples were evaluated in duplicate.

#### **Sample Preparation**

Samples were received and kept at 3 to 4°C. Within 24 hours of evaluation samples were cut into ~1/2-inch cubes and placed in 4-ounce lidded portion cups labeled with 3-digit random codes. Samples were tempered to 18-20°C prior to evaluation. "One month" cheeses were received at 7 weeks age, respectively.

#### **Statistical Analysis**

Data were evaluated by analysis of variance with means separation using the general linear model of SAS version 9.1 (Cary, NC).

### **Hand Evaluation Terms**

Procedure: Press the sample between your thumb and first two fingers.

**Hand firmness** – Press your fingers completely through the sample. Evaluate the amount of force required to completely compress the sample.

**Hand springiness** – Press the sample between your fingers until it is depressed 30%. (If you cannot depress the sample 30%, depress it as much as possible.) Evaluate the *total amount* of recovery of the sample. Note: If the sample fractures as it is depressed, the sample is *not* springy!

**Rate of Recovery**: Press the sample between your fingers until it is depressed 30%. (If you cannot depress the sample 30%, depress it as much as possible.) Evaluate the *rate of recovery* (i.e. how long it takes to recover to the original shape). Note: If the sample fractures as it is depressed, the sample does *not* recover!

### **Mouth Evaluation Terms, First Bite**

Procedure: Using your molars, take one complete bite through the sample.

**Firmness**: Evaluate the amount of force that is required to completely bite through the sample.

**Fracturability**: Evaluate the amount of fracturability in sample after biting.

Procedure: Chew the sample 5 times before evaluating the chewed mass.

**Degree of Breakdown**: Evaluate how much the sample breaks down during mastication. (Formerly Meltability-

Rate the amount of "melting" or "dissolvability" in the sample.)

**Cohesiveness**: Evaluate how well the mass sticks together.

**Adhesiveness**: Evaluate how much the chewed mass sticks to your mouth surfaces.

**Smoothness of Mass**: Evaluate how smooth the chewed mass surface is (i.e. evaluate for gritty or grainy particles).

### **Mouth Evaluation Terms, Residual**

Procedure: Expectorate sample and evaluate the residue in your mouth.

**Smoothness of Mouth Coating**: Evaluate the degree of smoothness felt in your mouth after expectorating.

## UTAH STATE UNIVERSITY CHEESES

	Trt 1 LF cheese, comminuted in bowl chopper, pressed into 20- lb. block	Trt 2 LF Cheese, Made with 1.5% fat, then comminuted, butter added, pressed	Control LF Cheese Non- mechanically treated
% moisture	54.4	55.6	51.2
% fat	6.0	4.5	6.0
% salt	2.39	2.87	2.12
pH	5.29	5.41	5.29
Make date	01/29/2008	02/05/2008	01/29/2008

### 1 month sensory evaluation

	LF Trt 1	LF Trt 2	LF control	FF Control System- atic Project 2-wk old	LF Control System- atic Project 2-wk old
hand firmness	13.5a	9.2b	13.8a	10.4	12.6
hand springiness	14.7a	13.8b	14.7a	10.4	13.9
hand rate of recovery	14.4a	13.5b	14.6a	13.1	13.3
firmness	10.8b	8.6c	11.8a	8.5	11.3
fracturability	8.8a	8.4a	7.5b	7.5	6.6
degree of breakdown	0.6b	1.2a	0.8b	3.5	3.1
cohesiveness	1.0a	0.8a	1.0a	3.8	3.3
adhesiveness	0.8b	1.4a	0.8b	3.8	3.3
smoothness of mass	0.7a	0.7a	0.6a	5.4	3.8
sm. of mouthcoating	1.0ab	0.7b	1.2a	5.3	5.8

means in a row followed by a different letter are significantly different  $p \leq 0.05$

### 3 month sensory evaluation

	LF Trt 1	LF Trt 2	FF Control Systematic Project 3 mo	LF Control Systematic Project 3 mo
hand firmness	12.9a	8.8b	9.2	12.3
hand springiness	14.6a	13.8b	10.1	13.3
hand rate of recovery	14.1a	13.3a	9.5	13.1
firmness	9.3a	6.7b	8.2	10.2
fracturability	6.0a	6.0a	5.6	6.2
degree of breakdown	1.1b	1.8a	9.4	3.9
cohesiveness	1.5a	1.9a	9.7	3.8
adhesiveness	2.1b	2.5a	8.2	3.8
smoothness of mass	1.6b	2.3a	8.4	4.5
sm. of mouthcoating	2.7a	2.8a	9.8	6.2

means in a row followed by a different letter are significantly different  $p \leq 0.05$

## DISCUSSION

Comminuting low fat cheese in a bowl chopper to particle size about 1 to 4 mm was not effective in improving the chew down properties of the cheese. The mechanically treated cheese still was far below the full fat cheese after 3 months of aging. These cheeses also had chew down properties below that obtained for low fat cheese in the previous systematic platform project.

# Low Fat Natural Cheese Strategic Platform Study - Objective 1

DONALD J. MCMAHON: Utah State University

## ABSTRACT

**Objective 1:** Manufacture four standardized cheeses (full fat, reduced fat and low fat) and six innovative “best-guess” low fat cheeses, store them for 9 months and distribute samples as needed for flavor, chemical, microbial, textural and functional analyses.

To make the scientific findings from this research applicable to a broad industry and to gain the most usable information from past (and future) research, low fat cheese with specified composition will be made at three different locations using standardized make procedures. A well-defined starter culture will be selected, and other than curd washing and slight pre-acidification, no other interventions will be used in making the reduced fat and low fat cheeses, that would drastically alter either cheese chemistry or microbial activities. To account for the influence of curd washing used in the low fat cheese, there will be two full fat cheddar cheeses made, one without using curd washing and the other using curd washing, so that any findings on flavor and texture can be referenced to the established scientific literature on cheddar cheese. The full fat and reduced fat standardized cheeses will be made at Utah State University and California Polytechnic State University, the standardized low fat cheese will be made at these two locations and at University of Wisconsin. In addition low fat cheeses will be made at each of the three institutions using various strategies proposed for improving the flavor and texture of low fat cheese. These may include such things as different starter cultures, addition of enzymes or other ingredients, specialized make procedures, etc., that will be chosen by the different institutions on a collaborative basis to avoid duplication of efforts. Four such cheeses will be made at the University of Wisconsin, and one each at Utah State University and California Polytechnic State University.

We will manufacture a total of fifteen cheeses in duplicate that will provide

- i. a basis for a systematic study of why the flavor and texture develops differently in low fat cheese compared to full fat cheese,
- ii. a benchmark for low fat cheese upon which all other research at the dairy research centers can be linked, and
- iii. a quick screening of possible interventions for improving the quality of low fat cheese suitable for table that will help to guide future research.

The cheeses will be stored at 8°C at their manufacturing location, and sampled made available to other investigators for analysis as needed at 2 wk, 6 wk, 3 mo, 6 mo and 9 mo storage times.

In developing high quality low fat cheeses, it is desirable to have flavor and textural properties as similar to full fat cheese as possible. This implies a complete understanding of the mechanisms responsible for flavor and texture. Determining *sensory texture* of full and low fat cheeses will establish which specific textural properties (sensory terms) are different among low fat and full fat cheeses. A complete analysis of fracture and rheological properties, in addition to pressure sensitive tack, is needed to know the molecular mechanisms responsible for textural. The instrumental measurement of these properties then need to be related to human sensory texture measurements to understand consumer perceptions of low fat cheese. To provide a linkage between this project and other cheese research the cheeses will also be tested using texture profile analysis.

**Key Deliverables.** Four standardized cheddar cheeses (full fat (control), full fat (washed), reduced fat and low fat) made in duplicate at two different locations using standardized make procedures, that will:

- i. allow a systematic study of why flavor develops differently in low fat cheese compared to full fat cheddar cheese,

- ii. increase our understanding of issues and possible solutions related to texture and functionality of low fat cheeses, and
- iii. provide a benchmark against which strategies and interventions for improving the quality of low fat cheddar cheese can be evaluated.

Six innovative low fat cheddar cheeses suitable for use as a table cheese, and made using the best currently available and tested strategies for improving the quality of low fat cheese, that will provide a quick determination of whether any of these “best-guess” cheeses work, and which directions to go for future

**Objective 4.** Understanding the molecular mechanisms responsible for textural properties of low fat cheese and their relationship to sensory perceptions and physical measurements of cheese texture.

## MATERIAL AND METHODS

### Objective 1.1

Step 1.1: A well defined *Lactococcus lactis* starter culture to be decided upon and obtained from a culture supplier.

Step 1.2: Confirm the composition targets for cheese at each fat level:

Cheese	%Fat	%FDB	%Moisture	%Salt	pH
Full Fat	31.5 – 33.5	51.0 – 52.5	36.0 – 38.0	1.50 – 1.70	5.10 – 5.30
Reduced Fat	16.0 – 18.0	32.0 – 34.0	48.0 – 50.0	1.70 – 1.90	5.10 – 5.30
Low Fat	5.0 – 6.0	10.5 – 13.0	52.0 – 54.0	1.90 – 2.10	5.10 – 5.30

Step 1.3: Manufacture at Utah State University, full-fat (control) cheddar cheese using the amount of starter culture estimated to produce an approx. 4-hour make time (set to salt) with a standard stirred-curd process so as to be within the target composition. Initial testing of make procedures will be using 300-lb of milk one 22.5 lb block of cheese. Modify this make procedure to use a stirred and washed curd method to produce a cheese with the same target composition. Moisture will be measured by microwave oven, salt by chloride analysis, fat by Babcock, pH by glass electrode, and protein by combustion N.

Step 1.4: Manufacture at Utah State University using 300-lb milk per vat, reduced fat and low cheeses using the same starter culture (Objective 1) using a stirred/washed curd method with compositions as described above. Modify the make procedure if necessary to reach the target composition.

Step 1.5: The standardized make procedures to be established and adapted for use at all three institutions based on cheese making equipment at each location.

### Objective 1.2

Step 2.1: The four standardized cheeses (full fat-control, full fat-washed, reduced fat, and low fat cheeses) to be manufactured at USU and CPSU, and the standardized low fat cheese to be manufactured at UW, based upon available cheesemaking times, and in coordination with the other investigators. Cheese at USU to be made using 1500-lb horizontal cheese vats, cheese at UW to be made using 600-lb open vats, cheese at CPSU to be made using 1,000-lb Kusel open vats. Cheese making to be scheduled on separate weeks at each institution so that testing for flavor, microbial, texture and functionality can be evenly distributed. Approximate planned times are the weeks beginning:

Replicate	Univ Wisconsin	Utah State Univ	Cal Poly SLO	make-up*
1	Sep 25, 2006	Sep 18, 2006	Oct 2, 2006	Oct 9, 2006
2	Oct 16, 2006	Oct 30, 2006	Oct 23, 2006	Nov 6, 2006

\*If any of the standardized cheeses do not meet the specified composition targets they will need to be remade.

Step 2.2: After manufacture the cheeses are sampled for proximate analysis to ensure they meet the specified targets, vacuum packaged and stored at 8±1°C. One week after manufacture, the cheese blocks are cut into the required number of 1-lb and 2-lb blocks, vacuumed packaged, the first set of samples



- shipped to other investigators as needed, with the remainder returned to  $8 \pm 1^\circ\text{C}$  storage. Further
- samples are shipped as needed, one week prior to 6 wk, 3 mo, 6 mo, and 9 mo timepoints.

### Objective 1.3

- Step 3.1: In consultation among the expert group, six “best-guess” strategies for potentially improving the quality of low fat cheese to be used as a table cheese are selected based on past research on other reduced fat, low fat and nonfat cheeses. This may include use of adjunct cultures, enzymes or other additives, different make procedures and/or different aging regimes.
- Step 3.2: Four of these cheeses to be made at UW, and one each at CPSU and USU using the above time line.
- Step 3.3: Each of the “best-guess” low fat cheeses to be stored at its preferred aging temperature, and then analyzed, packaged, and sampled on the same timeline as the standardized cheeses as outlined above.

### Objective 4.1

In developing high quality low fat cheeses, it is desirable to have flavor and textural properties as similar to full fat cheese as possible. This implies a complete understanding of the mechanisms responsible for flavor and texture. Determining sensory texture of full and low fat cheeses will establish which specific textural properties (sensory terms) are different among low fat and full fat cheeses. Previous research in the Foegeding laboratory on commercial low fat cheeses (Gwartney et al., 2002), and the effect of aging on Mozzarella and Monterey jack cheese (Brown et al., 2003), established the following general relationships between sensory texture and mechanical properties:

*Firmness, hardness and fracture terms* – These sensory terms are reflected in fracture properties of fracture stress and fracture modulus (fracture stress/fracture strain), and rheological properties of  $G'$  (elastic modulus) and  $J_{\max}$  (maximum compliance;  $\sim 1/G'$ ). These properties reflect the strength of the gel network and the amount of gel network. For example, when low fat cheese is made by decreasing fat and increasing protein, cheese firmness, fracture stress, fracture modulus and  $G'$  increase as a reflection of a greater amount of protein network.

*Chew-down terms of cohesiveness, adhesiveness and smoothness* – These tend to decrease with increasing protein content; however, the aging process causes an increase in these properties. The structural origin of these properties is more complex and they tend to reflect changes within the protein network and therefore are related to the fracture and viscoelastic properties of cheese. In addition, we have postulated that they are related to pressure-sensitive adhesion properties, which can be measured using a tack test.

Considering the aforementioned discussion, a complete analysis of fracture and rheological properties, in addition to pressure sensitive tack, is needed to understand the molecular mechanisms responsible for textural properties. Therefore we will use the following series of tests.

#### Rheological, fracture and pressure sensitive tack tests

##### a. Small-deformation oscillatory test using a controlled-strain instrument.

A Bohlin VOR rheometer (Bohlin Rheologi AB, Lund, Sweden) will be used to conduct frequency sweeps for measurement of mechanical spectra. Cheese slices, 2 mm thick, will be placed between 30 mm diameter serrated parallel plates. The initial test will be a strain sweep to determine the linear viscoelastic region. Once that is established, viscoelastic properties ( $G'$ , elastic stress/strain;  $G''$ , viscous stress/strain; and phase angle) will be measured within the linear viscoelastic region over a frequency range of 0.001 to 20 Hz at a controlled temperature. Mechanical spectra analysis at a standard 1% strain will accordingly generate shear strain rates from  $1 \times 10^{-5}$  to  $0.2 \text{ s}^{-1}$ .

##### b. Small-deformation oscillatory test using a controlled-stress instrument.

A Stresstech controlled-stress rheometer (Rheological Instruments AB, Lund, Sweden), with 20 mm diameter parallel plates will be used to conduct creep and recovery tests. Since serrated plates are not available, slip will be prevented by gluing the sample to the plates with cyanoacrylate glue. Creep tests involve applying a constant stress and measuring the resultant strain over time. After a given period of “creep,” the stress is removed and the “creep recovery” is measured as a function of time. Viscoelastic properties measured during creep will be instantaneous compliance ( $J_0$ , compliance at time zero) and maximum compliance ( $J_{\max}$ , peak compliance), where compliance is stress/strain or  $1/G'$ . Properties measured during recovery will be retardation time ( $t_{\text{ret}}$ , time required in recovery for the cheese to reach 63.2% of its final strain) and percent creep recovery (difference between  $J_{\max}$  and maximum recovered compliance expressed as a percentage of  $J_{\max}$ ).

### *c. Torsional large-deformation and fracture test*

Cylindrical cheese samples will be removed using a 19 mm internal diameter cork borer. Prior to torsion testing, cheese cylinders will be cut into 28.7 mm long cylinders, and notched styrene disposable disks will be glued with cyanoacrylate glue (Quik Tite, Loc Tite, Rocky Hill, CT) to each end of the samples. Next, the gel samples will be ground into a capstan shape, with a center diameter of 10 mm using a precision milling machine (Gel Consultants, Raleigh, NC USA). The gels will be mounted onto a Haake VT550 viscometer (Paramus, NJ) or twisting to failure at a specified rotational speed. During deformation of the samples, the time and corresponding torque is measured, and these data are used to calculate shear stress and strain using a modified version of the calculations originally proposed by Diehl et al. (1979). Torsion analysis will be performed on 4 to 6 samples at each treatment. Sample rotational speeds will vary to create shear rates ranging between  $1 \times 10^3$  to  $5.5 \text{ s}^{-1}$ . Besides fracture stress and strain, additional analysis will be performed on the entire force-deformation curve. First, the shape of the curve will be fit using various non-linear models to determine the degree of strain hardening or strain weakening. Second, force-deformation data will be integrated to compute areas under specific regions of the curve to correspond with stored and dissipated energies during the failure process.

### *d. Pressure sensitive tack testing.*

The adhesiveness of the cheese will be measured using a TAX-T2 Texture Analyzer (Texture Technologies, Scarsdale, NY) by the following approach. The cheese is cut into 4 cm squares and sliced to 6.35 cm thick by a modified wire cheese slicer. The cheese is then placed on a platform below a flat, 13 mm diameter, stainless steel probe, and the probe is brought to the surface of the cheese at a speed of 1 mm/s. Once the probe reaches the surface of the cheese, a force of 2.0 N is applied and held on the surface for five seconds. After five seconds, the probe is removed at a speed of 0.1 mm/s. As the probe is removed, the force-distance relationship is recorded. The maximum force needed to separate the probe from the surface as well as the energy required to separate the probe from the cheese are determined. The tack force is determined as the maximum force recorded during separation. The tack energy is measured from the area under the force-distance curve.

Treatment-associated changes in rheological and fracture properties will be analyzed in accordance with relevant polymer and colloidal models. This comparison should provide some insight into molecular mechanism associated with changes in cheese texture. In addition, correlations between mechanical (rheological and fracture) properties and sensory texture will be determined.

### **Objective 4.2**

Texture profile analysis (TPA) will be performed at USU and CPSU so as to provide a basis for relating other cheese research to the low fat cheeses produced during this study. At CPSU, TPA will be measured using a TAT22 texture analyzer (Texture Technologies Corp., NY) with a load of 500 N and a flat head plunger. Each sample will be cut into a cube shape ( $2 \text{ cm} \times 2 \text{ cm} \times 2 \text{ cm}$ ) will be subjected to 50 and 70% compression for measurement of textural parameters (hardness, springiness, cohesiveness, and gumminess) as described by Bourne et al. (1978). At USU, TPA will be measured using a TATX+ texture analyzer (Texture Technologies Corp., NY) with a 5-kg load cell and a flat head plunger. Samples will be cut into cylinders (height 2.0 cm, diameter 1.6 cm), obtained using a stainless steel borer and brought to room temperature then subjected to 25 and 60% two-bite compression for measurement of the same textural parameters.

A melt flowability test will be performed at USU on the cheeses using a UW-meltmeter. Cheese samples will be cut into disks (thickness 7 mm, diameter 30 mm), and then heated to  $65^\circ\text{C}$  and the change in height measured over 20 s as a constant force of 0.33 N is applied on top of the cheese disk.

Proteolysis in cheese will be studied at CPSU by urea-polyacrylamide gel electrophoresis (Urea-PAGE) of cheeses, determination of water-soluble N (WSN) and PTA-soluble N in the cheese and peptide profiles in the water-soluble N fraction by HPLC (Farkye et al., 1995). Sample preparation and electrophoresis will be done as described by Farkye et al., (1991). Extraction of WSN will be done as described by Kuchroo and Fox (1982) as follows: ten g cheese will be homogenized in 20 ml de-ionized water using a Stomacher. The homogenized mixture will be centrifuged and the supernatant analyzed for nitrogen content by Kjeldahl. Peptide profile of WSN extract will be analyzed by reversed-phase HPLC on an Ultrasphere™ (Beckman Instruments, Fullerton, CA) C-18,  $4.6 \times 45 \text{ mm}$  guard column attached to an Ultrasphere™ C-18,  $4.6 \times 250 \text{ mm}$  analytical column with 5 mm particle size. The System Gold™ (Beckman Instruments, Fullerton, CA) HPLC system consisting of two model 110B solvent delivery pumps and a diode array detector controlled with the Gold Nuveaux software will be used. Peptide peaks will be detected at a wavelength of 230 nm.

### **Objective 4.3**

Sensory perception of texture will be analyzed at NCSU on the set of cheeses made at USU. Briefly, cheese texture will be evaluated by trained panelists by both hand and mouth terms using a previously defined and anchored descriptive texture language for hard and semi-hard cheeses. The scaling technique is a 15-point product specific anchored and referenced scale. Panelists are provided with cheese references during evaluations in order to minimize variability. At each session, no more than 6 samples are evaluated. Each cheese is cut into 1.27 cm<sup>3</sup> cubes and presented at room temperature in lidded soufflé cups (to minimize moisture loss) with three-digit random codes. At each session, panelists have free access to spring water and unsalted crackers as well as appropriate references. Each cheese is evaluated in duplicate by each panelist.

## **RESULTS**

### **Objective 1**

Standardized Make Procedures:

#### ***Full Fat Cheese - Standard:***

Milk Fat Content: ca. 3.6% (True Protein/Fat = 0.83)  
Pasteurize: 161 F for 15 sec.  
Starter: 110% of recommended level of M70, set to salt time of ca. 3 h.  
Ripen for 45 min  
Setting temp: 88 F.  
Color: 60 ml single strength annatto per 1000 lb milk  
Calcium No calcium added  
Rennet: 40 ml DS chymosin per 1000 lb milk  
(adjust level based on rennet activity for 30 min cut at regular firmness)  
Cutting: standard cutting, healing, stirring for cheddar cheese  
Set to Start Cook: 50 min  
Cooking: Heat to 98 F over 25 min  
Drain pH 6.0  
Set to Drain: ca. 2 h 30 min  
Dry stir: Until curd pH = 5.45  
Set to Salt: ca. 3 h 10 min  
Add salt: 0.24 lb salt per 10 lb of curd, two applications 5 min apart.  
Hoop: 25.5 lb curd per 20-lb hoop.  
Press: 15 psi (calculated pressure on cheese) overnight.  
Moisture: 36.5 + 1.0%  
Salt: 1.60 + 1.0%  
Fat: 32.5 + 1.0% (FDB: 51.0 – 52.5%)  
pH: 5.20 + 0.10

#### ***Full Fat Cheese - Washed:***

Milk Fat Content: ca. 3.6% (True Protein/Fat = 0.83)  
Pasteurize: 161 F for 15 sec.  
Starter: 125% of recommended level of M70, set to salt time of ca. 3 h.  
Ripen for 45 min. Setting temp: 88 F.  
Color: 60 ml single strength annatto per 1000 lb milk  
No calcium added  
Rennet: 40 ml DS chymosin per 1000 lb milk  
(adjust level based on rennet activity for 30 min cut)  
Cutting: standard cutting, healing, stirring for cheddar cheese  
Set to Start Cook: 50 min  
Cooking: Heat to 98 F over 25 min  
Drain pH 6.0

Set to Drain: ca. 2 h 25 min  
After draining: add warm water at 95 F (10% of original milk volume).  
Leave wash water on for 5 min then drain.  
Dry stir: Until curd pH =5.45  
Set to Salt: ca. 3 h 10 min  
Add salt: 0.24 lb salt per 10 lb of curd, two applications 5 min apart.  
Hoop: 25.5 lb curd per 20-lb hoop.  
Press: 15 psi (calculated pressure on cheese) overnight.  
Moisture: 36.5 + 1.0%  
Salt: 1.60 + 1.0%  
Fat: 32.5 + 1.0% (FDB: 51.0 – 52.5%)  
pH: 5.20 + 0.10

***Reduced Fat Cheese - Standard:***

Milk Fat Content: ca. 1.5% (True Protein/Fat = 1.9)  
Pasteurize: 161 F for 15 sec.  
Starter: 125% of recommended level of M70, set to salt time of ca. 3 h.  
Ripen for 45 min. Setting temp: 88 F.  
Color: 60 ml single strength annatto per 1000 lb milk  
Calcium: No calcium added  
Rennet: 40 ml DS chymosin per 1000 lb milk  
(adjust level based on rennet activity for 30 min cut at regular firmness)  
Add same amount of rennet as used for full fat cheddar  
Cutting: 1/2" knives cut in vertical direction only  
Set to Start Cook: 60 min  
Cooking: Heat to 98 F over 20 min  
Drain pH 6.0  
Set to Drain: ca. 2 h 30 min  
Washing: After draining add cold water (ca. 55 - 60 F, sufficient to lower curd temperature to 78 F  
(ca. 10 % of original milk volume).  
Leave wash water on for 10 min then drain.  
Dry stir: Until curd pH =5.75  
Set to Salt: 3 h 5 min  
Add salt: 0.25 lb salt per 10 lb of curd, two applications 5 min apart.  
Hoop: 26 lb per 20-lb hoop.  
Press: ca. 8 psi (calculated pressure on cheese) overnight.  
Moisture: 47.0 + 1.0%  
Salt: 1.80 + 0.10%  
Fat: 17.0 + 1.0% (FDB: 30 - 33%)  
pH: 5.20 + 0.10

***Low Fat Cheese - Standard:***

Milk Fat Content: 0.55% (True Protein/Fat = 4.8-5.8)  
Pasteurize: 161 F for 15 sec.  
Pre-acidify: Add vinegar until milk pH = 6.25  
ca. 4000 ml per 1000 ml of cold milk then warm the milk to 90 F.  
Starter: 125% of recommended level of M70, set to salt time of ca. 3 h.  
Ripen for 45 min. Setting temp: 88 F.  
Color: 60 ml single strength annatto per 1000 lb milk  
Calcium No calcium added  
Rennet: 40 ml DS chymosin per 1000 lb milk  
(adjust level based on rennet activity for 30 min cut at regular firmness). Add same amount of  
rennet as used for full fat cheddar  
Cutting: 1/2" knives cut in vertical direction only  
Set to Start Cook: 50 min

Cooking: Heat to 96 F over 15 min  
 Drain pH 6.00  
 Set to Drain: ca. 1 h 40 min  
 Washing After draining: add cold water at 55-60 F, sufficient to lower curd temperature to 78 F  
 (ca. 10 of original milk volume).  
 Leave wash water on for 10 min then drain.  
 Dry stir: Until curd pH = 5.80  
 Set to Salt: 3 h 5 min  
 Add salt: 0.26 lb salt per 10 lb of curd, two applications 5 min apart.  
 Hoop: 27 lb per 20-lb hoop.  
 Press: ca. 8 psi (calculated pressure on cheese) overnight.  
 Moisture: 53.0 + 1.0%  
 Salt: 2.00 + 0.10%  
 Fat: 5.50 + 0.05%  
 pH: 5.20 + 0.10

Cheeses were made on the following dates with the composition as shown, samples were sent to our collaborators for analysis. The remainder of the cheese was stored at 8°C.

FAT LEVEL TREATMENT	MAKE DATE	% MOISTURE	% FAT	% SALT	pH
LOW FAT STANDARD 1	10/10/06	53.68	5.5	1.8	5.1
LOW FAT 850/LH32 1	10/11/06	54.3	5.07	1.92	5.07
RED FAT STANDARD 1	10/12/06	48.78	15.25	1.78	5.15
FULL FAT STANDARD 1	10/3/06	38.6	31.5	1.61	5.22
FULL FAT WASHED 1	10/4/06	38.4	31.75	1.6	5.2
LOW FAT STANDARD 2	11/1/06	53.13	4.5	1.53	5.3
LOW FAT 850/LH32 2	10/31/06	54.54	4	1.48	5.21
RED FAT STANDARD 2	11/28/06	47.98	16.5	1.95	5.14
FULL FAT STANDARD 2	11/15/06	47.98	32	1.83	5.2
FULL FAT WASHED 2	11/15/06	37.7	32.2	1.77	5.16

Cheeses have been sent for testing at 2 weeks, 6 weeks and 3, 6 and 9 months. This completes the Utah State University portion of Objective 1. Fig. 3. TPA graph of low fat cheese (standard) tested after 2 wk of storage at 8 C.

For the reduced fat and low fat cheeses, the stress induced by compression of the cheese sample continues to increase (exponentially) until the 60% compression is reached. These cheeses retain much of their internal integrity after the compression, and the sample returns to 80% or more of its original height once the strain is released. These cheeses thus have a higher cohesiveness value. This relates to the physical observations about the texture deficiency of low fat cheese in that they are too springy or rubbery, and do not break down in the mouth the same way as a full fat cheese. The interpretation of the TPA data is that to correct this problem there needs to be introduced into the low fat cheese protein matrix, points of interruption to the protein matrix that will allow slippage (fracture) to occur as the cheese is compressed.

Calcium, phosphate and protein were determined by ICP spectroscopy and combustion N analysis. Using a washing step in making the full fat cheese did not appear to influence protein, calcium or phosphate content of the cheese. It is interesting that there was a decrease in the phosphate to calcium ratio of the reduced fat cheese compared to the full fat cheese, and a further reduction in the low fat cheese. The reason for this is not known. The reduced fat cheese had similar Ca:protein ratios as the full fat cheeses while the low fat cheeses showed a decrease in Ca:protein ratio. This can be attributed to the pre-acidification step used in making the low fat cheeses and consequent solubilization and increased loss of calcium (in proportion to protein) in the whey upon draining the curd. The total amount of calcium in the low fat cheese was however higher than in the full fat cheese.

#### Objective 4

The differences in textural properties of the cheeses can be seen by the shape of the TPA force versus time curves as shown below. These are an indication of how the cheese responds to increasing strain as it is compressed. Full fat cheddar cheeses typically undergo fracturing during compression which is observed as a linear increase in stress as strain increases, a drop in stress at a specific point (as shown in Fig 1 at arrow 3), or a flattening of the force-time curve as stress is released. Figure 1 is a TPA graph of the full fat standard cheese tested using 60% compression, and shows a fracture strain occurring at 48% compression. Typically, as the cheese ages the fracture occurs at less strain. Such fracturing results in the sample regaining very little of its original height, and the second compression peak being much smaller than the first peak, this is measured as the cheese having a low cohesiveness value.

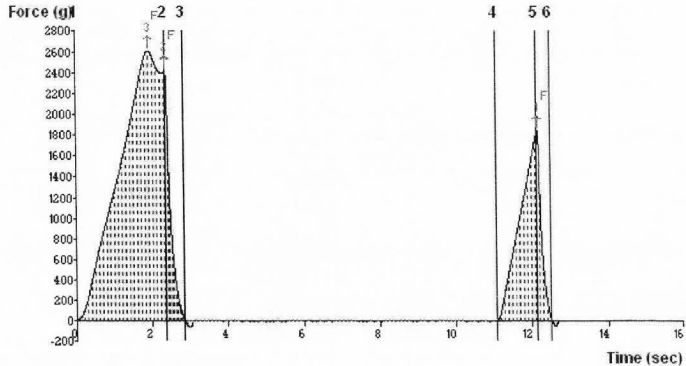


Figure 1. TPA graph of full fat cheddar cheese tested after 3 months of aging at 8 C.

When the fat content is reduced, the cheese is less likely to fracture and the cheeses having more cohesiveness. This is apparent from the shape of the TPA curves of the reduced fat and low fat cheeses shown in Fig. 2 and Fig. 3 respectively.

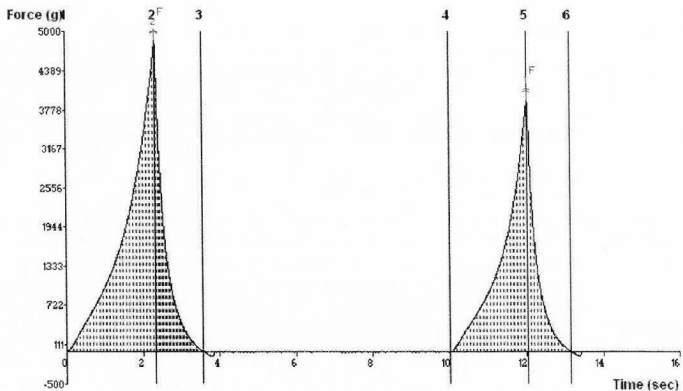


Figure 2. TPA graph of reduced fat cheese tested after 6 wk of storage at 8 C.

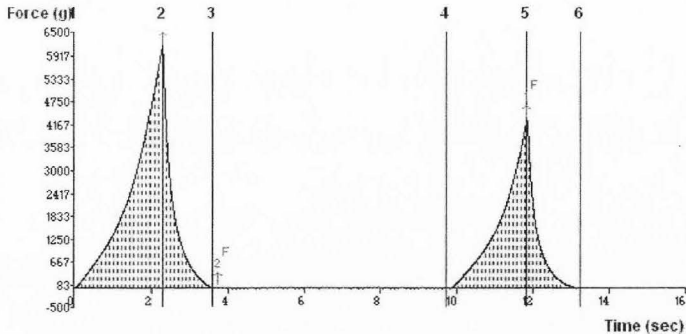


Figure 3. TPA graph of low fat cheese (standard) tested after 2 wk of storage at 8 C. For the reduced fat and low fat cheeses, the stress induced by compression of the cheese sample continues to increase (exponentially) until the 60% compression is reached. These cheeses retain much of their internal integrity after the compression, and the sample returns to 80% or more of its original height once the strain is released. These cheeses thus have a higher cohesiveness value. This relates to the physical observations about the texture deficiency of low fat cheese in that they are too springy or rubbery, and do not break down in the mouth the same way as a full fat cheese. The interpretation of the TPA data is that to correct this problem there needs to be introduced into the low fat cheese protein matrix, points of interruption to the protein matrix that will allow slippage (fracture) to occur as the cheese is compressed.

Calcium, phosphate and protein were determined by ICP spectroscopy and combustion N analysis. Using a washing step in making the full fat cheese did not appear to influence protein, calcium or phosphate content of the cheese. It is interesting that there was a decrease in the phosphate to calcium ratio of the reduced fat cheese compared to the full fat cheese, and a further reduction in the low fat cheese. The reason for this is not known. The reduced fat cheese had similar Ca:protein ratios as the full fat cheeses while the low fat cheeses showed a decrease in Ca:protein ratio. This can be attributed to the pre-acidification step used in making the low fat cheeses and consequent solubilization and increased loss of calcium (in proportion to protein) in the whey upon draining the curd. The total amount of calcium in the low fat cheese was however higher than in the full fat cheese.

	Protein	Ca	H <sub>2</sub> PO <sub>4</sub>	H <sub>2</sub> PO <sub>4</sub> /Ca	Ca/Protein
	%	%	%		
UFFST1	24.6	0.69	1.78	2.58	0.0281
UFFST2	24.6	0.66	1.75	2.65	0.0269
UFFWA1	24.9	0.71	1.91	2.69	0.0285
UFFWA2	24.6	0.61	1.88	3.08	0.0248
URFST1	29.9	0.91	1.60	1.75	0.0304
URFST2	29.3	0.79	1.60	2.02	0.0270
ULFST1	35.0	0.84	1.44	1.71	0.0240
ULFST2	33.4	0.84	1.38	1.64	0.0252
ULFSP1	33.9	0.76	1.50	1.98	0.0224
ULFSP2	31.8	0.89	1.28	1.44	0.0280

Data collection on texture properties of the cheeses made in Utah was completed.

## DISCUSSION

### *Objective 1*

The production of multiple cheeses at varying fat levels becomes very complex as the fat is eliminated the percent by weight of protein increases. As this occurs the moisture content also increases, resulting in a soft unusable cheese. Extensive manipulation of the cheese production must be undertaken to obtain a viable cheese at the various fat levels. After many variations to the standard make procedures, three variants have been generated.

### *Objective 2*

TPA data measures the physical structure of the cheese, as the fat is removed from the cheese the protein matrix becomes more interconnected with fewer disruptions in the matrix. This leads to a higher elasticity in the cheese and appears in the TPA results of the first and second peaks being very similar. Whereas the full fat cheese upon compression, micro-fracturing occurs along the discontinuities caused by the fat, resulting in reduced elasticity and more released energy. As low fat cheese is modified to enhance the textural quality, the TPA can record the relative elasticity. With such data, measured over time, several physical characteristics can be measured.

The major modifications in the finalized make procedures for the different fat levels of cheese are the pre-acidification of the low fat cheese and the higher pH obtained during dry stirring for the reduced fat and the low fat cheese. The pre-acidification may be responsible for the reduction of calcium vs. phosphate, and the reduction of phosphate vs. protein in the low fat cheese. With the lower initial pH, the bonding of the calcium phosphate to the casein mycelle would be weakened, resulting in the higher losses.

## CONCLUSION

### *Objective 1*

Three successful modifications of make protocols have been generated and are being evaluated. Data collection on texture properties of the cheeses made in Utah was completed.

### *Objective 2*

Analysis of the four cheeses from the new make procedures shows measurable changes in both physical and chemical properties.

## REFERENCES

- Bourne, M.C. 1978. Texture profile analysis. *Food Technology*. 32(7):63
- Brown, J.A., Foegeding, E.A., Daubert, C.R., Drake, M.A. and Gumpertz, M. 2003. Relationships among rheological and sensorial properties of young cheese. *J. Dairy Science* 86:3054-3067.
- Diehl, K.C., Hamann, D.D. and Whitfield, J.K. 1979. Structural failure in selected raw fruits and vegetables. *J. Texture Studies* 10:371-400.
- Farkye, N.Y., L.J. Kiely, R.D. Allshouse and P.S. Kindstedt. 1991. Proteolysis in Mozzarella cheese during refrigerated storage. *J. Dairy Sci.* 74:1433-1438
- Farkye, N.Y., Madkor, S.A. and Atkins, H.G. 1995. Proteolytic abilities of some lactic acid bacteria in a model cheese system. *Int. Dairy J.* 5:715-725.
- Gwartney, E.A., Foegeding, E.A. and Larick, D.K. 2002. Texture of commercial full-fat and reduced-fat cheese. *J. Food Science* 67:812-816.
- Kuchroo, C.N. and Fox, P.F. 1982. Soluble nitrogen in Cheddar cheese: comparison of extraction procedures. *Milchwissenschaft*, 37:331-335.



# Improve the Flavor of Low Fat Cheese by Adding Innovative Cultures and/or Flavoring Systems

Improving the flavor of low fat cheese by using cultures not usually used when making cheddar cheese and by adding flavorings while the cheese is being manufactured

DONALD J. MCMAHON: Utah State University

JEFF BROADBENT: Utah State University

SILVANA MARTINI: Utah State University

## ABSTRACT

A two pronged approach will be used to investigate ways to get a better flavored low fat cheese that includes the use of adjunct starter cultures that have been shown to increase, or provide specific flavor attributes to, the flavor level of other cheeses, as well as direct addition of complete flavor systems to the cheese curd prior to pressing the curd into a block. For the culture portion of the study, a variety of cultures will be tested, and the resultant cheese will be screened for flavor development, and those cheeses that show an improvement in flavor will be subjected to sensory analysis and those that do not will be discarded. Two sensory tests will be performed to obtain a description of the flavor profile, as well obtaining information on how well consumers like the low fat cheeses. The other part of the study involves working with flavor companies, and to use their expertise with flavors to obtain a flavoring system that provides the missing components of cheddar cheese flavor that are needed to produce a low fat cheese that is liked and acceptable to consumers.

## INTRODUCTION

Low fat cheese has a very mild flavor, the addition of various nontraditional bacterial cultures and/or additional flavor components may reduce this defect. Adjunct cultures that increase flavor production in cheese are readily available and have been used in full fat cheddar cheese. Several flavor enhancing components are commercially available and have been shown to enhance flavors and reduce ripening time in full fat cheese. The addition of such flavors has not been explored previously with low fat cheese. By utilizing nontraditional adjunct cultures and flavor additives a more expectable low fat cheese could be produced.

## MATERIAL AND METHODS

### *Objective 1*

The use of the addition of various nontraditional bacterial cultures used for non-cheddar varieties in a low fat cheese system was examined to determine if they have a positive effect on cheese flavor. The cheeses was screened for flavor improvement compared to the flavor of the cheese manufactured as part of the Low Fat Strategic Flavor Platform project, by cheese experts at Utah State University when the cheeses are 1mo old. If an improvement in flavor is evident then cheeses will be tested using consumer preference sensory evaluation when they are 2 month old. Any cheeses with high consumer preference scores will be sent to North Carolina State University for flavor profile analysis at 3 mo of age, and a consumer preference and acceptance sensory panel will be conducted at Utah State University. Any cultures that demonstrate a positive influence on flavor development will be used in combination and the above cheesemaking and tests repeated. Some of the cultures that will be tested include:

- A *Lactobacillus helveticus* CNRZ32, that has been shown to be effective in increasing the cheese flavor intensity and eliminating bitterness in cheddar cheeses (Broadbent and Steele, 2006; Sridhar et al. 2005).

- A *Lactococcus lactis* culture that was recently shown to increase the nutty flavor intensity of cheddar cheese (Carunchia Whetstone et al. 2006). This would be important in a low fat cheese because such cheeses in the past have tended to have a more brothy flavor than typically occurs in cheddar cheese.
- Other cultures recommended by culture suppliers but not usually used for cheddar cheese such as *Brevibacterium linens*, *Propionibacteria*, yeasts.

### **Objective 2**

Low fat Cheddar cheese with 7% fat and moisture 53-54%, was manufactured at WDC facility. The cheese was aged for 2 weeks at 5C and then ground using Urschel grinder with 1.5 mm grind size head. Different flavors (Cargill Flavors Inc.) were added and mixed thoroughly, and repressed. An informal sensory panel was conducted in Western Dairy Center with 4 panelists for flavor profile analysis of cheeses added with different levels of concentrated flavors using a 9-point hedonic scale of degree of liking. The three digit random codes were used on the sample cups to minimize the sample bias by the judges in informal descriptive panel for flavor profile of the formulated cheeses. (Table 1).

Based on the feedback or results obtained from first set of observations, cheeses were formulated with different combination of flavors. Another sensory flavor profile test was done with 4 panelists and best four among 8 cheeses were kept for further aging (Table 2). Proximate analysis were conducted on the best flavored cheese on the 5<sup>th</sup> day after cheese manufacture.

## **RESULTS**

### **Objective 1**

Low fat Cheddar cheese with *S.thermophilus* culture added has been made, and no flavor improvement was observe. There was also no improvement in flavor by adding *Lb. helveticus* LH32. Similar effects were observed by Mark Johnson (WCDR), and thus this objective has been discontinued until work on adding flavors is completed.

### **Objective 2**

An informal panel of five people from Western Dairy Center evaluated all formulated cheese samples on a 9-point hedonic scale for degree of liking where 1 = dislike extremely; 2 = dislike very much; 3 = dislike moderately; 4 = dislike slightly; 5 = neither like nor dislike; 6 = like slightly; 7 = like moderately; 8 = like very much; and 9 = like extremely. The judges or the panelists were given paper ballot to fill in their responses for degree of liking and their comments for each sample. Unsalted crackers and distilled water were provided to cleanse their palate in between tasting the samples. The responses received from five panelists (Table 1).

The second and third sets of flavor added cheese were evaluated following the above protocol (Table 2) (Table 3).

<b>S.No.</b>	<b>Code</b>	<b>Formulation</b>
1	607	Control (Ground and pressed Cheddar cheese)
2	131	1.5% DF Cream powder 153
3	637	2.0% DF Cream Powder 153
4	923	2.5% DF Cream Powder 153
5	305	3.0% DF Cream Powder 153
6	929	1.5% 145-00153 plus 2.0% NCF Swiss Cheese Powder 264
7	912	1.5% 145-00153 plus 1.5% NCF Cheddar cheese powder 22
8	465	1.5% 145-00153 plus 1.5% CPF Cheddar Cheese Paste 231
9	525	2.5% NCF Swiss Cheese Powder 264
10	550	2.5% NCF Cheddar cheese powder 22
11	267	2.5% CPF Cheddar cheese paste 231

**Table 1:** Formulation of flavor to be added in ground low fat Cheddar cheese

Code	Average degree of liking	Comments
607	5-6	Buttery, late bitter, rancid, lacks Cheddar flavor
131	4-5	Sweet milk flavor, buttery smell, weird after taste
637	4.5	Buttery, same as 550, slight rosey
923	7	Slight rancid, sour, rosy+buttery, more cheese flavor
305	6.5	Steady cheese flavor, Sharp flavor, rosy, slight rancid, cheesy
929	5.5	Very intense rosy favor, smell like old milk, romano-type flavor, rancid and buttery
912	7	Rosy smell, cheese like, buttery, Cheddar
465	8	Buttery with slight rosy, rancid, sour, waxy, slight acid, slight aged, better knit, more Cheddar, creamy after taste
525	8	Buttery, slight sweet, slight rancid, cooked flavor
550	5-6	Sour, old milk, buttery smell, Romano flavor, Cheesy rancid after taste
267	2-3	Off-flavor, lacking cheesy flavor, slight burnt, barny flavor

**Table 2:** Evaluation of formulated cheese with innovative flavor, singly or in combination, by the panelist

Date	Formulation	Comments
<b>Cheese Make – 07/02/08</b>	Control	Dry
	0.5% 010 paste	Fishy
	0.75% 010 paste	Oily
	1.00% 010 paste	Rancid
	1.00% 153 powder	No flavor
<b>Ground – 07/17/08</b>	1.5% 153	Sour
	2.0% 153	Buttery
	1.00% 153+0.25% 010 paste	Sulfury
	0.5% 153+0.5% 010 paste	Cheesy
<b>Evaluated for flavor – 07/30/08</b>	0.5% 010 paste + 0.5% butter	Rancid
	1.00% 153 + 0.5% butter	Buttery
	0.5% 153 + 0.5% 010 paste + 0.5% butter	Buttery

**Table 3:** Formulation of cheese with flavor and feedback from informal panel

Date	Cheese Type	Moisture%	pH	Fat%	Salt%
07/29/08	Stirred Curd	50.0	5.30	8	1.60
	Control cheese	48.4	5.28	8	1.72
	Cheddar cheese with 0.5% 010+0.5% 153+0.5% of butter	50.8	5.18	8	1.80

**Table 4.** Proximate composition on day 1 of Low Fat Low salt Cheddar cheese for Innovative flavor project. The addition of 2.0% 153 powder to the cheese enhanced the flavor, increasing the acceptability. When 1.0% 153 powder was used in combination with 0.5% butter, the resultant cheese also had improved acceptability. The addition of the flavor compound had a wide range of flavor modifications, The addition of the flavor compounds had little to no effect on the proximate results compared to the control cheese.

## DISCUSSION

### *Objective 1*

The addition of adjunct cultures has shown no improvement in flavor with all of the cultures used to this point. The variation from the microenvironment between low fat and full fat cheese may be producing an unbalanced growth in the low fat cheese, and thus leading to unusual flavor development.

### *Objective 2*

Based on the response received from five panelists, the Cheese powder 153 increases the cheesy flavor in low fat Cheddar at a 2.5% addition. However when the level is increased to 3.0%, it imparts rosy/floral flavor which is unacceptable in Cheddar cheese variety. Another observation from the informal tasting is that using Cheese paste 231 contributes to rancid and a odd after taste.

The NCF Swiss powder worked well with low fat ground Cheddar cheese but when added in combination with DF Cheddar cheese powder 153, it enhances the Cheddary flavor from 153 and also helps in knitting of ground cheese in better way. Given that the combination of NCF Swiss cheese flavor 264 and DF Cheddar cheese flavor 153 worked synergistically in first trial, the next trial would be to use 2% flavor mix (1% 264 plus 1% 153) in low fat Cheddar cheese to confirm the phenomenon. However, overall the only consistent trend was an increase in lipolytic flavor without any strong aged cheddar cheese flavor coming through.

## CONCLUSION

### *Objective 1*

Adjunct cultures have not had a positive effect on flavor to this point. Further work will be conducted at the conclusion of objective 2. Taste Panels at USU have been put on hold and cheese was not sent to NCSU, Mary Anne Drake.

### *Objective 2*

The combination of NCF Swiss cheese flavor 264 and DF Cheddar cheese flavor 153 worked synergistically in first trial, more study is needed.

# Investigating the Filled Gel Model for the Role of Fat in Cheese

DONALD J. MCMAHON: Utah State University

## ABSTRACT/SUMMARY

Cheese can be described as a protein network surrounded by an aqueous phase with milk fat globules inter-dispersed in the protein network. Based on this description, cheese can be modeled as a filled gel in which fat globules may act as an active or inactive filler based on its phase volume. This model will be tested by manufacturing cheese with fat levels from 32 to 4% fat, in 4% steps to determine the fat level at which the fat globules cease to perform as an active filler (Phase 1). Changes in rheological and sensory texture properties of the cheese will be determined as a function of filler volume fraction and storage time. The cheese will be tested using both rheological methods and sensory texture evaluation. The validity of such a model for understanding the texture in cheese will be verified by substituting fat with Sephadex beads (Phase 2).

## BACKGROUND

Cheese can be described as a protein network surrounded by an aqueous phase with milk fat globules inter-dispersed in the protein network. Based on this description, cheese can be modeled as a filled gel. The basic filled gel model predicts that the rheological ( $G'$ , elastic modulus) and fracture ( $s_f$  = fracture stress,  $g_f$  = fracture strain and  $G_f$  = fracture modulus =  $s_f / g_f$ ) properties of a filled gel depends on the following material properties:

*Properties of the protein network* (casein network in cheese)

- i.  $G'_N$  = Elastic modulus (stress/strain) of the network
- ii.  $G_m$  = Fracture modulus of the network

*Properties of the filler particles* (milk fat globules in cheese)

- iii.  $G'_p$  = Elastic modulus of the filler particle
- iv.  $f_p$  = Phase volume (v/v) of the filler particle

An additional factor is if the filler particle interacts or does not interact with the gel network. A particle that interacts with the network is called *active filler* whereas those that do not interact with the network are logically called *inactive filler*.

A hypothetical gel can be used to provide an overview of possible changes in textural properties associated with filler particles. One outcome is that as filler volume increases, texture changes such that the gel becomes firmer (higher  $s_f$ ), has a higher  $G'$  and is less deformable (lower  $g_f$ ). Another possibility is that an increase in filler volume caused a weakening of the gel such that  $s_f$  and  $G'$  decrease. These changes can be explained based on theoretical models of Kerner (1956) and van der Poel (1958) that account for the relative rigidities of the particle ( $G'_p$ ) and gel network ( $G'_N$ ) and on if the filler particle is an active or an inactive filler. These models are based on the mechanical properties of simple isotropic systems containing synthetic polymers and particles (mono-dispersed in size); therefore, they need to be validated in more complex systems where bio-molecules are used as fillers and gel networks.

The validity of this approach to biopolymer systems was tested by Ross-Murphy and Todd (1983). They used a very concentrated system containing 20% w/v gelatin and glass beads as filler particles ( $f_{\text{particle}} = 0$  to 80%). This can be considered one of the simplest cases where it is assumed that the dispersed particles are non-deformable and there is perfect adhesion between the particles and network at all strain levels (active filler). It was shown that an increase

in particle phase volume caused an increase in fracture stress and a decrease in fracture strain. Similar results were observed by Gwartney et al. (2004) with whey protein emulsion gels.

The gelatin system was further explored by Brownsey et al. (1987). They used gelatin as the protein network and added Sephadex beads as filler particles. Sephadex beads are made by chemical cross-linking a polysaccharide to form beads of different size (20 to 300 mm) and rigidity ( $G$ , this implies pure elasticity which may not be the case). The reinforcing effect of adding Sephadex filler particles to a 3% gelatin gel was shown by plotting the rigidity of the filled gel ( $G_{\text{filled gel}}$ ) divided by the rigidity of the gelatin network alone ( $G_N$ ) (i.e. reinforcement =  $G_{\text{filled gel}}/G_N$ ). This *reinforcing effect* is greater than 1 if the filled gel is more rigid than the un-filled gel, and less than 1 if the filled gel is weaker. As filler particle rigidity increased, the reinforcing effect increased (maximum of ~ 16 at  $f_f = 60\%$  v/v) and the phase volume where a filler effect was clearly observed decreased (this ranged from  $f_f = 10$  to 50% v/v). Increasing the gelatin concentration to 6% revealed other properties of the system. First, the three weakest filler particles caused a decrease in reinforcement (i.e., < 1). The particles showing a clear reinforcing effect in the 3% gelatin gel required a higher level of phase volume for the effect to be observed in the 6% gelatin gel, and the magnitude of the reinforcing effect was greatly reduced (maximum of ~5 at  $f_f = 60\%$  v/v). The general reinforcing effect can be described by the Kerner model (Kerner, 1956; Brownsey et al. 1987):

$$\frac{G_{\text{filled gel}}}{G_N} = \frac{1 + AB\phi_f}{1 - B\phi_f} \quad \text{eq. 1}$$

Where  $A$  is related to Poisson's ratio ( $\nu$ ) of deformation; assumed to be 0.5 for cheese.

$$A = \frac{7 - 5\nu}{8 - 10\nu} \quad \text{eq. 2}$$

And  $B$  describes the rigidity of the filler particle relative to the rigidity of the network.

$$B = \frac{(G_p/G_N) - 1}{(G_p/G_N) + A} \quad \text{eq. 3}$$

It was assumed that Sephadex beads did not interact with the gelatin network therefore this is a model for an *inactive* filler that bases the filler effect on the relative rigidities of the network and filler particles and the filler particle phase volume.

When  $G_{\text{filled gel}}/G_N$  from the Kerner model was compared with experimental results the general shape was the same but the Kerner model greatly under predicted the experimental data. Adjusting the equation to account for maximum filler packing volume moved the prediction closer but still below the predicted values (Brownsey et al., 1987). Assuming the model is valid, this could be due to several discrepancies. First, the actual rigidities of the filler particles and network could be different in the filled gel than when measured separately. Another possibility is that the phase volume occupied by the particles in the gel is greater than the particles alone.

Predicted trend lines for filler particles with various relative rigidity ratios ( $G_p/G_N$ ) are seen in Figure 1. The Brownsey et al. (1987) data suggested a critical phase volume requirement to observe a filler effect, and this is not seen in Kerner model. This could simply be due to the error in the data overshadowing a gradual increase or there actually could be a critical filler phase volume. This could be related to the filler particles altering gel structure and thereby rheological properties (see below for further discussion). In addition, and somewhat surprising, was that the filler particle size had no effect. Finally, when Sephadex particle surfaces had chemical groups that interacted with the gelatin network, the filler reinforcing effect increased. This showed that converting the inactive filler to an active filler caused a major increase in reinforcement.

A third key investigation is that of van Vliet (1988), who compared the properties of filled rubber-like gels (polyvinyl alcohol and Congo red) with casein gels. Both network types were filled with active or inactive filler particles. Both networks showed that inactive fillers decrease gel elasticity ( $G'$ ) and active fillers increased  $G'$ . An interesting observation was that active fillers in the rubber-like gel had a smaller reinforcing effect (maximum ~3) than casein gels (maximum ~ 6). It was speculated that the increased filler effect in the casein gels was due to flocculation of the active filler particles, forming an effective particle with a volume greater than the sum of the particles comprising the floc. This would increase the  $f$  in equation 1. It was proposed that inactive filler decreased rigidity but essential appearing as "fluid particles" with the rheological properties of water.

## Kerner Model

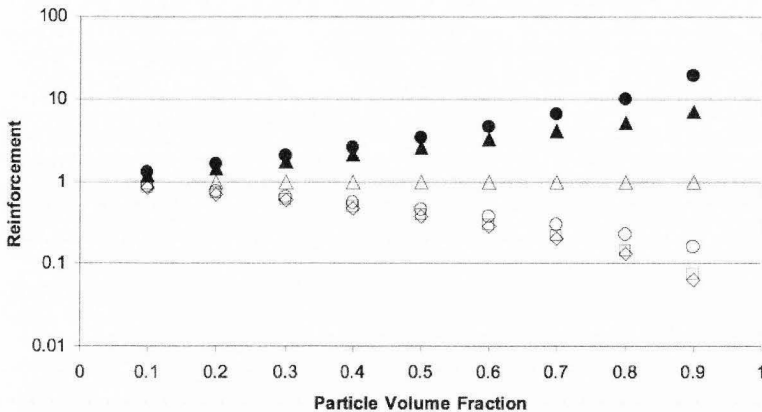


Figure 1. Theoretical reinforcement from particles with relative rigidity ratios ranging from 0.001 to 100 based in the Kerner Model.

These three fundamental studies on simple systems using food macromolecules (gelatin or casein) and simple filler particles to form networks established the following trends:

- v. Increasing the volume of interacting particles causes an increase in fracture stress and decrease in fracture strain. With cheese, this reflects an increase in firmness and a decrease in deformability (i.e., the texture becomes shorter).
- vi. The effect of particles will depend on the relative rigidities of the network ( $G_N$ ) and particles ( $G_p$ ). If a firmer network is desired, then the filler particles must be more rigid than the network; if less firmness is desired, then the opposite relationship is used.
- vii. The amount of filler needed to cause an effect will depend on the relative rigidities of the network and filler. In general, the greater the difference in rigidities, the lower amount of filler needed.
- viii. For a particle of given rigidity, causing the particle to interact with the network will greatly increase the filled gel rigidity.

These general principles will be used to evaluate other investigations in more complex systems.

Heat-set whey protein emulsion gels have been the subject of a series of investigations by Dickinson. The general system consists of a whey protein gel network filled with n-tetradecane emulsion particles (38% w/w). The choice of n-tetradecane is based on it being free of contaminating surfactants and remaining fluid over the range of experimental temperatures (Dickinson and Yamamoto, 1996). One key observation is that the critical concentration of whey protein needed to form a gel is greatly reduced in emulsion gels compared to whey protein alone (8.25% reduced to 4%) (Dickinson and Yamamoto, 1996). Supporting the trends listed above, the greater the relative filler rigidity ( $G_p/G_N$ ) the greater the reinforcing effect, and higher than predicted reinforcing effect can be explained by filler particle flocculation (Chen and Dickinson, 1998, 1999a). Additional filler effects established in these investigations were:

- ix. As phase volumes increase to high levels ( $f$  greater than  $\sim 45\%$ ), there is a possibility of particle-particle interactions greatly increasing rigidity or an inversion of structure where the network is formed by the particles (1999b).

- x. Adding filler particles can shift the type of network from a rubber-elastic to a particulate. The shift from rubber-elastic to particulate occurs as the  $f_{\text{particle}}$  is increased above 20 to 30% (Cher and Dickinson., 1999ab).
- xi. Larger active filler particles have less reinforcing effect because there is less surface area to interact with the gel network.

In the Low Fat Cheese Strategic Platform project it has been observed that when a low fat cheddar cheese is made so that it initially (first 30 days) has a similar hardness to a full fat cheese, that during storage the cheese loses rigidity. This is related to breakdown of the proteins that make up the protein matrix, and while similar protein hydrolysis occurs in full fat cheese, the full fat cheeses retain their rigidity during aging. This results in blocks of low fat cheese becoming deformed because of their own weight whereas the full fat cheeses retain their rectangular block shape. It is thought that the solidified fat globules in the cheese acting as a particulate filler to support the protein matrix, while in the low fat cheese as the proteins are hydrolysed the cheese loses some of its runner-elastic characteristics. By understanding the phase volume of particulate required to retain shape of the cheese block, it would provide information on how to improve texture of low fat cheeses.

The basic composition of reduced fat and low fat cheddar cheese is shown in Table 1.

Table 1. Composition of cheddar cheese.<sup>1</sup>

Cheese	Fat	Protein	Moisture
Normal	32%	25%	38%
Reduced-fat	16%	29%	48%
Low-fat	5%	33%	54%

<sup>1</sup>Data from DMI-funded project: *Low Fat Natural Cheese Strategic Platform*

Based on the previous discussion, fat can alter the texture of natural cheese by two possible mechanisms. One, is as an active or inactive filler particle. The second possibility is by altering the casein gel network structure. The network structure and filler effects ultimately determine sensory texture. If we decrease fat content by substituting with water, the cheese becomes more elastic and softer due to a lower particle phase volume (unpublished data from *Low Fat Natural Cheese Strategic Platform*). This suggests a possible change in gel type from particulate towards rubber-elastic, and this transition is supported by changes in force-deformation curves (unpublished data from *Low Fat Natural Cheese Strategic Platform*). This could be compensated for by increasing protein content and/or lowering water content, but then the relative firmness of the network and particles would modulate the change in texture. The most logical approach is to add a filler particle that has physical properties similar to milk fat globules. This would require being similar in size, shape, rigidity and interacting (or not interacting) with the protein network. Moreover, if an exact match is required, the rigidity of the particle should change in the temperature range experienced during mastication like that of a milk fat globule. This is clearly a difficult set of requirements to achieve and may for a great part explain why it has been so difficult to make a low-fat cheese where the particle phase is reduced from ~37% to 7%.

## MATERIAL AND METHODS

### **Objective 1. (Phase 1)**

**Cheese Manufacture.** Develop make procedures for making cheeses fat levels of 3, 8, 13, 18, 23, 28 and 33% fat using a stirred-washed curd method so that the protein matrix is similar in all cheeses with respect to pH, moisture and calcium content. Full fat (33%) and reduced fat (18%) cheeses will be virtually the same as used in the Low Fat Systematic Platform Project, the low fat cheese (3%) will be similar but will not include pre-acidification so as to maintain the protein to calcium ratio consistent. Adjustments to moisture content will be made by changing the



cook temperature used prior to draining, and the temperature and volume of wash water. Cheese will be made using 1500-lb enclosed cheese vats. Cheese will be pressed as 20-lb blocks, vacuumed packaged and stored at 4°C for 5 days, then cut into 4-lb blocks and re-packaged.

When the make procedures have been finalized, then the cheese for the two replicates will be made with three cheeses being made per week. The proximate analysis of the cheese will be performed on day 5, and if the cheese is not within the specified composition, the cheese manufacture will be repeated.

#### Chemical Analysis:

- Day 5: Measure pH by glass electrode, salt by chloride analysis, moisture by microwave oven, fat by Babcock, and water activity.
- 1 mo: Measure pH by glass electrode, moisture by vacuum oven, total protein and pH 4.6-soluble N by combustion N, calcium and other minerals by ICP spectroscopy, and water activity.
- 3, 6 and 9 mo: Measure pH by glass electrode, 4.6-soluble N by combustion N, and water activity.

#### Storage:

Store the cheeses at 6°C at Utah State University and sampled when the cheese is 1, 3, 6 and 9 mo of age. Cheese will be sent to North Carolina State University for mechanical and sensory testing, and internal structure examined at Utah State University using confocal scanning laser microscopy.

#### Internal Structure Analysis

Internal structural arrangements of the fat and protein in the cheeses will be examined by confocal scanning microscopy. Slices of cheese 5x5x3 mm will be cut from inside the block of cheese, and the protein stained using 0.02% fluorescein isothiocyanate and the fat stained using 0.02% Nile red. The cheese slice will be placed on a microscope slide and a cover slip placed on top and the edges sealed with silicone to prevent dehydration. The cheese will be examined using emission wavelengths of 500-535 and 600-635 nm, and 1024 x 1024 pixel images obtained simultaneously using two channels and then recombined with the protein colored as green and the fat colored as red.

#### Mechanical Testing:

Sensory testing starts with a sample at some set temperature (usually around room temperature) that is chewed at body temperature (~37°C). Therefore, mechanical tests will be done over the temperature range of 15 to 40°C to cover the range of temperature encountered during mastication.

##### *Small-deformation rheological tests.*

The basic method of Brown et al. (2003) will be used for rheological analysis. A controlled-stress Stresstech rheometer (Rheological Instruments AB, Lund, Sweden) equipped with parallel plates will be used for creep testing. Alternative, a controlled-strain Bohlin VOR rheometer (Bohlin Rheologi AB, Lund, Sweden) may be used for frequency sweeps and/or stress relaxation measurements. In either case, cheese will be attached to the plates using cyanoacrylate glue to prevent slip. Mechanical spectra analysis at a standard 1% strain will accordingly generate shear strain rates from  $1 \times 10^{-5}$  to  $0.2 \text{ s}^{-1}$ .

##### *Large-deformation rheological tests.*

**I. Torsion.** Cheese cylinders (19 mm diameter and 28.7 mm height) are cut from the cheese and mounted on notched styrene disposable disks with cyanoacrylate glue to allow for attachment to a grinder and rheometer. The cylinders are ground into a capstan shape, with a center diameter of 10 mm, using a precision milling machine (Gel Consultants, Raleigh, NC USA). Samples are mounted onto a Haake VT550 viscometer (Paramus, NJ) and twisted to failure at a specified rotational speed. Time and corresponding torque are measured and used to calculate shear stress and strain using a modified version of the calculations originally proposed by Diehl et al.<sup>75</sup> and Truong and Daubert.<sup>76</sup> Torsion analysis will be performed on 6 samples at each treatment. Sample rotational speeds will vary to create shear rates ranging between  $1 \times 10^{-3}$  to  $5.5 \text{ s}^{-1}$ . From the torsion test, force-deformation data will be integrated to compute areas under specific regions of the curve to correspond with stored and dissipated energies during the failure process. In addition, the shape of the curve will be fit to one or more non-linear models to calculate strain hardening parameters.<sup>1,4,15,48,72</sup>

**II. Compression.** Cheese cylinders (19 mm diameter and 28.7 mm height) will be compressed between lubricated plates to the point of fracture using a universal testing machine (Model 5565, Instron Engineering Corp., Canton, MA). Plates will be lubricated with mineral oil to prevent non-uniform expansion (barreling) during compression. The samples will be compressed at a constant cross-head speed from 2 to 400 mm/min, and 6 samples will be evaluated for each treatment. Under these conditions, strain rates may vary between  $1 \times 10^{-3}$  to  $0.4 \text{ s}^{-1}$ . Compressive stress and strain at fracture will be calculated as described by Truong and Daubert.<sup>76</sup> Similar to torsion analyses, the force-deformation data will be integrated to compute areas under desired regions of the curve to correspond with energies during deformation. In addition, the shape of the curve will be fit to one or more non-linear models to calculate strain hardening parameters.<sup>11,13,15,48</sup>

*Pressure sensitive tack.*

We have shown that the adhesive nature of cheese can be determined by measuring the pressure sensitive tack (Childs et al., 2007). Mechanical adhesiveness will be measured using a TAX-T2 Texture Analyzer (Texture Technologies, Scarsdale, NY) with a flat, 13-mm diameter, stainless steel probe.<sup>78</sup> Samples are cut into 4-cm squares and sliced to 6.35-cm thickness by a modified wire cheese slicer. The sample is placed on a platform below the probe arm and the probe is brought to the surface of the sample at a speed of 1 mm/s. Upon reaching the sample surface, a force of 2.0 N is applied, held for 5 s, and removed at a speed of 0.1 mm/s. The tack force is determined as the maximum force recorded during separation. The tack energy is measured from the area under the force-distance curve.

Sensory Analysis.

*Descriptive sensory analysis* with a highly trained panel will be used to document the specific texture characteristics Dr. MaryAnne Drake maintains two descriptive analysis panels (n=8 panelists each), each with more than 500 h of experience in the descriptive analysis of food flavor and food and non-food texture. Panelists are trained to using the Spectrum method with the universal Spectrum scale (all flavor, some texture) or product-specific scales (some texture). The texture attributes established by and Brown et al. (2003) will be used. Sample size and geometry is kept consistent to minimize variability.<sup>90</sup> Panelists will participate in a minimum of 5 2-h training sessions to specifically focus on gel texture. Briefly, panelists will be presented with a range of cheese samples and texture terms will be agreed upon to describe texture characteristics perceived in the mouth. The basic terms described in Table 1 will serve as core terms and new ones may be added. Product specific scaling will be applied<sup>91</sup> and specific cheese references will be identified to anchor 10-point category scales used for each attribute. Analysis of data collected from training sessions will be used to confirm that panelist and panel results are consistent and that terms are not redundant. Panelists will evaluate cheeses individually in sensory booths using computerized ballots (Compusense version Five Plus, Guelph, Canada). Red lighting may be used to mask differences in appearance. Four replications for each treatment will be conducted by each panelist. Data will be analyzed by univariate and multivariate statistical analyses using SAS (V 9.1, Cary, NC). Specifically, analysis of variance with means separation will be used to identify differences between treatments and attributes. Principal components analysis will be used to identify gross relationships between multiple treatments and attributes.

Table 1. Sensory terms used for descriptive analysis of gels.

<b>Evaluation Phase</b>	<b>Term</b>	<b>Definition*</b>	<b>Technique</b>
Pre-fracture	Smoothness <sup>b</sup>	Degree to which sample was perceived as smooth when evaluated with tongue	Move gel in mouth without chewing
	Small-strain <sup>c</sup> force	Force required to cause ~10% deformation	Compress to ~10 using molars
First bite	Firmness <sup>a,b</sup> (Fracture force) <sup>c</sup>	Force required to fracture sample with molars	Completely bite through sample using molars
	Moisture release <sup>b</sup>	Extent of moisture released	Same as above
	Crumbliness/fracturability <sup>a,b</sup>	Degree to which the sample fractures into pieces	Same as above
	Deformability <sup>c</sup>	Degree of deformation prior to fracture	Same as above
Chewdown	Degree of Breakdown <sup>a,b</sup> (Particle breakdown) <sup>c</sup>	Amount of breakdown	Chew the sample for 3 times, <sup>c</sup> 5 times, <sup>a</sup> or 8 to 10 times <sup>b</sup>
	Particle size <sup>b</sup>	Size after chews (small to large)	Same as above
	Particle size distribution <sup>b</sup>	Degree of homogeneity in distribution	Same as above
	Particle shape <sup>b</sup>	Degree of irregular particle shape (irregular means distinct edges)	Same as above
	Cohesiveness <sup>a,b</sup>	Degree to which the chewed mass holds together	Same as above
	Adhesiveness <sup>a,b</sup>	Degree to which the chewed mass sticks to mouth surfaces	Same as above
	Smoothness <sup>a,b</sup>	Degree to which the chewed mass surface is smooth	Same as above
	Number of <sup>a</sup> chews (Chewiness) <sup>c</sup>	Number of chews required for swallowing	Complete chewing
Residual	Smoothness of mouth coating <sup>a</sup>	Smoothness felt after expectoration	Evaluate after expectorating

A superscript letter indicated that the term was evaluated in the investigations of Brown et al.,<sup>8,10</sup> Gwarty et al.<sup>b,9</sup> or Barrangou et al.<sup>c,11</sup> \*Definition for terms used in Gwarty et al.<sup>9</sup>

## **Objective 2. (Phase 2) Validation of Model**

### Microparticles:

- Obtain Sephadex G-50 carbohydrate beads of three different sizes: medium (100 to 300 mm), fine (40 to 160 mm), and superfine (20 to 80 mm).

Cheese Manufacture: Make a control full fat (33%) control reduced fat cheese (16.5%), control low fat (6%) and corresponding microparticulate (MP) cheeses using the Sephadex G-50 beads.

- Manufacture cheddar cheese using 10 L of skim milk standardized with cream so that the resultant cheese contains 33% fat and 37% moisture.
- Manufacture cheddar cheese using 10 L of skim milk to which is added 370 g of hydrated medium, fine or superfine Sephadex G-50 beads. Make adjustments to the make procedure to obtain cheddar cheese with approx. 65% moisture [Assume 90% yield into cheese so that beads occupy about 33% of the volume of the final cheese, and that the beads consist of 86% moisture.]
- Manufacture 50% reduced fat cheddar cheese using 10 L of skim milk standardized with cream so that the resultant cheese contains 16.5% fat and 46% moisture.
- Manufacture 50% reduced fat cheddar cheese using 10 L of skim milk to which is added 185 g of hydrated medium, fine or superfine Sephadex G-50 beads. Make adjustments to the make procedure to obtain cheese with approx. 60% moisture
- Manufacture low fat cheddar cheese using 10 L of skim milk standardized with cream so that the resultant cheese contains 6% fat and 52% moisture.
- Manufacture low fat cheddar cheese using 10 L of reduced fat milk to which is added 65 g of hydrated medium, fine or superfine Sephadex G-50 beads. Make adjustments to the make procedure to obtain cheese with approx. 57% moisture
- Make 2 replicates of each cheese.

### Chemical Analysis:

- Measure pH by glass electrode, salt by chloride analysis, moisture by vacuum oven, fat by Babcock, protein by combustion N, calcium and other minerals by ICP spectroscopy at Utah State University, and measure water activity.

### Storage:

- The cheeses will stored at 6°C and physical testing performed when the cheese is 1 mo, and 4 mo old.

### Mechanical Testing

*As described above.*

## **References**

- J. Hamilton, B. Knox, D. Hill and H. Parr. Reduced fat products: Consumer perceptions and preferences. *British Food J.* **102**, 494 (2000).
- M. R. Heath and P. W. Lucas. Oral perception of texture. in *Food Structure – Its Creation and Evaluation*, edited by J. M. V. Blanshard and J. R. Mitchell (Butterworths, London, 1988), p 465
- J. X. Guinard and R. Mazzucchelli. The sensory perception of texture and mouthfeel. *Trends Food Sci. & Technol.* **7**, 213 (1996).
- C. Wilkinson, G. B. Dijksterhuis and M. Minekus. From food structure to texture. *Trends Food Sci. & Technol.* **11**, 442 (2000).
- A. van der Bilt. Human oral function: a review. *Braz. J. Oral Sci.* **1**, 7 (2002).
- T. van Vliet. On the relation between texture perception and fundamental mechanical parameters for liquids and time dependent solids. *Food Quality and Pref.* **13**, 227 (2002).
- E. A. Gwartzney, D. K. Larick and E. A. Foegeding. Sensory texture and mechanical properties of stranded and particulate whey protein emulsion gels. *J. Food Sci.* **69**, S333 (2004).
- J. A. Brown, E. A. Foegeding, C. R. Daubert, M. A. Drake and M. Gumpertz. Relationships among rheological and sensorial properties of young cheese. *J. Dairy Sci.* **86**, 3054 (2003).
- L. M. Barrangou, M. A. Drake, C. R. Daubert and E. A. Foegeding. Sensory texture related to large-strain rheological properties of agar/glycerol gels as a model food. *J. Texture Stud.* **37**, 241 (2006).
- J. Zhang, C. R. Daubert and E. A. Foegeding. Fracture analysis of alginate gels. *J. Food Sci.* **70**, E425 (2005).

- L. M. Barrangou, C. R. Daubert and E. A. Foegeding. Textural properties of agarose gels. II. Relationships between rheological and sensory texture. *Food Hydrocoll.* **20**, 184 (2006).
- L. M. Barrangou, C. R. Daubert and E. A. Foegeding. Textural properties of agarose gels. I. Rheological and fracture properties *Food Hydrocoll.* **20**, 196 (2006).
- J. F. Meullenet, M. L. Finney, M. Gaud. Measuring of biting velocity and predetermined and individual crosshead speed instrumental imitative test for predicting cheese hardness. *J. Texture Stud.* **33**, 45 (2002).
- T. van Vliet and P. Walstra. Large deformation and fracture behaviour of gels.
- D. D. Hamann. Structural failure in solid foods. in *Physical Properties of Foods*, edited by E. B. Bagley and M. Peleg (AVI Publishing, Connecticut 1983), p. 351
- J. Zhang, C. R. Daubert and E. A. Foegeding. A proposed strain-hardening mechanism for alginate gels. *J. Food Eng.* **80**, 157 (2007).
- H. T. Lawless and H. Heymann, *Sensory Evaluation of Food, Principles and Practices* (Chapman and Hall, New York 1998).
- R. Musampa, M. Alves and J. Maia. *Food Hydro.* **21**, 92 (2007).
- K. C. Diehl, D.D. Hamann, and J.K. Whitfield. Structural failure in selected raw fruits and vegetables. *J. Texture Stud.* **10**, 371 (1979).
- V. D. Truong and C.R. Daubert. Comparative study of large strain methods in assessing failure characteristics of viscoelastic food gels. *J. Texture Stud.* **31**, 335 (2000).
- J. Childs, E. A. Foegeding, C. Daubert, L. Stefanski. Factors regulating cheese shreddability. *J. Dairy Sci. In Press.* (2007).
- E. A. Foegeding and M. Drake. Sensory and mechanical properties of cheese texture. *J. Dairy Sci. In Press* (2007).
- A. M. Munoz, G.V. Civille. Universal, product, and attribute specific scaling and the development of common lexicons in descriptive analysis. *J. Sensory Stud.* **13**,57 (1998).
- M. E. Carunchia Whetstine, M.A. Drake, B.K. Nelson, and D. Barbano. 2006. Flavor Profiles of Full Fat, Reduced Fat and Cheese Fat made from Aged Cheddar with the Fat Removed using a Novel Process. *J. Dairy Sci.* **89**, 505 (2006).
- M. D. Yates, M. D., and M. A. Drake. Texture properties of Gouda cheese. *J. Sensory Stud. In Press* (2007).

## RESULTS AND DISCUSSION

### **Objective 1: (Phase 1)**

Determine the fat level in cheese in which fat globules changes from performing as an active filler into an inactive filler.

Cheese make procedures were developed for making cheeses with fat contents ranging from 3% to 33% that all had comparable protein matrix composition, i.e., only the fat volume in the cheese was different. The first replicate of cheeses were manufactured at Utah State University with target fat contents of 3, 8, 13, 18, 23, 28 and 33%. As shown below the proximate composition obtained at day 5 ( $\pm 1$ ) were close to the target composition, except for the salt levels. These were higher than planned in the first few cheeses made and so the remainder were made in a similar fashion with salt-in-water contents of about 4.7% which is more typical of regular full fat cheddar cheese anyhow. The cheeses are being stored at 6°C and analysis has started with rheological and texture studies being performed at North Carolina State University.

Proximate analysis data for Rep 1 Cheeses											
Make Date	% Fat		% Moisture		% Salt		% MNFS		% Salt in H2O		pH
	Target	Actual	Target	Actual	Target	Actual	Target	Actual	Target	Actual	
3/11/08	3.0	3.0	52.80	53.03	2.10	2.56	54.43	54.67	3.98	4.83	5.40
3/25/08	8.0	8.5	50.10	49.60	2.00	2.09	54.46	54.21	3.99	4.21	5.30
3/4/08	13.0	15.5	47.40	46.82	1.90	2.20	54.48	55.41	4.01	4.70	5.58
3/5/08	18.0	20.3	44.60	45.35	1.80	2.03	54.39	56.90	4.04	4.48	5.52
3/18/08	23.0	23.0	41.90	43.39	1.70	2.04	54.42	56.35	4.06	4.70	5.37
3/19/08	28.0	28.8	39.20	40.04	1.60	1.96	54.44	56.20	4.08	4.90	5.46
3/26/08	33.0	33.0	36.50	37.45	1.45	1.85	54.44	55.90	3.97	4.94	5.46

Q2 2008: All cheeses produced have uniform %MNFS and %salt in H2O concentrations as shown in Table 2. This represents a successful manufacture of all cheeses with water to protein ratios constant as % fat varies. Also pH and water activity over the 12 week period of sampling of rep 1 cheeses are very close, as predicted.

Table 2. Proximate analysis data for Rep 2 of Fat Model project											
Make Date	% Fat		% Moisture		% Salt		% MNFS		% Salt in H2O		pH
	Target	Actual	Target	Actual	Target	Actual	Target	Actual	Target	Actual	
3/11/08	3.0	2.5	52.80	53.42	2.10	2.13	54.43	54.79	3.98	3.99	5.29
3/25/08	8.0	11.0	50.10	47.18	2.00	2.09	54.46	53.01	3.99	4.44	5.23
3/4/08	13.0	15.0	47.40	46.48	1.90	2.17	54.48	54.68	4.01	4.68	5.42
3/5/08	18.0	20.0	44.60	43.73	1.80	1.87	54.39	54.66	4.04	4.27	5.30
3/18/08	23.0	24.0	41.90	41.24	1.70	1.87	54.42	54.27	4.06	4.53	5.27
3/19/08	28.0	29.0	39.20	39.66	1.60	2.07	54.44	55.86	4.08	5.21	5.29
3/26/08	33.0	33.0	36.50	37.94	1.45	1.75	54.48	56.63	3.97	4.60	5.19

Confocal microscope images show distribution of fat in the curd particles as shown in Figure 1. The low fat cheeses show smaller fat globules. As fat % increase, fat globules appear to increase in size.

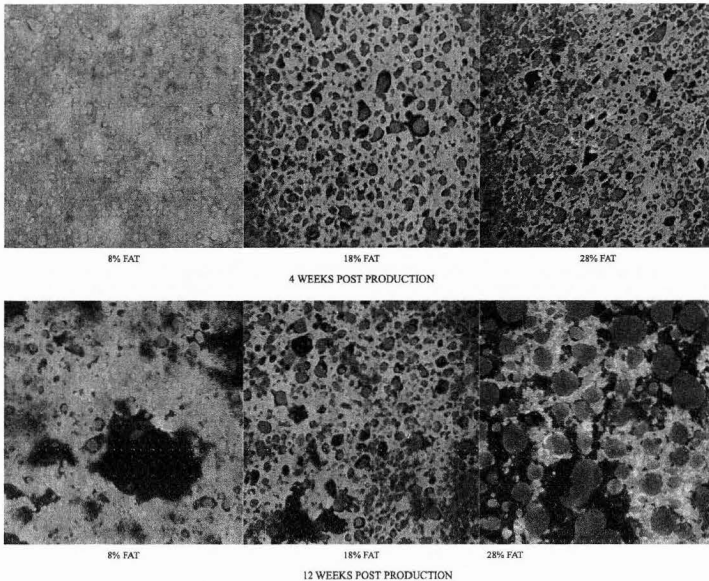


Figure 1. Confocal microscope images showing distribution of fat (orange) and protein (green).

Q3 2008: Rep 1 has been complete and rep 2 is at the 12 week sampling. pH and Moisture analysis show no significant differences through the 24 weeks. Confocal Microscopy analysis show no significant differences through the 24 weeks.

Q4: Rep 1 and 2 sampling has been complete. pH and Moisture analysis show no significant differences through the 24 weeks. Confocal Microscopy analysis show no significant differences through the 24 weeks.

**Objective 2: (Phase 2)**

Validate the filled gel model for cheese using Sephadex beads as filler particles in cheese.

Q4: 2008: Trial batches of low fat, reduced fat and full fat cheeses were made with adaption of the procedures used in Objective 1 for use in 10-kg vats. A set of low fat cheeses with addition of Sephadex beads as filler particles was made, however it was found that insufficient cheese curd was produced using the 10-kg vats for the cheese to be pressed properly. A set of larger containers that can hold 40 lb of milk were purchased so this can be repeated in 2009 Q1.

**CONCLUSIONS/NEXT STEPS**

Q4: 2008: During Jan –Feb 2009 manufacture Rep 1 of the low fat, reduced fat and full fat cheeses with Sephadex beads added. These will then be stored for aging and sent for rheological and texture studies at North Carolina State University.

***VALUE-ADDED DAIRY RESEARCH  
PROJECTS***



# Understanding How Salt Level Influences Perception of Cheese Flavor

How salty taste influences low fat cheese flavor

DONALD J. MCMAHON: Utah State University

MARYANNE DRAKE: North Carolina State University

## ABSTRACT

The role of salty taste in flavor profile and acceptance of low fat cheeses will be investigated. It is possible that salty taste modulates perception of other flavor attributes and such information is crucial. The primary goal of this study is to investigate this issue. Low fat cheeses with 1 % salt will be manufactured and aged for 4 months. Following aging cheese will then be divided into ten portions and sufficient salt added to produce cheese containing 1.0, 1.4, 1.8, 2.2, and 2.6% salt. Since the low fat cheese will still only have a mild flavor, half of the ground cheese material will also have a cheese flavoring added so as to produce an aged cheese flavor. A trained descriptive sensory panel will document flavor attributes and intensities of cheeses. Selected cheeses will subsequently be evaluated for consumer acceptance.

## INTRODUCTION

Salt content of cheddar cheese is recognized as playing an important role in suppressing unwanted microbial growth and activity. Typically such cheese has a moisture content of 36 to 38% moisture, and a salt-in-water content of 4.5 to 5.5%. When a low fat cheese, it is necessary to increase the moisture content to about 52%. This results in a much lower salt-in-water content unless the overall salt content is increased.

In the Low Fat Cheese Systematic Platform project, descriptive flavor analysis was performed on low fat, reduced fat and full fat cheeses. Cheese flavor development was much slower in the low fat cheeses and some flavor components were missing. There were also differences in the overall salt content and salt-in-water content between the cheeses, with the low fat cheeses having the highest salt content but the lowest salt-in-moisture content.

When performing a descriptive flavor analysis, the level of individual flavor attributes are determined. There is a question on how the level of one flavor attribute influences the perception of the other flavor attributes. Understanding this would help determine whether some of the insufficiencies in low fat cheese flavor result simply from a lack of production of the flavor compounds responsible for the attribute, or whether the flavor compounds are produced but the perception of those attributes by the panelist is altered by the levels of other flavor attributes in the cheese, such as the level of perceived saltiness.

Since salt-in-water content of cheese influences the ripening of cheese and development of cheese flavor, it will be necessary to manufacture cheese with the same low salt level and then after ripening, add extra salt at different levels. This can be done by grinding the low fat cheese, adding salt, and then repressing the cheese.

## MATERIALS AND METHODS

Manufacture low fat cheese using 1500-lb of milk in Scherping enclosed vat at Utah State University. The low fat cheese make procedure from the Low Fat Cheese Systematic Platform project was followed with the following exceptions:

- cheese was be pre-acidified to pH 6.4 instead of pH 6.25 as the previous cheese became too soft during aging.
- a starter culture recommended for making reduced fat and low fat cheeses was used instead of the single strain M70 culture (to minimize potential for bitter taste development).
- salt was added to produce a cheese containing only 1.0% salt.

- cheese was aged at 6°C instead of 8°C as the higher temperature was found to be unsuitable for low fat cheese (even though 8 to 10°C is now commonly used for aging full fat cheese).

The 20-lb cheese blocks were aged for 4 months, then cut into 12 –oz pieces and then further reduced in particle size to about 2 to 5 mm in a bowl chopper. The cheese was then divided into ten portions and sufficient salt added to produce cheese containing 1.0, 1.4, 1.8, 2.2, and 2.6% salt. Since the low fat cheese had only a mild flavor, half of the ground cheese material was had a cheese flavoring (Natural Cheese Flavor 6033, Chr. Hansen, Inc., Milwaukee, WI) added so as to produce an aged cheese flavor.

Cheese manufacture occurred twice on separate occasions with an experimental design as follows:

- Replicates: 2
- Cheese maturity levels: 2
- Salt levels: 5

After mixing the cheese was pressed into 6-lb blocks overnight, vacuum packaged and then stored for 3 weeks to allow the cheese particles to knit together. During this time the cheese was stored at 4°C to retard further flavor development. Moisture measured by microwave oven, salt by chloride analysis, fat by babcock and pH by glass electrode.

The cheeses was sent to North Carolina State University for descriptive flavor sensory analysis

## RESULTS

Low fat cheese with the same cheese flavor development but different levels of salt was manufacture. Two vats of low fat cheese were made using 1500 lb of milk on October 3 and 10, 2007 at Utah State University.

### Proximate Analysis (5 days after manufacture)

Target	Rep	% Moisture	% Salt	% Fat <sup>1</sup>	pH
1.0%	1	51.1	1.04	8	5.35
	2	51.8	1.12	6	5.44
1.0% + Flavor	1	51.4	1.16	8	5.36
	2	51.3	1.16	6	5.41
1.4%	1	50.5	1.28	8	5.33
	2	51.8	1.52	6	5.45
1.4% + Flavor	1	50.4	1.40	8	5.32
	2	51.4	1.56	6	5.42
1.8%	1	50.3	1.68	8	5.35
	2	51.7	1.87	6	5.44
1.8% + Flavor	1	49.7	1.81	8	5.33
	2	50.8	1.87	6	5.41
2.2%	1	50.1	2.20	8	5.37
	2	51.5	2.25	6	5.44
2.2% + Flavor	1	49.5	2.13	8	5.32
	2	51.0	2.24	6	5.42
2.6%	1	50.0	2.51	8	5.34
	2	51.5	2.57	6	5.45
2.6% + Flavor	1	49.6	2.49	8	5.34
	2	50.8	2.63	6	5.45

<sup>1</sup>Babcock measurements to nearest 0.5%

Replicate 1 cheese: 50.3% moisture, 6.0% fat, 0.96% salt and pH 5.12.

Replicate 2 cheese: 51.7% moisture, 4.5% fat, 0.91% salt and pH 5.26.

A small portion of the cheese was comminuted and tested for suitable flavoring to use and a level that would simulate some aged cheese flavor.

Replicate 1 was comminuted and salted on March 12, 2008.

Replicate 2 was comminuted and salted on March 19, 2008.

Informal taste panels were conducted

Cheeses sent to NCSU at the end of March 2008

## **DISCUSSION**

The cheeses were made at the 1.0% salt level. Storage at 38 F apparently provided adequate control over microbial growth that the cheese had a slightly aged cheese flavor even though it had low salt content. Adding a commercially available cheese flavoring increased the flavor intensity, although from informal tasting it still lacked the milkfat flavor typical of full fat cheese. The different salt levels in cheese were obtained for the targets 1.0, 1.4, 1.8, 2.2 and 2.6% salt levels.

## **CONCLUSIONS**

Cheese successfully prepared and delivered to NCSU, Mary Anne Drake for descriptive sensory analysis, and information on the results of that study are in reports from the Southeast Dairy Foods Research Center.

# Partitioning of Omega-3 Fatty Acids Between Cheddar Cheese Curd and Whey

CARL BROTHERSEN: Western Dairy Center  
DONALD J. MCMAHON: Utah State University  
BRIAN PETTEE: Western Dairy Center

## Objectives:

- Investigate different methods of incorporating omega-3 oils into cheese milk and partitioning of omega-3 fatty acids between cheese curd and whey.
- Conduct a sensory evaluation of cheese fortified with omega-3 fatty acids either by addition to milk or to cheese curd.
- Evaluate the sensory properties of dried whey obtained from manufacture of omega-3 fortified cheese.

## BACKGROUND

Currently there is interest in adding omega-3 fatty acids to cheese to produce a more healthful product. Commercial preparations of omega-3 fatty acids are available in two physical states, liquid and encapsulated powder; and two sources, plant and animal. There are also two methods of incorporating omega-3 fatty acids into the cheese, adding it to the cheese milk, and adding it to the finished curd.

This project will investigate how liquid omega-3 fatty acids from two different suppliers, representing animal and plant sources, partition between the curd and whey, when incorporated in the cheese milk and curd using different techniques.

## MATERIALS AND METHODS

### *Objective 1*

#### **Step A. Adding Omega-3 oils to milk at start of cheesemaking**

Cheese will be made in 10-L vats. Sufficient omega-3 fatty acids will be added to milk before renneting (a 80% yield will be assumed) to give at least 32 mg of DHA/EPA omega-3 fatty acids per ounce of cheese using the following methods:

1. Mixed encapsulated omega-3 oils into milk.
2. Mixing liquid omega-3 oil in a portion of the milk
3. Mixing liquid omega-3 oil in cream and adding to the cheese milk
4. Homogenizing liquid omega-3 oil in a portion of the milk
5. Homogenizing liquid omega-3 oil in a portion of the cream.

This experiment will be performed in duplicate. Omega-3 oil sourced from both algae (Martek Biosciences Corporation, Columbia MD), and fish (Ocean Nutrition Canada, Dartmouth, NS) will be used. Two pounds of cheese curd will be packed in round plastic hoops and pressed at 15 psi overnight. Whey will be collected and weighed during cheese making and pressing.

A portion of the whey will be frozen and stored for analysis. Cheese will be vacuum packaged and stored at 6°C. A sample will be frozen for analysis. The omega-3 fatty acid content of the cheese curd, the pressed cheese, the whey obtained during draining, and the whey obtained during pressing will be determined using GC methods. The partitioning of omega-3 fatty acids between whey and cheese determined.

#### **Step B. Adding Omega-3 oil to cheese curd at end of cheesemaking**

Cheese will be made from 600 lb of milk and the curd divided into ten portions. Sufficient omega-3 fatty acids will be added to cheese curd (a 100% yield will be assumed) to give at least 32 mg of DHA/EPA omega-3 fatty acids per ounce of cheese using different methods of incorporation along with a control cheese with no omega-3 oil added (in duplicate):

1. Mixing liquid omega-3 oil with salt and adding to curd
2. Mixed encapsulated omega-3 oils with salt and adding to curd.

Omega-3 oil from both algae and fish origins will be used. The cheese curd will be packed in round plastic hoops and pressed at 15 psi overnight into 5-lb blocks. Whey will be collected and weighed during cheese making and pressing.

A portion of the whey will be frozen and stored for analysis. Cheese will be vacuum packaged and stored at 6°C. A sample will be frozen at -30°C for analysis. The omega-3 fatty acid content of the cheese curd, the pressed cheese, the whey obtained during draining, and the whey obtained during pressing will be determined using GC methods. The partitioning of omega-3 fatty acids between whey and cheese determined.

### **Objective 2**

Full-fat Cheddar cheese will be manufactured from 1500 pounds of milk using a stirred curd procedure. The method from Objective 1a that has the highest retention of Omega-3 fatty acids in the cheese (i.e., lowest loss in the whey) will be used to add Omega-3 oil prior to renneting of milk to give at least 32 mg DHA/EPA of omega-3 fatty acid per ounce of cheese. Omega-3 oil sourced from algae and fish origins will be used. The cheese curd will be packed in 20 pound Wilson hoops and pressed at 15 psi overnight. A second treatment will use the control cheddar cheese and add omega-3 to the curd during salting (based on Objective 1b). The treated cheeses, along with controls not containing Omega-3 fatty acids will be done in duplicate.

The omega-3 fatty acid content of the cheese curd and the whey obtained during pressing, will be determined using GC methods.

The cheese will be vacuum packaged and stored at 8 °C for up to 270 days. Proximate analysis will be conducted on the cheese at 5 days of age. Samples will be evaluated for omega-3 fatty acid content at 1, 3, 6 and 9 months of age. Samples will be analyzed by a descriptive/trained taste panel at 3, 6, and 9 months of age. Samples will be analyzed by a consumer taste panel at 3 and 6 months of age.

### **Objective 3**

Whey will be collected from cheese made in Objective 2. The fat will be separated from the aqueous whey phase and then added to whey at levels representing good and poor whey cream separation. The whey will then be concentrated by reverse osmosis, and spray dried. The whey protein powder will be packaged in plastic, non-airtight bags and stored at 20° and 32° C for up to 9 mo of age. Samples will be analyzed for omega-3 fatty acid content, and by a descriptive/trained taste panel at 30, 90, and 180 days of age.

### **Results and Discussion**

Omega-3 fatty acid preparations from animal and plant sources in both liquid and encapsulated form have been obtained. Preliminary trials have been conducted to determine cheese make and sampling procedures. Distribution of liquid preparations in milk has proven difficult. Modification of procedures is being investigated in order to properly homogenize the preparations into milk and cream.

Cheddar cheese was made from 10 kg of milk. Omega-3 fatty acids were added to the cheese milk using the following techniques:

1. Encapsulated powder added directly into the cheese milk
2. Oil added directly into the cheese milk
3. Oil mixed in cream, a portion of the cream then mixed with skim milk to give the required fat level in the cheese.
4. Oil homogenized in milk and a portion of the homogenized milk mixed in the cheese milk to give the required fat level in the cheese.
5. Oil homogenized in cream and a portion of the homogenized cream added to the cheese milk to give the required fat level in the cheese.

Omega-3 fatty acids from both animal (Ocean Nutrition) and plant (Martek) sources were used; treatments were done in duplicate resulting in 20 vats of cheese. The omega-3 content of the cheese milk and the curd was determined. The recovery of the omega-3 fatty acid in the curd as a percent of the omega-3 in the cheese milk is shown in Figure 1, for both the vegetable and animal sourced products.

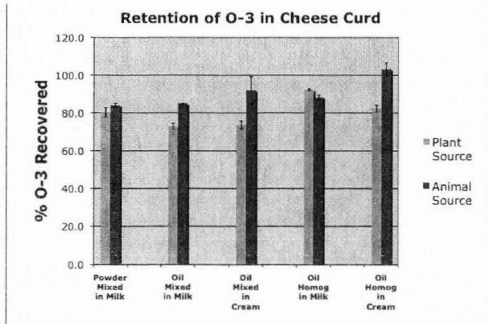


Figure 1.1. Retention of omega-3 fatty acids in cheese curd as a percent of the total omega-3 added to the cheese milk. Treatment 1: Encapsulated omega-3 powder added to the cheese milk. Treatment 2: Omega-3 oil added to the cheese milk. Treatment 3: Omega-3 oil mixed in cream and added to cheese milk. Treatment 4: Omega-3 oil homogenized in milk and added to cheese milk. Treatment 5: Omega-3 oil homogenized in cream and added to cheese milk.

### Next Steps

Objectives 2 and 3 are in progress and will be completed in 2009.

# Vitamin D Fortification of Cheddar Cheese

CARL BROTHERSEN: Western Dairy Center

SILVANA MARTINI: Utah State University

DONALD J. MCMAHON: Utah State University

MEGAN TIPPITS: Utah State University

BRIAN PETTEE: Western Dairy Center

Objective 1: Determine the best method for fortifying Cheddar cheese with Vitamin D

Objective 2: Generate sensory data on Vitamin D fortified cheese using two different Vitamin D preparations

Objective 3: Determine the best emulsification techniques to retain Vitamin D in cheese

## BACKGROUND

It is anticipated that FDA will increase the daily dietary Vitamin D recommendation for humans from 400 to 1000 IU. Cheese may be a good vehicle for increasing Vitamin D consumption. However, there is a potential for Vitamin D to contribute off flavors to cheese and whey, especially oxidized flavors. Data on the flavor profile of Vitamin D fortified cheese is not available.

In previous studies, Vitamin D retention in cheese ranged from 40-60%, depending on the vitamin preparation (C. Banville, et. al. 2000. *Int Dairy J.* 10:375-382.). More efficient techniques for adding Vitamin D to cheese milk need to be developed to improve Vitamin D retention in the cheese and reduce losses in the whey.

## MATERIAL AND METHODS

### *Objective 1*

A screening test will be performed using 10 L vats of milk, to determine the amount of Vitamin D that needs to be added to milk to produce cheese containing 100 IU and 200 IU per ounce. Vitamin D will be added to the cheese milk by one of three techniques: 1) Directly adding the liquid Vitamin D emulsion to cheese milk, 2) Homogenizing the liquid Vitamin D emulsion into a portion of the whole milk, and 3) by adding a water dispersible encapsulated Vitamin D powder into the whole milk. Samples of the cheese curd and whey obtained at draining will be taken, stored at -30°C until all cheeses are made, then analyzed for Vitamin D content.

### *Objective 2*

Six vats of full-fat Cheddar cheese will be manufactured from 600 pounds of milk using the standard WDC cheddar cheese make procedure. This consists of cheese made using the most effective method of Vitamin D addition from Objective 1, at 2 levels of addition, plus a control cheese (no Vitamin D added), each made in duplicate. Vitamin D will be added to the cheese milk to give 200 IU and 400 IU/ounce of cheese by adding a water dispersible encapsulated Vitamin D powder into the milk. The cheese curd will be packed in 20 pound Wilson hoops and pressed at 15 psi overnight, vacuum packaged, and stored at 6° C.

Samples of the cheese curd and whey obtained at draining will be taken, stored at -30°C until all cheeses are made, then analyzed for Vitamin D content. Proximate analysis will be conducted on the cheese at 5 days of age.

To determine the distribution of Vitamin D through out the block of cheese, two blocks will be randomly selected and Vitamin D analysis will be conducted on cheese samples taken from the following locations in the block: 1) the center of the surface of the front side as oriented in the press, 2) the center of the block, 3) the center of the surface of the top side as oriented in the press, 4) the center of the surface of the bottom side as oriented in press, 5) the corner of the block on the bottom side as oriented in the press.

Vitamin D content and descriptive taste panel analysis will be conducted at 90, 180, and 270 days of age. Vitamin D content in the cheese curd and whey will be determined using the AOAC official method 952.29. Samples will be sent to a private laboratory for determination (O'Neal Scientific, St. Louis, MO).

### **Objective 3**

**Part A.** To evaluate the effect of emulsification on the retention of Vitamin D in the cheese, emulsions will be formulated using different types of emulsifiers. The final pH of the emulsion will be maintained at 6.7 using buffer phosphate. Oil in water emulsions will be formulated using Vitamin D3 as the oil phase (5%) and 2% of the emulsifier in water. The emulsifiers to be tested are:

- o Sodium caseinate
- o Calcium caseinate
- o Skim milk powder
- o Whey protein concentrate (WPC-80)

Emulsions will be formed using a high shear device (Ultraturrax) which will generate emulsions of about 15  $\mu$ m.

Considering the previous description, Objective 3 will consist of 4 treatments (Vitamin D emulsions formulated with different emulsifiers) and 2 controls (Vitamin D emulsified in whole milk, and encapsulated Vitamin D as described in Objective 1).

The physicochemical stability of the emulsions will be studied using a vertical scan macroscopic analyzer (TurbiScan MA 2000). TurbiScan consists of a reading head moving along a flat-bottomed cylindrical cell while scanning the entire sample height. The reading head consists of a pulsed near-infrared light source and two synchronous detectors. Only the backscattering (BS) detector, which receives the light backscattered by the product (135 °), will be used due to the emulsion. The reading head acquires BS data every 40  $\mu$ m to a maximum height of 80 mm. The profile obtained characterizes the sample's homogeneity, particle concentration, and mean diameter. The parameters are represented by a curve showing the percentage of BS light as a function of the sample height in mm. The acquisition along the product is repeated with programmable frequency obtaining a superimposition of sample fingerprints, which characterize the stability or instability of the sample (e.g., the more identical the readings over time, the more stable the system). After forming the emulsions, they will be placed in an assay tube and held at room temperature. BS measurements will be taken as a function of time until the emulsions turn significantly unstable. Emulsions' destabilization kinetics will be measured by calculating the variation in BS as a function of time at half the maximum of the BS peak value with respect to the initial reading.

Droplet size distributions for all the emulsions will be determined using Beckman Coulter particle characterization instrumentation (LS20 Version 3.19, Beckman Coulter Inc.). Isolated droplets will be measured with this instrument as evidenced by the lack of flocculation in the emulsions when observed under a microscope.

Retention of the Vitamin D into cheese curd will be modeled on a laboratory scale by adding the emulsion to milk and performing a curd syneresis experiment. Appropriate amounts of the Vitamin D emulsion (to give the equivalent of approx. 200 IU per ounce of curd) will be added to 10 g of milk weighed into centrifuge tubes and heated to 35°C. Then 0.02 ml of rennet plus 0.2 g glucono-delta-lactone will be added and the milk incubated at 35°C for 30 min. After coagulation, two perpendicular cuts will be made in the curd and the samples centrifuged at 250 x g at 25 °C for 20 min. After centrifugation, the volume of released whey will be measured and expressed as a percentage of total moisture (g) initially present in the sample, and the Vitamin D content of the whey measured. Partitioning of the Vitamin D between the curd and the whey will then be calculated and used to predict its retention during cheesemaking.

Experiments will be performed in duplicate or triplicate as necessary. Data reported are the mean and standard deviation values calculated from the replicates. Significant differences will be analyzed using a Two- or One-way ANOVA test, as appropriate, and a Bonferroni post-test ( $\alpha = 0.05$ ). Statistical analysis will be performed using Graph Pad software (GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego, CA, USA, [www.graphpad.com](http://www.graphpad.com)).

Vitamin D content in the cheese curd and whey will be determined using the AOAC official method 952.29. Samples will be sent to a private laboratory for determination.

**Part B.** The best emulsification techniques obtained from the experiments described above will be validated in small scale (10 L of milk) cheese trials, with sufficient Vitamin D added to produce cheese with 200 IU per ounce. Vitamin D content of cheese curd and whey will be determined.



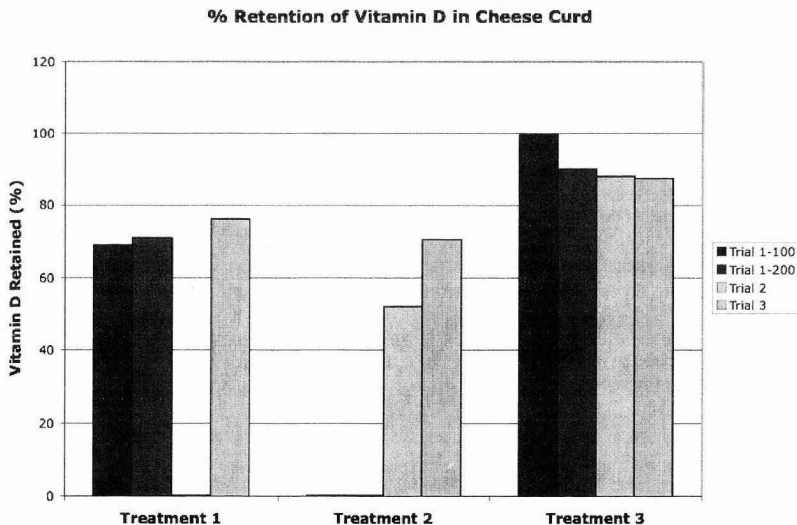
The emulsification procedure will be optimized to further increase the Vitamin D retention in cheese. Some of the optimization steps will include: (a) the addition of a harder fat, such as anhydrous milk fat, to improve the Vitamin D retention in the emulsion and therefore in the cheese; (b) formulation of emulsions with smaller droplets to evaluate how this parameter will influence the Vitamin D retention.

## RESULTS AND DISCUSSION

### Objective 1

Preliminary trials investigating the retention of Vitamin D in Cheddar cheese were conducted in 10 L vats in order to determine cheese make and sampling procedures. Vitamin D preparations consisting of the standard liquid used for fortification of milk, and encapsulated powdered were used. The three methods of Vitamin D addition were: 1) adding a commercially available emulsion of Vitamin D (VitaSystems VS-AD200, Continental Custom Ingredients, Inc.) directly to the cheese milk without homogenization; 2) homogenizing the Vitamin D emulsion in milk and adding the requisite amount of this fortified milk to 10 kg of cheese milk; 3) adding an encapsulated Vitamin D preparation in powdered form (BASF, Mount Olive, NJ) directly to the cheese milk. The curd and whey were collected, weighed and analyzed for Vitamin D content.

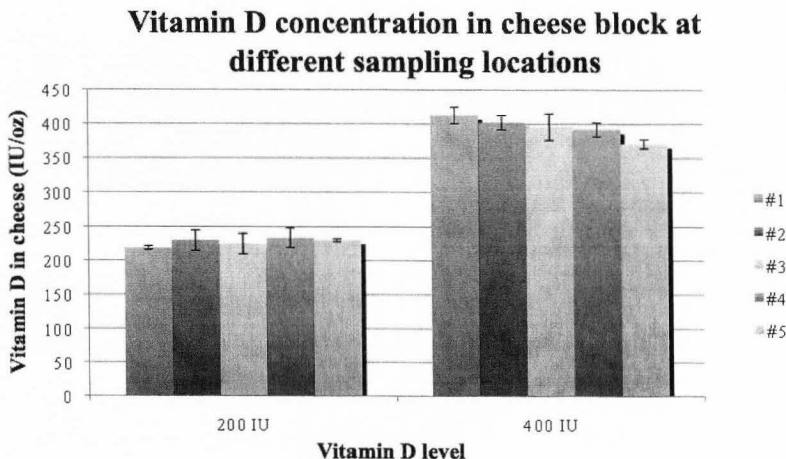
Figure 1 shows the amount of Vitamin D retained in the curd, as a percent of the total Vitamin D recovered in the curd and whey, for the three methods of addition listed above. Adding Vitamin D in powdered form resulted in the greatest retention in the curd, while adding the Vitamin D preparation directly into the cheese milk without homogenization resulted in the least retention.



**Figure 1.** Retention of Vitamin D in cheese curd as a percent of the total retained in the cheese in the whey. Treatment 1: homogenizing the emulsified Vitamin D preparation in a portion of the cheese milk. Treatment 2: Adding the emulsified Vitamin D directly to the cheese milk without homogenization. Treatment 3: Adding the encapsulated Vitamin D powder directly to the cheese milk.

### Objective 2

To determine the distribution of Vitamin D through out the block of cheese, two blocks at each Vitamin D level were sampled at 5 locations and Vitamin D analysis conducted by GC analysis. Figure 2.1 shows the Vitamin D concentration at different locations throughout the block of cheese. Vitamin D concentration appears evenly distributed throughout the block of cheese at both supplementation levels.



**Figure 2.** Vitamin D concentration in cheese block at different locations. : Location #1: the center of the surface of the front side as oriented in the press. Location #2: the center of the block. Location #3: the center of the surface of the top side as oriented in the press. Location #4: the center of the surface of the bottom side as oriented in press. Location #5: the corner of the block on the bottom side as oriented in the press.

### Objective 3

Droplet size and standard deviations for the four different emulsions are shown in Table 2. Stability of the emulsions as determined by vertical scattering turbidity is shown in Figure 2 Emulsions were very unstable, with separation occurring within two minutes.

Emulsifier	D <sub>(3,2)</sub> (µm)	SD
WPC	3.360	0.040
Skim milk	1.258	0.085
NaCas	4.454	1.062
CaCas	6.153	0.234

**Table 2.** Droplet size of the emulsions at time zero

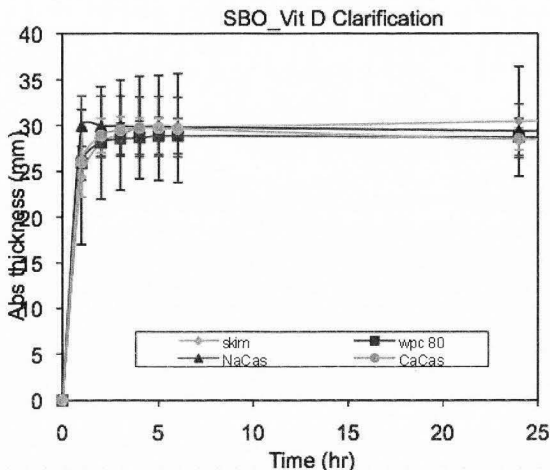


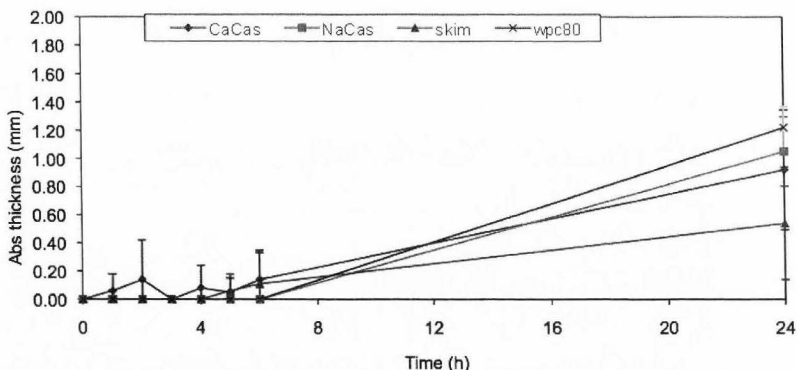
Figure 2. Destabilization kinetics (clarification) of Vitamin D emulsions formulated with different emulsifiers.

After formulation, the droplet size distribution of the emulsions was determined using Beckman backscattering equipment. Table 3 shows the average droplet size of all emulsions expressed as D(3,2) values.

D(3,2)	Average	std dev
CaCas	0.713	0.068
NaCas	0.730	0.094
WPC80	0.733	0.033
SMP	0.718	0.037

Table 3. Droplet size (D3,2) of emulsions formulated with different emulsifiers

From Table 3 it can be observed that different emulsifiers did not affect the droplet size of the emulsions under the processing conditions used. Figure 3 shows the stability of the emulsions as the thickness of the separating layer as a function of time. All the emulsions were destabilized through a creaming phenomenon when stored at 5°C. From Figure 3 it can be observed that all emulsions showed good stability for the first 6 hours after which they slowly destabilized with time. After 24 hours a creaming layer of approximately 1 mm was observed. When emulsions were stored for 7 days, the creaming layer increased to 3-5 mm. After 24 hours the most stable emulsion was the one formulated with SMP as emulsifier and the least stable was the one formulated with WPC80.



**Figure 3.** Physicochemical stability of emulsions formulated with SMP, WPC80, CaCas and NaCas as emulsifiers.

As mentioned in the materials and methods section, the volume of whey, the weight of curd, the moisture content, and the percent of curd yield was calculated. Results are presented in Table 4. The amount of whey obtained represented approximately 74-80% of the initial volume of milk with the curd equaling 20-26% of the initial volume.

	Volume	Std	Curd		Moisture		Curd	
SKIM	(mL)	E	(g)	std E	(%)	std E	(%)	std E
SMP	148.0	4.7	40.7	2.5	76.2	0.8	26.0	2.4
WPC80	143.7	10.2	47.3	6.5	75.6	1.8	28.2	5.1
NaCas	149.3	2.4	44.2	1.8	76.7	0.4	25.3	1.2
CaCas	155.2	2.5	37.5	2.2	75.3	0.6	22.4	1.3
Control	147.7	3.8	45.8	5.2	77.3	1.3	26.2	1.9

		Std	Curd		Moisture		Curd	
WHOLE	Volume	E	(g)	std E	(%)	std E	(%)	std E
SMP	150.7	5.6	46.0	5.7	67.6	2.0	24.7	2.1
WPC80	157.0	2.0	41.3	0.6	65.4	1.4	21.5	5.3
NaCas	153.3	0.9	44.0	2.3	67.8	0.4	23.3	0.9
CaCas	144.7	1.5	48.5	1.5	68.7	1.3	27.7	2.6
Control	149.0	2.5	49.4	4.4	69.5	1.4	25.5	2.2

**Table 4.** Volume of whey (mL), weight of curd (g), moisture content (%), and curd yield (%) for the bench-top cheeses.

The Vitamin D content present in the whey and curd obtained from the bench-top cheeses was determined by a private laboratory (O'Neil). Table 5 presents the values obtained, while Table 6 present the percentage of vitamin D that was retained by the curd and the percent lost in the whey.

	Whey			
	Average		Ave % of	std % of
	IU/g	std dev	IU/g	IU/g
<b>Whole milk</b>				
Ctrl	1.31	0.37	0.24	0.15
SMP	0.37	0.04	0.19	0.02
WPC80	0.56	0.05	0.18	0.02
NaCas	0.46	0.01	0.24	0.01
CaCas	0.62	0.19	0.32	0.08
<b>Skim milk</b>				
Ctrl	0.94	0.13	0.49	0.05
SMP	1.29	0.35	0.54	0.14
WPC80	1.32	0.65	0.37	0.26
NaCas	1.04	0.20	0.35	0.08
CaCas	0.86	0.07	0.35	0.03

	Curd			
	Average		Ave % of	std % of
	IU/g	std dev	IU/g	IU/g
<b>Whole milk</b>				
Ctrl	57.00	6.28	21.95	2.51
SMP	66.10	5.87	31.23	2.35
WPC80	66.30	8.45	28.21	3.38
NaCas	76.83	2.88	33.84	1.15
CaCas	73.47	5.00	25.01	2.00
<b>Skim milk</b>				
Ctrl	55.55	6.07	17.99	2.43
SMP	58.70	39.96	44.59	15.99
WPC80	92.57	15.53	38.73	6.21
NaCas	97.00	25.22	41.36	10.09
CaCas	114.33	4.46	42.39	1.78

Table 5. Vitamin D content of the curd and whey obtained from the bench-top cheeses. The expected vitamin D content in the curd is 250 IU per g of curd.

	% Whey recovery		% Curd recovery	
	Average	std E	Average	std E
<b>Whole milk</b>				
Ctrl	2.4	0.4	97.6	0.4
SMP	0.5	0.0	99.5	0.0
WPC80	0.8	0.1	99.2	0.1
NaCas	0.5	0.0	99.5	0.0
CaCas	1.0	0.2	99.0	0.2
<b>Skim milk</b>				
Ctrl	2.1	0.3	97.9	0.3
SMP	1.2	0.1	98.8	0.1
WPC80	1.3	0.3	98.7	0.3
NaCas	1.0	0.0	99.0	0.0
CaCas	0.8	0.1	99.2	0.1

Table 6. Percentage of recovery of vitamin D in the curd and whey.

***Next Step***

Objective 2. Descriptive and consumer sensory analysis will be conducted on the cheeses at the indicated time points as the cheese ages in 2009.

Objective 3 will be completed in 2009.

# Development of Cheddar Cheese Containing Probiotics

CARL BROTHERSEN: Western Dairy Center

DONALD MCMAHON: Utah State University

CRAIG OBERG: Weber State University

BART WEIMER: University of California, Davis

JEFF BROADBENT: Utah State University

Objective 1: Determine the survival of probiotic cultures in full-fat cheese during aging.

Objective 2: Determine the survival of probiotic cultures in 50% reduced-fat cheese during aging.

Objective 3: Determine the survival of probiotic cultures in low-fat cheese during aging.

## ABSTRACT/SUMMARY

This project will evaluate the survivability of 8 commercially available strains of probiotic cultures in full fat, 50% reduced-fat, and low-fat Cheddar cheese over nine months of aging. Cheese will be made with each of the cultures, plus a control cheese made without a probiotic culture. Treatment and control cheese will be made in duplicate. The cheeses will be analyzed at 0, 1, 2, 3, 4, 6, and 9 months of age for survival of the probiotic strains. The probiotic cultures will be enumerated using two techniques: 1) plating on selective media and 2) qPCR.

## BACKGROUND

Probiotic cultures are microbes, commonly lactic acid bacteria (LAB), which are believed to contribute to good health when ingested. Potential health benefits attributed to probiotic bacteria include prevention of colon cancer, lowering cholesterol, lowering blood pressure, improving immune function, modulating inflammatory response, preventing the reoccurrence of inflammatory bowel disease, and improving nutrient absorption.

Because many useful probiotics bacteria are LAB, dairy products have been used as a vehicle for introduction of the probiotic into the human diet. Several species of Bifidobacteria and Lactobacilli are available commercially for incorporation into foods. Yogurt has been used as a carrier for these microbes, however the higher pH of cheese may provide a less harsh environment and improve viability of the cultures. Several strains of Lactobacilli have been isolated as non-starter lactic acid bacteria (NSLAB) in aged Cheddar cheese. These strains grow to high numbers in the ripening cheese. However, there is some concern in the industry that the strains selected for use as probiotics are less hardy than the wild NSLAB of the same species.

The characteristics of the probiotic bacteria are strain specific. In addition, they are matrix specific, that is, the benefit expressed by a probiotic carried in plain yogurt, may not be the same as when carried in a yogurt containing fruit, or in cheese. Moreover, survivability of probiotic cultures in cheese, yogurt, or any other dairy product is also strain specific.

Currently there are 13 commercially available probiotic cultures: four strains of *Bifidobacterium lactis*, four strains of *Lactobacillus acidophilus*, two strains of *Lactobacillus paracasei* subsp. *paracasei*, one strain of *Lactobacillus casei*, and two strains of *Lactobacillus rhamnosus*.

There is considerable literature on the addition of probiotics to yogurt. However, there is little information on the impact of probiotics on the manufacture of semi-hard cheeses such as Cheddar, and the impact of the cheese matrix on the survivability of the probiotic. While there is little data on the survivability of probiotics in cheese, there is no data on the survivability of probiotics in reduced- and low-fat cheeses.

Preliminary work by our group has shown that the addition of Bifidobacteria to cheese milk causes an increase in the rate of acid production, necessitating changes in the cheese make procedure in order to obtain proper pH and moisture of the finished cheese.

This project will examine the impact of the probiotic culture on the cheese make procedure and determine the survival rate of the culture in low-, reduced- and full-fat Cheddar cheese.

## MATERIALS AND METHODS

### **Objective 1. Determine the survival of probiotic cultures in full fat cheese during aging.**

Individual vats of full-fat Cheddar cheese will be made from 350 pounds of milk with each of the probiotic cultures listed in Table 1, along with Chr. Hansen culture 850, using the standard Western Dairy Center make procedure. A control cheese containing only 850 will also be made. Each culture treatment will be made in duplicate, for a total of 16 vats.

The finished curd will be packed in 20 pound Wilson hoops, pressed, vacuum packaged, then stored at 8°C for four days. The blocks of cheese will then be cut into 11 pieces weighing approximately two pounds each, vacuum packaged, and stored at 8°C until analyzed. One package of cheese will be randomly selected at four days of age for proximate analysis and microbial enumeration. One package will be randomly selected at 0, 2, 3, 4, 6, and 9 months of age for microbial enumeration.

The cheese will be evaluated at 9 months of age by descriptive and consumer preference taste panels.

### **Objective 2. Determine the survivability of probiotic cultures in 50% reduced-fat cheese during aging.**

Individual vats of fifty percent reduced-fat Cheddar cheese will be made from 350 pounds of milk using the probiotic strains listed in Table 1 and Chr. Hansen 850 culture. A control cheese containing only 850 will also be made. Each culture treatment will be made in duplicate, for a total of 16 vats. Cheese will be sampled and analyzed as described in Objective 1 above.

### **Objective 3. Determine the survivability of probiotic cultures in low-fat cheese during aging.**

Individual vats of low-fat Cheddar cheese will be made from 350 pounds of milk using the probiotic strains listed in Table 1 and Chr. Hansen 850 culture. A control cheese containing only 850 will also be made. Each culture treatment will be made in duplicate, for a total of 16 vats. Cheese will be sampled and analyzed as described in Objective 1 above.

Table 1. Probiotic cultures and suppliers

SUPPLIER	ORGANISM	NAME
Cargill	<i>Bifidobacterium lactis</i>	BIF-6
Chr. Hansen	<i>Lactobacillus acidophilus</i>	LA-5
Chr. Hansen	<i>Bifidobacterium lactis</i>	BB-12
Chr. Hansen	<i>Lactobacillus casei</i>	CRL-431
DSM	<i>Lactobacillus acidophilus</i>	LAFTI L10
DSM	<i>Lactobacillus casei</i>	LAFTI L26
DSM	<i>Bifidobacterium lactis</i>	LAFTI B94
Medipharm/Chr. Hansen	<i>Lactobacillus paracasei</i> subsp. <i>paracasei</i>	F19



## Enumeration of Probiotic Strains by Plating Methods

Media preparation:

M17: Standard M17 media.

MRS-V: Just prior to pouring, add 2 ml of 0.5 mg/ml vancomycin to 1 liter standard MRS media.

MRS-S: Just prior to pouring, add 10 ml filter sterilized 10% sorbitol solution to 90 ml dextrose-free MRS media.

MRS+S: Just prior to pouring, add 10 ml filter sterilized 10% sorbitol solution to 90 ml standard MRS media.

MRS+C+ant: Just prior to pouring, add 5 ml antibiotic mixture to 100 ml MRS media containing 0.05% L-cysteine hydrochloride.

Antibiotic mixture: 2 g neomycin sulfate, 3 g nalidixic acid, 60 g lithium chloride, 4 g paromomycin sulfate prepared in 1 liter of deionized water and filter sterilized; store at 4°C.

Table 2. Media type and incubations condition for enumeration of starter and probiotic cultures by plating method.

Organism	Media	Incubation	Temperature	Time
Starter lactococci	M17	Aerobic	30°C	24 h
Total lactobacilli	MRS+S	Anaerobic	37°C	48 h
<i>Lactobacillus casei</i>	MRS-V	Anaerobic	37°C	48 h
<i>Lactobacillus paracasei</i>	MRS-V	Anaerobic	37°C	48 h
<i>Lactobacillus acidophilus</i>	MRS-S	Anaerobic	37°C	48 h
<i>Bifidobacterium</i>	MRSC+ant	Anaerobic	37°C	48 h

Each of the cultures listed in Table 1 were grown overnight in milk and dilutions were plated on the media listed in Table 2. The resulting colony counts are listed in Table 3.

## Enumeration of Probiotic Strains by qPCR Technique

A method for enumerating probiotic cultures by qPCR technique was developed and briefly described here. Known amounts of specific *L. acidophilus*, *B.lactis*, *L. casei* and *L. paracasei* subsp *paracasei* strains were added to buffer and subsequently cheese (Table 1). Initially, pure cultures of these organisms were used in buffer to determine the dose response with qPCR. Secondly, we created a standard curve in cheese for use with unknown samples.

Test strains were grown in MRS media overnight, collected by centrifugation, washed, and added to buffer or cheese in serial dilutions. Total genomic DNA was extracted from 0.25 g of cheese was added to 1 ml of Trizol, mixed, and treated with a bead beater for 30 sec. DNA was collected from the 400 µl fraction and resuspended in 100 µl of sterile deionized water. A portion (1 µl) was used as the template for the real time qPCR assay using a MJ Research DNA Engine Opticon2 PCR (Bio-Rad, Hercules, CA). The master mix (USB, Cleveland, OH) contained CYBR Green, FidelityTaq, and 10 ng of each primer. The total test time was 7 hours with a sensitivity of ~100 cells.

Serial dilutions of genomic DNA extracted from liquid cultures were used as template with four primer concentrations to optimize the q-PCR reaction condition. The results of this optimization indicated that a primer concentration of 10 ng was the optimal for all DNA template conditions tested. This primer concentration was used to generate standard curves of cheese spiked with different strains of *L. acidophilus*, *B.lactis*, *L. casei* and *L. paracasei*. Total Genomic DNA was extracted from cheese spiked with serial dilutions of bacteria and used as template for the qPCR reaction. The experiment was repeated 10 times.

The results indicate that the real time qPCR procedure detected ~100 cells in 0.25 grams of cheese with reliable and reproducible results.

## RESULTS AND DISCUSSION

Proximate analysis for all 48 vats of cheese made is shown in Table 4.

Table 3. Growth of probiotic cultures on selective media.

	MI17 30°C aerobic	MRS+S 37°C anaerobic	MRS-Y 37°C anaerobic	MRS+C+Ant 37°C anaerobic	MRS-S only 45°C anaerobic	RCA-BV 37°C anaerobic
	<i>Lactococcus</i>	Total LAB	<i>L. casei</i>	<i>Bifidobacterium</i> <i>L. acidophilus</i> <i>L. casei</i>	<i>L. acidophilus</i>	<i>L. casei</i> <i>L. acidophilus</i>
850 <i>Lactococcus</i>	2.2 x 10 <sup>9</sup>	1.9 x 10 <sup>9</sup>	<10 <sup>4</sup>	<10 <sup>4</sup>	<10 <sup>4</sup>	<10 <sup>4</sup>
LA 5 <i>L. acidophilus</i>	<10 <sup>4</sup>	9.2 x 10 <sup>7</sup>	<10 <sup>4</sup>	<10 <sup>4</sup>	8.0 x 10 <sup>8</sup> (small colonies)	9.2 x 10 <sup>7</sup>
L 10 <i>L. acidophilus</i>	<10 <sup>4</sup>	1.1 x 10 <sup>9</sup>	<10 <sup>4</sup>	7.0 x 10 <sup>8</sup>	9.0 x 10 <sup>8</sup> (small colonies)	
La L10 <i>L. acidophilus</i>	<10 <sup>4</sup>	1.8 x 10 <sup>9</sup>	<10 <sup>4</sup>	2.0 x 10 <sup>9</sup> (very small colonies)	3.4 x 10 <sup>9</sup>	<10 <sup>4</sup>
L 26 <i>L. casei</i>	<10 <sup>4</sup>	2.4 x 10 <sup>9</sup>	2.2 x 10 <sup>9</sup>	3.5 x 10 <sup>9</sup>	<10 <sup>4</sup>	1.9 x 10 <sup>9</sup>
431 <i>L. casei</i>	<10 <sup>4</sup>	2.2 x 10 <sup>9</sup>	2.2 x 10 <sup>9</sup>	2.6 x 10 <sup>9</sup> (very small colonies)	<10 <sup>4</sup>	2.4 x 10 <sup>9</sup>
F-19 <i>L. paracasei</i>	<10 <sup>4</sup>	6.0 x 10 <sup>8</sup>	1.2 x 10 <sup>9</sup>	2.0 x 10 <sup>9</sup> (very small colonies)		8.0 x 10 <sup>8</sup>
LH 32 <i>L. helveticus</i>	<10 <sup>4</sup>	5.1 x 10 <sup>7</sup>	<10 <sup>4</sup>	<10 <sup>4</sup>	5.1 x 10 <sup>7</sup>	<10 <sup>4</sup>
Bif 6 <i>Bifidobacterium</i>	<10 <sup>4</sup>	1.4 x 10 <sup>9</sup> (very small colonies)	<10 <sup>4</sup>	8.0 x 10 <sup>8</sup> (very small colonies)	<10 <sup>4</sup>	<10 <sup>4</sup>
BB 12 <i>Bifidobacterium</i>	<10 <sup>4</sup>	1.8 x 10 <sup>9</sup> (very small colonies)	<10 <sup>4</sup>	9.0 x 10 <sup>8</sup> (very small colonies)	<10 <sup>4</sup>	<10 <sup>4</sup>

Table 4. Average moisture, fat, salt and salt in moisture, for all 16 vats of each cheese type.

Cheese Type	% Moisture	% Fat	% Salt	% Salt in Moisture
Full-fat	38.8	31.5	1.22	3.17
Reduced-fat	45.8	17.1	1.91	4.2
Low-fat	50.5	7.5	2.0	4.0

Enumeration of probiotic cultures in reduced-fat cheese by both the plating and qPCR techniques are shown in Figures 1-15. The survival of probiotic cultures in the full-, and low-fat cheeses are similar to that in the reduced-fat cheese.

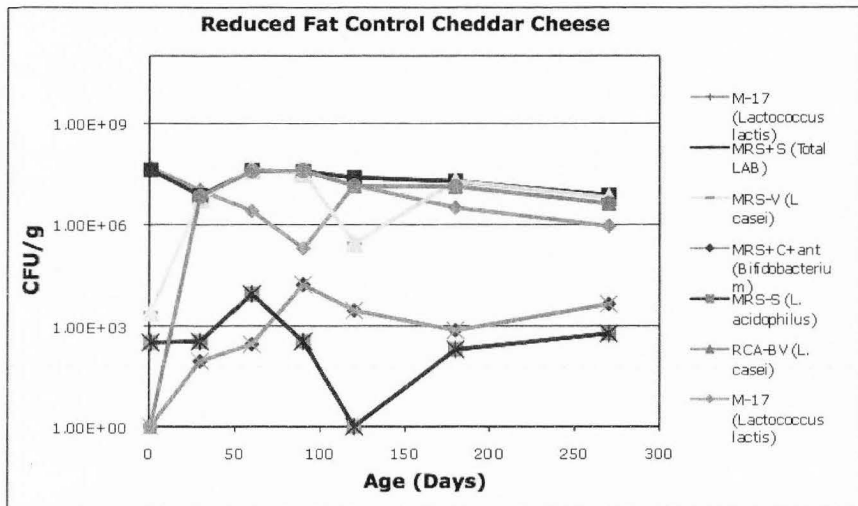


Figure 1. Enumeration of reduced-fat cheese containing *Lactococcus lactis* starter only, on selective media: M17, MRS+S, MRS+V, MRS+C+ant, MRS-S, and BCA-BV.

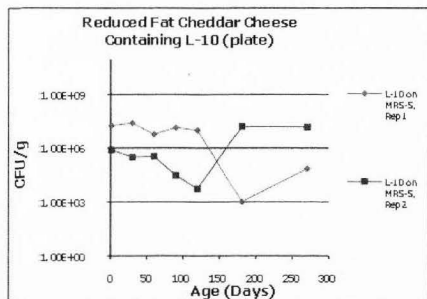


Figure 2. Enumeration of L-10 in reduced-fat cheese by plating on MRS-S media.

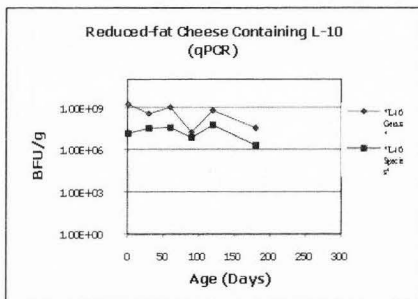


Figure 3. Enumeration of L-10 in reduced-fat cheese by qPCR technique.

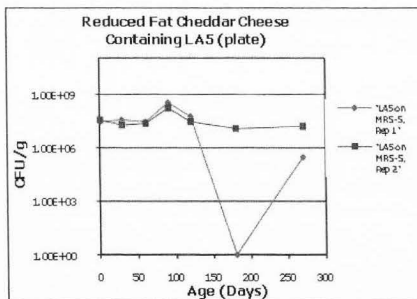


Figure 4. Enumeration of LA5 in reduced-fat cheese by plating on MRS-S media.

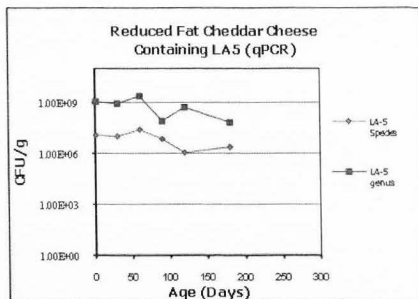


Figure 5. Enumeration of LA5 in reduced-fat cheese by qPCR technique.

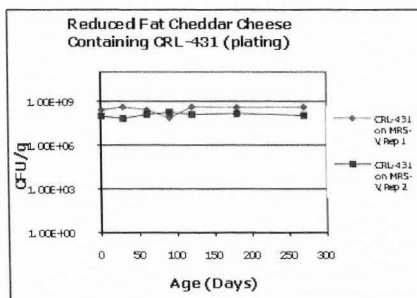


Figure 6. Enumeration of CRL-431 in reduced-fat cheese by plating on MRS-V media.

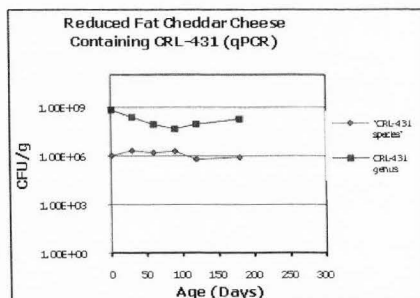


Figure 7. Enumeration of CRL-431 in reduced-fat cheese by qPCR technique.

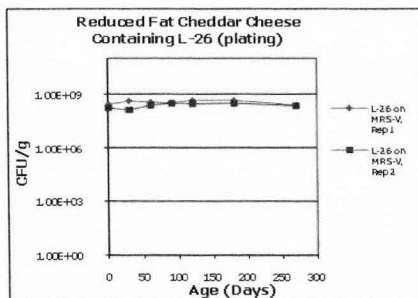


Figure 8. Enumeration of L-26 in reduced-fat cheese by plating on MRS-V media.

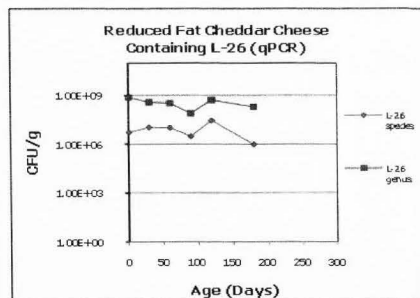


Figure 9. Enumeration of L-26 in reduced-fat cheese by qPCR technique.

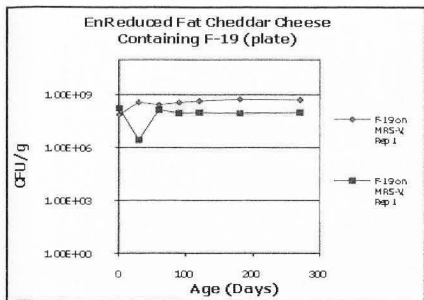


Figure 10. Enumeration of F-19 in reduced-fat cheese by plating on MRS-V media.

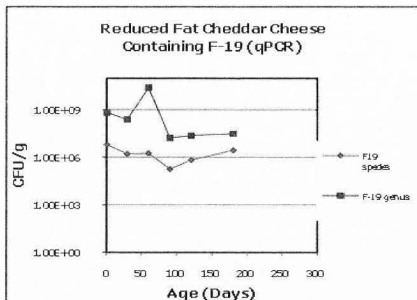


Figure 11. Enumeration of F-19 in reduced-fat cheese by qPCR technique.

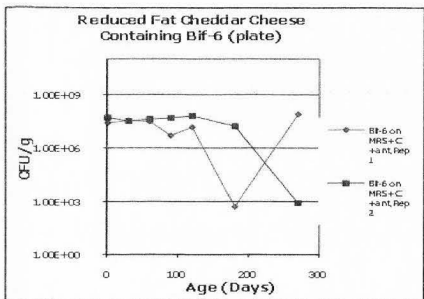


Figure 12. Enumeration of Bif-6 in reduced-fat cheese by plating on MRS+C+ant media.

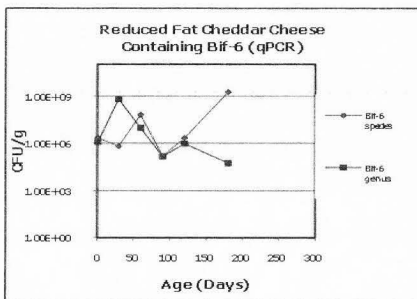


Figure 13. Enumeration of Bif-6 in reduced-fat cheese by qPCR technique.

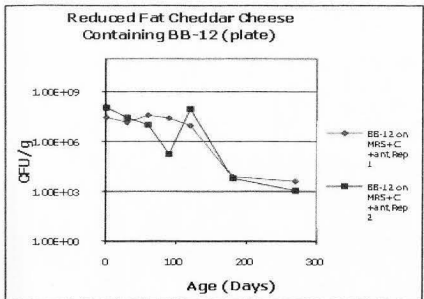


Figure 14. Enumeration of BB-12 in reduced-fat cheese by plating on MRS+C+ant media.

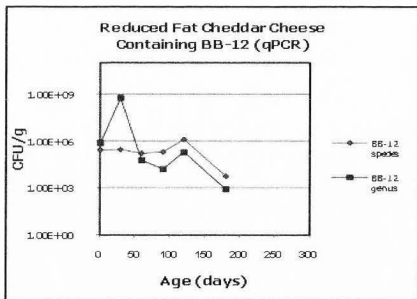


Figure 15. Enumeration of BB-12 in reduced-fat cheese by qPCR technique.

## CONCLUSIONS

There is good agreement between the two methods of enumeration. All strains survived well in reduced-fat cheese to six months of age except BB-12 and Bif-6. These bifidobacteria showed decreasing numbers with increasing age.

As shown in Figure 1, bacterial colonies from the control cheese, containing no probiotic cultures, grew on all media. This indicates that the media may not be as selective for the indicated probiotic strain as needed for accurate enumeration, or there are significant numbers of probiotic strains in our NSLAB population. While a high number of *L casei* are expected to be in the NSLAB population, *L acidophilus* and bifidobacteria are not. To address this, we are selecting colonies from the nine month old samples plates, and conducting 16S RNA analysis on them to identify the strains.

There is some concern with the qPCR technique that we are including DNA from lysed as well as intact cells. In developing the technique we treated the samples with DNAase to destroy the free DNA before lysing the cells. There was no difference in counts between the treated and non-treated samples. We are currently investigating the ability of the technique to distinguish between culturable and non-culturable cells.

***MILK AND WHEY RESEARCH  
PROJECTS***

# Synthesis, Characterization, and Bioactivity of Lactose Lauryl Esters

MARIE WALSH: Utah State University

Objective 1: Determine the optimum conditions for the synthesis of lactose lauryl esters.

Objective 2: Investigate the antimicrobial properties of lactose lauryl esters.

Objective 3: Data analysis and manuscript preparation.

## ABSTRACT

Fatty acid sugar esters are non-ionic detergents with multiple uses in the cosmetic, food, and pharmaceutical industries. Of the many different sugar esters synthesized, lactose, a by-product of cheese manufacture, has not been investigated. The objective of this research was to investigate the synthesis of novel lactose monolaurate (LML) and sucrose monolaurate (as a comparison) (SML) using four different immobilized lipases in three different solvents at constant sugar, vinyl laurate, temperature, and enzyme concentrations. Overall, the solvent 2-methyl-2-butanol gave the highest yields and reactions rates for the synthesis of both LML and SML. Of the immobilized lipases, those from *Pseudomonas cepacia*, *Mucor miehei* and *Thermomyces lanuginosus* were effective depending on the sugar/solvent combination. Higher overall yields were obtained for the synthesis of LML with the differences in yields presumably due to the decreased solubility of sucrose as compared to lactose in 3 of the solvents used. Response surface methodology was used to determine the optimal temperature, enzyme concentration and ratio of reactants for LML synthesis using the immobilized lipase from *Mucor miehei* in 2-methyl-2-butanol. Based on the analysis of ridge max, the optimal synthesis conditions were predicted to occur at 61 °C, with an enzyme amount of 32 mg/mL, and a molar ratio of lactose to vinyl laurate of 1:3.8; and the optimal actual yield was 99.3%.

The microbial inhibition (90%) of *Listeria monocytogenes* and *Staphylococcus Suis* was observed at LML 0.1%. There was a 70% inhibition of *S. suis* at LML concentrations of 0.01%. In addition to the two organisms mentioned above, there was limited (38%) inhibition of *Enterococcus faecalis* at 0.0% LML concentration. All of the organisms which showed growth inhibition were gram positive.

## Background Information

Carbohydrate esters are biodegradable and nontoxic and are currently used in the food and personal care industries. They have a variety of documented activities including antimicrobial, insecticidal, emulsification and foaming properties. The specific activities of a carbohydrate ester depends on the fatty acid esterified (carbon chain length), sugar moiety, and number of fatty acids esterified. The most commonly produced carbohydrate esters are sorbitol and sucrose esters which are being produced at about 20,000 and 4,000 Tm/year respectively. Sucrose esters are used in Japan as antibacterial agents in canned drinks (Ferrer et al., 2005). The use of lipases to synthesize carbohydrate esters has advantages including specificity and products are acceptable for use in foods, which is not the case for chemically synthesized carbohydrate esters.

The antimicrobial activity of carbohydrate esters (6-O-lauroylsucrose and 6'-O-lauroylmaltose, galactose laurate, and fructose laurate) has been demonstrated recently against the food spoilage bacteria including *Bacillus* sp. *Bacillus stearothermophilis*, *Lactobacillus plantarum*, *Escherichia coli*, (Ferrer et al., 2005) and *Streptococcus mutans* (Wantanabe et al., 2000) and *Streptococcus sobrinus* (Devulapalle et al., 2004) which are involved in the formation of dental caries. Over 30 different carbohydrate esters were screened in the above references with respect to antibacterial activity, yet there has been no publications to date on the synthesis, characterization and bioactivity of lactose esters. In general, carbohydrates esterified to lauric acid in the monoester form have shown the highest antimicrobial activity.

This proposal will investigate the synthesis and antimicrobial activity of lactose lauryl esters. The degree of esterification and the position of the fatty acid on the carbohydrate are dependent on the enzyme type, solvent composition, ratio of reactants, and temperature. Therefore, a response surface statistical design is described in the methods section to determine the optimum conditions for synthesis of lactose laurate monoesters. Specifically the



use of food grade solvents (ethanol, acetone, and ethyl methylketone), the type of immobilized enzyme (Lipozyme TL IM, Novozyme 435 and Lipase PS), the reactant concentrations of lactose:lauric acid (1:1, 1:3, and 1:5) and temperatures (40, 50 and 60C) will be investigated.

The antimicrobial activity of the lactose lauryl esters will be investigated by determining the minimum inhibitory concentration by the microbroth dilution method for *Streptococcus sobrinus*, *Streptococcus mutans*, *Bacillus subtilis*, *E. coli* K12, *Lactobacillus plantarum*, and *Pseudomonas fluorescense*. In addition the rate of antimicrobial action for lactose lauryl esters will be determined using a fluorescent dye that penetrates the cell wall of compromised bacteria.

Carbohydrate fatty acid esters are biodegradable, nontoxic and have broad applications in the food industry as well as other fields including cosmetics, detergents, oral-care products, agriculture, and pharmaceuticals. Their antimicrobial, insecticidal, and food functional properties have been reported. The specific properties of carbohydrate esters depends on the characteristics of the fatty acid esterified (generally carbon length), sugar moiety, and number of fatty acids esterified. Sucrose esters are tasteless, odorless, and are digested by pancreatic lipases to their components which are metabolized in the normal way. However they are resistant to degradation in the saliva due to the absence of lipases. Sucrose esters are stable at pH values between 4 and 8 and up to 180 C (Devulapalle et al., 2004). The most commonly produced sugar esters include sucrose, sorbitan and alkyl polyglycosides (Table 1) which are gaining increased attention due to advantages with regard to performance, health of consumers, and environmental compatibility.

**Table 1.** Production and use of common carbohydrate esters (Hill and Rhode, 1999)

Name	Manufacturers	Applications	Production (world tons annually)
Sorbitan esters	Akeros, Dai-ichi Kogyo Seiyaku, Henkel, Kao, ICI, PPG, SEPPIC	Pharmaceuticals, personal care, food, coatings	20,000
Sucrose esters	Croda, Dai-ichi Kogyo, Seiyaku, Mitsubishi	Food, personal care, pharmaceuticals	4,000
Alkyl polyglycosides	BASF, Henkel, ICI, Union Carbide	Personal care, detergents, agrochemicals	80,000

**Table 2.** Availability of carbohydrates as raw materials.

Carbohydrate	Price per Kg
Sucrose <sup>1</sup>	0.8
Glucose <sup>1</sup>	0.55-1.21
Sorbitol <sup>1</sup>	0.80-1.70
Lactose <sup>2</sup>	0.14

1 Hill and Rhode, 1999.

2 Glanbia Nutritionals, personal communication, May 2006.

As reviewed by Hill and Rhode in 1999, only a few carbohydrates fit the criteria of price for economical production of carbohydrate esters (Table 2). As of 1999, these included sucrose, glucose, and sorbitol. Interestingly, the current price of lactose is less than the above listed carbohydrates. Therefore, the production of lactose esters would be at least as economical as the currently produced carbohydrate esters.

The activity of carbohydrate esters depends on the type of carbohydrate uses as well as the type of fatty acid esterified (carbohydrate chain length) and the degree of esterification. For example, carbohydrate esters containing multiple long chain fatty acids (C12-C18) are commonly used in the food industry as emulsifiers (i.e. available from Degussa Food Ingredients, Danisco, Mitsubishi-Kagaku Foods Corporation) (Tual et al., 2006). The degree of esterification influences the hydrophilic-hydrophobic balance (HLB) that determines its usefulness in either a water in oil or oil in water emulsion.

In addition to the use of carbohydrate esters as emulsifiers, there has been significant research on the use of carbohydrate esters as antimicrobial or insecticidal agents. Recent research by Puterka et al. (2003) tested the activity of a variety of sugar esters on the insecticidal activity against a range of arthropod species. Overall, most of the carbohydrate esters examined had superior insecticidal activity compared to insecticidal soap. Specifically, sucrose octanoate high in monoester content had the highest activity against the range of arthropod pests at concentrations of 1200-2400 ppm. Sorbitol octanoate and xylitol decanoate also showed insecticidal activity. In addition to the research of Puterka et al. (2003), other investigators have also shown carbohydrate esters have insecticidal activity to soft bodied arthropods including mites, aphids, whitefly and psyllids (Chortyk et al., 1996; Chortyk, 2003; Puterka and Severson, 1995; Liu et al., 1996; Liu and Stansly, 1995).

The antimicrobial activity of carbohydrate esters (6-O-lauroylsucrose and 6'-O-lauroylmaltose, fructose laurate, and galactose laurate) has been demonstrated recently against the food spoilage bacteria *Bacillus* sp., *Bacillus stearothermophilus*, *Lactobacillus* (Ferrer et al., 2005) and two bacteria involved in formation of dental caries formation, *Streptococcus mutans* (Watanabe et al., 2000) and *Streptococcus sobrinus* (Devulapalle et al., 2004). Ferrer et al. (2005) studied the effect of sugar (glucose, sucrose, or maltose), length of fatty acid (lauric or palmitic) and degree of esterification (mono or diester) on antimicrobial properties. They synthesized 6 different carbohydrate esters including 6-O-lauroylsucrose, 6'-O-lauroylmaltose, 6'-O-palmitoylmaltose, lauroylsucrose, and lauroylsucrose and tested their antimicrobial activity against a variety of organisms including *Bacillus* sp., *Pseudomonas fluorescens*, *Staphylococcus aureus*, *Escherichia coli*, *Pichia jadinii*, *Bacillus stearothermophilus* and *Lactobacillus plantarum*. 6-O-lauroylsucrose and 6'-O-lauroylmaltose were successful at concentrations of 0.25 to 5 mg/ml against *Bacillus* sp., *E. coli*, *P. jadinii*, *B. stearothermophilus* and *L. plantarum*. For reference, sucrose esters are used as emulsifiers in foods at concentrations as high as 10 mg/ml (Ferrer et al., 2005).

Watanabe et al. (2000) synthesized 23 different carbohydrate esters from various sugars and fatty acids and evaluated their bacteriostatic activity against the cariogenic bacterium *Streptococcus mutans*. Galactose and fructose laurate showed the highest bacteriostatic activity. Generally, the carbohydrate esters containing lauric acid as the fatty acid were significantly more active than those containing capric, butyric, caproic, myristic or palmitic acids at concentrations as low as 0.01%.

Devulapalle et al. (2004) investigated the antimicrobial activity of lauroylsucrose, lauroylmaltose and lauroylmaltotriose against the cariogenic bacterium *Streptococcus sobrinus*. No bacterial growth was detected in the liquid medium supplemented with lauroylmaltose (0.5 mg/ml) or with lauroylmaltose (1 mg/ml) or lauroylsucrose (2 mg/ml). Other researchers have also demonstrated the antimicrobial activity of carbohydrate esters (Marshall et al., 1994; Bergsson et al., 2001; Kato et al., 1997).

In general with respect to antimicrobial activity, Gram-positive bacteria are more susceptible to carbohydrate esters than Gram-negative bacteria and fungi are more susceptible to carbohydrate esters than yeasts (Marshall, 1994). The mechanism of antimicrobial action of these carbohydrate esters has not been elucidated. It is possible that sugar esters disrupt the cell membrane, thereby altering its permeability and causing a selective leakage of glycolytic intermediates. In *B. subtilis* cells, there is a change in morphology and induced autolysis resulting in cell death (Ferrer et al., 2005) in the presence of carbohydrate esters.

Considering that lauric carbohydrate esters showed the highest antimicrobial activity, we are proposing to esterify lauric acid to lactose.

Various sugar esters have been synthesized from sucrose, maltose, leucrose, maltotriose with acyl chains ranging from 2 to 18 carbons. There are two methods of esterifying fatty acids to carbohydrates, one is chemical and the other is enzymatic using lipases.

The chemical process results in a high energy cost, chemical waste, undesirable by-products and heterogeneous esters (mixture of compounds differing in the degree of esterification on the position of the acyl group on the sugar

moiety). The chemical process is done under basic conditions and results disadvantages including low selectivity and yields and colored derivatives as side-products. The enzyme-catalyzed process is more selective and does not result in off products (Ferrer et al. 2000) and can result in esters that can be used as ingredients in foods.

By controlling the degree of esterification (enzyme type, solvent composition, ratio of reactants, and temperature) it is possible to modify their properties. The factors listed (enzyme type, solvent composition, ratio of reactants, and temperature) are interrelated with respect the degree of esterification. Therefore, a response surface statistical design is described in the methods section.

Lipases (EC 3.1.1.3) catalyze the hydrolysis of ester linkages in acylglycerols in aqueous environments at the oil-water interface. Under nonaqueous environments, lipases can catalyze the synthesis or interesterification of triacylglycerols. The optimum water levels for lipase-catalyzed reactions vary from 0.042 (%v/v) to as high as 50 (%v/v) depending on whether net esterification or net hydrolysis is sought (Balcao et al., 1996). The interesterification reactions can be divided into three different processes: 1) acidolysis is the exchange of fatty acids between acylglycerols and free fatty acids, 2) transesterification is the exchange of fatty acids between two acylglycerols, and 3) glycerolysis is a reaction between free fatty acids or acylglycerols and glycerol. These activities are used to produce trans-free and structured triacylglycerols. Lipases can also catalyze the transesterification reaction between free fatty acids and a hydroxyl group on sugars to form sugar esters. Recently, the use of the immobilized form of the lipase is preferred since immobilization improves the lipase stability and activity and the enzyme can be reused. The number of fatty acids esterified depends the type of solvent, the ratio of fatty acids to sucrose molecules and the type of enzyme used.

Plou et al. (2002) screened a variety of immobilized lipases for the acylation of di- and tri-saccharides with laurate and found four lipases which were capable of catalyzing this reaction efficiently. These lipases were from *Thermomyces lanuginosus*, *Candida antarctica* and *Pseudomonas*. Each lipase showed a different specificity based on the hydroxyl groups in the sugars. For example, with *C. antarctica* lipase and sucrose, both the 6C-OH and 6'C-OH groups were acetylated but with lipases from *T. lanuginosus* and *Pseudomonas* only the 6C-OH were acetylated. With maltose, each of the enzymes listed above was specific only for the 6C-OH. In general, the chemical reactivity of sucrose hydroxy groups follows the order of 6C-HO>6'C-OH>1'COH>secondary-OHs. The ratio of sucrose to fatty acids in these experiments was 1:1 and the solvent included dimethylsulfoxide. The authors also noted that increasing the concentration of dimethylsulfoxide resulted in the formation of diesters, not monoesters. The chemical, physical, and antimicrobial properties of carbohydrate esters changes with the degree of esterification as noted above.

Immobilized lipases are available commercially from a variety of vendors and vary in specificity from Sn-1,3 or nonspecific (Table 3). The commercial use of immobilized enzymes includes the production of trans-free fats, cocoa

**Table 3.** Characteristics of some commercially available immobilized enzymes (Walsh, 2006)

Name	Supplier	Organism	Specificity	Matrix
Lipozyme TL IM	Novozyme A/S	<i>Thermomyces lanuginosus</i> , TLL-1	Sn-1,3 specific	Silica granules
Lipozyme RM IM	Novozyme	<i>Rhizomucor miehei</i> , RML	Sn-1,3 specific	Macroporous ion exchange resin
Novozyme 435	Novozyme	<i>Candida antarctica</i> lipase B	nonspecific	Macroporous acrylic resin
Lipase PS-C	Amano	<i>Pseudomonas cepacia</i> lipase, PCL	nonspecific	Ceramic particles
Lipase AK-C	Amano	<i>Burkholderia cepacia</i> lipase (formerly <i>Pseudomonas fluorescens</i> lipase PFL)	Sn-1,3 specific	Ceramic particles

butter equivalents, and diacylglycerols (Enova™ Oil) (Archer Daniels Midland, Quincy IL) (reviewed in Walsh, 2006).

In order to synthesize carbohydrate esters it is necessary to find a medium that maintains the carbohydrate and the fatty acid soluble in the presence of the lipase. High aqueous systems favor ester hydrolysis, which limits sugar ester formation due to equilibrium. Common solvent systems for carbohydrate ester synthesis include dimethylsulfoxide, tert-butanol, propane, butane, hexane, ethyl methylketone, acetone, ethanol and propanol. Solvents permitted in the synthesis of a food ingredient include acetone, ethyl methylketone and ethanol (Gulati et al., 2003; Plou et al., 2002). For example, the yield of sorbitol monostearate in propanol and ethanol was lower than in other solvents (hexane, butanol) but it was still possible to achieve a 70% conversion. It is also possible to synthesize carbohydrate esters in aqueous environment although the yields are generally much lower than in a nonaqueous environment (Dossat et al., 2002). Therefore the methods section of this proposal describes the comparison of acetone, ethyl methylketone and ethanol for lactose lauryl ester synthesis.

It is common to include molecular sieves (10% w/v) for the removal of water produced during the reaction (Gulati et al., 2003) to maintain a low water activity ( $<0.5 a_w$ ). The low water activity shifts the equilibrium towards ester synthesis and prevents ester hydrolysis (Ducret et al., 1995; Ferrer et al., 1999). For example, Watanabe et al. (2000) esterified various fatty acids to carbohydrates using *P. cepacia* lipase in acetone at 60C for 48 hrs in the presence of molecular sieves.

In general, the monoesters of carbohydrates were shown to have greater antimicrobial activity compared to di- or tri-esters. This is a function of both the lipase used and the ratio of the carbohydrate to the fatty acid. Generally, molar ratios of 1:1 to 1:5 (carbohydrate:fatty acid) have been used to determine the optimum concentration of fatty acid resulting in monoester formation (Gulati et al., 2002; Ferrer et al., 1999; Reyes-Durate et al., 2005). For example, the synthesis of sorbitol monostearate was optimum at a sorbitol:stearic acid molar ratio of 1:4 at times of 12 h to 48 hours using a lipase from *Pseudomonas* (Gulati et al., 2002). Ferrer et al. (2005) used 2-methyl-2-butanol as a reaction medium when synthesizing antimicrobial carbohydrate esters (6-O-larylsucrose and 6'-O-laryoymaltose) with lipases from *T. lanuginosus* and *C. antarctica* at a sugar:fatty acid ratio of 1:5 at 40 C for at least 2 hrs.

In addition to the choice of solvent, ratio of reactants, and type of lipase, temperatures of esterification reactions vary from 40 to 60 C (Ferrer et al., 1999, Plou et al., 2002, Gulati et al., 2003) and the reaction times vary from 8 h to 7 days (Ferrer et al., 1999; Piao and Adachi, 2004).

Due to the interrelationship of the factors (solvent, enzyme, temperature, ratio of reactants) the methods section describes a response surface statistical design.

## MATERIALS AND METHODS

Lactose will be purchased from Glanbia Nutritionals (Monroe WI). Vinyl laurate and lauric acid will be purchased from (Sigma-Aldrich Fluka, St. Louis MO). Immobilized enzymes (Lipozyme TL IM from *Thermomyces lanuginosus* and Novozyme 435 from *Candida antarctica* B), which are Novozyme AS products will be purchased from Sigma-Aldrich (St. Louis MO). Lipase PS-C from *Pseudomonas cepacia* will be purchased from Amano Pharmaceuticals (Elgin IL). Molecular sieves (3 A, 8-12 mesh), tributyrin, sodium azide, Penicillin G, Polymyxin B, and reagents (acetone, methanol, ethanol, ethyl methylketone, acetonitrile, buffer salts) will be purchased from Sigma Chemical (St. Louis MO).

Microorganisms to be used for assaying the microbial inhibitory activity of the lactose lauryl esters are listed in Table 4. Microorganisms will be purchased from ATCC and the media and growth temperature listed for culturing the organisms will be purchased from Invitrogen-Gibco (Carlsbad, CA).

**Table 4.** Microorganisms to be used to test the microbial inhibitory activity of lactose lauryl esters.

Bacteria	Biosafety level	Media and ATCC Media Reference Number	ATCC #
<i>Streptococcus sobrinus</i>	1	ATCC 44: Brain heart infusion, 37 C	27351
<i>Streptococcus mutans</i>	1	ATCC 1169: Glucose tetrazolium medium, 37 C	31341
<i>Bacillus subtilis</i>	1	ATCC 18: Trypticase soy agar, 37 C	39088
<i>E. coli</i> K12	1	ATCC 1065: LB medium, 37 C	35695
<i>Lactobacillus plantarum</i>	1	ATCC 416: Lactobacillus MRS broth, 37 C	14431
<i>Pseudomonas fluorescense</i>	2	ATCC 368: Blood agar base, 37	25006

**Objective 1. Determine the optimum conditions for the synthesis of lactose lauryl esters**

**Statistical Analysis for Objective 1.** Response surface methodology (RSM) will be used to determine the optimum conditions for lactose lauryl ester synthesis based on literature conditions for the synthesis of sucrose, maltose and glucose esters of lactic acid and palmitic acid. When the goal is to determine treatment values for optimal responses (maxima or minima) RSM is a valuable tool. Although factorial-treatment structures can be used for these kinds of experiments, RSM is preferred when treatment factors are varied across a continuous range of values. The treatments (factors) that influence the production of sucrose, glucose and maltose esters include the type of solvent (generally acetone, ethyl methylketone or ethanol), temperature (from 40-60 C), the concentration of reactants (molar ratios of sugar:fatty acids from 1:1 to 1:5) the type of lipase (non-specific or sn-1,3 specific lipase) and the time (24-60 hrs). A response surface design (central composite design with orthogonal blocking) with 4 significant factors is shown in Table 5. Each condition will be analyzed in triplicate. Analysis of variance, regression and canonical analysis for the nature of the response variables will be done using SAS Design of Experiments (Cary NC). Response surface graphs will also be generated with SAS. The optimum treatment conditions predicted by RSM will be used to produce lactose lauryl esters to confirm the RSM outputs at various times (0, 8, 16, 32, 48 hrs) to define optimum conditions for synthesis. For example, a possible outcome from the RSM listed in Table 5 would be the use of acetone at 55 C with Lipase PSC at a molar ratio of 1:2 (note, RSM uses linear regression to determine the optimum conditions). These treatments would then be used at various times to determine the best possible time resulting in maximum lactose lauryl monoester synthesis.

**Lipase Activity.** Lipase activity will be determined to ensure the same units of enzyme activity are used for each of the three immobilized enzymes. Lipase activity will be determined using the tributyrin assay according to Ferrer et al. (1999) and Nam and Walsh (2005). Briefly, the reaction mixture (10 mL) will contain tributyrin (68 mM), 0.1 M NaCl, 0.1 M CaCl<sub>2</sub>, 1 mM Tris-HCl (pH 7) and 3% acetonitrile. Aliquots (vol amount) will be added to the reaction mixture and allowed to react at 25 C for 30 min while shaking on an orbital shaker (100 rpm). The reaction will be titrated with 1 M NaOH to determine the amount of fatty acids liberated. Activity will be expressed as microkat (amount of enzyme that liberates 1 micromol of fatty acid per minute), which is equal to 60 U.

**Lactose Lauryl Ester Synthesis.** Lactose lauryl esters will be synthesized according to Ferrer et al. (2005) by transesterification of lactose with vinyl laurate at the concentrations listed below (Table 6) in various solvents (acetone, ethyl methylketone or ethanol) in a 5 ml volume.

Immobilized lipases (Lipozyme TL IM from Novozyme, Novozyme 435 from Novozyme or Lipase PS from Amano Pharmaceuticals) will be used as biocatalysts (1060 units as determined as described above). Reactions will be performed at a set temperature of 40, 50 or 60 C on an orbital shaker (100 rpm) in the presence of 3 A molecular sieves (100 mg/ml).

Products will be monitored by HPLC using a Nucleosil 100-C18 reverse phase column (250 mm x 4.6 mm Beckman Cultor, Fullerton CA) maintained at 40 C on a Beckman System Gold HPLC with a 1255 solvent module (Beckman Cultor, Fullerton CA). The mobile phase will consist of a 90:10 (v/v) solution of methanol:water at a 1.1 ml/min flow rate. Detection will not be via refraction index as described by Ferrer et al. (2005) but instead we will use an evaporative light scattering detector (Altech ELSD 800, Deerfield IL) which is more sensitive and stable than

**Table 5.** Response Surface Central Composite Design with 4 Factors

Run Number	Block	Solvent	Temp (C)	Molar ratio	
				Lactose:Fatty Acid	Lipase type
1	1	Acetone	40	1:1	Lipase PSC
2	1	Acetone	40	1:5	Lipozyme
3	1	Acetone	60	1:1	Lipozyme
4	1	Acetone	60	1:5	Lipase PSC
5	1	Ethanol	40	1:1	Lipozyme
6	1	Ethanol	40	1:5	Lipase PSC
7	1	Ethanol	60	1:1	Lipase PSC
8	1	Ethanol	60	1:5	Lipozyme
9	1	EMK	50	1:3	Novozyme
10	1	EMK	50	1:3	Novozyme
11	2	Acetone	40	1:1	Lipozyme
12	2	Acetone	40	1:5	Lipase PSC
13	2	Acetone	60	1:1	Lipase PSC
14	2	Acetone	60	1:5	Lipozyme
15	2	Ethanol	40	1:1	Lipase PSC
16	2	Ethanol	40	1:5	Lipozyme
17	2	Ethanol	60	1:1	Lipozyme
18	2	Ethanol	60	1:5	Lipase PSC
19	2	EMK	50	1:3	Novozyme
20	2	EMK	50	1:3	Novozyme
21	3	Water	50	1:3	Novozyme
22	3	Water	50	1:3	Novozyme
23	3	EMK	30	1:3	Novozyme
24	3	EMK	70	1:3	Novozyme
25	3	EMK	50	0	Novozyme
26	3	EMK	50	1:7	Novozyme
27	3	EMK	50	1:3	None
28	3	EMK	50	1:3	None
29	3	EMK	50	1:3	Novozyme
30	3	EMK	50	1:3	Novozyme

**Table 6.** Concentrations of reactions

Lactose (M)	Laurate (M)	Lactose Laurate (M ratio)
0.03	0.03	1:1
0.03	0.09	1:3
0.03	0.15	1:5

refractive index detectors. Chromatography standards for identification and quantitation will include lactose, lauric acid, and vinyl laurate. The products will be quantified and collected after HPLC separation for future characterization by  $^1\text{H}$  NMR.

Characterization by  $^1\text{H}$  NMR will be done as described by Ferrer et al. (1999) by the USU Center for Integrated Biosystems (Logan, UT). Briefly,  $^1\text{H}$  NMR spectra will be recorded on a Varian INOVA (300 Mhz) spectrometer (Palo Alto, CA) at 30 C for samples.

Objective 1 will be completed in 15 months

### **Objective 2. Investigate the antimicrobial properties of lactose lauryl esters**

The minimum inhibitory concentration (MIC) for each of the organisms will be determined by the microbroth dilution method as described by the National Committee for clinical Laboratory Standards (Woods and Washington, 1995). The microorganisms will be grown overnight in their respective optimal growth media at the appropriate temperature (Table 4) from stock cultures stored in liquid nitrogen. Each culture will be sub-cultured twice, harvested in mid log phase (approximate based on dividing time for each organism) and washed with saline. Plate counts (using optimum growth media with agar as listed in Table 4) and OD600 measurements will be taken. Graphs of OD600 vs plate counts will be made for each organism. New stock cultures will be grown overnight in their respective optimal growth media at the appropriate temperature and sub-cultured twice, harvested in mid log phase and resuspended in optimal growth media to 105 CFU/ml (as determined by OD600 measurements of the same culture) containing lactose lauryl ester at concentrations of 0, 2, 6, 8, 10, 20, and 50 micrograms/ml in a total volume of 500 microliters in 48-well microplates (Corning NY). The plates will be incubated in optimal growth conditions for the respective organism and monitored for an increase in OD600 after 12, 24 and 48 hours using a Perkin-Elmer (HTS 7000) plate reader (Downers Grove IL). A positive control for inhibition of growth using Polymyxin B at 1000 micrograms/ml for Gram-negative organisms and Penicillin G at 1000 micrograms/ml for the Gram-positive organisms will be included in each microplate. Negative controls of organisms without lactose lauryl esters of each organism will also be included. The least concentration at which there is no increase in OD600 after 48 hours will be reported as the MIC. Each MIC will be determined in two replicates with triplicates test per replicate. The triplicates will be averaged for each replicate reported.

The rate of antimicrobial action for lactose lauryl esters will also be determined at the MIC value as well as at 0 and 2xMIC. Presuming the method of antimicrobial action involves the formation of pores in the microbial cell wall, the rate of uptake of propidium iodide (Fluoropure grade, Molecular Probes Inc Eugene OR) will be used as described by Haughland (2002). Briefly, all cultures will be grown overnight in their respective optimal growth media and temperature from liquid nitrogen stock cultures. Each culture will be sub-cultured twice, harvested in mid log phase, washed with saline and adjusted to an OD600 of 0.25 (as determined from experiment described above on OD600 vs plate counts) in saline. Propidium iodide, with an excitation wavelength of 535 and an emission wavelength of 617 will be added to the culture suspensions at final concentrations of 0, MIC and 2x MIC. The increase in fluorescence (RFU) will be measured with a Shimadzu RF 1501 spectrophotofluorometer (Columbia MD) at 15 s intervals for approximately 120 min. The rate of propidium iodide entering the pores in the cell walls will be determined in two replicates with triplicates test per replicate. The triplicates will be averaged for each replicate reported. The rate of antimicrobial action (pore formation) will be expressed as the inhibition rate (IR) using the following equation and the OriginPro Ver 7.0 (Natick MA) program. 
$$\text{IR} = ((\text{LogRFU}/\text{time}) - \text{C}) / \text{Time} \text{ (when } d\text{LogRFU}/dt > 0$$

Objective 2 will be completed after objective 1 in 9 months.

### **Objective 3. Data analysis and manuscript preparation**

The data generated in objectives 1 and 2 will be analyzed and a manuscript will be prepared.

## **RESULTS AND DISCUSSION**

### **3.1 Monoester Yields and Reaction Rates**

Fig. 1 shows the amount of LML (Fig. 1 A) and SML (Fig. 1 B) synthesized overtime (14 days) for 4 of the 12 enzyme/solvent combinations investigated at constant enzyme, temperature, and substrate concentrations. The temperature of 55 °C was chosen since previous researchers have shown that immobilized lipases are generally more active at temperatures of 50-70 °C [1]. We also wanted to stay below the evaporation temperatures of the solvents,

the lowest of which was acetone with a boiling point of 56.5 °C at ambient pressure.

Several points can be made from the graphs in Fig. 1. The highest monoester yields were obtained with lactose and it was possible to determine the synthesis rate based on the time of maximum ester synthesis. An example is the synthesis of LML in Fig. 1A with MM in 2M2B, which shows a maximum at day 3, compared to the continued production of LML and SML by PC in 2M2B over the 14 day time period. In contrast to monoester yield with PC in 2M2B, most enzyme/solvent combinations reached a maximum amount of monoester in about 10 to 14 days with some showing a decrease in monoester content (e.g. Fig. 1A, MM and TL in 2M2B) over the time course that will be discussed below.

Table 7 shows the % monoester yields and rates for each of the enzyme/solvent combinations used. The maximum theoretical yield was 21.95 mg/mL based on the amount of the limiting reactant (sugar). Overall, the solvent 2M2B showed the highest yields and reaction rates for both LML and SML synthesis except for TL in acetone in which LML synthesis was slightly higher. The solvent MEK was the least effective for each of the enzyme/solvent combinations. Cauglia and Canepa [16] showed that synthesis of glucosylmyristate with CA was dependent on the solvent with the highest yields in 2M2B, followed by acetone, hexane and finally diethylether. Other studies have also shown that high ester yields are obtained in 2M2B [1,4,9].

With respect to the enzymes, PC and TL showed the highest yields with sucrose and PC followed by MM and TL showed the highest yields with lactose. CA showed similar yields and rates with sucrose and lactose. PC was also similar with both sugars in MEK and acetone, as was TL in 2M2B. The lowest yields were obtained with MM with sucrose, and CA with lactose, depending on the solvent used. Lipase from CA is very popular in literature for the synthesis of sugar esters [1,6,8,9,11,17 are some of the most recent], yet we found this enzyme to be the least effective for LML synthesis depending on the solvent. SML yields for this enzyme were not as low as from MM. The source of our enzyme was different than the studies cited, which may account for the differences we observed.

Specifically for LML synthesis, MM in 2M2B had the highest reaction rate due to the shortest reaction time of 3 days. PC in 2M2B actually showed a slightly higher yield (56.6%) than MM in 2M2B (52.4%), but the rate is much slower due to the length of time (14 days) to reach maximum yield. Specifically for SML synthesis, TL and PC in 2M2B showed the highest synthesis rates and yields, but the yields were lower than those obtained with lactose. This difference may be due to the differing solubility of each sugar in the specific solvents, which is discussed below.

Monoester yields that are reported in the literature vary and depend on the conditions that were investigated in this study. Examples yields include 43% glucosylmyristate with CA in 2M2B [16], 13% sucrose laurate with CA in

**Table 7.** Reaction rates and yields of sucrose and lactose ester synthesis

Enzyme	Solvent	% Lactose ester yield	% Sucrose ester yield	Lactose ester rate (mmol/h/g enz)	Sucrose ester rate (mmol/h/g enz)
<i>Thermomyces lanuginosus</i>	2M2B	35.5 ± 3.10	32.9 ± 2.71	4.4	4.9
	acetone	43.1 ± 0.08	13.2 ± 0.66	5.9	2.4
	MEK	12.0 ± 0.01	1.6 ± 0.17	1.8	0.29
<i>Mucor miehei</i>	2M2B	52.4 ± 1.88	7.2 ± 0.78	26.7	0.81
	acetone	32.5 ± 2.06	0.8 ± 0.35	13.8	0.31
	MEK	12.2 ± 1.72	0	1.3	0
<i>Pseudomonas cepacia</i>	2M2B	56.6 ± 1.45	34.2 ± 1.66	10.4	4.8
	acetone	16.8 ± 0.21	10.0 ± 3.45	3.6	1.9
	MEK	1.3 ± 0.31	1.3 ± 0.31	0.89	0.13
<i>Candida antarctica</i>	2M2B	21.8 ± 1.57	20.9 ± 0.72	3.1	2.9
	acetone	1.3 ± 0.14	3.6 ± 0.51	0.17	1.3
	MEK	0.6 ± 0.15	0.5 ± 0.12	0.10	0.10



2M2B [1], and 30% sucrose laurate with TL in 2M2B [4].

The rates obtained for all enzyme/solvent combinations presented here are in the low mmol/h/g enzyme range. Other researchers synthesizing xylitol myristate [6, in acetone], fructose monopalmitate [11, in 2M2B], glucosylmyristate [16, in 2M2B], and glucose laurate [18, in 2M2B] obtained similar rates.

### 3.2 Solubilities of Sugars in Solvents

Fig. 2 shows the solubility of lactose and sucrose in MEK, acetone, acetonitrile and 2M2B. The solubility test was done with 5% sugar solutions to ensure complete solubility in water. Each sugar showed limited solubility in each solvent with the solubility in 2M2B being the highest at approximately 700-800 micro mole/L solvent. The solubilities in the other solvents were much lower at 50-200 micro mole/L solvent with sucrose about half as soluble as lactose. This difference in solubility may result in a generally higher LML synthesis than SML synthesis. The yield obtained for LML synthesis with PC in 2M2B is 56.6%; the amount of lactose solubilized over the synthesis time was greater than 50%, with is 100 times higher than the yield predicted based on the data in Fig. 2. Therefore, as the esters are synthesized, the insoluble sugars solubilize to maintain an equilibrium.

The limiting factors in the synthesis and yield may be a combination of the sugar solubility and inactivation of the enzyme. Flores et al. [18] showed that the initial synthesis rate of glucose laurate in 2M2B was dependent of the dissolved sugar concentration with a 70% conversion in the presence of molecular sieves. Reactions with the highest rates of conversion allow more of the sugar to be solubilized, hence, the reactions have a higher yield. The higher solubility of lactose and sucrose in 2M2B resulted in the highest yields and synthesis rates, excluding LML synthesis by TL in acetone.

If solubility were the only limiting factor, we would assume a higher, or at least equal yield for SML in 2M2B with each enzyme. But this was observed for only two of the four enzymes (TL and CA). The sugar type and enzyme specificity may also influence the rate of esters synthesized.

### 3.3 HPLC Chromatograms of Reactions

Figs. 3 (lactose reactions) and 4 (sucrose reactions) show HPLC chromatograms of the products synthesized for representative reactions. In both figures, peaks that have been identified include lactose, sucrose, SML/LML and lauric acid. With free fatty acids as substrates, product yields can be determined by the decrease in free fatty acid amount, while we found this not to be possible with the vinyl lipid with our HPLC conditions. The vinyl laurate did not elute from the column within the 30 min run time, presumable due to its hydrophobicity, which increased the retention time.

In each chromatogram in which the solvent was 2M2B (all except Fig. 3A), there is a sugar peak present, which supports the lactose solubility data in Fig. 2. Depending on the enzyme used, there are multiple products present that have greater hydrophobicity (e.g. retention times) than lauric acid. We assume these are sugar esters with multiple lauric acids esterified. The greatest number of these products is present in reactions with lactose as the substrate with TL, followed by reactions with either lactose or sucrose with PC, MM and CA. Peaks with same letter among the chromatograms have the same retention times and may be similar esters with multiple lauric acids esterified.

Doublet monoester peaks were observed for most enzyme/solvent reactions except with the lipase from PC. NMR analysis of purified LML esters synthesized by TL, MM and PC revealed that the LML products were all esterified at the C6' carbon with lactose in the alpha and beta configurations. The doublet peaks are presumably from the lactose in the alpha and beta configurations. Other reducing sugars, maltose and leucrose, were enzymatically esterified at the C6' and the C1 and C6' locations respectively [4,12]. The esterification of sucrose, a non reducing sugar, with lipids has been shown to occur most frequently in the C6 position but is dependent on the enzyme type. Ferrer et al. [4] showed that C6 and C6' SML were synthesized with CA but as minor products compared to the synthesis of C6, 6'di and only C6 SML was produced with TL with minor amounts of diesters. The difference may be due to the presence of DMSO in their reactions, which they have already shown changes the final degree of esterification and the site of esterification [4]. The doublet peaks for the sucrose monoesters are presumably from the presence of both the C6 and C6' products as previously determined [4,12].

The data in Fig. 1A shows that some of the enzyme/substrate combinations exhibit a decrease in yield over time. Specifically, reactions involving MM in 2M2B and acetone, TL in acetone, and PS in acetone showed a decrease in yield. It is possible that the monoester is being converted to di- or multi-ester sugar products for reactions that are synthesized by TL and PC since the chromatographs for these enzymes show multiple hydrophobic products. This is probably not the case for reactions with MM since the chromatograms show limited multi-ester peaks. We are not

sure why the yield decreases over time with this enzyme. There was no obvious decrease in any of the yields in the sucrose reactions in Fig. 1B.

### 3.4 RSM Analysis

The RSM analysis was conducted for the synthesis of LML using MM in 2M2B because this combination resulted in a high yield, fast rate, and the enzyme is more economical than the others. The experimental design and concentration of LML synthesized at each design point are given in Table 8. Among the various treatments, the highest yields were obtained with runs 4, 6 and 7, while runs 2 and 10 showed the lowest yields.

ANOVA results revealed that all three variables and the interactions of temperature x temperature and ratio x ratio exhibited statistically significant effects ( $p < 0.05$ ) on the yield of LML. The estimate response model equation, without the insignificant variables, was used to estimate the enzymatic synthesis of LML with MM and is as follows:

$$Y = -353.78 + 5.81 X_1 + 6.9 X_2 + 101.13 X_3 - 0.11 X_2X_2 - 13.50 X_3X_3 \quad (1)$$

where Y is the response factor in peak area and X1, X2, and X3 are the independent factors of temperature, enzyme concentration (mg/mL) and ratio of lactose to vinyl laurate. The coefficient of determination (R2) was 0.95 indicating that the model was suitable to represent the factors.

Canonical analysis of the three variables determined that the most critical factor was temperature, with the concentration of enzyme being the second most influential factor on the yield. Fig. 5 shows the effect of ratio, temperature and enzyme concentration on the amount of LML synthesized. The stationary point for maximum yield was determined to be a saddle point, therefore there was no unique optimum. This can be seen in Fig. 5 where there is a narrow range of ratios (3.7-3.8) at 61 °C that gives maximum LML yield. Fig. 5 also shows the influence of temperature on yield is linear, with increasing yields with an increase in temperature while the influence of substrate ratio and enzyme concentration have narrow optimum values.

The ridge maximum analysis was conducted as described by Chang et al., [14] which determines the optimal reaction conditions with the maximum, predicted yield. The conditions of 61 °C, 32 mg/mL of enzyme and a lactose:vinyl laurate ratio of 1:3.8 was predicted to yield 28 mg/mL LML. Our experimental results were in agreement with a concentration of 27.8 mg/mL obtained with conditions listed above. Therefore RSM was successful in determining the optimal conditions for LML synthesis in 2M2B with MM.

### Microbial Inhibition Studies

**Table 8.** Response surface design and experimental results

Run	Temperature (°C)	Enzyme amount (mg/mL)	Substrate molar ratios (lactose:vinyl laurate)	Lactose ester yield (mg/mL)
1	40	30	1:5.83	11.00
2	40	30	1:0.17	0.43
3	25	10	1:0.17	2.13
4	55	10	1:4.41	21.43
5	25	50	1:4.41	4.22
6	55	50	1:4.41	21.33
7	61	30	1:1.59	22.00
8	18	30	1:1.59	1.70
9	40	58.28	1:1.59	4.02
10	40	1.72	1:1.59	0.81
11	40	30	1:3	13.95

The microbial inhibition (90%) of *Listeria monocytogenes* and *Staphylococcus Suis* was observed at LML 0.1% (Fig 6). There was a 70% inhibition of *S. suis* at LML concentrations of 0.01%. In addition to the two organisms mentioned above, there was limited (38%) inhibition of *Enterococcus faecalis* at 0.0% LML concentration. All of these organisms are gram positive. Other studies investigating the growth inhibitory effects of sugar esters have reported that gram positive organisms are more sensitive than gram negative.

## CONCLUSIONS

The enzymatic synthesis of LML was conducted with 4 different immobilized lipases in three different solvents and compared to the enzymatic synthesis of SML under the same conditions. The yields of the monoesters was dependent on the type of solvent and enzyme, with the solvent 2M2B generally showing the highest yields and MEK showing the lowest yields for both sugars. Of the enzymes used, CA showed the lowest yields for both sugars. RSM was successfully used to optimize the synthesis of LML using MM lipase in 2M2B. The activity of LML can now be investigated and compared to the cited activities of other sugar esters.

## References

- Balaco, VM, A Kempainen, FX Malcata, PJ Kalo. 1998. Lipase-catalyzed acidolysis of butterfat with oleic acid: characterization of process and product. *Enzyme Microb Technol* 23:118-128.
- Bergsson, G, J Arnfinnsson, O Steingrimsdottir, H Thormar. 2001. Killing of gram-positive cocci by fatty acids and monoglycerides. *APMIS* 109:670-678.
- Chortyk, OT, Pomonis JG, Johnson AW. 1996. Synthesis and characterization of insecticidal sucrose esters. *J Agric Food Chem* 44:1551-1557.
- Chortyk, OT. 2003. Chemically synthesized sugar esters for the control of soft-bodied arthropods. US patent 6,608,039.
- Devulpalle, KS, A Gomez de Segura, M Ferrer, M Alcalde, G Mooser, FJ Plou. 2004. Effect of carbohydrate fatty acid esters on *Streptococcus sobrinus* and glucosyltransferase activity. *Carbohydrate Res.* 339:1029-1034.
- Dossat, V, D Combes, A Marty. 2002. Lipase-catalysed transesterification of high oleic sunflower oil. *Enzyme Microb Technol.* 30:90-94.
- Ducret, A, A Giroux, M Trani, R Lortie. 1996. Enzymatic preparation of biosurfactants from sugars or sugar alcohols and fatty acids in organic media under reduced pressure. *J Am Oil Chem Soc* 73:109-113.
- Ferrer, M., MA cruces, M Bernabe, A Ballesteros, FJ Plou. 1999. Lipase-catalyzed regioselective acylation of sucrose in two-solvent mixtures. *Biotechnol Bioeng* 65:10-16.
- Ferret, M, MA Cruces, FJ Plou, E Pastor, G Fuentes, M Bernabe, JL Parra, A Ballesteros. 2000. Chemical versus enzymatic catalysis for the regioselective synthesis of sucrose esters of fatty acids. *Surface Sci and Catalysis* 30:509-514.
- Ferrer, M., J. Soliveri, FJ Plou, N Lopez-Cortes, D Teyes-Duarte, M Christensen, JL Copa-Patino, A Ballesteros. 2005. Synthesis of sugar esters in solvent mixtures by lipases from *Thermomyces lanuginosus* and *Candida antarctica* B and their antimicrobial properties. *Enzyme Microb Technol* 36:391-398.
- Gulati, R, P Arya, B Malhotra, AK Prasad, RK Saxena, J Kumar, AC Watterson, VS Parmar. 2003. Novel biocatalytic esterification reactions of fatty acids: synthesis of sorbitol monostearate. *ARKAT* 30:159-1780.
- Haughland, RP. 2002. Handbook of fluorescent probes and research chemicals, 9th ed. Molecular Probes Inc. Eugene Oregon.
- Hill, K, O Rhode. 1999. Sugar based surfactants for consumer products and technical applications. *Fett/Lipid* 101:25-33.
- Kato A, Arima K. 1971. Inhibitory effect of sucrose ester of lauric acid on the growth of *E. coli*. *Biochem Biophys Res Commun* 42:596-601.
- Kato, S, H Kobayashi, T Wantanabe. 1987. Antimicrobial action of sucrose monolaurate on *Staphylococcus aureus*. *J Food Hyg Soc Japan* 28:261-266.
- Liu, TX, and PA Stansly. 1995. Toxicity and repellency of some biorational insecticides to *Bemisia argentifolii* on tomato plants. *Entomol Exp Appl* 74:137-143.
- Liu, TX, PA Stansly, OT Chortyk. 1996. Insecticidal activity of natural and synthetic sugar esters against *Bemisia argentifolii*. *J Econ Entomol* 89:1233-1239.

- Marshall, DL, LB Bullerman. 1994. Antimicrobial properties of sucrose fatty acid esters. In Carbohydrate Polyesters as Fat Substitutes. CC Akoh, BG Swanson Eds. Marcel Dekker, New York. Pp149-167.
- Niam, S.-H. and M.K. Walsh. 2005. Covalent immobilization of bovine phospholipase A2. J. Food Biochem. 29:1-12.
- Piao, J and S Adachi. 2004. Enzymatic preparation of fatty acid esters of sugar alcohols by condensation in acetone using a packed-bed reactor with immobilized *Candida antarctica* lipase. Biocat and Biotransformation 22:269-274.
- Plou, FJ, MA Cruces, M Ferrer, G Fuentes, E Pastor, M Bernabe, M Christensen, F comelles, JL Parra, A Ballesteros. 2002. Enzymatic acylation of di- and trisaccharides with fatty acids: choosing the appropriate enzyme, support and solvent. J Biotechnol 96:55-66.
- Puterka, GJ, RF Severson. 1995. Acitivity of sugar esters isolated from the trichomers of *Nicotiana glauca* to pear psylla. J. Econ. Entomol 88:615-619
- Puterka, GJ, W Farone, T Palmer, A Barrington. 2003. Structure-function relationships affecting the insecticidal and miticidal activity of sugar esters. Exotox 96(3):636-644.
- Reyes-Duarte, D, N Lopez-Cortes, M Ferrer, FJ Plou, A Ballesteros. 2005. Parameters affecting productivity in the lipase-catalyzed synthesis of sucrose palmitate. Biocat and Biotransform 23:19-27.
- Tual, A, E Bourles, P Barey, A Houdoux, M Despraïres, JL Courthaudon. 2006. Effect of surfactant sucrose ester on physical properties of dairy whipped emulsions in relation to those of O/W interfacial layers. J. Colloid and Interfaces Sci. 295:495-503.
- Wash, M.K. 2006. Immobilized enzyme technology for food applications. In Novel Enzyme Technology for Food Applications. R. Rastall Ed.
- Watanabe, T, S Katayama, M Matsubara, Y Honda, M Kuwahara. 2000. Antibacterial carbohydrate monoesters suppressing cell growth of streptococcus mutans in the presence of sucrose. Curr Microb 41:210-213.
- Woods, GL and JA Washington. 1995. Antibacterial susceptibility tests: Dilution and disk diffusion methods. In PR Murray (ed) Manual of Clinical Microbiology 6th edition. ASM Press. Herndon VA. pg 1327-1341.

## FIGURES

**Fig. 1.** Synthesis rates of LML (A) and SML (B) with the immobilized lipase from *Thermomyces lanuginosus* in 2M2B (■); *Candida antarctica* in 2M2B (○); *Mucor miehei* in 2M2B (●); *Pseudomonas cepacia* in 2M2B (▲).

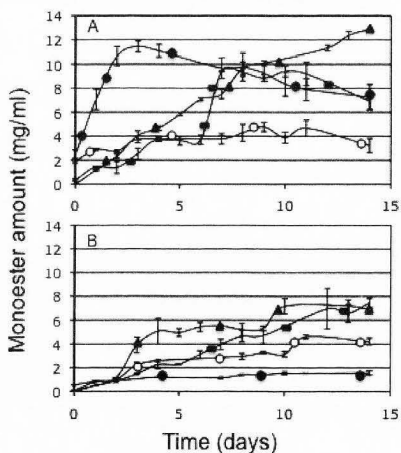


Fig. 2. Lactose and sucrose solubilities in various solvents.

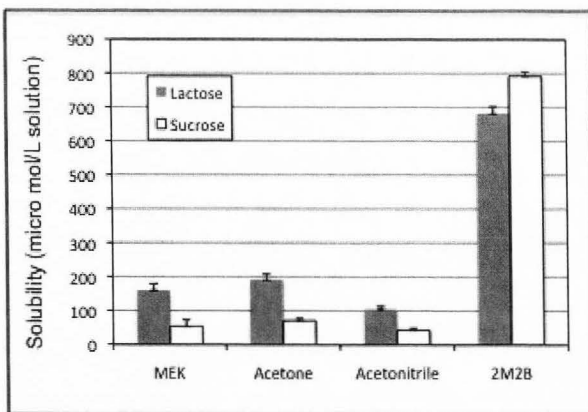
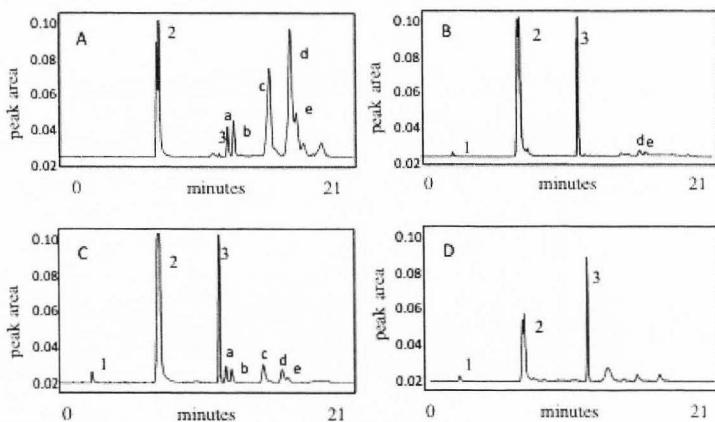
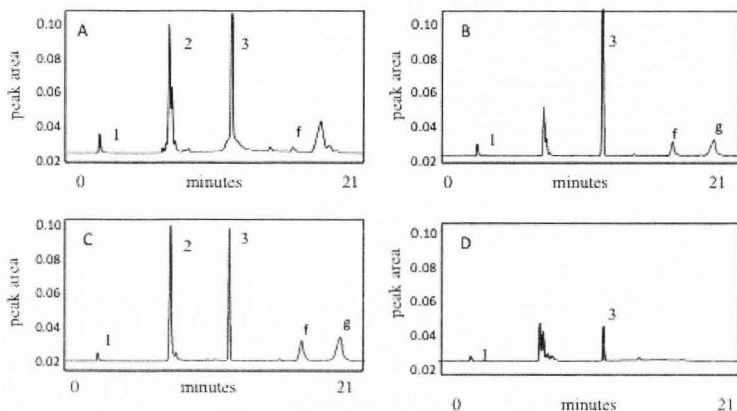


Fig. 3. HPLC chromatograms of lactose ester reactions with various enzymes and solvents. A. Reaction in acetone with lipase from *Thermomyces lanuginosus* after 7 days. B. Reaction in 2M2B with lipase from *Mucor miehei* after 3 days. C. Reaction in 2M2B with lipase from *Pseudomonas cepacia* after 14 days. D. Reaction in 2M2B with lipase from *Candida antarctica* after 9 days. Identified peaks; 1, lactose (2.2 min), 2, lactose monoester (6.8-7.9 min), 3, lauric acid (11.4 min). Peaks sharing the same letter have the same retention times.



**Fig. 4.** HPLC chromatograms of sucrose ester synthesis with various enzymes in 2M2B. A. Reaction with lipase from *Thermomyces lanuginosus* after 10 days. B. Reaction with lipase from *Mucor miehei* after 14 days. C. Reaction with lipase from *Pseudomonas cepacia* after 14 days. D. Reaction with lipase from *Candida antarctica* after 8 days. Identified peaks; 1, sucrose (2.2 min), sucrose monoester (6.8-7.9 min), 3 lauric acid (11.4 min). Peaks sharing the same letter have the same retention times.



**Fig. 5.** Response surface plots showing the mutual effects of substrate ratios with temperature (A at a constant enzyme concentration of 32 mg/mL) and with enzyme concentration (B at a constant temperature of 61 °C) on the synthesis of lactose monolaurat in 2M2M with *Mucor miehei* lipase.

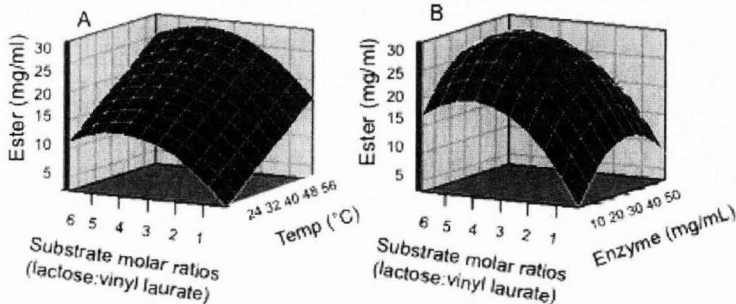
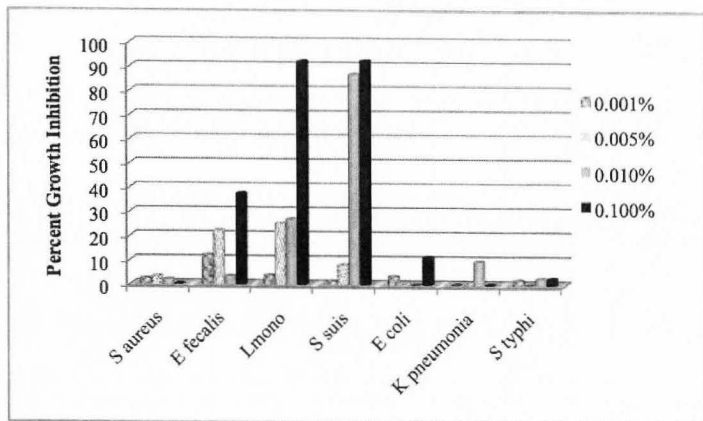


Fig. 6. Microbial growth inhibition of lactose monolaurate at concentrations from 0.001 to 0.1% on selective microorganisms.



# Development of Descriptive Taste Panel for Dairy Application

SILVANA MARTINI: Utah State University  
CARL BROTHERSEN: Western Dairy Center  
DONALD J. MCMAHON: Utah State University

## ABSTRACT/SUMMARY

A descriptive sensory panel is being developed in the Dairy Technology Innovation Lab at the Western Dairy Center. Panelists will be selected, and trained to evaluate the flavor of dairy products. Techniques and standards will be used mainly from existing bibliography. The descriptive panel will be ready for tasting dairy products by May 2008.

## BACKGROUND

Development of value-added cheeses and cheese research in general requires that sensory flavor analysis be conducted in a way that allows comparison with past research or work conducted at other locations. This can only be done by use of descriptive analysis using a panel that has been trained using standardized methods with flavor anchors and descriptors.

## MATERIAL AND METHODS

A schematic diagram of the panel selection and training process is given in Figure 1.

### *Pre-Selection*

Panelists will be recruited from the local community and asked to complete Questionnaire 1. Participants will be selected from this pool according to their responses and availability. Subjects with health conditions, allergies to dairy products or other foods will be discarded from the pool.

### *Selection*

Subjects chosen from the pre-selection will be asked to identify and rank the intensity of tastes and odors as shown on Questionnaire 2. Aqueous solutions of the following compounds will be used for taste identification: caffeine (0.07%) for bitterness, sucrose (2%) for sweetness, sodium chloride (0.2%) for salty, monosodium glutamate (1%) for umami and citric acid (0.07%) for sourness. The concentrations of compounds used for intensity ranking is listed in Table 1.

The following compounds will be used for odor recognition: butyric acid for free fatty acids, diacetyl for buttery, pineapple for pineapple/fruity, omega-3 fatty acids for fishy, wheat germ for nutty, hexanal for grassy and eucalyptus oil for floral.

Panelists with the best scores will be selected based on the following criteria:

1. One point awarded for each taste that was correctly identified.
2. One to five points awarded for recognition of odors.
3. An ANOVA test will be used to eliminate the judges that are significantly different from the group in intensity ranking.



**Table 1:** Attributes used for intensity ranking exercise

Taste	Concentration (%)
Bitter (caffeine)	0.035
	0.07
	0.14
Sweet (sucrose)	1.00
	2.00
	4.00
Salty (sodium chloride)	0.10
	0.20
	0.40
Sour (citric acid)	0.035
	0.07
	0.14

### Training

One hour training sessions will be conducted four times a week until training is complete. Flavor attributes, representative compounds, and concentrations are listed in Table 2. Panelist will first be trained to recognize these flavor attributes, then trained in quantification of the attributes. Milk will be used as the carrier. When available, references and scales from the Spectrum Method (rating of intensity using a 15 cm scale) will be used. Mixtures of attributes will also be used for training. The intensity of each attribute will be standardized to a specific rating in the scale as described in the Spectrum Method. An example of the scale used is presented in Table 3.

Panelists were presented with ten Ricotta cheese samples, in a random order, and asked to evaluate them for some of the attributes found in dairy products: bitter, brothy, salty, sour, sulfur, sweet, and umami flavor attributes. Two of the samples presented to each panelist were control samples, and the remaining eight samples were spiked with the attributes of interest and prepared as follows:

4. Bitter: 1 g caffeine + 425 g Ricotta
5. Brothy: 40 g low-sodium chicken broth + 415 g Ricotta
6. Salty: 5.5 g salt + 425 g Ricotta
7. Sour: 4.35 g 88% lactic acid + 425 g Ricotta
8. Sulfur: (0.47 ppm H<sub>2</sub>S) Formulated sample by adding 0.0225 g saturated hydrogen sulfide water (0.0 g H<sub>2</sub>S/100ml at 25 °C) to 100 ml skim milk. Then, added 1 ml of that milk to 415 g Ricotta.
9. Sweet: 25 g sugar + 380 g Ricotta
10. Umami: (0.1% MSG) 0.5 g monosodium glutamate + 425 g Ricotta

Following the criteria stated above, the best 13 panelists were chosen.

### Panel Evaluation

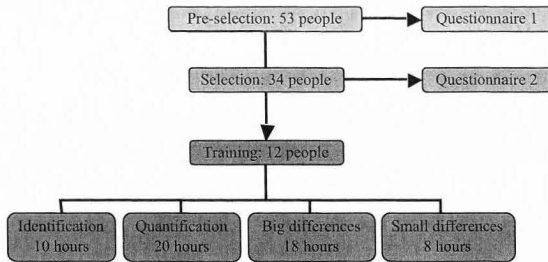
Panelists identified and quantified on a scale of 0-15, the following 18 attributes in commercial cheeses: bitter brothy, cooked, fruity, fatty acid, metallic, nutty, oxidized, pineappely, rancid, rosy/floral, salty, sour, sulfur, sweet umami, whey and buttery. The cheeses used were: Precious Mozzarella Whole Milk, Cache Valley Sharp Cheddar Gossner Unsalted Mild Cheddar, Utah State University Aggiano, Gossner Salted Mild Cheddar, Cracker Barrel Vermont Sharp-White, Aggie Extra Sharp Cheddar, Cache Valley Mozzarella Skim, Aged Cheddar Cheese.

The panel as a whole, and individual panelist performance were analyzed with "PanelCheck" software. PanelCheck is free software that provides graphical interpretation of descriptive panels performance. The panelist names were deleted from the Figures (Figures 1-3) to maintain confidentiality.

**Table 2:** Scaling references for training. References marked with an (S) mean that they correspond to the Spectrum method.

Attribute	Reference Set(s)
Sour	0.05, 0.08, 0.15, and 0.20% citric acid in water (S) or 0.035, 0.07, and 0.014% lactic acid in milk or water
Fishy	2, 5, or 7 drops fish oil supplement/ 900 ml skim
Oxidized	0.2 ml of 1% copper sulfate solution in 900 ml of non-homogenized milk (stored at 5 °C for 1 week). Stock solution and following dilutions, prepared with fresh pasteurized non-homogenized milk: 1/3 oxidized, 2/3 oxidized, and unoxidized.
Bitter	0.05, 0.08, 0.15, and 0.20% caffeine in water (S) or 0.02, 0.05, and 0.08% caffeine in water or milk
Salty	0.2, 0.35, 0.5, and 0.7% NaCl in water (S) or 0.1, 0.2, 0.4, and 0.75% NaCl in water or milk
Sweet	2, 5, 10, and 16% sucrose in water (S)
Lactone/Fatty Acid	skim, 1/3 whole, 2/3 whole, whole milk
Cooked	Cooked on stove top at 80-90 °C for 45 min and presented this to panelists along with the dilutions: skim, 1/4 cooked (3/4 uncooked skim), 1/2 cooked, 3/4 cooked; also given in dilutions of 1/3, rather than 1/4
Umami	0.1, 0.25, or 0.75% monosodium glutamate (MSG) in skim milk
Nutty	0.5 ml, 2 ml, and 5 ml hazelnut extract in 950 ml skim
Whey	(skim milk:whey) 6:1, 3:1, 1:1
Buttery	2g, 10g, 20g melted butter in warmed milk (~350ml total) ultra turraxed for up to 3 minutes until blended well enough to have the butter droplets remain dispersed in the milk. Stirring immediately before presentation reduces visual difference between the samples. Must present immediately.
Fruity	2, 5, 10, and 20 g fruit juices blend in 1000 ml skim milk (Blend consists of ½ 100% apple juice frozen concentrate and ½ 100% peach/white grape juice frozen concentrate.)
Rosy/Floral	0.01505 g 2-Phenethylamine to 1000 ml (or 1050-75 g) skim (stock solution), a 1/2 dilution of this, and 1/10 dilution
Sulfur0	Stock solution: $4 \times 10^{-7}$ g H <sub>2</sub> S. The references are formulated from this as follows: 25% stock in water/skim, 37% stock in water/skim, and 50% stock in water/skim. Water dilutions are stable; whereas milk one's must be stored for less than two days in order to avoid degradation and loss of the attribute to other flavors typically found in UHT milk (i.e. cabbagey).
Brothy	2, 10, and 20 g organic low sodium chicken broth in 1000 ml skim
Pineapple	2, 10, and 20 g 100% pineapple juice frozen concentrate
Metallic	0.04, 0.08, and 0.16 g ferrous sulfate/1000 ml skim.

**Figure 1.** Process during the pre-selection, selection and training of panelists



**Table 3:** Scale used to train the panelists. Adapted from the Spectrum Method

<b>NAME:</b> _____	<b>DATE:</b> _____
<b>Sample:</b> _____	
<b>Attribute:</b> _____	
<b>Intensity:</b> 0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15	
<b>Comments:</b> _____	

## RESULTS AND DISCUSSION

After a lengthy advertising and interview period, we hired descriptive panel coordinator. This person will continue training and maintenance of the panel, maintain the sensory laboratory, work with industry and researchers to conduct necessary tests, perform statistical analysis and write reports.

The descriptive panel met once or twice a week to practice (maintenance) the sensory skills developed during training. The panel performance as a whole and the individual performance were evaluated to assure that panelists are able to correctly identify and rate flavor attributes in dairy products. Panelists were evaluated for their ability to identify and rate all 19 flavor attributes in skim milk, and in the following cheeses: Aggie Squeaky Cheese Curd; store-bought mild, medium, and sharp Cheddar cheeses; Cracker Barrel-Vermont White Cheddar, store-bought Monterey and Colby Jack cheeses, Precious Mozzarella, Cache Valley Mozzarella, aged Aggiano, week-old USU Feta, and year-old USU Feta.. Panelist received additional training as needed.

### *Ricotta Controls*

The reproducibility of panelist ratings between the two controls was evaluated. Differences between replicates were not significant with a p-value of 0.6060. As would be expected, there was a significant interaction between the panelist and flavor attribute variables, with a p-value of 0.0058. When a mean comparison was performed, no significant differences were found between the panelists' ratings for each attribute. Though insignificant, there is room for the group to improve their ability to rate the intensity of brothy and sweet attributes in this food system. A summary of the results is in Table 4.

**Table 4:** Cheeses used for the panel evaluation

Cheese	Abbreviation
Precious Mozzarella Whole Milk	WMozz
Cache Valley Sharp	Sharp
Gossner Unsalted Mild Cheddar	NS Mild
Aggiano	Aggiano
Gossner Salted Mild Cheddar	S Mild
Cracker Barrel Vermont Sharp-White	White
Aggie Extra Sharp Cheddar	E.Sharp
Cache Valley Mozzarella Skim	SMozz
Aged Cheddar Cheese	Aged

**Spiked Ricotta Samples:**

1. Bitter sample: Judge ratings were not significantly different from one another ( $p = 0.1389$ ). Sample was rated significantly more bitter than all other samples ( $p \leq 0.0001$ ). Bitter sample had an lsmean of 8.4 with all other samples having means  $< 1.0$ .
2. Brothy sample: Judge ratings were not significantly different from one another ( $p = 0.3201$ ). Sample was rated significantly more brothy than all other samples ( $p \leq 0.0001$ ). Brothy sample had an lsmean of 7.35 with all other samples having means  $< 3.0$ .
3. Salty sample: Judge ratings were not significantly different from one another ( $p = 0.4358$ ). Samples were rated significantly more salty than all other samples ( $p \leq 0.0001$ ). Salty sample had an lsmean of 6.95 with all other samples having means  $\leq 1.5$ .
4. Sour sample: Judge ratings *were* significantly different from one another ( $p \leq 0.0001$ ). One of the judges rated samples significantly lower than other judges (lsmean 0.3794), and two other judges rated samples significantly higher than other judges with lsmeans of 3.179 and 2.779, respectively. Even though no problems were found during the control testing, the tasting with real samples show that the group could be further trained in rating sourness in ricotta cheeses. Samples were also significantly different from one another ( $p \leq 0.0001$ ). Sour sample had an lsmean of 7.55 with all other samples having means  $< 1.8$ .
5. Sulfur sample: Unfortunately, judge ratings *were* significantly different from one another ( $p = 0.0109$ ), and sample ratings *were not* ( $p = 0.9539$ ). Only three or four judges could taste any sulfur flavor in this sample. One judge could significantly taste more sulfur in this sample ( $p < 0.05$ ). All samples, except sweet (lsmean 0.3), had lsmeans between 0.9 and 1.5. The amount of sulfur added did not significantly increase the intensity of sulfur flavor in the spiked samples. Therefore, the performance of these judges on this attribute could not be evaluated. Changes to this reference will be made in the future.
6. Sweet sample: Judge ratings *were* significantly different from one another ( $p \leq 0.0001$ ). Three judges rated samples significantly less sweet when compared with the rest of the group with lsmeans of 0.798, 0.998, and 0.0798, respectively. The lsmeans for all other panelists ranged from 1.89 to 3.49. Evidenced by these results, the group could use some more work on this attribute as well. Samples were also significantly different from one another ( $p \leq 0.0001$ ). Sweet sample had an lsmean of 9.0 with all other samples having means  $< 2.1$ .

7. Umami sample: Judge ratings *were* significantly different from one another ( $p \leq 0.0001$ ). This difference was created by a single panelist who rated the umami flavor significantly higher than the rest of the panelist. While the rest of the group had an lsmean rating for umami no greater than 1.025, this panelist had an lsmean of 2.925. This panelist could use a little help with toning down umami ratings in order to rate consistently with the rest of the group. Samples were also significantly different from one another ( $p \leq 0.0001$ ). Sour sample had an lsmean of 3.6 with all other samples having means  $< 0.9$ .

A summary of the results is presented in the following table:

Attribute	Significance between judges	Significance between samples	Attribute LSmean	Attribute LSmean in other samples	Comments
Bitter	0.1389	0.0001	8.4	<1.0	Good identification and rating
Brothy	0.3201	0.0001	7.3	<3.0	Good identification and rating
Salty	0.4358	0.0001	6.9	<1.5	Good identification and rating
Sour	0.0001	0.0001	7.5	<1.8	Judges need more training
Sulfur	0.0109	0.9539	0.3-1.5	0.3-1.5	Attribute concentration too low.
Sweet	0.0001	0.0001	9.0	<2.1	Judges need more training
Umami	0.0001	0.0001	3.6	<0.9	Judges need more training

### Line Plots

In the line plots, the score for each panelist, represented by colored circles, is plotted for each taste attribute. The average score of the panel, for each attribute is indicated by the blue line. Samples of line plots for selected cheeses are shown in Figure 2.

### Correlation Plots

With correlation plots, the score of each panelist is plotted against the panel average for each flavor attribute. The score of the individual panelist being examined is represented by a colored circle, the score of the other panelists is represented by an open circle. A perfect correlation between an individual panelist's score, and the panel average is represented by a dashed blue line. Selected plots for individual flavor attributes are shown in Figure 3.

Performance of the panelists was evaluated using a well-characterized Cheddar Cheese of medium age. The test cheese was evaluated by each panelist in triplicate, along with a control cheese. The line plots of these evaluations are shown in Figure 4, wherein the replicate analyses are labeled CVMED1, CVMED2, and ControlMedium. A spider web plot of the results is shown in Figure 5, and indicates good reproducibility among the panelists.

## CONCLUSION

The panel is conducting tests on products developed in WDC research projects, as well as products submitted by industry. We continue to conduct training exercises to maintain panel efficiency. In addition we are recruiting and training replacement panelists.

Figure 2: Line plots for several cheeses (mozzarella, Aggiano, Cheddar)

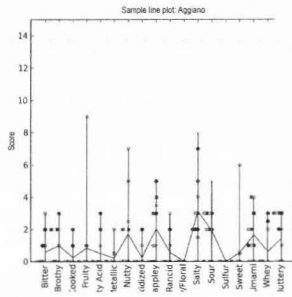
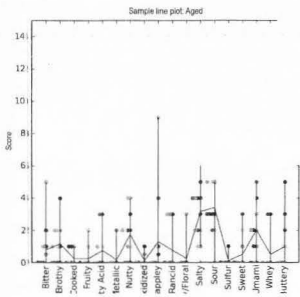
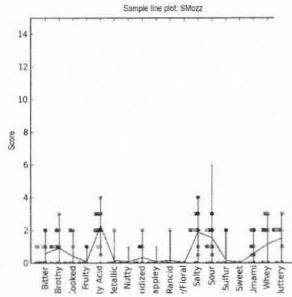
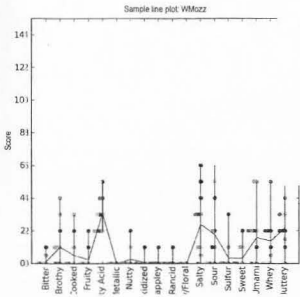


Figure 3: Panelist correlation plots

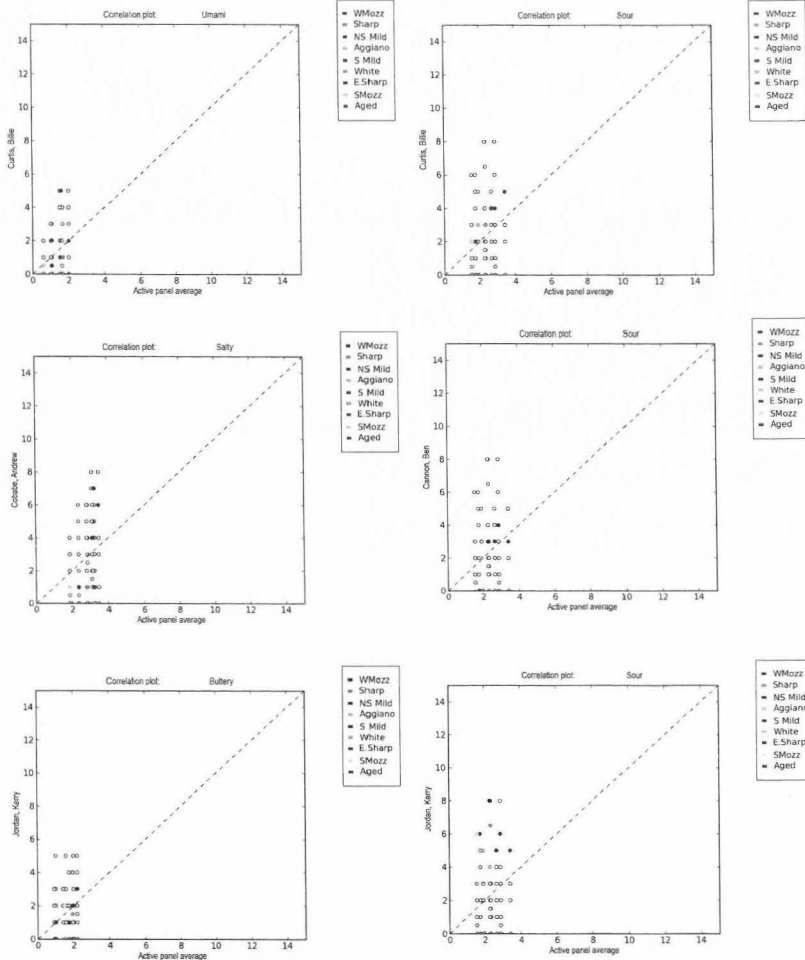


Figure 4. Line plots showing individual panelist performance evaluating Cheddar cheese.

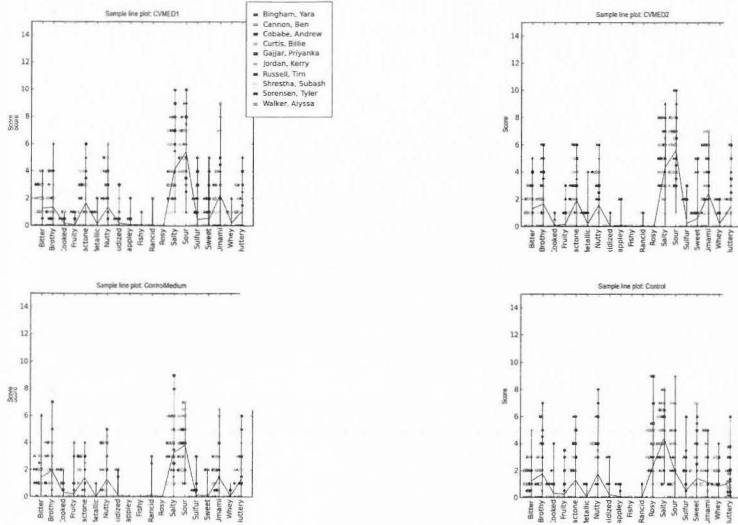
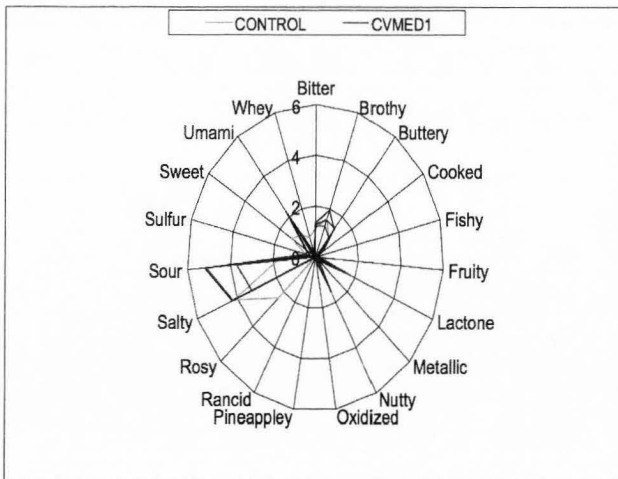


Figure 5. Spider web graph showing triplicate analysis of Cheddar cheese.





# Iron-Binding by Milk Mineral

## A Possible Antioxidant and Anti-Microbial Mechanism

DAREN CORNFORTH: Utah State University

### SUMMARY/ABSTRACT

Milk mineral (MM), the dried mineral fraction from whey, is a good calcium source, and has powerful antioxidant properties in cooked ground meats. Recent studies show that MM also prevents browning in raw ground beef. To receive acceptance for MM as a food ingredient, further studies are needed on antioxidant mechanism, possible anti-microbial effects, and sensory evaluation of products with added MM. Our hypothesis is that MM tightly binds trace levels of iron, preventing iron-stimulated rancidity, browning, and microbial growth in meat products. Demand for MM and related dairy products (nonfat dry milk) will increase as a result of a better understanding of MM properties.

### BACKGROUND

To receive acceptance of MM as a food ingredient, further studies are needed on antioxidant mechanism, possible anti-microbial effects, and sensory evaluation of products with added MM.

It is proposed that iron binding is the mechanism by which MM prevents lipid oxidation (rancidity) and meat pigment oxidation (browning) in meat products. Iron binding may also have anti-microbial effects in raw meat products. Since antioxidants have known health benefits, demonstration of antioxidant effects of MM will create a positive health image for all dairy products. Demand for MM and related dairy products (nonfat dry milk) will increase as a result of a better understanding of MM properties.

MM iron-binding capacity will be measured as the ability of MM particles to absorb iron from a solution of ferrous chloride. Iron remaining in solution will be measured by the ferrozine colorimetric assay. Iron-binding of MM will be compared to that of various calcium phosphate salts. It is hypothesized that the large MM particles with numerous phosphate groups will bind iron more effectively than the water-soluble calcium phosphate compounds. Iron binding to MM particles will also be examined by scanning electron microscopy, using energy dispersive X-ray spectrometry. Minerals (calcium, phosphate, iron) have different x-ray absorption properties that can be used to determine the position of each element in the MM particle. It is hypothesized that the bulk of the MM particle is calcium phosphate. Iron-binding to MM particles may result in detection of iron atoms on the surface of MM particles. Since many bacteria require iron, addition of MM to raw ground beef may inhibit microbial growth. To test this possibility, total plate count and Enterobacter numbers will be monitored in ground beef with and without MM, using dried media on petri film, available from 3M Corp (St. Paul, MN).

Recent work here has shown that MM also prevents browning of raw ground beef during storage. This may be due to anti-microbial effects of MM, or to iron-binding to prevent iron-catalyzed myoglobin (Mb) oxidation to brown metmyoglobin (metMb). Preliminary work here has shown that iron indeed stimulates Mb oxidation in buffered (pH 5.6 or 7.2) model systems consisting of purified horse heart Mb (Sigma Chemical, St. Louis, MO), and 35-350  $\mu$ M iron. MM counteracts the effects of iron addition. This work will be replicated in order to compare treatment means by analysis of variance. Further work will also be done to determine the level of MM needed to prevent oxidation in presence or absence of phospholipids, and at high(80%) or low (1-2%) oxygen levels, as might exist in different meat packaging methods. MM from two commercial sources (Glanbia, Twin Falls, Id) and First District Assn (Litchfield, MN) will be compared in the myoglobin model system. Finally, MM will be compared with recognized antioxidants (Trolox, eugenol, rosemarinic acid) for their ability to prevent lipid oxidation and rancid flavor development in cooked ground beef, as measured by thiobarbituric acid (TBA) assay and trained panel

sensory measurement, respectively. PI Cornforth will supervise all laboratory experiments and be responsible for data analysis, preparation of reports, and submission of results for publication in appropriate journals.

Milk mineral is the dried permeate of ultra-filtered skim milk or whey, and typically contains 24% calcium and 36-39% phosphate. It is commercially available from at least three sources in the USA (Glanbia Foods, Twin Falls, ID; First District Assn, Litchfield, MN; and DMV International, Fraser, NY). Its primary market is as a natural calcium source for sports drinks, power bars, and as a nutritional supplement. It is also a powerful antioxidant in cooked ground beef, pork, and turkey (Cornforth and West, 2002; Jayasingh and Cornforth, 2003; Vasavada and Cornforth, 2005; Vasavada, Dwivedi, and Cornforth, 2006.). It was originally used to test the hypothesis that calcium could displace iron from phospholipid binding sites, thereby slowing lipid oxidation. MM at 1.5-2% of raw meat weight indeed worked extremely well to prevent lipid oxidation, but control ground meat samples with the equivalent levels of calcium from calcium chloride were rancid. Control samples with equivalent levels of sodium mono-phosphate were also rancid, but addition of polyphosphates inhibited lipid oxidation as well as MM. Thus, Cornforth and West (2002) concluded that the polyphosphate fraction of MM, rather than calcium, was responsible for the antioxidant actions of MM, probably by chelating soluble iron and preventing iron catalysis of lipid oxidation. However, the specific antioxidant mechanism of MM has not been conclusively determined.

Recently, 0.75% MM has been shown to stabilize red color (prevent browning) in raw ground beef in 80% oxygen modified atmosphere packaging (MAP; Vissa and Cornforth, 2006). Preliminary experiments at USU indicate that MM prevents myoglobin oxidation to brown metmyoglobin by iron in lipid-free model systems. These results are of interest from both a commercial and chemical standpoint. From a commercial standpoint, MM may reduce the losses due to surface browning of fresh meats in retail markets. From a chemical standpoint, there appears to be a novel role for ionic iron in myoglobin oxidation. Previously, it has been accepted that the role of iron was to catalyze lipid oxidation, and that oxidation products such as nonenal were responsible for binding to myoglobin, and thereby increasing metmyoglobin formation and browning (Alderton and others, 2003). Thus, it is the goal of the present work to clarify the interactions of MM, iron, and oxygen levels on rates on metmyoglobin formation and browning in both model systems and ground beef.

Many consumers equate freshness of raw meats with color, and often base their purchase decisions on this characteristic (Issanchou 1996). Indeed, studies suggest the presence of brown colors in raw meats result in reduced sales (Hood 1994), with industry estimates on the subsequent financial losses ranging from \$700 million to \$1 billion (Hoffman-La Roche Inc. 1993, Liu et al. 1995). The preservation of red color in raw beef products could reduce these losses.

High-oxygen modified atmosphere packaging (MAP) has been shown to delay the onset of browning in fresh beef products (Jakobsen and Bertelsen 2000, Ho et al. 2003). However, while red color may be preserved, the high oxygen atmosphere increases lipid oxidation, leading to the development of rancid flavors and odors (Jayasingh et al. 2002).

Much attention has therefore been focused on antioxidants, which can be added to decrease the level of lipid oxidation. The results of a study examining the use of milk mineral (MM) as the antioxidant source (Vissa and Cornforth 2006) in MAP ground beef has raised an interesting question: Why does MM preserve redness in raw ground beef so well? (see Photo 1, below). While it has been suggested that MM is a Type II (iron-chelating) antioxidant (Cornforth and West 2002), to adequately answer this question, additional information on the mode of action of MM is needed.

## REFERENCES

- Alderton A.L., Faustman C., Liebler D.C., Hill D.W. 2003. Induction of redox instability of bovine myoglobin by adduction with 4-hydroxy-2-nonenal. *Biochem.* 42:4398-4405.
- Cornforth DP, West EM. 2002. Evaluation of the antioxidant effects of dried milk mineral in cooked beef, pork, and poultry. *J. Food Sci.* 67(2):615-618.
- Ho C.P., Huang N.Y., McMillin K.W. 2003. Microflora and color of ground beef in gas exchange, modified atmosphere packaging with abusive display temperatures. *J. Food Sci.* 68(5): 1771-1776.
- Hoffman-La Roche Inc. 1993. Vitamin E: Better Beef Color and Better Retail Profits. Roche Animal Nutrition and Health Fact Sheet, Nutley, NJ.
- Hood D.E. 1980. Factors affecting the rate of metmyoglobin accumulation in pre-packaged beef. *Meat Sci.* 4:247-265.

- Hunt M.C. and Hedrick B. 1977. Chemical, physical and sensory characteristics of bovine muscle from four quality groups. *J. Food Sci.* 42:716.
- Issanchou S. 1996. Consumer expectations and perceptions of meat and meat products. *Meat Sci.* 43:S5-S19.
- Jakobsen M., Bertelsen G. 2000. Color stability and lipid oxidation of fresh beef. Development of a response surface model for predicting the effects of temperature, storage time, and modified atmosphere composition. *Meat Sci.* 54(1):49-57.
- Jayasingh P., Cornforth D.P., Brennan C.P., Carpenter C.E., and Whittier D.R. 2002. Sensory evaluation of ground beef stored in high-oxygen modified atmosphere packaging. *J. Food Sci.* 67:3493-3496.
- Jayasingh, P. and Cornforth, D. P. 2003. Comparison of antioxidant effects of milk mineral, butylated hydroxytoluene and sodium tripolyphosphate in raw and cooked ground pork. *Meat Sci.* 66:83-89.
- Liu Q., Lanari M.C., Schaefer D.M. 1995. A review of dietary vitamin E supplementation for improvement of beef quality. *J. Animal Sci.* 73(10):3131-3140.
- Vasavada NM, Cornforth DP. 2005. Evaluation of milk mineral antioxidant activity in beef meat balls and nitrite-cured sausage. *J. Food Sci.* 70:C250-253.
- Vasavada MN, Dwivedi S, Cornforth DP. 2006. Evaluation of garam masala spices and phosphates as antioxidants in cooked ground beef. *J Food Sci.* In press.
- Vissa A, Cornforth DP. 2006. Comparison of milk mineral, sodium tripolyphosphate (STP) and vitamin E as antioxidants in ground beef in 80% oxygen modified atmosphere packaging. *J Food Sci.* 71: C65-68.

## MATERIAL AND METHODS

### **Objective 1 - Iron Binding Column Preparation**

Milk mineral, sodium tripolyphosphate, calcium phosphate monobasic, and calcium pyrophosphate will be used as the test materials. Columns will be prepared using small (14.5 cm length) disposable borosilicate Pasteur-type pipettes. Columns will be plugged with glass wool, then filled with test material to a depth of 2.5 cm. The amount of test material added to each column will be determined by weight difference. Columns will be pre-wetted with 1 ml of distilled water, then 0.5 ml of 1 mg/ml ferrous chloride (FeCl<sub>2</sub>) standard (in 0.1N HCl) will be added. Columns will be rinsed with distilled water to a total volume of 10 ml. Ten replicates will be performed for each test material.

**Percent Packing Loss.** Spent iron binding columns will be dried overnight at 90° C, then cooled in a desiccator. Columns will be weighed to determine the amount of packing solubilized.

**Iron Retention.** Total iron content of the filtrates will be determined using the Ferrozine assay (Carter, 1971). Briefly, an aliquot of each filtrate at basic pH (~9) will be reduced with an ascorbic acid solution. Ferrozine will be added, and the chromogen allowed to develop in the dark. Total iron will be assayed at 562 nm. Each filtrate will be assayed in duplicate. Iron retention, in mg iron / g packing compound, will be calculated based on a target value of 0.05 mg iron / ml filtrate, the expected concentration where no iron is retained by the column.

### **Objective 2 – Light and Scanning Electron Microscopy (SEM)**

Lean ground beef (90%) will be obtained from the USU Meat Lab. Samples will be prepared by adding MM or STP at 0.75% and 1.5% levels to 50 g of meat. Samples will be mixed thoroughly (kneading 25 times), wrapped in plastic film, then placed in resealable sandwich bags and held under refrigeration for three days. Samples will be prepared for light microscopy by dehydrating, embedding in paraffin, sectioning, and re-hydrating. To obtain information on milk mineral solubility (or insolubility), Von Kossa staining (Sheehan and Hrapchak, 1980) will be performed on the re-hydrated sections to test for the presence of undissolved calcium. To obtain information regarding the suspected association of iron with the calcium phosphate of milk mineral, x-ray dispersive SEM will be done on MM samples with and without treatment with ferrous chloride. MM will be prepared in PF's lab and shipped to the SEM Lab, Materials Science and Engineering Dept, University of Utah (Salt Lake City, UT) for x-ray dispersive SEM mineral localization photography.

### **Objective 3 – Effect of MM on Microbial Load and Enterobacter Levels of Ground Beef.**

**Preparation of ground beef.** USDA select grade beef shoulder clods will be purchased and used for preparation of ground beef within 1 week postmortem. Clods will be first cut into 2.5 cm thick strips. Beef trim will be prepared

by coarsely (0.60 cm plate) then finely (0.32 cm plate) grinding the strips. pH of freshly ground beef will be measured, but will not be controlled. MM (1-2%) will be manually mixed with the meat, and re-ground through the fine plate. Portions (130 g each) will be wrapped in oxygen permeable polyvinyl chloride film in a Styrofoam tray, and held for 1, 4, 7, or 14 days at 2° C. Three replicates will be performed.

**Microbial load.** Total aerobic counts and Enterobacter counts will be taken on beef samples after grinding, and at days 1, 4, 7, and 14 based on AOAC method 990.12 (AOAC 1995). Briefly, 11 g of sample will be stomached in Butterfield's phosphate diluent. Subsequent dilutions will be plated using Petrifilm™ aerobic count plates and Enterobacter specific plates (3M Corporation, St. Paul, MN), then incubated at 32°C for 48 hours. Colony counts will be taken and results interpreted per the manufacturer's guidelines. All samples will be plated in duplicate.

### **Objective 5. Interaction of Milk Mineral, Phospholipids, & Oxygen Levels on Mb Oxidation**

**Reagent and buffer preparation.** FeCl<sub>2</sub> solution will be prepared at a concentration of 0.1 mg/ml in 0.1 N HCl. Bathophenanthroline solution (BPS) will be prepared in a 5% ethanol / 95% hexane mixture. MES buffer solution (0.04 M) will be prepared in distilled water (DI), and adjusted to a final pH of 5.6 using a supersaturated sodium hydroxide solution. Tris buffer (0.1M) will be prepared as MES, with a final pH of 8.0. Residual iron will then be removed from buffers using a bathophenanthroline extraction (Schlit 1969). Briefly, 100 ml of buffer will be extracted three times with 10 ml aliquots of BPS, using a separatory funnel. Extracted buffer will be heated to approximately 95°C while being stirred rapidly to remove residual ethanol. Buffer will be cooled, and final volume adjusted back to 100 ml with distilled, deionized water (DDI).

**Iron content of myoglobin and phospholipid.** Since myoglobin and lipid cannot be purified of contaminating iron using the bathophenanthroline extraction, their total iron content will be determined and taken into consideration when designing model systems and interpreting their results. Samples will be dry ashed in triplicate, then assayed for iron using the Ferrozine procedure, as outlined previously.

**Generation of MbO<sub>2</sub> stock solutions.** A concentrated solution of horse heart myoglobin (~20 mg/ml) will be prepared in DDI water (Brown and Mebine 1969), and will be reduced by adding a small volume (~100  $\mu$ l) of a concentrated dithionite solution (Sage et al. 1991). To remove excess dithionite, the myoglobin will be passed through a Sephadex G25 column and eluted with Tris buffer (pH 8.0). Deoxymyoglobin will be converted to MbO<sub>2</sub> by bubbling air through the solution with a Pasteur pipette. Conversion to MbO<sub>2</sub> will be confirmed spectrophotometrically, based on the presence of the characteristic MbO<sub>2</sub> peaks at 545 and 580 nm (Bowen 1949). The concentration of the MbO<sub>2</sub> stock solution will be adjusted to ~0.1 mM with MES buffer (pH 5.6). Fresh MbO<sub>2</sub> stock will be generated for each experiment and replicate.

**Preparation of model system samples to examine the effect of lipids.** Lipid-free model systems will be prepared in MES (pH 5.6) using a combination of MbO<sub>2</sub>, FeCl<sub>2</sub>, and/or antioxidant to observe the effect of free iron on the conversion of MbO<sub>2</sub> to MetMb in the absence of lipid. Lipid-containing model systems will be prepared in MES (pH 5.6) using a combination of MbO<sub>2</sub>, phospholipid, FeCl<sub>2</sub>, and/or antioxidant to examine the effect in the presence of lipid.

Systems will be prepared in disposable 3.5 ml spectrophotometer cuvettes, covered with parafilm, inverted 10x to mix, then scanned from 400 to 650nm. Scans will be repeated for each system at 15, 30, 45, and 60 minutes, and at 1 and 2 days. Samples will be held at room temperature (23°C). Five complete replicates will be performed. Preparation of samples to clarify role of hydrogen peroxide. Samples will be prepared as outlined above for lipid-free model systems, using a combination of MbO<sub>2</sub>, FeCl<sub>2</sub>, catalase, and/or antioxidant to examine the potential effect of hydrogen peroxide on the conversion of MbO<sub>2</sub> to MetMb. Thymol-free catalase will be added at a level of 3mmol per mol heme (Watkins et al. 1985, Brantley 1993). After preparation, samples will be handled and spectra obtained as outlined above. Five complete replicates will be performed.

**Preparation of samples to examine the effect of partial pressure of oxygen.** Samples will be prepared as outlined for lipid-free model systems. To obtain samples with 4 initial oxygen levels (none = 0 mm Hg partial pressure = vacuum; low = 2 mm Hg = 2.6% oxygen; atmospheric = 150 mm Hg = 20% oxygen; high = 600 mm Hg = 80% oxygen), model systems will be flushed with the appropriate gas then sealed with oxygen impermeable caps. Gas cylinders certified to contain the desired oxygen concentration (2.6%, 20%, 80% oxygen, with the remainder as nitrogen) will be used.

Preliminary studies will be conducted to determine the time needed to bubble the gas through the solution in order to obtain the desired oxygen concentration in the headspace and in solution. Actual headspace oxygen concentrations will be measured with a benchtop oxygen headspace analyzer (Illinois Instruments, Ingleside, IL). Spectra will be obtained as outlined above. Five complete replicates will be performed.

**Preparation of samples for examination of concentration dependence.** Samples will be prepared containing varying iron concentrations (35, 180, and 350  $\mu\text{M}$  added iron). Control (0  $\mu\text{M}$  added iron) will consist of equal parts  $\text{MbO}_2$  and MES. Additional “control” samples will be prepared containing 2 mg/ml MM or STP, to chelate any “free” iron in the  $\text{MbO}_2$  stock, and to provide for an essentially iron-free system. Representative spectra (400 – 650nm) will be obtained every 5 minutes for 1 hour, for a total of 13 scans per sample.

#### **Objective 6 – Effect of various antioxidants (MM, Trolox, Eugenol, Rosemarinic Acid) on Raw Ground Beef Appearance and Cooked Ground Beef Sensory Acceptability.**

**Preparation of ground beef.** USDA select grade beef shoulder clods will be purchased and used for preparation of ground beef within 1 week postmortem. Clods will be first cut into 2.5 cm thick strips. Beef trim will be prepared by coarsely (0.60 cm plate) then finely (0.32 cm plate) grinding the strips. pH of freshly ground beef will be measured, but will not be controlled. Antioxidants (milk mineral, Trolox (water-soluble vitamin E), eugenol, rosemarinic acid) will be manually mixed with the meat at appropriate levels to be determined in preliminary studies, and re-ground through the fine plate. Four portions (130 g each) for each treatment will be flattened to a thickness <12 mm, to allow for complete oxygenation of the sample interior in the high oxygen atmosphere. The flattened samples will then be flushed and sealed in 80% oxygen MAP and held for 1, 4, 7, or 14 days at 2° C. The gas cylinder containing 80% oxygen and 20% carbon dioxide will be certified to be within  $\pm 0.5\%$  of the indicated mixture. Actual oxygen concentration in MAP will be directly measured using an oxygen gas analyzer. MAP samples will be held under refrigeration until analyzed for  $\text{MbO}_2$  content, Hunter color, and thiobarbituric acid reactive substances (TBARS) at 1, 4, 7, or 14 days; these analyses will also be conducted on the fresh ground beef the day of preparation. A final portion (100 g) from each treatment will be placed in a vacuum bag and frozen at  $-20^\circ\text{C}$  for later determination of fat and non-heme iron content. Five complete replicates will be performed.

**Oxymyoglobin determination.** Conversion of  $\text{MbO}_2$  to MetMb will be confirmed spectrophotometrically, based on the presence of the characteristic  $\text{MbO}_2$  peaks at 545 and 580 nm (Bowen, 1949). Reflectance spectra (400-650 nm) of meat samples will be obtained in duplicate. Due to varying fat and total myoglobin content between meat batches, it may be necessary to normalize reflectance values for statistical analysis. Normalization will be based on the initial total myoglobin concentration, to be determined immediately after the initial grinding step (prior to antioxidant addition).

**Hunter color measurement.** The  $L^*$ ,  $a^*$  and  $b^*$  values will be measured using a Hunter lab Miniscan portable colorimeter (Reston, VA), standardized through the packaging film using a white and black standard tile. Five color measurements will be taken per sample.

**TBA analysis.** The thiobarbituric acid reactive substances (TBARS) assay will be performed as described by Buege and Aust (1978). Briefly, duplicate ground beef samples (random 0.5g cores) will be mixed with 2.5 ml of stock solution containing 0.375% thiobarbituric acid, 15% trichloroacetic acid, and 0.25 N HCl. The mixture will be heated for 10 min in a boiling water bath (100°C) to develop a pink color, cooled in tap water and then centrifuged (4,300 x g for 10 min). The absorbance of the supernatant will be measured spectrophotometrically at 532 nm.

**Microbial load.** Total aerobic counts will be taken on beef samples after grinding, and at days 1, 4, 7, and 14 based on AOAC method 990.12 (AOAC 1995). Briefly, 11 g of sample will be stomached in Butterfield’s phosphate diluent. Subsequent dilutions will be plated using Petrifilm™ aerobic count plates (3M Corporation, St. Paul, MN), then incubated at 32°C for 48 hours. Colony counts will be taken and results interpreted as per the manufacturer’s guidelines. All samples will be plated in duplicate.

For each objective 1-6, separate experiments will be done. Experiments 1 (iron binding by MM) and 4-5 (myoglobin model systems) will be done in a minimum of 5 replications per treatment. SEM microscopy of MM particles (experiment 2) will be done in triplicate. Experiments 3 and 6 (MM addition to ground beef) will also be done in triplicate for each treatment. For each experiment, treatment means will be calculated and compared by analysis of variance (ANOVA), using the proc GLM function in SAS version 9.0 (SAS Institute, Inc., Cary, NC). Statistical significance will be identified at the 95% confidence level, and post-hoc means comparisons will be made based on p-values obtained using the Tukey-Kramer adjustment

#### **Sensory Evaluation**

A 6-member panel is currently active for evaluation of various flavors (beef flavor, rancid flavor, rancid odor) in cooked ground beef. All panelists have had previous sensory panel experience with cooked beef products. The panelists were trained in two sessions. In the first session, panelists were familiarized with the 5-point intensity scale

and its usage. Panelists were also familiarized with cooked beef flavor (both fresh and rancid samples) in cooked ground beef. For this study, a training session will be held to familiarize the panelists with the study. Cooked ground beef (fresh and rancid, as verified by TBA values) will be served, and panelists will be re-evaluated for their consistency and accuracy of sample identification and scoring.

**For cooked ground beef panel evaluation, a set of 3 or 4 samples (6 g each) will be served to each panelist in each session. Cooked (internal temperature of 82°C) ground beef samples will be coded and microwave re-heated for 25 seconds to attain a temperature of 80-85°C immediately before serving. Samples will be evaluated in individual booths under red lights. The serving order will be randomized to avoid positional bias. Panelists will be asked to evaluate samples for intensity of rancid odor, rancid flavor, and beef flavor on a 5- point scale, where 1 = no flavor or odor, 2 = slightly intense, 3 = moderately intense, 4 = very intense, and 5 = extremely intense flavor or odor. Panelists will be also asked to provide additional qualitative comments for each sample. Before evaluating the next sample, ballot instructions will specify that the previous sample be expectorated into cups provided for that purpose. Panelists will be instructed to rinse their mouths with tap water. Unsalted crackers will also be provided to cleanse the palate.**

### **Statistical Analysis**

Where appropriate, measured values will be evaluated by analysis of variance using the proc GLM function in SAS version 9.0 (SAS Institute, Inc., Cary, NC). Statistical significance will be identified at the 95% confidence level, and post-hoc means comparisons will be made based on p-values obtained using the Tukey-Kramer adjustment.

### **References**

- AOAC. 1995. Aerobic plate count in foods: Dry rehydratable film. Sec. 17.02.07, Method a990.12. In: *Official Methods of Analysis of AOAC International*, 16<sup>th</sup> ed., P.A. Cunniff Ed., 10-11. AOAC International, Gaithersburg, MD.
- Buege J.A., Aust S.D. 1978. Microsomal lipid peroxidation: The thiobarbituric acid assay. *Meth. Enzymol.* 52:302-310.
- Bowen W.J. 1949. The absorption spectra and extinction coefficients of myoglobin. *J. Biol. Chem.* 179:235-245.
- Brantley R. E., Smerdon S.J., Wilkinson A.J., Singleton E.W., Olson J.S. 1993. The mechanism of autooxidation of myoglobin. *J. Biol. Chem.* 268:6995-7010.
- Brown W.D., Mebine L.B. 1969. Autoxidation of oxymyoglobins. *J. Biol. Chem.* 244:6696-6701.
- Carter P. 1971. Spectrophotometric determination of serum iron at the submicrogram level with a new reagent (ferrozine). *Anal. Biochem.* 40:450-458.
- Pazos M., Alonso A., Fernandez-Bolanos J., Torres J.L., Medina I. 2006. Physicochemical properties of natural phenolics from grapes and olive oil by-products and their antioxidant activity in frozen horse mackerel fillets. *J. Agric. Food Chem.* 54:366-373.
- Sage J.T., Morikis D., Champion P. M. 1991. Spectroscopic studies of myoglobin at low pH: Heme structure and ligation. *Biochem.* 30:1227-1237.
- Schlitz A.A. 1969. *Analytical applications of 1,10-phenanthroline and related compounds*, 1<sup>st</sup> ed. Pergamon Press Oxford, New York.
- Sheehan D.C. and Hrapchak B.B. 1980. *Theory and Practice of Histotechnology*, 2<sup>nd</sup> ed. Battelle Press, Columbus, OH.
- Watkins J.A., Kawanishi S., Caughey W.S. 1985. Autoxidation reactions of hemoglobin A free from other red cell components: a minimal mechanism. *Biochem. Biophys. Res. Commun.* 132:742-748.

## **RESULTS AND DISCUSSION**

Funding for this project was received September 1, 2006. Technician Karin Allen has completed objective 1 (iron-binding measurement of MM and other calcium phosphate compounds), objective 2 (microscopy of MM added to ground beef, and scanning electron microscopy of MM particles with regard to iron binding, and objective 4 (Effects of added iron on Myoglobin (Mb) Oxidation in a Model system), as follows:

## **Objective 1 and 2 Results**

MM was compared to sodium tripolyphosphate (STPP), calcium phosphate monobasic (CPM), and calcium pyrophosphate (CPP) to determine iron-binding capacity, sample solubility, and eluate soluble phosphorus after treatment of samples in glass columns with 1 mg/ml ferrous chloride. Scanning electron microscopy with energy dispersive x-ray analysis was used to localize minerals on iron-treated MM particle surfaces. Histochemical staining for calcium was also performed on raw and cooked ground beef samples with added MM. MM bound more iron per gram ( $P < 0.05$ ) than the other compounds, and was much less soluble ( $P < 0.05$ ) than either STPP or CPM. Mineral localization showed an even distribution of calcium, phosphorus, oxygen and iron across the MM particle surface, directly demonstrating iron binding to MM particles. Unlike other common chelating agents, such as STPP and citrate, histochemical staining demonstrated that MM remained insoluble in ground beef, even after cooking. The ability of MM to bind iron and remain insoluble may enhance its antioxidant effect, by removing iron ions from solution. However, MM particles must be small and well distributed in order to adequately bind iron throughout the food system.

## **Objective 4 Results**

A model system was used to study the effect of nonheme iron on myoglobin oxidation at pH 5.6 and pH 7.2 at 23 °C. The addition of ferrous iron significantly ( $p < 0.05$ ) increased the rate of myoglobin oxidation in the absence of lipid, demonstrating that iron promoted myoglobin oxidation independent of the effect of lipid oxidation. The addition of the type II, iron chelating antioxidants sodium tripolyphosphate (at pH 7.2) or milk mineral (at pH 5.6) negated the effect of added iron, slowing oxidation of myoglobin. A clear concentration dependence was seen for iron-stimulated myoglobin oxidation, based on both spectral and visual evidence. Further investigation is needed to determine the possible role for nonheme ferrous iron on myoglobin oxidation *in vivo* or in meat.

## **Q4 2008**

Rossarin Tansawat conducted control studies on the stability of MM or STP after autoclave heating, to better understand previous results. She found that when solutions of 0.5% STP in distilled water or tryptic soy broth (TSB) were autoclaved, orthophosphate concentrations increased to 3.79 mg Pi per ml in TSB, but remained at zero for STP in water solution. Thus, there was some effect of TSB solution to degrade STP to orthophosphate (Pi) during autoclave heating. When MM (0.75%) was added to water, measurable Pi was found even at room temp (1.65 mg Pi/ml), but after autoclave heating, Pi levels increased to 2.89 mg Pi/ml. When MM was added to TSB solution to a level of 0.75% MM, Pi levels were 1.43 mg Pi/ml, and increased to 4.02 mg Pi/ml after autoclave heating. Again, there was some effect of TSB solution to increase Pi levels after heating. The degradation of STP to Pi in heated TSB does not appear to be an acid hydrolysis effect, since TSB solutions were buffered to pH 7.3. We postulate that microbial phosphatases were present and active in the warm-up time prior to reaching autoclave temperatures, and that STP was degraded during this period. The increase in Pi after heating MM solutions was probably a heat solubilization of MM phosphates, although the possibility that some enzymatic degradation of MM was occurring cannot be ruled out. Rossarin also conducted an experiment to measure aerobic plate count (APC) and inorganic orthophosphate phosphate (Pi) in ground beef patties formulated with 0.5% STP or 0.75% MM, and incubated at 20-22 °C for 2 days. There was almost no increase in APC or Pi for 2 days at °C. However, both APC and Pi increased significantly in patties held at 22°C. MM was more stable (Pi increase was less) for MM vs STP patties. There was a high correlation ( $r = 0.77$ ) between aerobic plate count and Pi levels of ground beef patties at 22°C. Thus, there is good indication that MM is more stable than STP added to fresh ground beef patties, particularly at higher temperatures (22°C) conducive to rapid bacterial growth. It also appears that microbial phosphatases are responsible for at least some of the degradation of STP that occurs when this compound is added to ground beef patties. Next steps include publication of this data in the MS thesis of Rossarin Tansawat, and submission of a manuscript on this topic to an appropriate journal.

## **CONCLUSIONS**

One new student (Rossarin Tansawat) has just begun working on objective 3 (possible anti-microbial effects of milk mineral (MM)). Fish filets (tilapia) were dipped and held for 30 seconds or 24 hr in solutions of 0.9% sodium chloride (control), 2% lactate/diacetate (a widely used anti-microbial solution) or 1% milk mineral. Aerobic plate



counts (APC) were conducted on filets after 1, 5 and 9 days storage at 3°C. APC were not different from controls for any treatment when held only 30 sec in the treatment solution. However, APC were 1.5 and 0.5 log lower for samples held 9 days in lactate diacetate and milk mineral, respectively.

So, MM had only a small anti-microbial effect in marinated fish filets.

A similar experiment was conducted in ground beef. In trial 1, APC of control ground beef after 4 days storage at 2°C was  $2.3 \times 10^6$ . In comparison, samples with 1% MM had lower APC of  $1.5 \times 10^5$  (about a 1-log reduction with MM treatment). Samples with 3% lactate/diacetate, a widely used anti-microbial in meat products, had APC of  $4.1 \times 10^5$ . After 7 days storage, APC of all treatments was  $10^6 - 10^7$ , and there were no significant differences among treatments. In a second ground beef trial with low initial microbial load ( $10^1$  APC/g), there were no significant differences in APC among treatments at any storage time (1, 4, 7 days).

Further work will continue on possible antimicrobial effects in model systems and other food systems. New student Rossarin Tansawat will initiate the portion of objective 3, regarding possible anti-microbial effects of MM against food pathogens (*Listeria*, *E. coli*). She will initially inoculate test organisms (*Listeria innocua*, *E. coli* K-12, *Pseudomonas viridescens*) into culture media with and without 1% MM. It will be necessary to determine the iron level of media, before addition of MM. It will also be necessary to conduct preliminary experiments with inoculated media with variable levels of MM (0.1 – 2%), to determine the minimum levels of MM needed for possible anti-microbial effects.

Rossarin Tansawat has completed the USU lab safety training course, and also completed training with Ms. Becky Thompson, Lab Manager, USU NFS Microbiology Labs, on procedures for culturing, transferring, and plating the microorganisms of interest in this study. She has initiated tests of MM effects on growth of *Listeria innocua*, *E. coli* DH5 $\alpha$ , and *Pseudomonas fluorescens* in Brain Heart Infusion broth, Tryptic Soy broth, and Nutrient broth, respectively. Each broth was chosen as the best media for growth of each organism, respectively. Each organism was inoculated into appropriate broth to obtain a vigorously growing culture, which was serially diluted and plated onto 3M aerobic plate count petrifilm. Vigorous cultures have been obtained for *Listeria innocua* and *E. coli* DH5 $\alpha$ , but not *Pseudomonas fluorescens*. We have placed an order for a new frozen culture of *Pseudomonas fluorescens*, from American Type Culture Collection (ATCC; Manassas, VA). In the meantime, experiments will continue with *Listeria innocua* and *E. coli* DH5 $\alpha$ , to compare organism growth in control media, compared to media containing 0, 0.75, and 1.5% milk mineral (MM).

Karin Allen has begun experiments on objective 6 – Effect of various antioxidants (MM, Trolox, Eugenol, Rosemarinic Acid) on Raw Ground Beef Appearance and Cooked Ground Beef Sensory Acceptability. She has found that rosmarinic acid is a very fast reductant of ferric iron to the ferrous form aqueous solutions. In a model system containing myoglobin (Mb), iron (Fe), and various antioxidants, myoglobin remaining after 60 min was as follows:

- Mb + Fe + Rosmarinic acid – 9.9% Mb remaining (a).
- Mb + Fe + Eugenol – 37.3 % Mb remaining (b).
- Mb + Fe – 53.8% c
- Mb + Fe + Phytate – 60.4% d
- Mb only – 63.3% d
- Mb + Fe + sodium tripolyphosphate (STP) – 67.7% Mb remaining (d).

This result is quite interesting regarding the mechanism of Mb oxidation. The type 1 antioxidants (hydrogen donors: rosmarinic acid and eugenol) act to rapidly reduce ferric iron to ferrous. The ferrous iron in turn forms a putative complex with oxygen that facilitates the transfer of an electron from the heme iron of Mb to oxygen, forming superoxide and Met Mb (brown pigment formation). The type 2 antioxidants (iron chelators: Phytate and STP) are much more effective at preserving Mb, and preventing formation of metMb. So, this experiment supports the hypothesis that soluble ferrous iron greatly stimulates Mb oxidation in aerobic environments. Also, this result again demonstrates the powerful antioxidant effects of MM to prevent brown color formation in fresh meats.

Karin has completed one additional experiment comparing the effect of antioxidants on myoglobin oxidation as affected by oxygen concentration in the solution. She has constructed an apparatus to obtain oxygen concentrations of zero (under nitrogen), 2% oxygen, 20% oxygen (atmospheric), and 80% oxygen. To obtain these oxygen concentrations, she purchased gas cylinders of 100% nitrogen, 2% oxygen + 98% nitrogen, compressed air (20% oxygen), and 80% oxygen + 20% nitrogen. The apparatus consists of tubing connecting the gas cylinders with appropriate regulators to glass jars, fitted with glass inlet and out tubes, and stop cocks, so that the jar may be flushed with the desired gas. The sample cuvettes containing myoglobin + antioxidant solutions are placed in the jar, the lid



is sighted, the air is replaced by flushing with the desired gas, and then the inlet and outlet stopcocks are closed to maintain the desired atmosphere for up to 24 hrs. She has completed one run with myoglobin + rosemarinic acid in both 80% oxygen and 2% oxygen. She verified with an oxygen probe analyzer that the desired oxygen atmosphere was obtained. She found that in presence of myoglobin + iron + rosemarinic acid, myoglobin oxidation proceeded rapidly, at both 2% or 80% oxygen. Thus, 2% oxygen level was not limiting to myoglobin oxidation in presence of ferrous iron. She is currently conducting further experiments to evaluate possible interactions of oxygen level, with or without added iron, and with or without various antioxidants, on myoglobin oxidation rate (measurements taken at 15 min, 60 min, 2 hr, 4 hr, 8 hr, and 24 hr in the appropriate atmosphere).

Karin Allen has completed her final experiment (described above). In summary, she evaluated the interaction effects of oxygen level (2.6, 20, 80 % oxygen) on rate of myoglobin oxidation to metmyoglobin (browning) in a control solution (200 micromolar myoglobin, pH 5.6), a myoglobin solution with 2 micrograms/ml added ferrous chloride, or myoglobin + iron + 0.3% sodium tripolyphosphate (STP – an iron-binding compound), for 15, 30, 45, and 60 min at 20°C. Desired oxygen levels were obtained by flushing (bubbling) the solution with the desired oxygen level. The 3 oxygen levels were purchased as gas cylinders from PraxAir (Salt Lake City, UT), certified to contain oxygen at the desired levels. The remainder of the gas in each cylinder was nitrogen.

Karin repeated each treatment interaction 5 times, and is currently conducting statistical analysis of the results. It was apparent that iron addition stimulated myoglobin oxidation at 2.6% and 20% oxygen, but at 80% oxygen, metmyoglobin formation was lower. Sodium tripolyphosphate addition also lowered metmyoglobin formation at 2.6 and 20% oxygen, but at 80% oxygen, there was no combined effect of STP and high oxygen to lower metmyoglobin levels. High oxygen levels alone were about as effective as high oxygen + STP to prevent metmyoglobin formation in the model system during the 1 hr incubation period. These results are important in understanding the factors (iron, oxygen levels) affecting browning in fresh meats packaged by various modified atmosphere packaging methods.

Rossarin Tansawat has recently completed one replication of a study with lean ground beef packaged in high (80%) oxygen modified atmosphere packaging, to compare the stability of added STP or milk mineral (MM). Inorganic phosphate levels increased after 2-3 days in meat + 0.3% STP, compared to meat + 0.75% MM. This result indicated that phosphatase enzymes from either the meat or from bacterial growth in meat were degrading the added STP. This provided partial explanation for the increased antioxidant effects of MM, compared to STP, when added to ground meats. If the STP is degraded to inorganic orthophosphate, it would have less affinity for soluble iron. Thus, at longer storage times, meat + STP would have higher free iron, and thus more lipid oxidation, compared to meat + MM. The added MM appeared to be less susceptible to degradation by phosphatase enzymes, compared to added STP.

# Effect of Milk Fat Globular Membrane (MFGM) in Providing Protection Against Gastrointestinal Stress

KORRY HINTZ: Utah State University

ROBERT WARD: Utah State University

## ABSTRACT

Milk fat globular membrane is an intriguing component of milk, and is available in large quantities as a potential functional ingredient. The MFGM likely interacts extensively with the gut mucosa during digestion, both physically and biochemically, and we predict some benefit may imparted to the gut via this interaction. Isolated MFGM is a relatively rich source of Zn, and induces genes involved in Zn metabolism when provided to gastrointestinal cells in culture. In addition, in other preliminary work conducted in the labs of the PIs, MFGM provided protection against EGTA induced disruption of epithelial cell tight junctions, and the effect was enhanced after the material was subjected to an *in vitro* digestion process. Based on the composition and structure of MFGM, and on these preliminary data, we propose to test the ability of this material to improve the resistance of the gut to stress imparted by LPS in a mouse model. To assess the efficacy in gut mucosal protection we plan to look at the effects on a) gut permeability b) systemic inflammation and c) markers of Zn status.

MFGM will be isolated from buttermilk via microfiltration in the pilot plant in the department of the PIs, and chemically characterized. The isolated MFGM will be incorporated into animal diets at the level of 10%, and other components adjusted as per the compositional analysis.

An animal study will be conducted with mice in four treatment groups, supplemented with 10% MFGM or control, and either challenged with LPS injection or injected with the vehicle control. Tissues will be collected for analysis in third objective.

## MATERIALS AND METHODS

Objective 1. Milk Fat Globular Membrane (MFGM) was isolated from cream buttermilk by microfiltration in the USU Nutrition and Food Sciences pilot plant. MFGM was incorporated into animal diets by a commercial vendor.

Objective 2. Protocols and diets are now in place for the proposed animal which will begin Jan. 12, 2009

### **Study Design of Upcoming Animal Study**

To test the hypothesis that MFGM confers protective effects against LPS induced inflammation and improves gut barrier protection; a 2 X 2 factorial design will be used. Weanling, male BALB/c mice (n=72) will be randomly assigned to one of the following treatments (n=18/trt): 1) Control diet (AIN 93G), saline vehicle control injection. 2) Control diet, lipopolysaccharide (LPS) injection. 3) Control + 10% MFGM diet, saline vehicle control injection. 4) Control + 10% MFGM diet, LPS injection. Mice will be fed experimental diets for 4 weeks. On day 29, mice will be injected (intraperitoneal) with a dose of LPS (10 µg/g BW) known to induce inflammatory cytokines and compromise gut barrier integrity. To assess intestinal permeability, inulin conjugated with fluorescein will be gavaged (1 mL/100g/bw gavage of 1.43 mmol/L inulin-fluorescein suspended in PBS) immediately after LPS or vehicle control injection. After injection and gavage, animals will be serially harvested at 0, 6, and 24 hrs (n=6/ timepoint/trt) by CO<sub>2</sub> asphyxiation. To control for food intake, animals will be pair-fed after injection.

Table 1. Macro and Micronutrient Composition of Experimental Diets

Macro-Nutrient Composition of Test Diets

Ingredient (g/kg diet)	Control Diet	10% MFGM Diet
Protein (casein)	200	200 (50)*
Fat (soy oil)	70	70 (50)*

\*Amount derived from MFGM portion of the diet (g/kg diet)

Micro-nutrient Composition of Test Diets

Ingredient (mg/kg diet)	Control Diet	10% MFGM Diet
Zn	30	30 (12.6)*
Mg	507	507 (24.6)*
Ca	5000	5000 (88)*
P	1561	1561 (14.5)*
Cu	6	6 (2.5)*

\*Amount derived from MFGM portion of the diet (mg/kg diet)

## RESULTS AND DISCUSSION

Objective 1. We were successful in isolating sufficient MFGM to formulate diets for animal studies. Therefore, objective is now complete.

Objective 2. All of the preliminary work to begin animal studies is now complete and animal studies will begin January 12, 2009.

Objective 3. Work will begin on objective 3 once the animal study has been completed.

## CONCLUSIONS

Discuss your conclusions on your research findings up to the current reporting period.

We have successfully completed Objective 1 clearing the way to begin animal studies early in the new year. The bulk of the experimental data will be generated in Objective 3 so there is little data to report during the first quarter of this project. The investigators feel that we are on schedule to complete the project in the allotted time and are enthusiastic about the upcoming experiments.

## NEXT STEPS

Provide specific details on potential deviations from proposed research. If there are no changes please state "no change" to eliminate ambiguity. PIs must follow-up with Center Directors and/or DMI on delays or problems.

# ***PAPERS***

# Acid Coagulation of Milk

Ultrastructural changes in casein micelles during acidification of skim milk

D. J. MCMAHON: Utah State University

H. DUJ: Utah State University

W. R. MCMANUS: Utah State University

K. M. LARSEN: Utah State University

## ABSTRACT

Pasteurized skim milk was acidified using different levels of glucono- $\delta$ -lactone at 10, 20, 30, and 40°C to give slow, medium and fast rates of acidification. Milk coagulation was monitored by measuring turbidity and curd firmness, and ultrastructural changes during acidification were observed on glutaraldehyde-fixed agar-solidified milk samples using transmission electron microscopy. Rate of acidification had little influence on changes observed during acidification, except at 10°C when slower acidification caused an initial increase in turbidity while the milk was still fluid (pH 6) followed by a decrease to a minimum at pH 5.2, after which the turbidity increased as aggregation and gelation occurred. At 40°C, the casein micelles were spherical throughout acidification, while at lower temperatures they became progressively more ragged in appearance. All of the milks gelled at the same pH (pH 4.8), as measured by curd firmness. Whereas, increases in turbidity typically associated with aggregation and gelation, were observed to start at about pH 5.2 to 5.4. Different gel structures were formed based on temperature of acidification with a coarse-stranded gel network formed at 40°C and a fine-stranded gel network at 10°C.

As the milk was acidified, aggregates of loosely entangled proteins were observed, presumably originating from proteins that had dissociated from the casein micelles. These aggregates were often as large as the casein micelles, particularly as the pH of the milk approached the isoelectric point of the caseins. Larger aggregates were observed at 40°C than at the lower temperatures, suggesting the involvement of hydrophobic interactions between the proteins. As pH dropped below pH 5.3, the loosely entangled proteins became more compact and began to aggregate with the casein micelles and form a gel network.

A three-phase model for acid-induced gelation of milk is proposed. Phase 1 involves temperature-dependent dissociation of proteins from the casein supramolecules, with more dissociation occurring as temperature is decreased. When warm (40°C) the modified casein micelles remain spherical while in cold milk (10°C), the dissociated proteins are present as loosely entangled aggregates. Dissociation continues as milk pH is lowered with the released proteins forming into loosely-entangled aggregates, some as large as the casein micelles. Phase 2 occurs between pH 5.3 and pH 4.9 and involves the loosely-entangled protein aggregates forming into more compact colloidal particles or to re-associate with any remaining casein micelles. Phase 3 involves rapid aggregation of the colloidal casein particles into a gel network at about pH 4.8.

(Key words: acid coagulation, casein micelle, gelation)

## INTRODUCTION

Acid coagulation of milk is the foundation of many dairy products. Formation and physical properties of acid milk gels have been reviewed by Lucey and Singh (1998) and their rheological properties have been extensively studied (Lucey et al., 1998; Lucey, 2001; Tranchant et al., 2001), and mathematical models describing acid coagulation have been developed (Novakovic et al., 2000). Milk can be acidified and coagulated when warm (typically in the range of 30 to 45°C) or it can be acidified while cold (e.g., 5°C) and then warmed to induce gel formation.

It has been observed that different gel properties are obtained based on acidification temperature (Lucey et al., 1997). In the manufacture of yogurt, high incubation temperatures have been associated with excessive syneresis and a coarser gel texture (Lucey, 2001). Depending on the type of product being manufactured, the milk may or may not be given a high heat treatment prior to acidification. Examples of this would be yogurt and American-style cottage cheese respectively. High heat treatments will denature whey proteins and bring about their co-coagulation with the caseins.

During acidification of milk, calcium phosphate contained in the casein micelles is solubilized, and proteins dissociate from the casein micelles (Roefs et al., 1985; van Hooydonk et al., 1986; Vreeman et al., 1989). Such protein dissociation is temperature-dependent, with more casein becoming non-sedimentable at 5°C than at higher temperatures (Dalgleish and Law, 1988; Singh et al., 1996). Maximum protein dissociation occurs at about pH 5.5, then as milk is further acidified, the amount of non-sedimentable casein decreases to zero at pH 4.8 at which stage all of the casein is incorporated into the gelled network. Even though acid-coagulated milk gels are observed as a network of aggregated colloidal casein supramolecules (Tamime et al., 1984), it cannot be assumed that these particles have the same structure as the casein supramolecules present in milk before it was acidified (Heertje et al., 1985; Roefs et al., 1985). What is recognized is that when the pH of milk is sufficiently lowered and passes through the isoelectric point of the caseins, their solubility decreases bringing about a re-association and subsequent coagulation (Kauzmann, 1959).

In the traditional manufacture of fermented milk products such as yogurt, cottage cheese, quark, and cream cheese, a bacterial culture is used at temperatures of 38 to 42°C to generate lactic acid and bring about acid coagulation. Other approaches to acid gelation of milk include direct acidification of warm milk using glucono- $\delta$ -lactone (GDL) to gradually lower the pH of milk and cause coagulation, or cold acidification followed by warming to induce coagulation. Our objective was to observe ultrastructural changes of casein supramolecules at various acidification rates and temperatures, and monitor turbidity and gelation, to increase our understanding of the processes occurring prior to milk gelation.

## MATERIALS AND METHODS

### Preparation of Milk

Raw whole cows' milk was obtained from the Gary Haight Richardson Dairy Products Laboratory of Utah State University, centrifuged (Sorvall® RC-5C, Du Pont Company, Ontario, CA) at 3000 g for 60 min at 4°C, and then filtered through glass fiber filter paper under suction. The resultant skim milk was then heat treated in a water bath at 63°C for 30 min. If the milk was to be chemically acidified, 0.02% (w/w) sodium azide (Mallinckrodt, Paris, KY), 0.01% chloramphenicol (Sigma-Aldrich Co., St. Louis, MO), and 0.01% benzylpenicillin potassium salt (Sigma-Aldrich) were added to inhibit bacteria growth.

### Milk Acidification

Microbial acidification with 1.2 g of freeze-dried yogurt starter culture *Lactobacillus delbruekii ssp. bulgaricus* and *Streptococcus thermophilus* (Danisco USA, New Century, KS) per 100 g of skim milk at 40°C was used to lower the pH to 4.6 in 125 min. Similar chemical acidification to pH 4.6 in 125 min at 10, 20, 30 and 40°C was obtained by addition of 4.0, 2.5, 2.0 and 1.5 g GDL (Sigma-Aldrich Co.) per 100 g of milk. Faster and slower rates of acidification were obtained by adding more or less GDL.

### Electron Microscopy

Before and during the acidification process, 5-ml samples were taken and fixed by the addition of 0.2 ml of a 50% glutaraldehyde solution (Electron Microscopy Sciences, Fort Washington, PA). After 5 min, the milk was solidified by mixing with an equal volume of warm (50°C) 3% (wt./wt.) agar (Bacto-Agar, DIFCO Laboratories, Detroit, MI). The samples were cut into small strips 1x1x10 mm and preserved in 0.1 M phosphate buffer solution, pH 6.8, containing 2% (wt./wt.) glutaraldehyde and refrigerated.

The glutaraldehyde-fixed agar-solidified samples were reduced in size to 1 mm<sup>3</sup> cubes, rinsed with 0.1 M phosphate buffer, and post-fixed in 2% (wt./wt.) osmium tetroxide (Electron Microscopy Sciences). Dehydration was performed by transferring samples through an increasing concentration of graded ethanol (30%, 50%, 70%, 95%, and 100%). After dehydration, the samples were transitioned into propylene oxide and mixed in varying proportions ranging from 25% ethanol in propylene oxide to 100% propylene oxide alone in four steps. The cubes were infiltrated with a solution containing propylene oxide and epoxy resin (Electron Microscopy Sciences) stepwise in various proportions ranging from 25% propylene oxide in epoxy resin to 100% epoxy resin. The infiltrated samples were embedded in Beem capsules (Electron Microscopy Sciences) and incubated at 45°C followed by 60°C for 24 h each. The hardened epoxy resin was removed from the Beem capsule, and excess epoxy trimmed to expose the sample. Ultrathin sections were cut using an Ultratcut E ultramicrotome (Leica, Inc., Gilroy, CA) and collected

on 300 hex grids (Electron Microscopy Sciences). Sections were post-stained with uranyl acetate and lead citrate and examined using a Zeiss CEM 902 transmission electron microscope (Zeiss Inc., Thornwood, NY) at 80 KV. Images were recorded on Kodak electron image film SO-163 (Eastman Kodak Co., Rochester, NY).

### **Turbidity**

Turbidity changes brought about by changes in particle size, aggregation and gelation were monitored using a single beam spectrophotometer (model DU-8B; Beckman Instruments, Inc., Fullerton, CA) as described by McMahon et al. (1984). Milk was warmed to 10, 20, 30, or 40°C for 30 m, and a 3-ml portion filled into a 1-cm pathlength cuvette and placed into the spectrophotometer and turbidity at 600 nm set to zero. Appropriate amounts of GDL or starter culture were added to aliquots of milk, and filled in duplicate into 3-ml cuvettes and turbidity measured while the milk was maintained at 10, 20, 30, or 40°C. At each temperature, one cuvette was monitored for changes in turbidity at 600 nm and one for changes in pH using a glass microelectrode (Microelectrodes, Inc., Londonderry, NH). Measurements were performed in triplicate.

### **Gelation**

The pH at which gelation occurred was determined in triplicate using a Formagraph (Dick-John Corp., Fishkill, NY) with a modification of the method described by McMahon and Brown (1982). Milk was warmed to the appropriate temperature (10, 20, 30, or 40°C) for 30 min and the Formagraph 10-well sample holder was set to the same temperatures. For each temperature, the required amount of GDL or starter culture was added to the milk simultaneously as the Formagraph recorder module was started. About 10 ml of milk was then deposited in each sample well, and the sample holder transferred to the recorder module. Detection of curd formation was defined as the point where the baseline of firmness versus time diagram began to increase in width. Changes in pH of the milk samples were recorded using a glass microelectrode.

## **RESULTS**

### **Prior to Acidification**

Baseline images of milk prior to acidification are shown in Fig. 1. The measured pH of the milks at 10, 20, 30 and 40°C were pH 6.96, 6.75, 6.64, and 6.55 respectively. This shift in pH as milk is cooled is related to uptake of protons as colloidal calcium phosphate is solubilized when milk is cooled, and their release when milk is warmed. The ultrastructure of the casein micelles that was observed (Fig. 1) is similar to that previously reported. (Kalab et al., 1976), however, other methods of imaging casein micelles (McMahon and McManus, 1998; McMahon and Oommen, 2008) have shown that casein micelles have a more open structure than when using glutaraldehyde fixation. However, there were strong interactions between the casein micelle and agar that caused structural artifacts if acidified milk was mixed with the warm agar without prior glutaraldehyde fixation (data not shown). Strands of protein that appeared to radiate from the casein micelles were observed in non-glutaraldehyde fixed acidified milks that were similar to what had been observed by Kalab et al. (1976). Thus, while the fine internal supramolecular structure of the casein micelles could not be retained, the dissociation of material from the casein micelles that occurred when milk was cooled or acidified could be observed. Thin fibrous strands observed in the micrographs were attributed as artifacts relating to solidifying of the milk using agar and were assumed to be agarose fibers (Aleyne et al., 1993).

At 40°C, the casein micelles were observed to have predominantly smooth spherical surfaces and had a range of sizes. There appeared to be very few casein micelles that were < 80 nm in diameter. Some casein micelles had appendages, but the majority of the electron dense areas in the micrographs were the colloidal casein micelles with little non-particulate protein being observed (Fig. 1A). At lower temperatures there appeared to be more electron dense material that was only loosely attached to the casein micelles with no extensive dissociation of the casein micelles, except for milk at 10°C.

At 30°C (Fig. 1B), the casein micelles were spherical in shape but many of them had a raspberry-like surface appearance similar to that initially reported by Kalab et al. (1982). In some cases, the surface material seemed only loosely attached to the rest of the colloidal particle. At 20°C (Fig. 1C), the casein micelle surface had a more terrillar appearance with electron-dense areas protruding from the particle surface.

At 10°C (Fig. 1D), the casein micelles appeared close together and grouped in clumps, however this was attributed to rapid solidification of the warm agar before it could be well dispersed throughout the cold milk. The casein micelles had a more ragged structure, and there was a greater proportion of smaller particles, including some that were no longer spherical in appearance. Also, there were some particles that had an absence of material in their core. Compared to the casein micelles at 40°C, the casein micelles at 10°C were less electron-dense (i.e., there was less heavy metal-staining), and their peripheral edges were less distinct with a relatively open structure. There was also more protein material dispersed as loose aggregates among the casein micelles at 10°C than at higher temperatures.

### **Acidification Rate**

Microbial acidification of milk at 40°C using starter culture began slowly (apart from a small drop caused by adding the starter culture itself). After approximately 15 min the rate increased, as the starter culture moved into log phase and begin to multiply, generating lactic acid (Fig. 2A). Then the pH gradually decreased with a pH of 4.6 being reached after 125 min. In contrast, during chemical acidification, there was an initial rapid drop in pH as GDL was hydrolyzed to gluconic acid, then acidification rate slowed as the concentration of remaining GDL decreased (Fig 2B).

### **Microstructure of Warm Acidified Milk**

**GDL-Acidified Milk.** In 40°C milk, ultrastructural changes in the casein micelle structure were observed during acidification by GDL (Fig. 3). At pH 5.5 (Fig. 3A), there were numerous small casein micelles, many being as small as 30 nm diameter. Casein micelles up to 200 nm diameter were still present, although on a number basis the smaller casein micelles were predominant. There were no protuberances on the periphery of the casein micelles, which appear more spherical than prior to acidification. As acidification continued to pH 5.2 (Fig. 3B), the very small casein micelles (30 to 40 nm diameter) were no longer present, being replaced by loosely-entangled proteins dispersed amongst the larger spherical casein micelles. Some of these loosely-entangled proteins were present in clumps of up to 100 to 200 nm in size.

At pH 4.95 (Fig. 3C) the dissolution of the casein micelles continued, with only casein micelles greater than 100 nm in diameter remaining. Casein micelles were present that had only low electron density (gray spherical particles) and were mainly in the size range of 100 to 200 nm diameter. Larger, denser casein micelles (black spherical particles) were also present, ranging from 150 to 250 nm in diameter. In addition to the spherical casein micelles there were also clumps of loosely-entangled protein dispersed throughout the sample. Compared to the milk at pH 5.2, this loosely-entangled protein at pH 4.95 tended to be present as very low electron density particle-like aggregates.

When the 40°C milk had been acidified to pH 4.8 (Fig. 3D), the casein protein appeared as an interconnecting colloidal mass, indicative of a three-dimensional gel network being formed. When using TEM to examine thin sections of a gel, only a cross-sectional view is obtained which underestimates the extent of strand formation that makes up the gel network (Kalab et al., 1976). Even what appears to be individual colloidal particles would most likely be a cross-section of a strand that is traversing the thin section at a perpendicular angle. The strands of the gel network appeared to be about 180 to 300 nm in thickness. Some clumps of loosely-entangled protein were also evident but the majority of this protein had become associated with the casein micelles and could be observed as filamentous material on the periphery of the gel network strands. Heertje et al. (1985) had observed that in warm acidified milk, the coagulum that formed consisted of clusters of aggregated micelles that had joined together to form a three dimensional network.

**Microbially-Acidified Milk.** Changes in microstructure during microbial-acidification of warm milk (Fig. 4) were similar to that observed when using GDL (Fig. 3). The only difference was that dissociated of protein from the casein micelles seemed to occur earlier. By pH 5.4 (Fig. 4A) there was an increase in number of small (40 to 60 nm diameter) casein micelles and a considerable amount of loosely-entangled protein, (while in the GDL-acidified milk this was not apparent until pH 5.2). At pH 5.0 (Fig. 4B) there were no longer any intact casein micelles less than 70 nm diameter in size. As was observed with the GDL-acidified milk, the smaller casein micelles tended to be less electron dense than the larger casein micelles. The loosely-entangled protein was more interconnected than was observed at higher pH.

While the dissociation of proteins from the casein micelles occurred earlier when using cultures, formation of large network strands was delayed. Also, the increase in turbidity that occurs just prior to gel formation was



observed at a lower pH compared to GDL-acidified milk, even though there was no difference in detection of coagulation by the Formagraph, which is based on development of curd firmness. At pH 4.75, considerable re-association had occurred with virtually no colloidal particles being less than 100 nm diameter, with some clumps of loosely-entangled proteins still present. The large network strands developed upon further acidification with the gel network formed (Fig. 4D) being similar in appearance to the gel formed in warm GDL-acidified milk (Fig. 3D). The loosely-entangled protein and the smaller casein micelles present at this pH were mostly observed to be fused to the periphery of the strands making up the gel network.

### **Microstructure of Cold Acidified Milk**

When cold (10°C) milk was acidified, there was a large increase in the amount of loosely-entangled protein present, even at pH 5.9 (Fig. 5A). The same artifact described above for addition of warm agar to cold milk samples was also present in the micrographs with the agar solidifying before complete mixing with the milk. The increased dissociation of the casein micelles was not unexpected because there is more dissociation of the casein micelles in cold milk prior to acidification. At pH 5.3 the proportion of loosely-entangled protein appeared to have decreased (Fig. 5B) suggesting that a re-association of proteins back into colloidal particles had already commenced.

Cold acidified milk at pH 5.0 (Fig. 5C) was similar to that at pH 5.3, with the colloidal particles having numerous appendages on their peripheral surface with some small chains and clumps of particles. At pH 4.8 (Fig. 5D), virtually all the protein had become associated back into spherical particulate form and incorporated into a fine-stranded gel network. There was no evidence of the very large and electron dense colloidal particles observed when warm milk was acid coagulated. Differences in size of the colloidal particles forming the aggregated protein network as temperature was lowered, were more obvious when the gelled milks were examined at lower magnification (Fig. 6). When coagulated at 10°C and 20°C, the gel network was made up strands of aggregated colloidal particles that were mostly 100 nm or less in diameter. At 30°C the colloidal particles of the network strands were about 100 to 200 nm diameter, while at 40°C they were in the range of 300 to 400 nm diameter. Similarly the spacing between the network strands decreased as temperature was lowered, with spacings of 1 to 2  $\mu\text{m}$  regularly occurring at 40°C, and at spacings of a few hundred nanometers at 10 and 20°C. At the lower temperatures the network strands appeared to be made of a clusters of small particles rather than being a single linear strand as implied by the cross-sectional view of the gel network at 40°C.

### **Turbidity**

Though none of the treated milks formed a firm coagulum until pH 4.8, changes taking place as described above were also detected by monitoring turbidity (Figs. 7 and 8). For warm milk (30 and 40°C) the turbidity remained constant down to pH 6.2, after which turbidity gradually increased by approximately 0.02 absorbance units. This was followed by a rapid turbidity increase at pH 5.3 and 5.5, respectively (Fig. 7). In contrast, for cooler milk (10 and 20°C) the increase in turbidity commenced sooner at approximately pH 6.5 and followed the same general pattern as above, continuing to a maximum at pH 5.9 and 5.6, respectively.

Directly after acidification, increase in turbidity was more pronounced at 10°C. As acidification proceeded, the turbidity at 10°C dropped below its original level with a minimum occurring at about pH 5.3 (Fig. 7). The depth of this turbidity minimum in the 10°C milk was larger if the acidification rate was slower (Fig. 8A). No such turbidity drop was seen for the other temperatures (data not shown). After further acidification, a rapid increase in turbidity was observed in all samples. At 40°C, after this increase the turbidity decreased from its maximum, starting at pH 5.0, leveling off at pH 4.8. Similar changes in turbidity of warm milk during acidification had earlier been reported by Bringe and Kinsella (1990) at 25°C, and Banon and Hardy (1991, 1992) at 30 and 42°C. Recently, Alexander and Dalgleish (2004) and Dalgleish et al. (2004) using diffusing wave spectroscopy to monitor GDL-acidification of 30°C milk, reported changes in inverse photon free path, that were representative of the changes in turbidity we observed.

If the inflection point of the turbidity curve is used as a measure of the pH at which a change in the system is occurring, we observed that this change occurred earlier as temperature was increased. The average turbidity inflection point for milks acidified using GDL was calculated at pH 4.96, 4.98, 5.05 and 5.20 at 10, 20, 30 and 40°C, respectively. Kim and Kinsella (1989) similarly observed that turbidity changes during acidification of pasteurized skim milk started between pH 5.1 and pH 5.2 at 35 to 50°C, and increased to pH 5.6 at 55°C. However, as shown by Dalgleish et al. (2004), during acidification of milk (at 30°C) the measured average particle radii did not increase until pH 5.0 was reached. Prior to this, starting at pH 5.5, the change that occurs is the average distance between

light scattering events decreases implying that the number of colloidal particles increases. This could come about as protein that had initially dissociated from the casein micelles re-associates into colloidal form. The pH at which turbidity increased was not influenced by acidification rate (Fig 8A). Similar observations were made by Alexander and Dalgleish (2004) who observed that changes in apparent particle size during acidification of skim milk (at 30°C) was dependent on pH of the milk with a sharp increase in apparent radius occurring at pH 5.0.

When milk at 40°C was acidified using starter culture, similar turbidity changes were observed except that starting at pH 5.5 the rate at which turbidity increased was slower (Fig. 3B). Thus, the turbidity infection point occurred at pH 5.0, rather than pH 5.2 when the milk was acidified using GDL (Fig. 8B).

## DISCUSSION

If the casein micelles in milk at 40°C prior to pH adjustment are considered to be in their native supramolecular form, then at lower temperatures or at lower pH, they should be considered as having a non-native structure. In general, this is seen as an increase in the number of small colloidal particles, and the presence of loosely formed non-spherical protein aggregates. Throughout the acidification process, differences in size and physical form of the casein particles and aggregates occur depending on temperature (through its influence on hydrophobic interactions) and pH (through its influence on protein charge and calcium phosphate solubility). However, the actual physical gelation of the milk appears to override these differences as gelation occurs at the same pH (pH 4.8) at all temperatures tested, regardless of the differences in the gel networks.

### *Temperature-Induced Changes*

Changes in physical and chemical environment can alter the tendency of colloidal particles to remain dispersed, aggregate, precipitate, or coagulate. As temperature of milk is lowered, the interactions between various components of the casein supramolecules that maintain its integrity (especially hydrophobic liaisons) are weakened, and calcium phosphate increases in solubility. This allows some casein molecules (especially  $\beta$ -casein), calcium, and phosphate to dissociate from the casein micelles (Qvist, 1979; Pierre and Brule, 1981; Dalgleish and Law, 1989) and for the caseins to be less tightly bound to each other. This appears to bring about loosening of the casein supramolecule structure as milk temperature is lowered from 40 to 20°C. At 10°C, a large number of smaller, less electron dense and loosely entangled protein particles are present. When the micrographs were digitally modified through pixel binning to remove artifacts caused by agar solidification of the milk during sample preparation, to show only electron dense casein micelles (black) and less electron dense (grey) entangled protein aggregates (Fig. 9), these changes become more apparent. At 40°C (which is the closest temperature to their native state), most of the casein supramolecules were spherical in shape and electron dense, while at 10°C, they were smaller, more ragged in appearance, and less electron dense. However, the casein micelles were still predominantly present as colloidal particles, although some had tendrils and appendages extending from the particle periphery. This would be expected as Dalgleish and Law (1988) showed that as milk is cooled from 30 to 5°C, the fraction of casein not associated with the colloidal supramolecules (i.e., not sedimenting at 70,000 g) increases only slightly, from 5% to 13%.

The light scattering properties of these loosely entangled proteins could be expected to be different from that of the casein micelles, and so calculations of particle size by light scattering techniques (i.e. using transformation equations) would need to account for the presence of a complex mixture of particles. It cannot be assumed that the light scattering properties of milk (or diluted milk) are the same at cold temperatures as they are at warm temperatures.

### *Acid-Induced Changes*

***Dissociation of Protein from Casein Micelles.*** As milk is acidified, a number of chemical changes are taking place. This includes a decrease in magnitude of net negative charge on individual protein molecules and the overall net charge of casein micelles (Heertje et al, 1985), and solubilization of calcium phosphate (Evanhuis and de Vries, 1959; Visser et al., 1986). The calcium phosphate nanoclusters that provide an interlocking aspect to the casein micelle supramolecule structure (McMahon and Oommen, 2008) are gradually solubilized and therefore proteins (such as some  $\alpha$ s1-,  $\alpha$ s2-, and  $\beta$ -casein molecules) that are only bound into the supramolecule structure by the sequestering action of their phosphoserine groups towards the calcium phosphate nanoclusters could dissociate.

In contrast, casein that is bound via Ca-mediated salt bridges between two different casein molecules would remain attached to the supramolecule until the negative charge of the proteins is neutralized. About 3.5 mM of

calcium in milk is directly bound to the caseins (Singh et al., 1996) and this represents an average of three Ca<sup>++</sup> bound per protein molecule. Proteins bound via hydrophobic liaisons would also remain in the casein micelles supramolecule structure, provided the temperature is sufficiently high for the increase in entropy gained by hydrophobic groups remaining together to make a significant contribution to lowering free energy of the acidified milk system.

As pH is lowered, there is some dissociation of protein from the casein micelle supramolecule, but much of it apparently still remains in an aggregated form. At high temperature (e.g., 40°C) most of the proteins retain a spherical shape, whereas at low temperature (e.g., 10°C) the non-supramolecular proteins are present as what appears to be loosely-entangled non-spherical aggregates that range in size upward to that of casein micelles. Even though the average particle size has been reported to remain relatively constant until the rapid aggregation stage (Singh et al., 1996; Dalgleish et al., 2004), there are major changes taking place to the casein supramolecule particles, and it does not appear that the structural framework of the casein micelles is maintained as earlier postulated.

As shown in Fig. 9, especially for the milk at 40°C, there would appear to be a decrease in size of the casein micelles with many more small casein micelles being present at pH 5.7 compared to milk prior to acidification. This corresponds to the pH at which Dalgleish and Law (1988) observed maximum dissociation of casein from the casein micelles at 30°C. However, the quantity of dissociated casein at 30° they measured was still less than 10% of the total casein in milk, which supports our observation that at 30°C there are loosely-entangled protein aggregates present at pH 5.7 as well as many casein micelles in the 50 to 100 nm range. At 40°C and this pH, the loosely-entangled protein aggregates were not evident but there were many very small casein micelles in the 30 to 50 nm range.

Dalgleish and Law (1988) also observed that at 30°C the dissociation of casein upon acidification of milk to pH 5.5 had a constant proportion of  $\alpha$ s1-,  $\beta$ - and  $\alpha$ -casein, suggesting that these proteins may have dissociated as an intact complex. The smallness of these particles also agrees with them being enriched in  $\alpha$ -casein, and having less  $\alpha$ s1-casein than the original casein micelles. This would be expected as  $\alpha$ -casein is needed to stabilize the particles because neither  $\beta$ -casein nor  $\alpha$ s-caseins can exist as monomers at these conditions (van Hooydonk et al., 1986).

As milk temperature is further lowered, there is more dissociation of proteins from the casein micelles. Dalgleish and Law (1988) reported 30% and 55% being dissociated at 20°C and 4°C respectively, while Singh et al. (1996) reported only 7% and 22% dissociation at 22°C and 5°C, respectively, as they used a higher centrifugal force (88,000 g compared to 70,000 g) which would sediment more of the very small casein micelles and aggregates. It was apparent from our microstructural observations that even at temperatures above 25oC there was dissociation of proteins from the casein micelles during acidification. Although these dissociated proteins still existed in an aggregate or particulate form that would allow them to sediment during centrifugation.

Dalgleish and Law (1988) proposed three primary factors influencing the dissociation of protein from the casein micelles as milk is acidified. These are hydrophobic effects (which are temperature dependent), solubilization of calcium phosphate (which is pH dependent), and isoelectric precipitation (which is both pH and temperature dependent). It appears that between pH 6.7 and pH 5.5, the dominant influence on protein dissociation comes from a combination of hydrophobic effects and calcium phosphate solubilization.

**Loosely-Entangled Protein Aggregates.** Warm temperatures increase hydrophobic interactions and compensate for solubilization of calcium phosphate, thus more intact casein micelles remain during acidification, and the dissociated caseins are mainly present as large loosely entangled aggregates (Fig. 8). This dissociated material was prevalent in the 10°C milk before and during the initial acidification and could account for the observed turbidity increase in cold milk (Fig. 6) and observed slower acidification rates, thus allowing more dissociation to occur (Fig. 7).

At cold temperatures, the dissociation of  $\alpha$ s1-,  $\beta$ - and  $\alpha$ -caseins from casein micelles are independent of each other. As acidification occurs from pH 6.4 to pH 5.2, the proportion of  $\beta$ -casein in the non-casein micelle fraction decreases while that of  $\alpha$ s1-casein increases (Dalgleish and Law, 1988). They suggest this difference in behavior may be either the greater hydrophobicity of  $\beta$ -casein, or its ability to form larger aggregates than  $\alpha$ s1-casein as temperature is increased. Apparently, these loosely entangled protein aggregates are sufficiently large that they will sediment when centrifuged. This explains why it was thought that no dissociation of proteins from the casein micelles occurs during acidification of milk as long as the temperature is above 25°C.

**Colloidal Protein Re-Associations.** As discussed by Heertje et al. (1985) and Dalgleish and Law (1988), when pH of milk approaches pH 5.2, the isoelectric precipitation of  $\beta$ -casein can occur. Above this pH it would be expected that  $\beta$ -casein would be readily released from the casein micelles at low temperatures but would then begin to either re-associate with the casein micelles or aggregate into large polymers and precipitate. Thus, as temperature

increases (and there is a corresponding increase in importance of hydrophobic interactions), the return of non-sedimentable casein into large aggregates would occur at higher pH. Larger aggregates of loosely entangled protein were observed at pH 5.3 when the milk was acidified at 40°C than at 20 and 30°C.

At low temperatures (10°C) and slow acidification (using 2.0% GDL, in which it required 175 min to reach pH 5.3 and 265 min to reach pH 5.0) the protein dissociation as shown by the turbidity increase occurred well before aggregation. The turbidity minimum appeared to coincide with a reduction in the loosely entangled protein aggregates and reincorporation of protein back into the casein micelles. With rapid acidification, (using 4.0% GDL in which it only required 60 min to reach pH 5.3, and 85 min to reach pH 5.0) the turbidity change was smaller suggesting that dissociation from the casein micelles is relatively slow and as the pH passes through the  $\beta$ -casein isoelectric point, re-association is induced before dissociation is completed.

**Gel Formation.** In the 40°C-milk, large spherical colloidal particles were observed throughout the acidification process (see Figs. 3 and 8) showing the importance of hydrophobic interactions in maintaining a supramolecular structure (McMahon and Oommen, 2008) even in the absence of calcium phosphate nanoclusters. By pH 5.0, the dissociated proteins appear to be forming into more densely packed aggregates. Then by pH 4.8, when a gel has formed, all of the caseins have either re-associated back into the remaining colloidal casein (micelles), or co-aggregated as part of the three-dimensional protein network that forms as the milk coagulates. It is, however, unlikely that this re-association would occur in the same locations from which they dissociated.

At pH 4.8, the protein molecules carry different charges than they do at the native neutral pH of milk, and so their conformational structure would be different, as well as how they interact with each other. Less calcium bridging and ion-pairing will occur because they will carry fewer negatively-charged amino acid side groups. The casein-casein interactions that do occur will result from hydrophobic liaisons (if the temperature is high enough) and a decrease in electrostatic repulsion. In comparison, during initial mammary gland synthesis of milk it is a combination of hydrophobic liaisons and attractive interactions with calcium phosphate nanoclusters that orient casein molecules in forming native casein micelle supramolecules (McMahon and Oommen, 2008).

At 40°C, the large size (~200 nm diameter) of the aggregating colloidal casein particles (Fig. 8A) results in a coarse stranded gel being formed which is in agreement with Lucey et al. (1997). The importance of hydrophobic liaisons in forming large colloidal particles below pH 5.1 has been shown by Herbert et al. (1999), and consequently at 10 and 20°C (Fig. 8C and 8D), the aggregating particles are much smaller (<100 nm) and a fine-stranded gel is formed.

## CONCLUSION

We propose a three-phase model for acid-induced gelation of unheated milk that is influenced by temperature as shown in Figure 9. Phase one involves a temperature-dependent dissociation of proteins from the casein supramolecules with less protein being released when the milk is warm (40°C). At cold temperatures (e.g., 10°C) these dissociated proteins are present as loosely entangled aggregates. At 40°C, there appears to be sufficient hydrophobic interactions to maintain the proteins as (small) spherical colloidal particles (many with diameters < 50 nm). This hydrophobic interaction would require a rearrangement and consolidation of the supramolecule interior structure with a predominance of  $\kappa$ -casein remaining on their periphery.

As acidification occurs, this dissociation continues with proteins forming into large loosely-entangled protein aggregates. Such aggregates are nonspherical and do not have the compact nature observed for casein micelles, although some are as large as the casein micelles and occasionally are attached to the casein micelles.

Phase two occurs between ~pH 5.3 and pH 4.9 and involves the loosely-entangled proteins forming into more compact colloidal particles. At 40°C, the loosely-entangled aggregates become quite large (up to 200 to 300 nm) while at 10°C, they are much smaller and have already started to re-associate with the casein micelle remnants.  $\beta$ -Casein with its net positive charge in this pH region can act as a polymerizing agent with the negatively charged  $\alpha_{s1}$ - and  $\alpha_{s2}$ -caseins.

Phase three involves the rapid aggregation of the colloidal casein particles into a gel network as the pH drops to pH 4.8 and lower. This gel network consists of chains of compact spherical particles that consist of those casein micelle remnants that remained in colloidal form during acidification, as well as those formed during re-association of the casein molecules. The nature of the gel is dependent on temperature with the gel at low temperature being composed of smaller particles that form thinner strands and have more crosslinking. Acid coagulating milk at 10°C forms a fine-stranded gel, while at 40°C a coarse-stranded gel is formed.

## ACKNOWLEDGMENTS

This research was funded by Dairy Management Inc. and the Utah Agricultural Experiment Station, Utah State University, and approved as journal paper number 8029.

## REFERENCES

- Alexander, M., and D. G. Dalgleish. 2004. Application of transmission diffusing wave spectroscopy to the study of gelation of milk by acidification and rennet. *Colloids Surfaces B: Interfaces* 38:83-90.
- Alleyne, M.C., McMahon, D.J., Youssef, N.N. and S. Hekmat. 1993. An apparatus for a new microcube encapsulation of fluid milk in preparation for transmission electron microscopy. *Food Struct.* 12:21-30.
- Banon, S., and J. Hardy. 1991. Study of acid milk coagulation by an optical method using light reflection. *J. Dairy Res.* 58:75-84.
- Banon, S., and J. Hardy. 1992. A colloidal approach of milk acidification by glucono-delta-lactone. *J. Dairy Sci.* 75:935-941.
- Bringe, N. A., and J. E. Kinsella. 1990. Acidic coagulation of casein micelles: mechanisms inferred from spectrophotometric studies. *J. Dairy Res.* 57:365-375.
- Dalgleish, D. G., and A.J.R. Law. 1988. pH-induced dissociation of bovine casein micelles. I. Analysis of liberated caseins. *J. Dairy Res.* 55:529-538.
- Dalgleish, D. G., and A.J.R. Law. 1989. pH-induced dissociation of bovine casein micelles. II. Mineral solubilization and its relation to casein release. *J. Dairy Res.* 56:727-735.
- Dalgleish, D. G., M. Alexander, and M. Corredig. 2004. Studies of the acid gelation of milk using ultrasonic spectroscopy and diffusing wave spectroscopy. *Food Hydrocolloids* 18:747-755.
- Evanhuis, N., and Th. R. de Vries. 1959. The condition of calcium phosphate in milk. *Neth. Milk Dairy J.* 13:1-15.
- Heertje, I., J. Wisser, and P. Smits. 1985. Structure formation in acid milk gels. *Food Microstructure* 4:267-278.
- Herbert, S., A. Riaublanc, B. Bouchet, D. J. Gallant and E. Dufour. 1999. Fluorescence spectroscopy investigation of acid- or rennet-induced coagulation of milk. *J. Dairy Sci.* 82:2056-2062.
- Kalab, M., D. B. Emmons and A. G. Sargant 1976. Milk gel structure V. Microstructure of yoghurt as related to the heating of milk. *Milchwissenschaft* 31:402-408.
- Kalab, M., B. E. Phibbs-Todd, and P. Allan-Wojtas. 1982. Milk gel structure XIII. Rotary shadowing of casein micelles for electron microscopy. *Milchwissenschaft* 37:513-518.
- Kauzmann, W. 1959. Some factors in the interpretation of protein denaturation. *Adv. Protein Chem.* 14:1-63.
- Kim, B. Y., and J. E. Kinsella. 1989. Rheological changes during slow acid induced gelation of milk by D-glucono- $\delta$ -lactone. *J. Food Sci.* 54:894-898.
- Lucey, J. A. 2001. The relationship between rheological parameters and whey separation in milk gels. *Food Hydrocolloids* 15:603-608.
- Lucey, J. A. and H. Singh. 1998. Formation and physical properties of acid milk gels: a review. *Food Res. Int.* 30:529-5442.
- Lucey, J. A., van Vliet, T., Grolle, K., Geurts, T. and P. Walstra. 1997. Properties of acid casein gels made by acidification with glucono- $\delta$ -lactone. 2. Syneresis, permeability and microstructural properties. *Int. Dairy J.* 7:389-3977.
- Lucey, J. A., M. Tamehana, H. Singh and P. A. Munro. 1998. A comparison of the formation, rheological properties and microstructure of acid skim milk gels made with bacterial culture or glucono- $\delta$ -lactone. *Food Res. Int.* 31:147-1555.
- McMahon, D. J., and R. J. Brown. 1982. Evaluation of formagraph for comparing rennet solutions. *J. Dairy Sci.* 65:1639-11642.
- McMahon, D. J., R. J. Brown, and C. A. Ernstrom. 1984. Enzymic coagulation of milk casein micelles. *J. Dairy Sci.* 67:745-7448.
- McMahon, D.J.J. and W.R. McManus. 1998. Rethinking casein micelle structure using electron microscopy *J. Dairy Sci.* 81:29985-2993.
- McMahon, D. J. and B. S. Oommen. 2008. Supramolecular structure of the casein micelle. *J. Dairy Sci.* 91:1709-1721.

- Novakovic, P., T. Petrak, J. Kordic and V. Slacanac. 2000. Application of mathematical models in milk coagulation process during lactic acid fermentation. I. Relation between enzymatic and acidic milk coagulation. *Acta Alimentaria* 29:241-254.
- Pierre A., and G. Brule. 1981. Mineral and protein equilibria between the colloidal and soluble phases of milk at low temperature. *J. Dairy Res.* 48:417-428.
- Qvist, K. B. 1979. Reestablishment of the original rennetability of milk after cooling. I. The effect of cooling and LTST pasteurization of milk and renneting. *Milchwissenschaft* 34:467-470.
- Roefs, S.P.F.M., P. Walstra, D. G. Dalgleish, and D. S. Horne. 1985. Preliminary note on the change in casein micelles caused by acidification. *Neth. Milk Dairy J.* 39:119-122.
- Singh, H., M. S. Roberts, R. A. Munro and C. T. Teo. 1996. Acid-induced dissociation of casein micelles in milk: effects of heat treatment. *J. Dairy Sci.* 79:1340-1346.
- Tamime, A. Y., M. Kalab, and G. Davies. 1984. Microstructure of set-style yoghurt manufactured from cow's milk fortified by various methods. *Food Microstructure* 3:83-92.
- Tranchant, C. C., D. G. Dalgleish and A. R. Hill. 2001. Different coagulation behaviour of bacteriologically acidified and rennet milk: the importance of fine-tuning acid production and rennet action. *Int. Dairy J.* 11:483-494.
- van Hooydonk, A.C.M., H. G. Hagedoorn, and I. J. Boerrigter. 1986. pH-induced physico-chemical changes of casein micelles in milk and their effect on renneting. I. Effect of acidification on physico-chemical properties. *Neth. Milk Dairy J.* 40:281-296.
- Visser, J., A. Minihan, P. Smits, S. B. Tjan, and I. Heertje. 1986. Effects of pH and temperature on the milk salt system. *Neth. Milk Dairy J.* 40:351-368.
- Vreeman, H. J., B. W. van Markwijk, and P. Both. 1989. The structure of casein micelles between pH 5.5, and 6.7 as determined by light-scattering, electron microscopy and voluminosity experiments. *J. Dairy Res.* 56:463-470.

## FIGURES

Figure 1. Transmission electron micrographs of skim milk, glutaraldehyde-fixed and agar-solidified at (A) 40°C, (B) 30°C, (C) 20°C, and (D) 10°C; short arrows = agarose fibers, d = dissociated protein, bar = 500 nm.

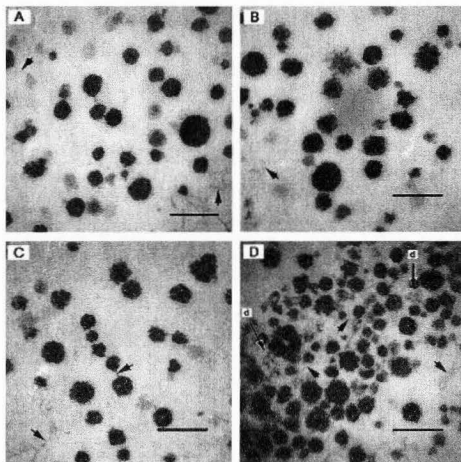


Figure 2. Reduction of pH in skim milk as a function of temperature based on (A) amount of glucono- $\delta$ -lactone (GDL) added and (B) addition of 1.2% freeze-dried starter culture.

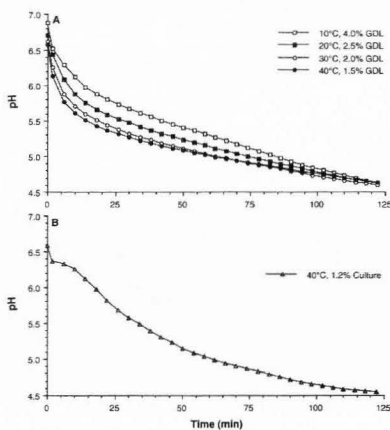


Figure 3.

Transmission electron micrographs during warm acidification at 40°C of skim milk by 1.55% glucono- $\delta$ -lactone, glutaraldehyde-fixed and agar-solidified at (A) pH 5.5, (B) pH 5.2, (C) pH 4.95, and (D) pH 4.8; d = loosely-entangled protein, short arrows = agarose fibers, a = aggregated particles, bar = 500 nm.

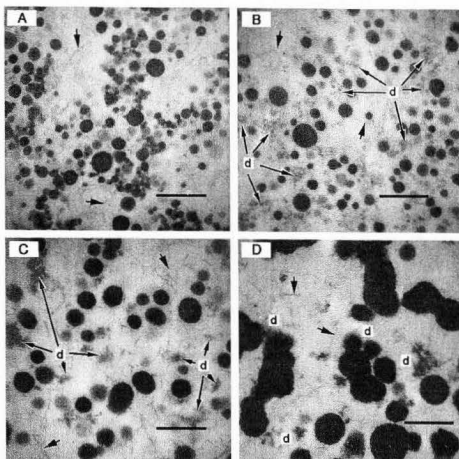


Figure 4.

Transmission electron micrographs during warm acidification at 40°C of skim milk by addition of 1.2% starter culture, glutaraldehyde-fixed and agar-solidified at (A) pH 5.4, (B) pH 5.0, (C) pH 4.75, and (D) pH 4.6; d = loosely-entangled protein, short arrows = agarose fibers, a = aggregated particles, bar = 500 nm.

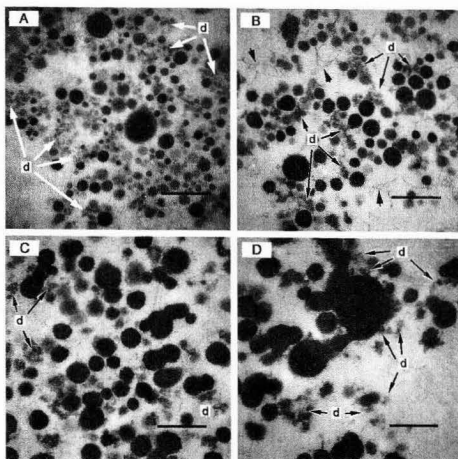




Figure 5.

Transmission electron micrographs during cold acidification at 10°C of skim milk by 4.0% glucono- $\delta$ -lactone, glutaraldehyde-fixed and agar-solidified at (A) pH 5.9, (B) pH 5.3, (C) pH 5.0, and (D) pH 4.8; d = loosely-entangled protein, short arrows = agarose fibers, bar = 500 nm.

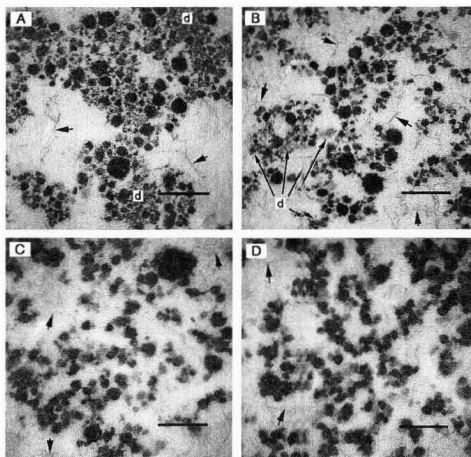


Figure 6.

Transmission electron micrographs of acid milk gels formed after acidification of skim by glucono- $\delta$ -lactone at (A) 40°C, (B) 30°C, (C) 20°C, and (D) 10°C, bar = 1  $\mu$ m.

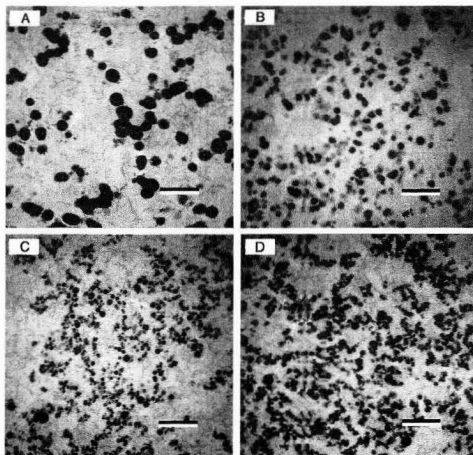


Figure 7

Influence of temperature on changes in turbidity upon acidification of skim milk by 4.0% (, 2.5%, 2.0%, and 1.5% (w/w) glucono- $\delta$ -lactone (GDL) at 10, 20, 30, and 40°C, respectively.

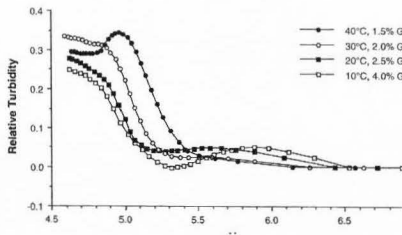


Figure 8.

Change in turbidity of skim milk as a function of slow, medium and fast acidification rates using glucono- $\delta$ -lactone (GDL) at (A) 10°C, and (B) 40°C, as well as by using 1.2% starter culture at 40°C (B).

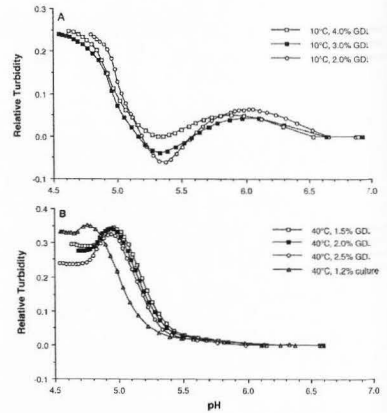
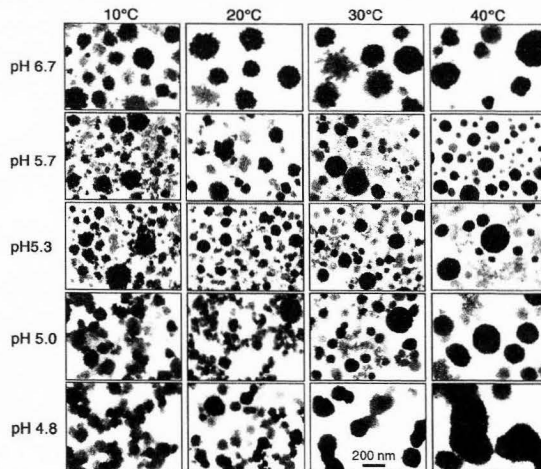


Figure 9.

Microstructural changes occurring during chemical acidification of milk as a function of temperature and pH. Images derived from transmission electron micrographs with colloidal casein micelles depicted in black and loosely-entangled protein aggregates depicted in grey, and all background digitally converted to white.



# Influence of Brine Concentration and Temperature on Composition, Microstructure, and Yield of Feta Cheese

D. J. MCMAHON: Utah State University

M. M. MOTAWEE: National Organization for Drug Control and Research

W. R. MCMANUS: Utah State University

## ABSTRACT

The protein matrix of cheese undergoes changes immediately following cheesemaking in response to salting and cooling. Normally, such changes are limited by the amount of water entrapped in the cheese at the time of block formation but for brined cheeses, such as feta cheese, brine acts as a reservoir of additional water. Our objective was to determine the extent to which the protein matrix of cheese expands or contracts as a function of salt concentration and temperature, and whether such changes are reversible. Blocks of feta cheese made with overnight fermentation at 20 and 31°C yielded cheese of pH 4.92 and pH 4.83 with 50.8 and 48.9 g/100 g moisture, respectively. These cheeses were then cut into 100-g pieces and placed in plastic bags containing 100 g of whey brine solutions of 6.5, 8.0 and 9.5% salt, and then stored at 3, 6, 10, and 22°C for 10 d. After brining, cheese and whey were re-weighed, whey volume measured and cheese salt, moisture and pH determined. A second set of cheeses were similarly placed in brine (n=9) and stored for 10 d at 3°C, followed by 10 d at 22°C, followed by 10 d at 3°C, or the complimentary treatments starting at 22°C. Cheese weight and whey volume (n=3) were measured at 10, 20 and 30 d of brining. Cheese structure was examined using laser scanning confocal microscopy. Brining temperature had the greatest influence on cheese composition (except for salt content), cheese weight and cheese volume. Salt-in-moisture content of the cheeses approached expected levels based on brine concentration and ratio of brine to cheese, i.e., 4.6, 5.7 and 6.7%. Brining at 3°C increased cheese moisture, especially for cheese with initial pH of 4.92, producing cheese with moisture up to 58 g/100 g. Cheese weight increased after brining at 3, 6 or 10°C. Cold storage also prevented further fermentation and the pH remained constant, whereas at 22°C the pH dropped as low as pH 4.1. At 3°C the cheese matrix expanded (20 to 30%) whereas at 22°C there was a contraction and a 13 to 18 g/100 g loss in weight. Expansion of the protein matrix at 3°C was reversed by changing to 22°C. However, contraction of the protein matrix, was not reversed by changing to 3°C and the cheese volume remained less than what it was initially.

**Keywords:** cheese, brining, structure, yield

## INTRODUCTION

Feta cheese is a pickled cheese, traditionally made from sheep's milk, that has its origins in Greece where its annual consumption is still the highest in the world at 12 kg/person (Anifantakis and Moatsou, 2006). Its usage spread with the Greek diaspora to other countries (such as Australia, United States, Canada), where feta became a generic term for white pickled cheese made from cow's milk. There are many different types of pickled cheeses that are preserved, or stored, in brine, and their names vary from one region to another. In the 1980s to 1990s, cow's milk feta cheese was the most significant type of pickled cheese exported and marketed in the world (Tamime et al., 1996). Global production of feta-type cheeses is currently about  $1 \times 10^9$  kg/y, which is about 7% of total global cheese production (Anonymous, 2009). Feta is a major cheese type in the Asia-Pacific-Middle East-Africa region and accounts for one-third of total cheese produced in this region. During 2008, there was a large increase in new feta cheese products, especially in Africa, United States, and Greece (Anonymous, 2009). Since 2002, the name 'Feta' has a Product of Designation of Origin registration such that in the European Union it can only be applied to cheeses manufactured in certain geographical areas of Greece using sheep's or sheep/goat's milk (Anonymous, 2002; Anifantakis and Moatsou, 2006).

In traditional manufacture of feta cheese, after initial whey drainage and block formation, cheese blocks are dry salted over a number of days with the cheese held at 16 to 18°C and exposed to air so that a slimy growth of

bacteria and yeast forms on the cheese surface (Anifantakis and Moatsou, 2006). This is later washed off before final packaging into a 7% brine solution, but contributes to cheese maturation and flavor development during further storage at 16 to 18°C for about 2 wk before being transferred into cold storage at 3 to 4°C. A milder flavor feta cheese can be made by reducing or eliminating the dry-salting time and immersing the cheese in brine after overnight fermentation and gravity drainage of the curd blocks. Such early brining interrupts the development of the secondary microflora (Bintsis, 2006).

There are variable ways in which pickled cheeses are salted, and brining gives faster rates of salt absorption, a more even distribution of salt in the cheese, and produces cheeses with higher retention of moisture and hence higher yields (Bines and Holmes, 1994) which along with the milder flavor is an incentive for its use in large production facilities (Bintsis, 2006). Traditionally, a crude rennet obtained from abomas of lambs and kids was used as the coagulant for making feta cheese which contributed to flavor development by lipase and pregastric esterases (Anifantakis and Moatsou, 2006). These enzymes are not present in modern commercial rennets, and so lipases are added to the milk prior to renneting to provide the aroma and peppery taste characteristic of feta cheese.

In general, salting promotes syneresis of whey from the curd, reducing the moisture content of the cheese, although this can be influenced by other intrinsic and extrinsic factors such as the calcium content of the cheese (Paulson et al., 1998) and temperature (Turhan and Kaletunç, 1992). A small amount of salt (<0.8%) can cause a physical change in the proteins in cheese by increasing their water solubility and hydration as described by Paulson et al. (1998). Calcium concentrations of 0.5 to 0.6% in brine are needed to prevent leaching of calcium from the cheese surface and its increased hydration resulting in 'soft rind' defects (Geurts et al., 1972). Increasing the salt concentration of brine will give faster salt diffusion and lower final cheese moisture (Prasad and Alvarez, 1999) but at very high levels, such as in saturated brine, 'salting-out' can occur causing a contraction of the protein matrix and reduced salt diffusion (Melilli et al., 2003).

The objective of our research was to investigate cheese weight and volume during storage of feta cheese in brine at warm and cold temperatures and to determine their reversibility. Then to relate these changes to changes in the cheese microstructure.

## MATERIALS AND METHODS

### Materials

The starter culture used was Choozit Feta A (Danisco USA Inc, New Century, KS) which consisted of a lyophilized blend of various strains of *Lactococcus lactis*, *Lactobacillus* species, and *Streptococcus thermophilus* and Holdbac LC (Danisco USA Inc.) a *Lactobacillus rhamnosus* culture was used as an adjunct. Kid goat lipase powder (#71444) was obtained from Chr. Hansen Inc (Milwaukee, WI) and double strength (~650 International milk clotting units/ml) chymosin rennet (Maxiren) was obtained from DSM Food Specialties USA Inc. (Eagleville, PA) Cow's milk was obtained from the Utah State University's Caine Dairy Research and Teaching Center (Wellsville, UT).

### Cheesemaking

Milk was pasteurized at 73°C for 15 s and 136 kg filled into an open cheese vat in the Gary H. Richardson Dairy Products Laboratory at Utah State University. Milk was warmed to 35°C, 4 g of starter culture and 1 g of adjunct culture were added and allowed to ripen for 60 min. Ten grams of lipase and 12 ml of rennet (57 international milk clotting units/ kg milk) were added, the curd was cut after 30 min using wire knives (16 mm spacing) and then stirred after 15 min of healing. About one-third of the whey was removed after 30 min and then curd and whey were transferred into 4 perforated rectangular stainless steel molds and the curd allowed to mat together and press under its own weight. The molds were turned 4 times on 20-min intervals and then 2 forms were transferred to a warm room (31°C) with the other 2 remaining at room temperature (~20°C) for overnight fermentation and continued whey drainage. The next day the cheese blocks were removed from the forms and cut into 100 ± 1-g pieces in preparation for brining.

### Brining

Whey that was removed during cheese making was used to prepare brine solutions of 6.5, 8.0 and 9.5% (wt./wt.) salt by adding NaCl, and then adjusting the pH of each to pH 4.7 by adding vinegar. Each 100-g piece of

cheese was placed into a reclosable plastic bag (Hefty One Zip Quart Freezer bag; Pactiv Corp., Lake Forest, IL) in triplicate and  $100 \pm 1$  g of brine added. Air was expelled from the bag, the bag closed, and then stored at its designated temperature. One set of cheeses was stored at 3, 6, 10 or 22°C for 10 d then analyzed. A second set of cheeses (from cheese fermented at 31°C) underwent brining with the temperature alternating between cold and warm storage starting with 10 d at 3°C, followed by 10 d at 22°C, then 10 d at 3°C. Brining of the third set of cheeses started warm with 10 d at 22°C, followed by 10 d at 3°C, then a final 10 d at 22°C.

### **Cheese Analysis**

Moisture content was determined in triplicate by weight loss using a microwave oven (CEM Corp., Indian trail, NC) at 70% power with an endpoint setting of <0.4 mg weight change over 2 s. Fat content was determined in duplicate using a modified Babcock method (Richardson, 1985). Salt was measured by homogenizing grated cheese with distilled water for 4 min at 260 rpm in a Stomacher 400 (Seward, England). The slurry was filtered through a Whatman #1 filter paper, and the filtrate was analyzed for sodium chloride using a chloride analyzer (model 926, Corning, Medfield, MA). Salt-in-moisture (S/M) content was calculated as the percentage (by weight) of salt in these cheese based on combined moisture plus salt content. The pH was measured using a glass electrode after stomaching 20 g of grated cheese with 10 g of distilled water for 1 min at 260 rpm. After brining, the weight of each cheese block, and residual brine solution was measured, as well as brine volume. The change in cheese volume was considered to be equal to the change in brine volume, using initial densities of 1.02, 1.03, 1.05, and 1.07 g/ml for the cheese, 6.5, 8.0 and 9.5% brines, respectively.

### **Microstructure**

Cheese samples at 3 and 22°C were prepared for examination by laser scanning confocal microscopy (LSCM) based on the method of McManus et al. (2009) so that their structure did not change during sample staining and viewing by LSCM. Small pieces of cheese about 8 x 8 x 2 mm were immersed in 0.10 g/ml osmium tetroxide (Ted Pella, Redding, CA) in whey, and protein and fat fixation performed by placing the sample in a Pelco 3470 microwave system (Ted Pella) at power 6 for 20 min and then holding the sample in the fixative overnight. The fixed cheese sample was rinsed three times in water (distilled deionized) then soaked in 0.20 g/ml Nile Red (Sigma-Aldrich, Inc., Saint Louis, MO) in dimethylsulfoxide (Sigma-Aldrich, Inc) for 5 min to stain for fat. It was then rinsed twice in water, and stained with 0.050 g/ml fluorescein isothiocyanate (FITC) (Sigma-Aldrich, Inc.) in acetone-water (1:1) solution to stain for protein. The stained sample was mounted on standard microscope slides with glycerin jelly, cover slipped and placed on an inverted microscope (Model MRC 23, Biorad, Hercules, CA) with a Kr/Ar laser, exciting FITC at 488 nm and Nile Red at 568 nm. Fluorescent emissions were from 488 to 650 nm, and 550 to 750 nm for FITC and Nile Red, respectively, and captured sequentially using exclusion filters of 512 to 532 nm, and  $\geq 585$  nm. Images were false colored with fat as orange and protein as light green.

### **Experimental Design**

Differences in composition of cheese after fermentation (i.e., before brining) were analyzed using the 2-tailed Student's t-Test function in Excel 2008 (Microsoft Corp., Seattle, WA). Cheese composition, cheese and brine weight and volume after brining for 10 d were analyzed as a 2-way factorial of temperature and concentration with three replicates. The experiments in which cheese was brined at alternating temperatures for periods of 10 d, were analyzed separately as 2-way factorials of brine concentration and storage time with three replicates. Analysis of variance was performed after log transformation to normalize the data using PROC GLM in SAS (version 9.1, SAS Institute, Cary, NC) with REGW multiple range test and Tukey Least Squares Means.

## **RESULTS AND DISCUSSION**

### **Cheese Composition – Before Brining**

Allowing the cheese blocks to ferment overnight at different temperatures, 20°C (overnight room temperature) and 331°C, produced cheese with different moisture and pH. At the lower temperature, fermentation by the starter culture was retarded and after 20 h the cheese had only reached pH 4.92 compared to pH 4.83 when stored at 31°C ( $P = 0.002$ ). There was also a difference in moisture, with more syneresis occurring at the higher temperature

resulting in significantly ( $P < 0.001$ ) lower moisture contents, that is, 48.9 and 50.8 g/100 g for cheeses fermented at 31 and 20°C respectively. Salt contents of the cheeses were the same (0.11 g/100 g) and there was a slight difference in fat content with cheese fermented at the higher temperature having a slightly higher fat level of 51.9 g/100 g compared to 50.9 g/100 g fat on a dry basis (FDB) ( $P < 0.001$ ). Although both cheeses were made from the same vat of milk, this increased FDB probably results from higher losses of nonfat solids (such as lactose, whey proteins, and minerals) with the higher syneresis that occurred during overnight fermentation at 31°C.

### **Cheese Composition - After Brining**

**Moisture.** As shown in Table 1, the final moisture content of the cheese was influenced by the temperature of overnight fermentation. Those cheeses that were fermented at 31°C, in general, had lower moisture content after brining compared to those fermented at 20°C. This was dependent on both brining temperature and brine salt concentration with both factors and their interaction having significant effects on moisture ( $P < 0.001$ ). The average differences in moisture were 7.4, 6.8, 4.4, and 0.9 g/100 g lower for cheeses brined at 3, 6, 10 and 22°C, respectively. Whether this was a function of the initial lower moisture or pH could not be determined, although lower moisture loss is normally associated with a lower cheese pH (Guinee, 2004). Cheese with increased syneresis during fermentation (and hence lower moisture) would be expected to have allowed more crosslinking to occur between protein strands within the protein matrix. Such crosslinking would then act to restrict the level of changes taking place during brining. Having a lower pH could also play a role although it has been shown that below pH 5.0 it is the protein solubility that becomes the controlling factor for determining the physical properties of the cheese protein matrix (Pastorino et al., 2003).

Cheese texture mirrored moisture content and was more dependent on temperature during brining than salt content. Cheeses brined at 3°C were the softest, especially for those from the 20°C fermentation that had the higher initial pH and moisture contents. Such cheese brined at 3°C had final average moisture content of 57.2 g/100g and were very soft. Cheese brined at 6°C (with 54.9 g/100 g average moisture) was considered soft and comparable to what would be expected for a relatively young white brined cheese such as feta or Domiat cheese.

Moisture uptake from the brine during cold storage is typical of white-brined cheeses (Alichanidis et al., 1991; Pappas et al., 1996). Cheeses brined at 10°C (50.7 g/100 g average moisture) were firmer than those brined at 6°C, whereas those brined at 22°C (41.0 g/100 g average moisture) were quite hard and were more typical of white brined cheese that had been aged for a year or more. There was a slight effect of salt concentration of the brine on final moisture of the cheese although this varied based on storage temperature and initial pH and moisture of the cheese (Table 1).

In comparison, the average gross composition of feta cheese obtained from different retail outlets in Greece was 52.9 g/100 g moisture, 55.6 g/100 g FDB, 16.7 g/100 g protein, and 5.27 g/100 g S/M (Anifantakis and Moatsou, 2006). In the United States, there are no standards of identity for feta cheese and it can have a broad range of composition, with moisture being as high as 55 to 60 g/100 g and FDB < 40 g/100 g (M.E. Johnson, Wisconsin Center for Dairy Research, Madison, WI, personal communication). This is a reflection of how companies standardize their milk composition and optimization of their cheesemaking process on an economic basis.

**pH.** After 10 d of brining, cheese pH had decreased depending on brine concentration, temperature and their interaction ( $P < 0.001$ ) as shown in Table 1. Lactic acid bacteria (either starter or nonstarter bacteria) grow at a fast rate during the fermentation and early stages of cheese maturation (Anifantakis and Moatsou, 2006) provided the temperature remains sufficiently high. When brined at 3°C, there was very little drop in pH (average of 0.05 units) whereas a large decrease in pH (average of 0.61 units) occurred when the cheese was stored at 22°C with the cheeses reaching as low as pH 4.1 after 10 d after brining. Cheeses brined at 6 and 10°C had intermediate pH drops of 0.14 and 0.25 units, respectively. This corresponds with the known interruption in biochemical activity of feta cheese during maturation (and consequent milder flavor) that occurs when feta cheese is placed in brine without the week-long traditional dry salting period at 16 to 18°C (Bintsis, 2006).

Lower salt concentrations also allowed more generation of lactic acid and larger pH drops during brining. Although, with the limited range of salt concentrations used in this experiment, effect of salt was not as great as the effect of temperature. After 10-d brining, the average pH drop was 0.27, 0.25 and 0.18 units for cheeses brined in 6.5, 8.0 and 9.5% salt brines. There was little difference between use of the 6.5 and 8.0% brines, but significant retardation of acid development occurred in the 9.5% brine.

**Salt.** During brining, salt is absorbed into the cheeses with a concomitant decrease in concentration of salt in the brine. This net movement of Na<sup>+</sup> and Cl<sup>-</sup> ions occurs as a consequence of osmotic pressure differences between

cheese and 1 brine (Guinee and Fox, 2004). Brine used for these experiments was prepared from whey obtained during cheesemaking, thus, minerals other than  $\text{Na}^+$  and  $\text{Cl}^-$  would have been similar in both cheese and brine and so no movement of minerals ions, such as  $\text{Ca}^{2+}$  and  $\text{H}_2\text{PO}_4^-$  was expected to occur during brining. The observed softening of the cheeses when brined at low temperature was therefore considered to result from the decreased strength of protein interactions allowing an infusion of moisture into cheese rather than from having too little calcium in the brine.

Salt concentration in the brined cheeses was influenced by salt content ( $P < 0.001$ ) and temperature of the brine ( $P < 0.001$ ) as shown in Table 1. For cheeses fermented at  $31^\circ\text{C}$ , there was not a significant interaction between temperature and salt content of the brine ( $P = 0.35$ ) but there was for the cheese fermented at  $20^\circ\text{C}$  ( $P < 0.001$ ). When pooled over all temperatures for both warm and cool fermentation conditions, average salt content of cheeses after 10 d in 6.5, 8.0 and 9.5% brines were 2.30, 2.88 and 3.37 g/100 g respectively. The cheeses made using the warmer fermentation temperature were an average 0.26 g/100 g lower in salt, which was attributed to these cheeses being lower in moisture and therefore having less aqueous phase into which salt could diffuse from the brine.

Taking into account initial cheese moisture (48.9 and 50.8 g/100 g) and salt (0.11 g/100 g) contents, and that equal weights (100 g) of cheese and brine were mixed together (and assuming the salt content throughout all the water phase was equalized after 10 d of brining) the overall S/M content of the cheese/brine system would on average have been 4.6, 5.7 and 6.7 g/100 g for the 6.5, 8.0 and 9.5% brines respectively. And on average these were the S/M levels measured in the cheeses after 10 d of brining. Mean cheese S/M for cheeses brined in the 6.5, 8.0, and 9.5% brines were 4.60, 5.60 and 6.40 g/100 g, respectively. As salt concentration increased there was slightly less salt absorbed into the cheese than theoretically expected, although at 9.5% salt brine concentration, the final S/M in the cheese was still only 0.3 g/100 g less than predicted. Thus, a desired S/M level in feta cheese can be easily obtained by controlling the cheese weight to brine weight ratio and taking into account the moisture content of cheese before brining and salt content of the brine. In our experiment, the brine to cheese ratio was 1:1 which for the 9.5% brine produced a cheese with S/M similar to the 6.27 g/100 g reported for feta cheese by Abd El-Salam and Alichanidis (2004). For salt uptake not to be influenced by the amount of cheese being brined, the brine to cheese ratio needs to be increased to 5:1 (Bintsis, 2006).

Temperature had only a slight effect on final S/M contents and this was only evident in cheeses fermented at  $20^\circ\text{C}$  ( $P = 0.03$ ) and not in cheeses fermented at  $31^\circ\text{C}$  ( $P = 0.76$ ). There was no significant interaction between salt concentration and temperature ( $P = 0.87$ ) and when averaged over all salt concentrations, mean S/M for each temperature was within the range of  $5.53 \pm 0.05$  g/100 g. Usually increasing brining temperature increases both diffusion rate of salt into cheese and amount of salt absorbed (Turhan and Kaletun, 1992; Prasad and Alvarez, 1999). This would only be apparent with shorter brining times and our brine concentrations were much lower than the  $>20\%$  salt solutions required to produce salting-out of the proteins and loss of porosity at the cheese surface.

*Lactococcus* starter cultures are typically inhibited in growth, and acid production slows down as S/M levels increase (especially at  $\text{S/M} > 5.0$  g/100 g), but lactobacilli nonstarter bacteria found in cheese are usually more salt tolerant and require a  $\text{S/M} > 6$  g/100 g to retard their activity (Guinee and Fox, 2004). Thus, the observed average pH drops of 0.37, 0.25 and 0.18 units for cheeses brined to S/M levels of 4.6, 5.6 and 6.4 g/100 g, respectively, correspond to extent of expected inhibition of bacterial activity with salt concentration. A combination of high salt (9.5% brine, 6.7 g/100 g S/M) and low temperature ( $3^\circ\text{C}$ ) was sufficient to completely inhibit acid development with zero change in pH occurring during brining. At the other extreme, with low salt (6.5% brine, 4.6 g/100 g S/M) and high temperature ( $22^\circ\text{C}$ ), there was little inhibition of bacterial growth and a 0.73 unit drop in pH occurred.

### **Cheese Yield**

Both brine temperature ( $P < 0.001$ ) and salt concentration ( $P < 0.001$ ) and the temperature x concentration interaction ( $P = 0.0004$ ) influenced changes in cheese weight during brining (Table 2). Salt concentration in the range used for this study had a minor effect with those cheeses being stored in 8.0% or 9.5% salt brine having a mean 5 g/100 g additional increase in weight compared to those brined in 6.5% salt brine. The cheeses that started out with lower moisture contents (that is, those subjected to warm fermentation) also had about 5 g/100 g less change in weight.

The dominant factor influencing cheese weight was the temperature at which they were brined, with weight being gained at lower temperatures and weight lost when cheese was brined at  $22^\circ\text{C}$ . The colder the temperature, the greater the weight gain with mean changes in weight of +20.0, +14.3 and +7.0 g/100 g occurring at 3, 6 and  $10^\circ\text{C}$ , respectively, compared to a mean change in weight of -14.2 g/100 g when the cheese was brined at  $22^\circ\text{C}$ .

So if a cheese is sold in a brine solution, the temperature at which it is expected to be stored needs to be taken into consideration when determining the net weight of cheese.

Part of the weight increase results from absorption of salt during brining, but most of the weight increase occurs because of absorption of brine into the cheese and a subsequent increase in volume (Table 2). The lower the temperature and salt concentration of the brine, the greater the volume of brine absorbed into the cheese ( $P < 0.001$ ,  $P = 0.001$ , respectively). For cheeses fermented overnight at 20°C (with pH of 4.92), the mean absorption of brine into the cheese was 9.35, 14.7, and 21.6 ml for cheese brined at 10, 6 and 3°C, respectively. When brined at 22°C, there was a mean transfer of serum into the brine of 11.3 ml/100 g cheese. The cheeses fermented at 31°C (with pH of 4.83) demonstrated less weight change but with the same trend with 3.7, 8.4 and 13.8 ml of the brine being absorbed into 100 g of cheese at 10, 6 and 3°C, respectively, and a loss of 3.0 ml/100g cheese at 22°C.

Brine concentration had less influence on volume of brine absorbed into the cheese than temperature, with a general trend of slightly more brine absorption as brine concentration increased from 6.5 to 9.5%. These changes were attributed to a decrease in protein-protein interactions as temperature is lowered and salt concentration increased. Paulson et al. (1998) showed that when cheese curd is salted there is an increase in water holding capacity of cheese protein matrix, so that absorption of water into cheese would be expected when cheese is brined. Pastorino et al. (2002) reported that heating cheese promotes hydrophobic interactions between proteins as  $\beta$ -casein and calcium become less soluble, resulting in increased protein aggregation as shown by Metzger et al. (2000).

At 3°C, hydrophobic interactions are minimized, whereas at 22°C they are of considerable importance and can cause both an expulsion of serum from the cheese and a drop in cheese moisture content. It was interesting that if cheese pH had decreased to 4.8 before brining, there was less absorption (or expulsion) of brine (or serum) during the 10-d brining period. This occurred at all temperatures and salt concentrations. For example, increase in cheese moisture content when pH 4.92 cheeses were brined at cold temperatures (3 and 6°C) was about double that for pH 4.83 cheeses. According to Pastorino et al. (2003), as pH is lowered below pH 5.0, physical properties of cheese change from being dependent on calcium level in the cheese to being dependent on casein insolubility as cheese pH approaches the casein isoelectric point. Thus, in the pH 4.83 cheese, decreased solubility of the caseins apparently limits the physical changes that can occur within the protein matrix as the cheese is immersed in brine and cooled.

The overall magnitude of effects of brining temperature (3 to 22°C), salt concentration (6.5 to 9.5%) and pH (4.83 and 4.92) on change in cheese volume were: temperature  $\gg$  salt concentration  $>$  pH. For cheeses stored in the 6.5% brine, the crossover from contraction of the cheese to an expansion occurred at 10°C. For cheeses stored in the 8.0 and 9.5% brines, it was estimated that at 15°C there would be no net change in volume.

### **Reversibility of Changes During Brining**

When brining was alternated between cold (3°C) and warm (22°C) storage conditions, the extent that weight and volume changes were reversible depended on whether they had first been stored cold or warm, and to some extent the salt concentration of the brine. When cheese was stored cold for 10 d, there was about a 15% increase in cheese weight in all the cheeses (Fig. 1), and similar change in volume (Fig. 2). Then if such cheeses were then warmed to 22°C and stored for 10 d more, their weight and volume decreased. Cheeses stored in the 6.5 and 8.0% brines had an overall weight loss (about -14%) which was comparable to just 10 d storage in 22°C brine (as shown in Table 2, for cheese with starting pH of 4.83). However, the weight loss in the cheese stored in 9.5% brine was much less and the cheese remained heavier than its initial weight. The effect of salt concentration on inhibiting the reversibility of the changes caused by brining cheese is better observed by looking at changes in cheese volume (Fig. 2). The higher the salt concentration, the less change in cheese volume occurs during the second 10-d brining period. In 6.5% brine, the cheese underwent a 13% increase in volume during the first 10-d storage at 3°C, then after a subsequent 10-d storage at 22°C the cheese volume had dropped to 12% less than its starting size. Cheese stored in 8.0% brine shrunk less, and the cheese stored in 9.5% brine hardly at all and was still 5% larger than originally.

When cheese was stored cold for 10 d, then warm for 10 d, then stored cold for a third 10-d period, they increased in weight and volume compared to cheese that had only the first 20 d of storage, but did not reach the levels obtained by cheeses that had just the first 10-d of cold storage. Cheeses in each of the three brine concentrations increased about 6% in volume compared to their volume after 20 d. Their overall changes in volume of -4%, +7%, and +11% for cheeses in 6.5, 8.0 and 9.5% brines, respectively, after 30 d of brining were dependent on the extent of changes that had occurred during the 10 d of warm storage.

So whereas it appears that the expansion of cheese volume that occurs during storage in cold brine can be partly reversed by storage in warm brine, the contraction of the cheese in warm brine is not as reversible. If cheese was



first brined at 22°C so that cheese volume decreased (along with a concomitant decrease in cheese weight), there was no large increase in weight or volume when the cheese was subsequently stored at 3°C. Such cheese remained below its original cheese weight (Fig. 3) and volume (Fig. 4) compared to cheese and well below that after brining for 10 d at 3°C (Figs 1 and 2). Such a scenario of warm brining followed by cold brining is more like the traditional method for making feta cheese in which the cheese blocks are held at 16 to 18°C to allow flavor development (Anifantakis and Moatsou, 2006). Brining at warm temperatures induces syneresis of whey that is normally associated with salting (Bines and Holmes, 1994) with the quantity of water lost being about twice the quantity of salt absorbed (Guinee, 2004). Likewise, early brining of cheese (and subsequent cooler temperature of storage) not only produces a more even distribution of salt in the cheese but also higher retention of moisture and higher cheese yield (Bintsis, 2006).

### **Cheese Microstructure**

The process for making feta cheese has common features with most other rennet set cheeses such as initial milk coagulation, curd cutting, curd firming stages, block formation and continued acid development. There are changes in microstructure as curd is converted into cheese as has been shown by Kimber et al. (1975) using transmission electron microscopy and by Oberg et al. (1993) using scanning electron microscopy. The cheese curd consists of a protein matrix with thick strands of protein making up a continuous network with fat globules (either individually or as aggregates) and pools of serum (whey) interspersed throughout. During overnight fermentation of the cheese blocks there is further whey syneresis that occurs and so the fat and protein components of the cheese matrix become more closely associated with less retained serum.

Differences in microstructure of the cheeses were observed depending on the brining temperature as shown in Fig 5. In these images, the areas corresponding to protein are designated as light green and the fat as orange. The protocol used for preparing the cheese samples for viewing using LSCM included an osmium-fixation step that fixes the proteins and the fat in their relative location at the time of fixation. Cheese brined at 3°C (Fig. 5a) had an even distribution of fat droplets throughout the cheese, except for those areas corresponding to curd particle junctions that were devoid of fat. This results from loss of fat from the curd at the time the initial coagulum was cut as previously shown by Paquet and Kalab (1988). The fat droplets had the typical size distribution of fat globules in milk and most appeared spherical in shape.

Some fat droplets appeared as duplets but this was considered to be an artifact of LSCM. Assignment of pixels in the micrograph as fat is based upon fluorescence of Nile Red when it is excited by the 568-nm band of the laser light. This only occurs when Nile Red is in a lyophilic environment and is much greater in the presence of fat than in the presence of proteins. It is the light emitted from the Nile Red molecules that is then recorded by the LSCM. Each fluorophore will emit light in all directions (when excited at the appropriate wavelength) and the optics of the microscope captures the cone of light directed towards the aperture of the objective lens as the LSCM scans across the field of view of the sample. The observed size of each group of emitting fluorophores, such as fat droplets with their attached Nile Red molecules, will appear larger than their actual size. Taking this into account, the observed duplets would probably be individual fat globules that are adjacent to each other but have not coalesced. There are some fat droplets that do appear to have lost the spherical shape of native milk fat globules but there is little evidence of large pools of free coalesced fat within the cheese microstructure after brining at 3°C.

Cheese that had been brined at 22°C was different from that at 3°C in both the extent to which the protein matrix occupied the cheese microstructure and the arrangement of fat (Fig. 5b). Rather than there being a continuous protein matrix (as observed in cheese brined at 3°C), interspersed with the protein matrix (light green) were numerous small and large areas that were relatively devoid of protein (dark green). These areas represent serum droplets and pools, and frequently appear around the fat droplets. Such a change in microstructure as a function of temperature have previously been observed in nonfat cheese (Pastorino et al., 2002). Under cool conditions (10°C) nonfat cheese had a homogeneous protein matrix without any free serum, as we observed for cheese brined at 3°C. In contrast, nonfat cheese at 50°C had large pools of free water that Pastorino et al. (2002) concluded was a result of contraction of the protein matrix under the influence of increased protein-protein hydrophobic interactions at the higher temperature, and an internal micro-syneresis of serum from within the protein matrix.

These microstructural changes induced by a change in temperature, do not always result in an observable macroscopic change, such as the changes in weight or volume that occurred in this experiment. For nonfat cheese, a change in opacity can be observed with the cheese being translucent at cold temperatures and becoming opaque as it is heated (Paulson et al., 1998). This change in opacity is less apparent in full fat cheeses because the fat globules are

the major light scattering centers. A block of cheese that is vacuum packaged, however, cannot increase in weight, although a loss of weight can occur if there is any syneresis during storage. McMahon et al. (1999) have shown that during cold storage of mozzarella cheese, the water contained in serum pockets in the cheese after manufacture is absorbed into the protein matrix. Concomitantly, the protein matrix expands and eventually occupies all the space except for that occupied by the fat globules. This corresponds to the microstructure observed in feta cheese when brined at 3°C (Fig. 5a).

As well as the contracted appearance of the protein matrix after brining at 22°C, the fat also appeared less evenly dispersed. Some fat was present as individual droplets dispersed throughout the protein matrix, but there were also larger pools of coalesced fat droplets. These seemed associated with the serum pockets, and possibly occur as the protein matrix contracts away from the fat allowing any fat droplets with mechanically-damaged fat globule membranes to coalesce.

## CONCLUSIONS

During brining of feta cheese there is a change in cheese weight that is influenced by temperature, salt concentration of the brine, and initial cheese composition. Absorption of salt from brine accounted for some of the weight change, but this can be overshadowed by changes in cheese volume. All cheeses absorbed salt, and reached a moisture S/M level based on the amount of salt in the brine and the total moisture present in the brine and cheese, which for equal starting weights of cheese and brine was 4.6, 5.7 and 6.7 g/100 g for the 6.5, 8.0 and 9.5% brines, respectively. Cheeses brined at 3°C increased in moisture content whereas those brined at 22°C decreased in moisture content with intermediate changes occurring at 6 and 10°C.

Fermenting cheese to a lower pH (4.83 versus 4.92) before brining partially prevented moisture uptake by the cheese such that when brined at 6°C, the cheeses had mean changes in moisture content of -0.8 and +4.1 g/100g, respectively. This trend would be expected to continue if initial pH of cheese dropped below pH 4.83. Cheese pH decreased during brining based on salt concentration and temperature, with no change occurring at 3°C in 9.5% brine and pH dropping to pH 4.15 + 0.03 in 6.5% brine at 22°C.

Volume changes were mainly dependent on temperature with only slight increases in cheese volume as brine concentration increased. At 3°C, the cheeses expanded with increases in volume of 11 to 28% and had a cheese microstructure consisting of only a well hydrated protein matrix containing dispersed fat globules. In contrast, when brine at 22°C, the cheese shrunk from 1 to 17% as a consequence of a contraction of the protein matrix presumably because of increased protein-protein hydrophobic associations. Such cheeses had numerous serum pockets interrupting the protein matrix and increased presence of coalesced fat droplets.

These changes in cheese during brining are only partially reversible when temperature is changed. In general, the cheeses will expand when the temperature is lowered, but initial exposure to 22°C for 10 d, inhibited expansion of the protein matrix when temperature was subsequently lowered to 3°C and the cheeses remain below their original weight and volume. Thus, temperature history as well as cheese pH before brining, brining temperature, and brine salt concentration all impact yield of feta cheese obtained after brining.

## ACKNOWLEDGMENTS

The authors thank Nidhi Gharg for performing statistical analysis. This research was supported by the Utah Agricultural Experiment Station, Utah State University, and approved as journal paper No. 8028.

## REFERENCES

- Abd El-Salam, M. H., and E. Alichanidis. 2004. Cheese varieties ripened in brine. Pages 227-240 in *Cheese Chemistry, Physics and Microbiology*, Vol. 2., P F. Fox, P. L. H. McSweeney, T. M. Cogan and T. P. Guinee, eds, Elsevier Academic Press, London.
- Alichanidis, E., A. Polychroniadou, N. Tzanetakis, and A. Vafopoulou. 1981. Teleme cheese from deep-frozen curd. *J. Dairy Sci.* 64:732-739.
- Anifantakis, E. M., and G. Moatsou. 2006. Feta and other Balkan cheeses. Pages 43-76 in *Brined Cheeses*, A. Tamime, ed., Blackwell Publishing, Oxford.

- Anonymous. 2002. European Union Commission Regulation No. 1829-Amending to the Annex to Regulation (EC) No. 1107/96 with regard to the name "Feta". Official J. Eur. Communities, L277:10-14.
- Anonymous. 2009. Chr. Hansen launches two new culture blends for feta cheese. Chr. Hansen. A/S. [http://www.chr-hansen.com/press/news/show\\_news/chr-hansen-launches-two-new-culture-blends-for-feta-cheese.html](http://www.chr-hansen.com/press/news/show_news/chr-hansen-launches-two-new-culture-blends-for-feta-cheese.html). Accessed Jan 12, 2009.
- Bines, V., and D. Holmes. 1994. Brine salting of cheese. *Dairy Industries Int.* 59(6):33-38.
- Bintsis, T. 2006. Quality of Brine. Pages 264-301 in *Brined Cheeses*, A. Tamime, ed., Blackwell Publishing, Oxford..
- Geurts, T. J., P. Walstra, and H. Mulder. 1972. Brine composition and the prevention of the defect 'soft rind' in cheese. *Neth. Milk Dairy J.* 26:168-179.
- Guinee, T. P. 2004. Salting and the role of salt in cheese. *Int. J. Dairy Technol.* 57:99-109.
- Guinee, T. P., and P. F. Fox. 2004. Salt in cheese: physical, chemical and biological aspects. Pages 207-259 in *Cheese Chemistry, Physics and Microbiology*, Vol. 1. General Aspects, 3rd ed., P. F. Fox, P. L. H. McSweeney, T. M. Cogan, and T. P. Guinee, eds., Elsevier Academic Press, Oxford.
- Kimber, A. M., B. E. Brooker, D. G. Hobbs, and J. H. Prentice. 1975. Electron microscope studies of the development of structure in Cheddar cheese. *J. Dairy Res.* 41:389-396.
- McMahon, D. J., R.L. Fife, and C. J. Oberg. 1999. Water partitioning in mozzarella cheese and its relationship to cheese meltability. *J. Dairy Sci.* 82:1361-1369
- McManus, W. R., K.M. Larsen and D. J. McMahon. 2009. Identification of starch in milk protein gels using confocal microscopy. *Microscopy Today*, 17(3) In press.
- Melilli, C., D. M. Barbano, G. Licitra, G. Portelli, G. Di Rosa, and S. Carpino. 2003. Influence of the temperature of salt brine on salt uptake by Ragusano cheese. *J. Dairy Sci.* 86:2799-2812
- Metzger, L. E., D. M. Barbano, M. A. Rudan, P. S. Kindstedt, and M. R. Guo. 2000. Whiteness change during heating and cooling of Mozzarella cheese. *J. Dairy Sci.* 83:1-10.
- Oberg, C. J., W. McManus, and D. J. McMahon. 1993. Microstructure of Mozzarella cheese during manufacture. *Food Structure* 12:251-258.
- Pappas, C. P., E. Kondyli, L. P. Voutsinas, and H. Mallatou. 1996. Effects of salting method and storage time on the composition and quality of Feta cheese. *J. Soc. Dairy Technol.* 49:113-118.
- Paquet, A., and M. Kalab. 1988. Amino acid composition and structure of cheese baked as a pizza ingredient in conventional and microwave ovens. *Food Microstructure* 7:93-103.
- Pastorino, A. J., R. I. Dave, C. J. Oberg, and D. J. McMahon. 2002. Temperature effect on structure-opacity relationships of nonfat mozzarella cheese. *J. Dairy Sci.* 85:2106-2113.
- Pastorino, A. J., C. L. Hansen, and D. J. McMahon. 2003. Effect of pH on the chemical composition and structure-function relationships of cheese. *J. Dairy Sci.* 86:2751-2760
- Paulson, B. M., D. J. McMahon and C. J. Oberg. 1998. Influence of salt on appearance, functionality, and protein arrangements in nonfat mozzarella cheese. *J. Dairy Sci.* 81:2053-2064
- Prasad, IN., and V. B. Alvarez. 1999. Effect of salt and chymosin on the physicochemical properties of Feta cheese during ripening. *J. Dairy Sci.* 82:1061-1067.
- Richardson, G. H., ed. 1985. Page 351 in *Standard Methods for the Examination of Dairy Products*. 15th ed. Am. Publ. Health Assoc., Washington, DC.
- Tamime, A. Y., D. G. Dalglish, and W. Banks. 1996. Introduction. Pages 11-48 in *Feta and Related Cheeses*, R. K. Robinson and A. Y Tamime, eds, Woodhead Publishing Limited, Cambridge.
- Turhan, M., and G. Kaletunç. 1992. Modeling of salt diffusion in white cheese during long-term ripening. *J. Food Sci.* 57:1082-1085.

## FIGURES

Figure 1. Percent change in cheese weight ( $\pm$ SEM) after brining feta cheese in 6.5 (●), 8.0 (■), and 9.5% (▲) salt brine solutions for either 10 d at 3°C, 10 d at 3°C plus 10 d at 22°C, or 10 d at 3°C plus 10 d at 22°C then another 10 d at 3°C.

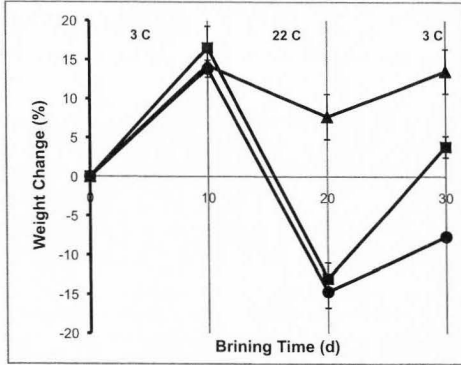


Figure 2. Percent change in cheese volume ( $\pm$ SEM) after brining feta cheese in 6.5 (○), 8.0 (□), and 9.5% (△) salt brine solutions for either 10 d at 3°C, 10 d at 3°C plus 10 d at 22°C, or 10 d at 3°C plus 10 d at 22°C then another 10 d at 3°C.

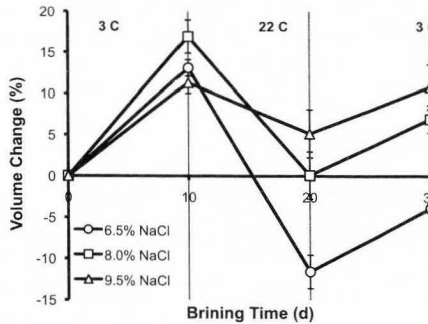


Figure 3. Percent change in cheese weight ( $\pm$ SEM) after brining feta cheese in 6.5 (●), 8.0 (■), and 9.5% (▲) salt brine solutions for either 10 d at 22°C, 10 d at 22°C plus 10 d at 3°C, or 10 d at 22°C plus 10 d at 3°C then another 10 d at 22°C.

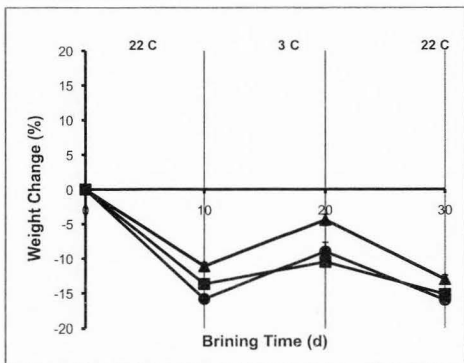


Figure 4. Percent change in cheese volume ( $\pm$ SEM) after brining feta cheese in 6.5 (○), 8.0 (□), and 9.5% (△) salt brine solutions for either 10 d at 22°C, 10 d at 22°C plus 10 d at 3°C, or 10 d at 22°C plus 10 d at 3°C then another 10 d at 22°C.

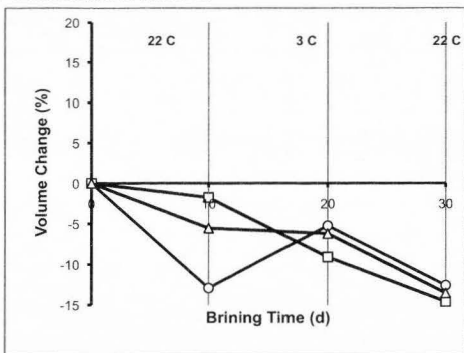
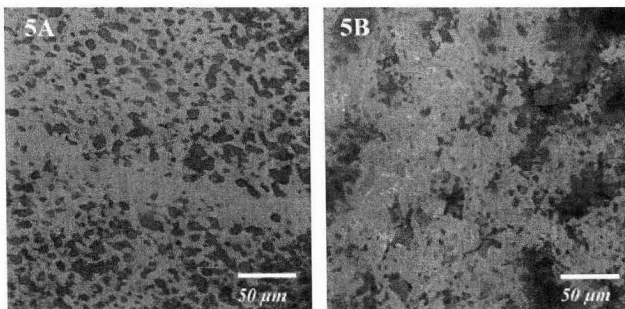


Figure 5. Microstructural images obtained using laser scanning confocal microscopy with fluorescent staining of protein (light green) by fluorescein isothiocyanate and fat (orange) with Nile Red for feta cheese after 10 d of storage in 8.0% salt brine at 3°C (A) or 22°C (B).



**Table 1.** Mean pH, moisture and salt content of feta cheeses after 10 d brining in 6.5, 8.0, and 9.5% (wt./wt.) salt brine at 3, 6, 10, and 22°C

Cheese	Brine Temperature (°C)	Brine Concentration (%)	Moisture (g/100 g)	Salt (g/100 g)	S/M <sup>1</sup> (g/100 g)	pH
Cheese fermented overnight at 20°C 50.8 g/100 g moisture pH 4.92	3	6.5	58.3 <sup>a</sup>	2.74 <sup>ef</sup>	4.48 <sup>f</sup>	4.83 <sup>bc</sup>
		8.0	57.3 <sup>ab</sup>	3.36 <sup>f</sup>	5.54 <sup>de</sup>	4.90 <sup>a</sup>
		9.5	56.0 <sup>bc</sup>	3.79 <sup>a</sup>	6.35 <sup>b</sup>	4.92 <sup>a</sup>
	6	6.5	54.3 <sup>d</sup>	2.61 <sup>fg</sup>	4.58 <sup>f</sup>	4.72 <sup>de</sup>
		8.0	56.0 <sup>bc</sup>	3.23 <sup>f</sup>	5.46 <sup>de</sup>	4.82 <sup>bc</sup>
		9.5	54.3 <sup>d</sup>	3.75 <sup>a</sup>	6.47 <sup>ab</sup>	4.85 <sup>ab</sup>
	10	6.5	48.0 <sup>e</sup>	2.32 <sup>ij</sup>	4.61 <sup>f</sup>	4.57 <sup>h</sup>
		8.0	52.0 <sup>c</sup>	2.99 <sup>g</sup>	5.43 <sup>e</sup>	4.72 <sup>de</sup>
		9.5	52.0 <sup>c</sup>	3.59 <sup>ab</sup>	6.46 <sup>ab</sup>	4.81 <sup>bc</sup>
	22	6.5	40.2 <sup>h</sup>	1.97 <sup>j</sup>	4.53 <sup>f</sup>	4.17 <sup>i</sup>
		8.0	40.4 <sup>h</sup>	2.41 <sup>hi</sup>	5.64 <sup>de</sup>	4.29 <sup>k</sup>
		9.5	42.3 <sup>g</sup>	2.97 <sup>g</sup>	6.57 <sup>ab</sup>	4.39 <sup>ij</sup>
Cheese fermented overnight at 31°C 48.9 g/100 g moisture pH 4.83	3	6.5	50.3 <sup>f</sup>	2.47 <sup>gh</sup>	4.68 <sup>f</sup>	4.70 <sup>de</sup>
		8.0	50.0 <sup>f</sup>	3.02 <sup>d</sup>	5.69 <sup>cd</sup>	4.77 <sup>cd</sup>
		9.5	49.2 <sup>fg</sup>	3.43 <sup>bc</sup>	6.52 <sup>ab</sup>	4.83 <sup>bc</sup>
	6	6.5	45.5 <sup>h</sup>	2.20 <sup>jk</sup>	4.61 <sup>f</sup>	4.52 <sup>h</sup>
		8.0	49.0 <sup>fg</sup>	2.91 <sup>de</sup>	5.60 <sup>de</sup>	4.70 <sup>de</sup>
		9.5	49.7 <sup>f</sup>	3.58 <sup>ab</sup>	6.72 <sup>a</sup>	4.79 <sup>bc</sup>
	10	6.5	43.5 <sup>i</sup>	2.12 <sup>kl</sup>	4.65 <sup>f</sup>	4.44 <sup>i</sup>
		8.0	45.3 <sup>h</sup>	2.74 <sup>f</sup>	5.70 <sup>cd</sup>	4.54 <sup>h</sup>
		9.5	50.0 <sup>f</sup>	3.42 <sup>bc</sup>	6.39 <sup>b</sup>	4.65 <sup>g</sup>
	22	6.5	41.6 <sup>j</sup>	1.97 <sup>j</sup>	4.70 <sup>f</sup>	4.12 <sup>j</sup>
		8.0	39.3 <sup>k</sup>	2.40 <sup>hi</sup>	5.76 <sup>c</sup>	4.26 <sup>k</sup>
		9.5	39.4 <sup>k</sup>	2.50 <sup>hi</sup>	5.73 <sup>c</sup>	4.34 <sup>k</sup>

<sup>abcdeghijkl</sup> Means within a column with a common letter were not significantly different ( $\alpha = 0.05$ ).

<sup>1</sup> Salt-in-moisture = salt/(salt + moisture) x 100.

Table 2. Change in cheese weight and volume after 10 d brining of 100 g of cheese in 100 g of 6.5, 8.0, or 9.5% (wt./wt.) salt brine at 3, 6, 10, and 22°C

Brining Conditions		pH 4.92-Cheese		pH 4.83-Cheese	
Salt (%)	Temperature (°C)	Weight Change (g/100 g)	Volume change (%)	Weight Change (g/100 g)	Volume change (%)
6.5	3	+23.6 <sup>ab</sup>	+23.6 <sup>ab</sup>	+13.8 <sup>bcd</sup>	+12.8 <sup>ef</sup>
	6	+14.0 <sup>bcd</sup>	+17.4 <sup>bcd</sup>	+4.2 <sup>def</sup>	+4.0 <sup>efghi</sup>
	10	+0.7 <sup>ef</sup>	+1.1 <sup>ghij</sup>	-0.9 <sup>f</sup>	-1.2 <sup>ijk</sup>
	22	-17.7 <sup>g</sup>	-16.7 <sup>l</sup>	-15.8 <sup>g</sup>	-1.2 <sup>ijk</sup>
8.0	3	+29.7 <sup>a</sup>	+28.0 <sup>a</sup>	+16.5 <sup>abc</sup>	+16.5 <sup>bcd</sup>
	6	+23.8 <sup>ab</sup>	+20.5 <sup>abcd</sup>	+10.4 <sup>cdef</sup>	+9.0 <sup>efgh</sup>
	10	+13.3 <sup>bcd</sup>	+11.6 <sup>def</sup>	+3.9 <sup>def</sup>	+3.1 <sup>ghij</sup>
	22	-14.1 <sup>g</sup>	-13.5 <sup>kl</sup>	-13.7 <sup>g</sup>	-1.9 <sup>hijk</sup>
9.5	3	+21.9 <sup>abc</sup>	+20.1 <sup>abc</sup>	+14.3 <sup>bcd</sup>	+11.1 <sup>cde</sup>
	6	+19.0 <sup>abc</sup>	+15.8 <sup>bcd</sup>	+14.4 <sup>bcd</sup>	+11.6 <sup>bcd</sup>
	10	+13.5 <sup>bcd</sup>	+8.3 <sup>efg</sup>	+11.4 <sup>bcd</sup>	+9.0 <sup>ef</sup>
	22	-12.8 <sup>g</sup>	-12.9 <sup>kl</sup>	-11.1 <sup>g</sup>	-5.6 <sup>ijk</sup>

<sup>abcde fghijk</sup> Means within weight or volume columns with a common letter were not significantly different ( $\alpha = 0.05$ ).

# Fortification of Reduced-Fat Cheddar Cheese with $\omega$ -3 Fatty Acids: Effect on Off-Flavor Generation

**SILWANA MARTINI:** Western Dairy Center, Utah State University, Nutrition and Food Sciences Department  
J.E., T.Thurgood:

**CARRL BROTHERSEN:** Western Dairy Center Associate Director, Nutrition and Food Sciences, Utah State University

**ROBERT WARD:** Utah State University, Nutrition and Food Sciences, Assistant Professor

**DONALD J. MCMAHON:** Utah State University, Nutrition and Food Sciences Department, Professor

## ABSTRACT

The objective of this study was to fortify 50% reduced fat Cheddar cheese with  $\omega$ -3 fatty acids and evaluate whether this fortification generated specific off-flavors in the cheese. Docosahexaenoic (DHA) and eicosapentaenoic (EPA) fatty acids were added to the cheese to obtain three final fortification levels (18, 35 and 71 mg of DHA/EPA per serving size [28g] of cheese) representing 10, 20 and 40% of the suggested daily intake level for DHA/EPA. The presence of oxidized, rancid and fishy flavors as a function of fortification level and cheese aging (6 mo) was evaluated using a sensory descriptive panel. No differences ( $p > 0.05$ ) were found in the oxidized and rancid flavors as a consequence of DHA/EPA fortification with only slight intensities of these flavors. The presence of fishy off-flavor was dependent on the fortification level. Cheeses with low fortification levels (18 and 35 mg of DHA/EPA per serving size) did not develop significant ( $p > 0.05$ ) fishy off-flavor when compared to the control, while in the highest fortification level (71 mg of DHA/EPA per serving size) the fishy off-flavor was significantly stronger ( $p < 0.05$ ) in young cheeses. The fishy flavor decreased as a function of age and became non-significant from the control at 3 mo of storage. Even though fishy flavors were detected in the fortified cheeses, the DHA/EPA content during storage remained constant and complied with the suggested values for food fortification. Results obtained from this research indicate that 50% reduced fat Cheddar cheese aged for 3 mo can be used as a vehicle for  $\omega$ -3 fatty acids delivery without off-flavors generation.

**Key words:**  $\omega$ -3 fatty acid, DHA, EPA, reduced fat cheddar cheese, sensory evaluation

## INTRODUCTION

Lipids are vital components of our diet; they are a source of energy and essential fatty acids. Some  $\omega$ -6 fatty acids such as linoleic and  $\gamma$ -linolenic are essential fatty acids that need to be incorporated into the diet as they cannot be synthesized by the body. The adequate intake recommended by United States Department of Agriculture (USDA) for  $\omega$ -3 and  $\omega$ -6 fatty acids is estimated at 1.0-1.6 g/d and 12-17 g/d, respectively (USDA, 2005). Docosahexaenoic (DHA) and eicosapentaenoic fatty acid (EPA) are  $\omega$ -3 fatty acids that show beneficial health effects (Williams, 2000). The average intake of DHA and EPA fatty acids in western societies in 2003-2004 was approximately 0.09 and 0.14 g/day for females and males, respectively (USDA, 2007) showing a marginal intake in the consumption of these fatty acids. DHA and EPA consumption has been associated with several health benefits. These fatty acids can help reduce the risk of coronary heart disease and reduce cancer; they also have anti-inflammatory activity and proper neural activity (Kris-Etherton et al., 2003; Ruxton et al., 2004; Williams, 2000; Rose and Connolly, 1999; Sperling et al., 1993). Typically, DHA and EPA fatty acids are contained in oily fish, such as salmon, lake trout, tuna and herring. To meet the adequate intake recommended by USDA, oral supplements of  $\omega$ -3 fatty acids are available to consumers. Typically, oral supplements for adults contain 300 mg EPA, 200 mg DHA and 100 mg other  $\omega$ -3 fatty



acids; while supplements for children contain 100 mg DHA. An additional alternative to increase the consumption of  $\omega$ -3 fatty acids is to fortify foods with DHA and EPA. Several foods have been supplemented with DHA/EPA, from confectionery to dairy products (Avramis et al., 2003; Kolanowski and Weifbrodt, 2007), including sausages, margarine (Scollan et al., 2006), milk (Lock and Bauman, 2004), margarine spreads (Metcalf et al., 2003; Kolanowski et al. 2004), cookies (Borneo et al., 2007) and bread (Murphy et al., 2007). Fortification of cheese has also been performed using either DHA/EPA fortified milk or by directly incorporating the DHA/EPA during cheesemaking. Avramis et al. (2003) made Cheddar cheese using milk from cows fed with fish meal and observed no sensory impact in the cheeses; however levels of DHA/EPA in the cheese were not measured. Aryana (2007) substituted 100, 50, 25 and 0% of the fat in cheese with OmegaPure™, a commercial oil rich in  $\omega$ -3 fatty acids, and did find a significant difference between the flavors of  $\omega$ -3 fatty acids-fortified cheeses when compared to the control (0% DHA).

DHA and EPA are polyunsaturated fatty acids. These highly unsaturated fatty acids tend to oxidize, generating typical oxidized, rancid and/or fishy off-flavors. When incorporating  $\omega$ -3 fatty acids in foods it is vital to achieve the desired concentration in the food product without originating the typical off-flavors associated with polyunsaturated fatty acids. Therefore, the sensory quality of foods supplemented with DHA/EPA needs to be closely evaluated to ensure good consumer acceptance.

The objective of this research was to manufacture 50% reduced-fat Cheddar cheese with increased levels of DHA and EPA using an encapsulated fish oil powder and evaluate the presence of oxidized, rancid and fishy flavors in the fortified cheeses using a sensory descriptive panel. The levels of fortification used were 18, 35 and 71 mg of DHA/EPA per serving size (28 g), which represent approximately 10, 20 and 40% of the suggested daily intake level for DHA/EPA. The oxidized, rancid and fishy flavor intensities as a function of DHA/EPA fortification level and cheese age (6 mo) were evaluated by a sensory descriptive panel.

## MATERIALS AND METHODS

### *Cheese Manufacturing Method*

Reduced fat cheddar cheese curd was made from 227 kg of pasteurized (73°C, 15 s) milk (Gary Haight Richardson Dairy Products Laboratory at Utah State University) standardized to a protein to fat ratio of 1.9. Sixty grams of *Lactococcus lactis* starter culture (DVS850, Chr. Hansen Laboratories, Milwaukee, WI) was added to the milk at 31°C, ripened for 40 min and then set using 20 ml double strength chymosin (Maxiren, DSM Food Specialties, Menomonee Falls, WI). The curd was cut using 16-mm curd knives in the vertical direction only, heated and then cooked to 36°C over 15 min. Whey was drained when curd pH reached 6.20 (set-to-drain time ~90 min) and curd was washed by adding enough water (at 18°C) to lower the curd temperature to 29°C. After 10 min, the wash water was drained and the curd stirred until pH reached 5.9 and then divided into portions to manufacture the fortified and control cheeses.

### *DHA/EPA Source*

DHA/EPA samples were provided by Ocean Nutrition (Dartmouth, N.S., Canada). The material consisted of microencapsulated fish oil. In this product, the fish oil is encapsulated using the Powder-loc microencapsulation technology resulting in a powder of approximately 60  $\mu$ m of diameter. The certificate of analysis of the powder indicates that 95.3% passes through a 140 mesh sieve while 99% of the powder passes through a 100 mesh. For more detail on the product characteristics, please refer to: <http://www.ocean-nutrition.com/Products/Fl/finmeg3real.php#Tech>

### *$\omega$ -3 Fatty Acids Addition*

Cheese fortification was performed by grinding the curd with the DHA/EPA encapsulated material. To obtain a fortification level of 18, 35 and 71 mg of DHA/EPA per serving size (28 g), 3 portions 1.4 Kg of ground salted curd obtained as described below (ground control), were blended with 4.8, 9.6, or 19.2 g of encapsulated DHA/EPA powder (MEG-3, Ocean Nutrition, Dartmouth, N.S., Canada) in a bowl mixer (KitchenAid, model KSM5, St. Joseph, MI) and pressed overnight. The encapsulated DHA/EPA material contained 179 mg of DHA/EPA per gram. All cheeses (controls and fortified) were then vacuum packaged, and stored at 8°C. Cheeses were made in triplicate over a 3-week period. Sensory evaluation was performed at 1, 7 d, 1, 3 and 6 mo of age.

## Controls

**Ground Control.** A ground control cheese (without the addition of DHA/EPA) was obtained by grinding 13.6 kg of fresh, unsalted cheese curd with 340 g of salt for 30 seconds (Hobart, model VCM 25). A 2.7 kg portion of this salted curd was packed into round hoops and pressed overnight at 20.7 kPa. The rest of the curd was used to manufacture the fortified cheeses as described above ( $\omega$ - fatty acids addition).

**Non-ground Control.** To corroborate that the grinding process did not incorporate off-flavors in the cheese, a non-ground control cheese was also included in the experimental design. This control was obtained by adding salt (68 g) to the curd (2.7 kg) in 3 applications, 5 minutes apart and then pressed in a round plastic hoop overnight at 20.7 kPa. All control cheeses were made in triplicate over a 3-week period

## Proximate Analysis

The pH, moisture, fat, and salt content were determined for all cheeses at 5 days of age. Cheese pH was determined by combining 20 g finely-grated cheese with 10 g water in a plastic bag and stomaching (Stomacher model 400, Seward, city state) at 260 rpm for 1 min. The pH of this slurry was measured using a Xerolyt combination electrode (model HA405, Mettler Toledo, Columbus, OH) and an Accumet pH meter (model AR 25, Fisher Scientific, Pittsburgh, PA). Moisture content was measured in triplicate using a CEM microwave oven (CEM Corp., Indian trail, NC). Fat content was determined using the Babcock method (method 15.8.A; [American Public Health Association, 1992](#)). Salt content was measured by combining 5 grams of finely grated cheese with 98.2 g water and mixing for 4 min at 260 rpm in a Stomacher 400 (Seward, England). The slurry was filtered through a Whatman #1 filter paper, and the filtrate was analyzed for sodium chloride using a chloride analyzer (model 926, Corning, Medfield, MA).

## Sensory Evaluation

The presence of oxidized, rancid, and fishy flavors in fortified and control cheeses were evaluated using a descriptive panel. Thirteen panelists, selected according to their availability and interest in participating in this study, received an initial 13 h of training, where they were taught to correctly identify and quantify oxidized, rancid and fishy flavors. During the tasting period (180 d), panel maintenance consisted of 1 training session before each tasting session. The panel consisted of 7 women and 6 men, between 24 and 60 years old. The training and tests were performed in the Sensory Evaluation Facilities in the Department of Nutrition and Food Sciences at Utah State University. These facilities consist on a kitchen where cheese samples were cut, placed in 2 oz. cups, covered with a lid and left at room temperature until tasting. Samples were always served at room temperature. Trainings were performed in a round table in the kitchen, while tasting was performed in individual booths under fluorescent light.

For the training and maintenance sessions, reference cheeses were prepared to represent the attributes of interest. Reference cheeses were prepared using 1.4 kg of freshly prepared cheese curd which was ground with salt using the method listed above (Ground Control section). Two different levels of oxidized flavor in the cheese were obtained by thoroughly mixing (KitchenAid model KSM5, St. Joseph, MI) 5 or 12 mL of a 1% solution of  $\text{CuSO}_4$  to the ground curd (Shipe et al. 1978). Cheeses were aged for at least 2 weeks to allow flavor development before tasting. Rancid flavor references were prepared by thoroughly mixing 60 or 120 g Feta cheese (USU Dairy Products Laboratory, Logan, UT) to the 1.4 kg of freshly made cheese curd. Finally, three levels of fishy flavored cheese was prepared by thoroughly mixing 4.8, 9.6, or 9.2 g of encapsulated DHA/EPA powder (MEG-3, Ocean Nutrition, Dartmouth, N.S., Canada) (which on its own has an intense fishy flavor attribute) in 1.4 kg of the freshly made cheese curd.

During training and tasting sessions, panelists were asked to take a bite of the sample, keep it in their mouth for a couple of seconds, expectorate, and evaluate the intensity of oxidized, rancid, and fishy flavor using a 5-point category scale with the following categories: no flavor, slight flavor, moderate flavor, strong flavor, and extremely strong flavor. For statistical analysis, numerical scores of 0 = no flavor, 1 = slight flavor, 2 = moderate flavor and 4 = extremely strong flavor were assigned to the category scale. Panelists were instructed to follow good sensory practices as described by Meilgaard et al. (2007). Unsalted crackers and water were provided to rinse the palate between samples.

Of the original 13 panelists that volunteered to participate in the study, the attribute ratings from 2 of the judges were discarded based on poor attendance. Additionally, as some panelists demonstrated inability to correctly identify and accurately rate 1 or more of the flavor attributes being studied, they were removed from the analyses of

such. Therefore, the number of panelists included in the statistical analyses of the oxidized, rancid, and fishy flavor attribute ratings, respectively, were 10, 9, and 9.

Prior to training and tasting, approval from the Utah State University Institutional Review Board was obtained to use human subjects in the panel. Sensory evaluation of the experimental cheeses was performed at 1 (same day as cheese was removed from the press), 7 d, 1, 3 and 6 mo after manufacture.

### ***Fatty acid Composition***

Fatty acids were measured in the cheese samples quantitatively as methyl ester derivatives by the method of Ocean Nutrition (Curtis et al. 2008). According to the method, the C19:0 synthetic triglyceride, trionadecanoin, was used as a surrogate spike to calculate analyte recovery, and the C23:0 methyl ester, tricosanoic acid, was used as an internal standard. In addition, 3 gas chromatographic reference standards, GLC 409, GLC 461, and GLC 603 were used to identify peaks and to establish instrument response factors for each fatty acid methyl ester (FAME) of interest. All lipid reference materials were purchased from Nu Chek Prep Inc. (Elysian, Minnesota). Response factors are shown in Table 3.

Fatty Acid Methyl Esters (FAME) were analyzed using a Shimadzu QP2010s gas chromatograph/mass spectrometer equipped with an Agilent HP-88 column (100 m × 0.25 mm i.d. × 0.2 μm film thickness) and a flame ionization detector (FID). The injector was operated in split mode (50:1 split ratio), and hydrogen was used as the carrier gas at a fixed linear velocity of 41.1 cm/s. The injector temperature was maintained at 250°C. The following temperature program was used: initial temperature 50°C, hold 1 min, ramp at 40°C/min to 175°C and hold for 4 minutes, ramp at 3.5°C to 250°C and hold for 3 minutes. The total program time was 32.55 minutes. The FID was operated using the following parameters: 250°C temperature, nitrogen makeup flow at 30 ml/min, air at 450 ml/min and hydrogen at 40 ml/min.

### ***Statistical Analysis***

For statistical analysis, numerical scores of 0 = no flavor, 1 = slight flavor, 2 = moderate flavor and 4 = extremely strong flavor were assigned to the category scale. Cheeses were made in triplicate and a repeated measures design using an autoregressive correlation structure was used to analyze the effect of ω-3 fatty addition and age on the intensity of oxidized, rancid, and fishy flavors. Samples were presented to panelists in a randomized complete block design. Sensory data was collected using SIMS 2000 and statistical analysis was performed using SAS 9.1.3 (SAS Institute, Cary, NC).

## **RESULTS AND DISCUSSION**

### ***Proximate Analysis***

No differences ( $p > 0.05$ ) were found between the proximate analysis of the controls (non-ground and ground) and the ω-3 fortified cheeses (different fortification levels). The non-ground cheese was slightly lower in moisture content than the ground cheeses (data not shown). Table 1 summarizes the average values and standard errors obtained for the controls and the fortified cheeses. Standard of identity for full fat Cheddar cheese indicate that these types of product should contain approximately 37% of moisture and 33% of fat. As expected for 50% reduced fat Cheddar cheeses, moisture, fat, salt and pH values were approximately 47%, 16%, 1.8% and 5.2, respectively.

### ***Sensory Evaluation***

No differences ( $p > 0.05$ ) were found between the ground and non-ground controls for any of the attributes examined over the time of the study, with  $p$ -values of 0.72, 0.69 and 0.76 for oxidized, rancid and fishy, respectively. Therefore, only the data for the ground control cheese is discussed from this point forward.

Figures 1-3 show the descriptive panel scoring for oxidized, rancid and fishy attributes, respectively. No differences ( $p > 0.05$ ) were found between the oxidized flavor as a function of DHA/EPA addition and age (Figure 1). Average rating for all samples remained below or equal to 1.0, indicating a very slight level of this flavor. Even though differences were not significant, a slight increase in oxidation scores was observed with aging, especially for the control cheese which score increased from 0.3 to 0.7 for 1 d and 6 mo of age, respectively.

Figure 2 shows the scores for rancid flavor obtained for the fortified and control cheeses. The fortification level did not affect the rancid flavor intensity ( $p > 0.05$ ). However, a significant increase in the rancid flavor was observed as a function of aging ( $p = 0.0007$ ). Sensory scores were slightly higher for rancid than for oxidized with values between 0.5 and 1.0. The increase in rancid flavor notes in cheeses as a function of aging is expected and not necessarily a defect. Figure 3 shows the sensory scores obtained for fishy flavors detected in the fortified and control cheeses. Both fortification level and age significantly ( $p < 0.05$ ) affected the fishy flavors' intensities in the cheeses. The higher the amount of  $\omega$ -3 fatty acids added, the stronger the fishy flavor, especially for short aging times. The fishy notes decreased as a function of aging and no significant differences were found between any of the fortified cheeses and the control at 3 mo of age. Table 2 shows a summary of the differences in fishy flavor detection between the fortification level and aging times. Significance levels between the samples and the average values for fishy flavor (diagonal values) are shown in this table. For clarity, standard errors are omitted in this table. Standard errors values were low and varied from 0 to 0.1. Table 2 shows that no differences ( $p > 0.05$ ) between the control and the cheeses with 18 and 35 mg of DHA/EPA were observed for any aging time. Average and standard error scores for the fishy attribute were  $0.2 \pm 0.1$ ,  $0.3 \pm 0.1$  and  $0.8 \pm 0.2$ , respectively for d 1, and decreased with aging. Differences were found between the control and the cheese fortified with 71 mg of DHA/EPA ( $p < 0.001$ ), with an average score for the fishy flavor of 1.2 for d 1. However, this value was not significantly different from the fishy flavor obtained for the cheese with 35 mg of DHA/EPA ( $p > 0.05$ ), but became significant at 7 d of aging ( $p < 0.005$ ) as a consequence of a decrease in the fishy flavor intensity for the cheese fortified with 35 mg of  $\omega$ -3. As aging time increased, the fishy flavor intensity decreased for all samples, with ratings between 0 and 0.5 for 3 and 6 mo of storage. The greatest decrease in fishy flavor intensity was observed for the highest fortification level (71 mg) going from  $1.2 \pm 0.2$  at 1 d to  $1.0 \pm 0.2$ ,  $0.5 \pm 0.1$  and  $0.3 \pm 0.1$  for 1, 3 and 6 mo respectively. Consequently, differences between the 71 mg fortified cheeses and the control became non-significant at 3 mo of age. It is interesting to note here, that even though a fishy note was detected for the fortified cheeses, the intensity was never rated above 1.2, indicating only a slight intensity of this off-flavor.

#### **DHA/EPA Content**

The encapsulated powder used for  $\omega$ -3 fortification of the cheeses was listed as containing 179 mg of DHA/EPA per gram of powder and the quantities added to the cheese curd was selected to provide 18, 35, and 71 mg DHA/EP/A per 28-g (1-oz) serving of cheese. Fatty acid analysis of the cheeses showed that on average, fortified cheeses contained 0, 18, 35 and 71 mg DHA/EPA per 28 g of cheese for the control and  $\omega$ -3 fortified cheeses, respectively, which is in accordance to the target values and well within the ranges proposed for being 10, 20 and 40% of the recommended daily level for DHA/EPA (Table 3). No differences ( $p > 0.05$ ) were found in the cheeses' DHA/EPA content with aging. Even though the highly fortified cheeses had a fishy flavor at early aging times, which we had assumed to be a consequence of the oxidation of the DHA/EPA fatty acids, the degree of oxidation was not enough to significantly affect the total content of these fatty acids in the cheese. Most importantly, the decrease in the fishy flavor with age was not due to a lack of substrate for oxidation but might be attributed to other causes such as other flavor developments during cheese age, changes in texture, decrease in moisture content, etc.

### **CONCLUSIONS**

Fortification of Cheddar cheeses with low amounts (18 and 32 mg/serving size) of  $\omega$ -3 fatty acids does not produce any off-flavor in the cheese. Higher fortification levels (71 mg/serving size) result in a slight fishy flavor at early stages of age. This off-flavor diminishes as a function of cheese age, with no differences found between the control and the fortified cheeses at 3 mo of aging. Even though the fishy off-flavor decreased during aging, DHA/EP/A remained constant as shown by the GC data indicating that 50% reduced-fat Cheddar cheese can be fortified with  $\omega$ -3 fatty acids, specifically DHA/EPA without affecting the flavor profile of the cheese, if aged for 3 mo or longer. Fishy off-flavors are most likely originated as a consequence of oxidation of the  $\omega$ -3 polyunsaturated fatty acids. This chemical reaction is catalyzed by the presence of oxygen, light and metals. Briefly, the oxidation of lipids is a self-propagating reaction mediated by the generation of free radicals. The reaction ends when free radicals combine and generate non-free-radicals compounds. These compounds are short chain aldehydes, alcohols, and esters that, due to their volatility, impart the characteristic off-flavor of oxidized products (White, 2000; Ganeko et al., 2008; Venkateshwarlu et al., 2004 a, b). The decrease in fishy off-flavor with aging might be due to (a) a reduction in the oxidation rate of the polyunsaturated fatty acids present in the cheese due to the low redox potential

of cheese, (b) the disappearance of fishy off-flavors as a consequence of bacterial metabolism, or (c) the masking effect of flavors developed during cheese ageing. More research is needed to evaluate the factors that control fishy off-flavor generation in reduced fat Cheddar cheeses and to understand its dissipation during storage. Such experiments should include SPME analyses of the cheeses as a function of aging and a complete characterization of the flavor profile of the fortified cheeses.

## ACKNOWLEDGEMENTS

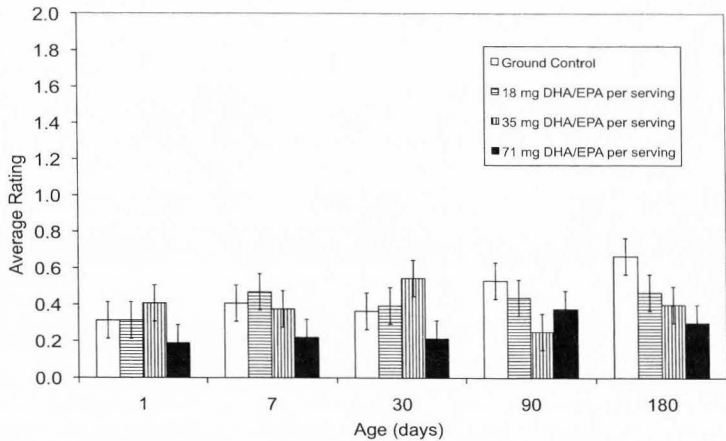
Financial support for this project was provided by DMI Inc., The Western Dairy Center and Utah Agricultural Experiment Station. The authors thank, Mr. Steve Larsen for his technical assistance in cheesemaking, the panelists involved in this study for their commitment and willingness to participate, SIMS2000 personnel for their technical support and advice, Mr. Brian Pette for Proximate Analysis and Mr. Michael Young for helping in the chromatographic analysis. This paper was approved by the Utah Agricultural Experiment Station as paper # 8018.

## REFERENCES

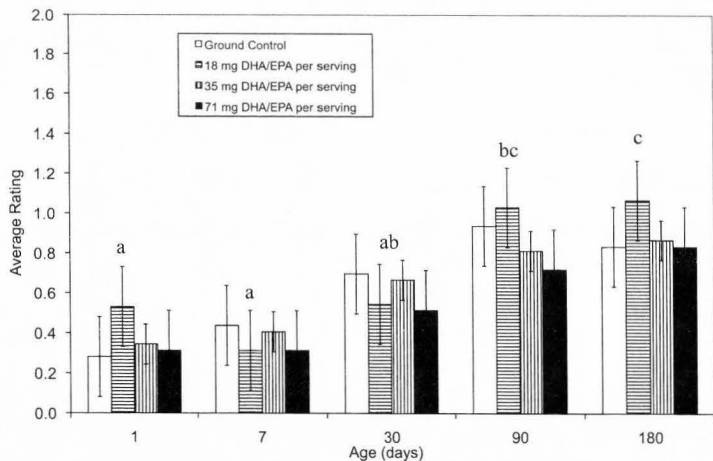
- Agrawal, P., and A.N. Hassan. 2007. Ultrafiltered milk reduces bitterness in reduced-fat Cheddar cheese made with an exopolysaccharide-producing culture. *J. Dairy Sci.* 90:3110-3117
- Agricultural Research Service (ARS) Food Surveys Research Group 2003-2004 [http://www.ars.usda.gov/SP2UserFiles/Place/12355000/pdf/0304/Table\\_1\\_NIF.pdf](http://www.ars.usda.gov/SP2UserFiles/Place/12355000/pdf/0304/Table_1_NIF.pdf)
- American Public Health Association. 1992. *Standard Methods for the Examination of Dairy Products*. 16th ed. R. T. Marshall, ed. Am. Publ. Health Assoc. Inc., Washington, DC.
- Aryana, K. J. 2007. Cheddar cheese manufactured with oil high in omega-3 fatty acids. *Milchwissenschaft-Milch Science International* 62:167-170.
- Association of Official Analytical Chemists. 2000. *Official Methods of Analysis*. 17th ed. AOAC, Gaithersburg MD.
- Avramis, C. A., H. Wang, B. W. McBride, T. C. Wright, and A. R. Hill. 2003. Physical and processing properties of milk, butter, and cheddar cheese from cows fed supplemental fish meal. *J. Dairy Sci.* 86:2568-2576.
- Borneo, R., D. Kocer, G. Ghai, B. J. Tepper, and M. V. Karve. 2007. Stability and consumer acceptance of long chain omega-3 fatty acids (eicosapentanoic acid 20:5, n-3 and docosahexaenoic acid, 22:6, n-3) in cream-filled sandwich cookies. *J. Food Sci.* 72:49-54.
- Chen, Y. C., J. Nguyen, K. Semmens, S. Beamer, and J. Jaczynski. 2006. Enhancement of omega-3 fatty acid content in rainbow trout (*Oncorhynchus mykiss*) filets. *J. Food Sci.* 71:383-389.
- Curtis J. M., N. Berrigan, and P. Dauphinee. 2008. The determination of n-3 fatty acid levels in Food Products containing microencapsulated fish oil using the one-step extraction method. Part 1: measurement in the raw ingredient and in dry powdered foods. *J. Am. Oil Chem. Soc.* 85:297-305.
- Ganeko, N., M. Shoda, I. Hirohara, A. Bhadra, T. Ishida, H. Matsuda, H. Takamura, and T. Matoba. 2008. Analysis of volatile flavor compounds of sardine (*Sardinops melanosticta*) by solid phase microextraction. *J. Food Sci.* 73:S83-S88.
- Kolanowski, W., F. Swiderski, D. Jaworska, and S. Berger. 2004. Stability, sensory quality, texture properties and nutritional value of fish oil-enriched spreadable fat. *J. Sci. Food Agric.* 84:2135-2141.
- Kolanowski, W., and J. Weißbrodt. 2007. Sensory quality of dairy products fortified with fish oil. *International Dairy Journal* 17:1248-1253.
- Kris-Etherton, P. M., W. S. Harris, L. J. Appel, and American Heart Association Nutrition Committee. 2003. Omega-3 fatty acids and cardiovascular disease: New recommendations from the American Heart Association. *Arterioscler. Thromb. Vasc. Biol.* 23:151-152.
- Lock, A. L., and D. E. Bauman. 2004. Modifying milk fat composition of dairy cows to enhance fatty acids beneficial to human health. *Lipids.* 39:1197-1206.
- Metcalfe, R. G., M. J. James, E. Mantziaris, and L. G. Cleland. 2003. A practical approach to increasing intake of n-3 polyunsaturated fatty acids: use of novel foods enriched with n-3 fats. *European Journal of Clinical Nutrition* 57:1605-1612.
- Murphy, K. J., B. J. Meyer, T. A. Mori, V. Burke, J. Mansour, C. S. Patch, L. C. Tapsell, M. Noakes, P. A. Clifton, A. Barden, I. B. Puddey, L. J. Beilin, and P. R. C. Howe. 2007. Impact of foods enriched with n-3 long-chain

- polyunsaturated fatty acids on erythrocyte n-3 levels and cardiovascular risk factors. *British Journal of Nutrition* 97:749-757.
- Rose D. P., and J. M. Connolly. 1999. Omega-3 fatty acids as cancer chemopreventive agents. *Pharmacol. Ther.* 13:214-244.
- Ruxton, C. H. S., S. C. Reed, M. J. A. Simpson, and K. J. Millington. 2004. The health benefits of omega-3 polyunsaturated fatty acids: a review of the evidence. *J. Hum Nutr. Dietet.* 449-459.
- Scollan, N., J. Hocquette, K. Nuernberg, D. Dannenberger, I. Richardson, and A. Moloney. 2006. Innovations in beef production systems that enhance the nutritional and health value of beef lipids and their relationship with meat quality. *Meat Science* 74:17-33.
- Shipe, W.F., R. Bassette, D.D. Deane, W.L. Dunkley, E.G. Hammond, W.J. Harper, D.H. Kleyn, M.E. Morgan, J.H. Nelson, and R.A. Scanlan. 1978. Off flavors of milk: Nomenclature, standards and bibliography. *J. Dairy Sci.* 61:855-869.
- Sperling, R. I., A. I. Benincaso, C. T. Knoell, J. K. Larkin, K. F. Austen, and D. R. Robinson. 1993. Dietary omega-3 polyunsaturated fatty acids inhibit phosphoinositide formation and chemotaxis in neutrophils. *J. Clin. Invest.* 91:651-660.
- USDA. 2005. Dietary Reference Intakes for Energy, Carbohydrate, Fiber, Fat, Fatty Acids, Cholesterol, Protein, and Amino Acids (2002/2005). [http://www.ion.edu/Object\\_File/Master/7/300/Webtablemacro.pdf](http://www.ion.edu/Object_File/Master/7/300/Webtablemacro.pdf)
- USDA. 2007. Nutrients Intakes from Food: Mean amounts Consumed per Individual, One day, 2003-2004. [http://www.ars.usda.gov/SP2UserFiles/Place/12355000/pdf/0304/ Table\\_1\\_NIF.pdf](http://www.ars.usda.gov/SP2UserFiles/Place/12355000/pdf/0304/ Table_1_NIF.pdf)
- Venkateshwarlu, G., M.B. Let, A.S. Meyer, C. Jacobsen. 2004a. Chemical and olfactometric characterization of volatile flavor compounds in a fish oil enriched milk emulsion. *J. Agric. Food Chem.* 52:311-317
- Venkateshwarlu, G., M.B. Let, A.S. Meyer, C. Jacobsen. 2004b. Modelling the sensory impact of defined combinations of volatile lipid oxidation products on fishy and metallic off-flavors. *J. Agric. Food Chem.* 52:1635-1641
- White, F.J. 2000. Flavor quality of fats and oils. In *Introduction to fats and oils technology*. Second Edition. Eds: O'Brien, R., Farr, W.E. and Wan, P.J. AOCS Press, Champaign, IL. pp 341-370.
- William, C. M. 2000. Dietary fatty acids and human health. *Ann. Zootech.* 49:165-180
- Wright, T. C., B. J. Holub, A. R. Hill, and B. W. McBride. 2003. Effect of combinations of fish meal and feather meal on the milk fatty acid content and nitrogen utilization in dairy cows. *J. Dairy Sci.* 86:861-869.

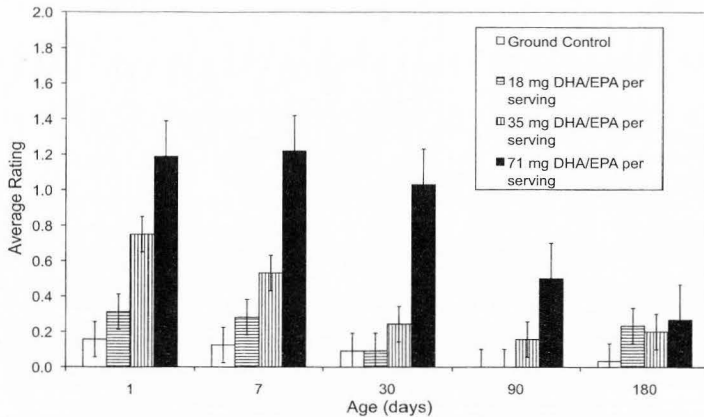
## FIGURES



**Figure 1:** Average sensory panel score for oxidized attribute as a function of level of fortification level and age. Bars represent standard errors. Sensory scores are: 0- no flavor, 1- slight flavor, 2- moderate flavor, 3-strong flavor and, 4- extremely strong flavor.



**Figure 2:** Sensory panel score for rancid attribute as a function of fortification level and age. Bars represent standard errors. Sensory scores are: 0- no flavor, 1- slight flavor, 2- moderate flavor, 3-strong flavor and, 4- extremely strong flavor.



**Figure 3:** Sensory panel score for fishy attribute as a function of fortification level and age. Bars represent standard errors. Sensory scores are: 0- no flavor, 1- slight flavor, 2- moderate flavor, 3-strong flavor and, 4- extremely strong flavor. Significant differences between samples and ageing times are shown in Table 2.

**Table 1:** Proximate analysis (mean values  $\pm$  standard error) for the non-aged control and fortified cheeses. Data presented are averages of 3 replicates.

	Control	O-3
<b>Moisture</b>	47.20 $\pm$ 1.35	48.12 $\pm$ 0.17
<b>Fat</b>	16.17 $\pm$ 0.33	16.17 $\pm$ 0.17
<b>salt</b>	1.83 $\pm$ 0.10	1.98 $\pm$ 0.12
<b>pH</b>	5.20 $\pm$ 0.08	5.27 $\pm$ 0.06



**Table 2:** Significant differences in the fishy flavor for cheeses fortified with  $\omega$ -3 fatty acids and aged for a period of 180 days

AGE (days)	FORTIFICATION	1				7				30				90				180					
		C	18	35	71	C	18	35	71	C	18	35	71	C	18	35	71	C	18	35	71		
1	Control	0.2			**				**				*										
	18 mg DHA/EPA		0.3		*				**				*										
	35 mg DHA/EPA			0.8									*	*	*				*				
	71 mg DHA/EPA	**	*		1.2	**	**	*		**	**	*		**	**	**		**	**	**	**	**	**
7	Control				**	0.1			***				**										
	18 mg DHA/EPA				**		0.3		**				*										
	35 mg DHA/EPA				*			0.5	*														
	71 mg DHA/EPA	**	**			***	**	*	1.2	***	***	**		***	***	**		***	**	**	**	**	**
30	Control				**				***	0.1			**										
	18 mg DHA/EPA			*	***				***		0.1		**										
	35 mg DHA/EPA				*				**			0.2	*										
	71 mg DHA/EPA	*	*			**	*			**	**	*	1.0	***	***	*		***	*	**	*	*	*
90	Control			*	***				***				***	0.0									
	18 mg DHA/EPA			*	***				***				***		0.0								
	35 mg DHA/EPA				**				**				*			0.2							
	71 mg DHA/EPA																0.5						
180	Control			*	***				***				***				0.0						
	18 mg DHA/EPA				**				**				*							0.2			
	35 mg DHA/EPA				**				**				**									0.2	
	71 mg DHA/EPA				**				**				*										0.3

Control (C) = Ground Control, Fortification Level is 16, 32 and 64 mg per 28 g serving of cheese,

Significant Difference of the means \* =  $p < 0.05$ , \*\* =  $p < 0.001$ , \*\*\* =  $p < 0.0001$

**Table3:** Response factors and fatty acid composition of the 50%-reduced Cheddar cheeses fortified with different levels of omega-3 fatty acids. Data reported are milligrams of fatty acids per gram of dried cheese.

Fatty acid	Response Factors	Control		18 mg/serving size		35 mg/serving size		71 mg/serving size	
		AVRG	STD	AVRG	STD	AVRG	STD	AVRG	STD
C4:0	0.50	12.7	0.44	12.4	0.57	12.5	0.48	12.2	0.76
C6:0	0.66	7.14	0.19	6.95	0.29	6.80	0.25	6.61	0.28
C8:0	0.72	4.34	0.09	4.21	0.08	4.18	0.10	4.13	0.15
C10:0	0.82	9.36	0.15	9.03	0.08	9.00	0.12	8.89	0.17
C11:0	0.86	0.23	0.00	0.22	0.00	0.22	0.00	0.22	0.00
C12:0	0.88	10.7	0.17	10.3	0.03	10.3	0.13	10.2	0.17
C13:0	0.90	0.40	0.01	0.39	0.00	0.39	0.01	0.39	0.01
C14:0	0.93	36.3	0.53	35.6	0.43	35.9	0.32	35.9	0.49
C14:1t	0.84	0.63	0.01	0.63	0.01	0.64	0.01	0.64	0.01
C14:1 c	0.89	3.00	0.05	2.92	0.04	2.91	0.06	2.86	0.05
C15:0	0.92	3.83	0.05	3.77	0.04	3.78	0.04	3.77	0.04
C15:1 t	0.86	0.79	0.02	0.78	0.01	0.78	0.02	0.78	0.01
C16:0	1.00	92.8	1.09	91.2	1.86	91.8	1.26	91.5	1.46
C16:1 t	0.88	0.14	0.04	0.14	0.04	0.15	0.06	0.17	0.02
C16:1 c	0.94	4.46	0.04	4.72	0.08	5.02	0.03	5.39	0.09
C17:0	0.96	1.94	0.03	1.90	0.05	1.91	0.04	1.91	0.06
C18:0	1.00	37.6	0.36	36.7	0.74	36.8	0.56	36.4	0.57
C18:1 t9	0.95	1.03	0.07	1.03	0.05	1.05	0.02	1.05	0.05
C18:1 t 11	0.92	3.67	0.18	3.56	0.12	3.67	0.18	3.60	0.11
C18:1 c7	0.86	3.46	0.07	3.32	0.04	3.33	0.10	3.39	0.11
C18:1 c9	1.02	66.1	0.85	64.7	1.51	65.1	1.20	64.4	1.28
C18:1 c 11	0.90	2.02	0.03	2.09	0.03	2.20	0.03	2.34	0.06
C19:1t	0.96	0.23	0.10	0.27	0.14	0.22	0.11	0.16	0.01
C19:1c	0.94	0.48	0.09	0.43	0.02	0.42	0.02	0.41	0.00
C18:2 e9c12	0.74	8.79	0.08	8.61	0.10	8.67	0.08	8.58	0.08
C20:0	0.98	0.48	0.05	0.48	0.06	0.49	0.06	0.49	0.05
C18:3 n6	1.01	0.14	0.05	0.14	0.07	0.15	0.06	0.15	0.06
C18:3 n3	0.96	1.82	0.05	1.81	0.05	1.82	0.04	1.83	0.04
C20:1c11	0.96	0.13	0.09	0.24	0.07	0.28	0.04	0.34	0.05
9-11 CLA	0.97	1.38	0.09	1.36	0.03	1.33	0.03	1.31	0.03
C20:2 n6	1.00	0.13	0.05	0.13	0.06	0.14	0.06	0.14	0.06
C22:0	0.99	0.19	0.06	0.18	0.06	0.19	0.06	0.19	0.06
C20:3 n6	1.02	0.46	0.06	0.46	0.05	0.47	0.05	0.47	0.06
C20:3 n3	1.01	0.06	0.02	0.14	0.03	0.08	0.01	0.14	0.09
C20:4 n6	1.02	0.52	0.01	0.49	0.02	0.47	0.03	0.50	0.13
C20:5 n3	1.02	0.20	0.05	0.90	0.08	1.57	0.06	2.39	0.10
C22:5 n3	1.01	0.24	0.09	0.34	0.10	0.41	0.08	0.48	0.07
C22:6 n3	0.96	0.05	0.04	0.57	0.11	1.09	0.15	1.83	0.33

# Hardening of High Protein Nutrition Bars and Sugar/Polyol-Protein Phase Separation

DONALD J. MCMAHON: Utah State University

S. L. ADAMS: Utah State University

W. R. MCMANUS: Utah State University

## ABSTRACT

Use of hydrolyzed proteins is known to delay hardening of high protein nutrition bars. Bars were formulated using ratios of 0, 25, 50, 75, or 100% partially hydrolyzed whey protein isolate (HWPI) to non-hydrolyzed whey protein isolate (WPI) in one experiment, and either WPI or HWPI combined with high fructose corn syrup (HFCS) or sorbitol syrup (SS) in a second experiment along with vegetable shortening such that initial  $a_w$  was 0.59 for HWPI bars and 0.64 for WPI bars. After mixing, the dough was extruded into bars and stored at 32 °C for accelerated shelf life testing. Hardness, color and microstructure were measured during 42 d of storage. Bars initially had similar hardness of ~3.4 N that increased during storage. Bars with HWPI were softest with hardness at 37 d of 10 to 15 N compared to almost 100 N for bars with WPI. Water activity increased for WPI bars to 0.69 by 34 d. Bars became darker during storage depending on amount of Maillard browning reactants, i.e., HWPI/HFCS bars >> HWPI/SS > WPI/HFCS bars > WPI/SS bars. Bar microstructure at d 2 showed protein and fat dispersed in particulate form throughout the carbohydrate syrup within the bar matrix. During storage, a single non-lipid phase developed in HWPI bars while in WPI bars a phase separation occurred between protein and carbohydrate. We propose such phase separation initiates bar hardening and promotes subsequent protein aggregation. Successful formulation of HPN bars depends on cosolvent properties of the polyol/sugar towards the proteins and their preferential exclusion from the solvation layer surrounding the proteins.

## Practical Application

High protein nutrition bars can be formulated so they remain soft during storage by selecting proteins and sugars that are compatible with each other. Otherwise, the protein and sugar will separate from each other which can then lead hardening.

**Key words:** whey, protein, sugar, phase, separation

## INTRODUCTION

In foods, texture has a significant effect on product acceptance by consumers because texture perception influences overall sensory appreciation (Wilkinson and others 2000). This is especially true in the food bar industry where the hardening of nutrition bars during storage results in consumer avoidance (Stefan 2003). The market for nutrition bars in the United States grew to \$3 billion in 2007 (Wright 2008) although the initial rapid growth in sales of high protein nutrition (HPN) bars leveled out as interest in low-carbohydrate diets dwindled, and because of their higher cost compared to other food bars (Wright 2008).

In general, HPN bars consist mainly of protein, fat, carbohydrates, and water with a few other components including flavors, stabilizers, and inclusions such as peanuts and dried fruit. Whey proteins are often used as the protein source as whey protein isolate (WPI) or a 70 to 80% whey protein concentrate. Soy and other plant protein isolates are also used in HPN bars, often as mixtures with whey proteins. The fat sources most often used are vegetable shortening, cocoa butter or vegetable oil. A blend of high fructose corn syrup (HFCS) and a polyol (glycerol, sorbitol, or maltitol) syrup are common sources of carbohydrate, and there use as a syrup provides the water required to form a dough. Glycerol is used in HPN bars as often as sorbitol although usually at lower levels.

High protein bar components are mixed in approximately 30:30:40 (w/w) ratios of protein, fat and carbohydrate syrup, respectively, resulting in a dough that is soft, malleable, and readily formed into bars, yet firm enough to retain its bar shape for packaging and distribution. The onset of hardening in such HPN bars can begin soon after production (depending on bar formulation) and the bars may become hard and unstable over time resulting in a shelf life of less than 6 months at room temperature (23°C) (Gautam and others 2006). One strategy for keeping HPN bars soft has been to use a mixture of proteins, or to use hydrolyzed proteins (Gottschalk 2006).

Companies have developed products to address the hardening problem based on increasing the hydrophobicity of the proteins or hydrolyzing proteins. It has been suggested that this helps prevent the proteins from absorbing water and causing sugar crystallization (Halliday 2005) or lower their glass transition temperature (Labuza 2008). By including a portion of hydrolyzed protein in the bar formulations, HPN bars have been manufactured that stay soft throughout their 6-month shelf life of about 6 months, even though a mechanism of action for this has not been determined.

It has been suggested that bar hardening occurs because of moisture-induced whey protein aggregation via disulfide crosslinking (Zhou and others 2008a) and non-covalent interactions (Labuza 2008). Hardening is influenced by the polyol used in the bar formulation (Liu and others 2009) as well as the combination of protein and polyol (Li and others 2008). Liu and others (2009) reported that rate of hardening increased as the glass transition temperature of the polyol increased (maltitol > sorbitol > glycerol) with the exception of propylene glycol which interacts hydrophobically with proteins and causes rapid protein aggregation. Proteins that have higher nuclear magnetic resonance relaxation times usually cause less hardening, suggesting that hardening is a function of the amount of water in the bar able to act as a plasticizer (Li and others 2008). Maillard browning may also be involved in hardening when a reducing sugar is used in the bar formulation (Labuza 2008).

This study was carried out with the aim of learning about the mechanism of HPN bar hardening by visualizing differences in microstructure between bars that become hard during storage compared to those that remain soft. This was achieved by using different ratios of WPI and partially hydrolyzed WPI (**HWPI**) in combination with HFCS or sorbitol syrup (**SS**).

## MATERIALS AND METHODS

### *Materials*

Protein powders used were a WPI (Provon 190, 4.5% moisture, 86% protein) and HWPI (BarFlex 191, 5.0% moisture, 86% protein) and were donated by Glanbia Nutritionals, Inc. (Twin Falls, ID). The other HPN bar ingredients were vegetable shortening (Crisco, The J. M. Smucker Co., Orrville, OH) made from soybean oil, fully hydrogenated cottonseed oil, partially hydrogenated cottonseed and soybean oils, and mono and diglycerides; and HFCS (Cornsweet 55, 23% moisture, 42% fructose, 32% glucose, 3% other saccharides) and SS (30% moisture, 70% sorbitol) from Archer Daniels Midland Company (Decatur, IL). Storage of the HPN bars was in moisture-barrier foil-lined Mylar pouches (Sorbent Systems, Los Angeles, CA). Fluorescent stains for laser scanning confocal microscopy (**LSCM**) were Nile Red and fluorescein isothiocyanate (**FTIC**) and were obtained from (Sigma-Aldrich, Inc., Saint Louis, MO).

### *Bar Manufacture and Storage*

Bars were made in 1200-g batches with each batch starting with 456 g of protein powder in a 5-L mixing bowl. In one experiment the WPI and HWPI powders were mixed to prepare protein powder blends containing 0, 25, 50, 75, and 100% HWPI. In the second experiment, the protein powder was either 100% WPI or 100% HWPI. Then 512 g of saccharide/polyol syrup (HFCS in the first experiment, HFCS or SS in the second experiment) was added and mixed into the protein powder, after which 232 g of vegetable shortening was added. These ingredients were mixed using a flat metal attachment at speed setting "1" (Kitchen Aid, St. Joseph, MI) for the minimum time (~1 to 4 min) required to mix the ingredients into a homogenous mass and form a smooth nougat-like texture.

The resultant doughs were formed into small cylindrical masses and fed into a bar former (Bepex Hut, Leingarten, Germany) which rolled the dough out and cut it into bars (6.5 x 3 x 1 cm). The finished bars were placed in the moisture barrier pouches, then sealed and labeled for storage. The packaged bars were stored in a controlled temperature room at 32 ± 1 °C which provides accelerated storage testing in which storage for 42 d corresponds to ~12 months storage at ambient temperatures (~22°C) (Li and others 2008).

## Hardness

Hardness was measured as the maximum load recorded during 8.5-mm penetration of a 45° chisel knife blade (TA-42, Texture Technologies, Ramona, CA) at a speed of 1 mm/s into the HPN bar. We used a TA.XT Plus texture analyzer (Texture Technologies) with a 30-kg load cell and an activation force of 5 g-force. Bars were laid on the platform and a hardness test performed at three locations along each bar, and an average hardness value calculated.

## Color

Color changes during storage of bars made were measured using a colorimeter (Miniscan XE Plus Model 45/O-S, Hunter Associates Laboratory Inc., Reston, Va.) using the method of Vissa and Cornforth (2006). The colorimeter was standardized using black and white tiles covered with plastic wrap. The HPN bar were removed from their package and covered in plastic wrap, then three measurements were taken at different places on the sample and average  $L^*$ ,  $a^*$ , and  $b^*$  values calculated by the colorimeter.

## Water Activity Testing

Water activity ( $a_w$ ) was measured using a Pawkit  $a_w$  meter (Decagon Devices Inc., Pullman, WA). The meter was calibrated using 6.0 molal NaCl and 13.41 molal LiCl standards that were individually placed in the sample cup and a reading was taken. Water activities of HPN bars were measured by placing a sufficient portion of the bar in a sample cup to cover the bottom of the cup and placing the meter on top of the cup and recording  $a_w$ .

## Confocal Microscopy

The CLSM method used was similar to that of Libaek and others (2006) with a few modifications. Squares of bar with dimensions of 8 x 8 x 2 mm were sliced from the middle of the bar at room temperature (~22 °C) and placed on a pre-washed microscope slide (FisherBrand, Loughborough, UK). One drop of a 0.02% (w/w) FITC solution in absolute acetone was applied to the sample and allowed 60 s to penetrate. One drop of a 0.02% (w/w) Nile Red solution in absolute acetone was then applied and allowed to penetrate for 60 s. The piece was covered with a glass cover slip and sealed with air impermeable petroleum gel (Taylor Lube, Haynes Manufacturing Co., Westlake, OH) sealing in the moisture and preventing dehydration during sample analysis.

An inverted microscope (Biorad, Hercules, CA) with an Ar/Kr laser was used to excite the FITC at a wavelength of 488 nm and the Nile Red at 568 nm. This yielded emissions with peak wavelengths of 520 nm for the FITC and 640 nm for the Nile Red. Fluorescence was captured sequentially using filters of wavelengths 512 to 532 nm for the FITC and  $\geq 585$  nm for the Nile Red. In the final false colorized images, the fat (Nile Red fluorescence) appears as red and the protein (FITC fluorescence) appears as green, while areas without protein or fat appear as black. In order to confirm that the black areas of the images were carbohydrate and not air or water, the plane of focus was moved up and down within the black areas to determine if the bottom of this region could be brought into sharp focus (which it would if it was a water droplet or air bubble).

Because of time and resource constraints during the first experiment, 12 fields of image of each bars containing 0% and 100% HWPI were taken at each week as they were expected to have the greatest differences, but only 2 images were acquired for the 25, 50, and 75% HWPI bars. Micrographs were further analyzed using AnalySIS (Olympus Soft Imaging Solutions, Lakewood, CO) with arbitrary color values using the "Set Thresholds" function, by looking at one image and inputting red, green, and black values for each assigned color. The "Phase Analysis" function of the software was then used to calculate the percent red, green, and black areas in the images.

## Statistical Analysis

Bars were made from duplicate batches of dough. In the first experiment, a completely randomized design was used to analyze the data with the source of variation being the level of HWPI in the bar formula, with storage time nested within the replication. The second experiment was a 2 x 2 factorial with protein and carbohydrate types in a completely randomized design. Data were analyzed using SAS 9.1 (SAS Inst. Inc., Cary, NC) using the Proc Mixed function, with saccharide/polyol, and/or protein, and day as the fixed variables and the batch as the random variable. Statistical significance was declared at  $P \leq 0.05$  for treatment effects and between means using differences of least squares means.

## RESULTS

### **Dough Manufacture**

As the doughs were mixed, differences were observed in the amount of mixing time required to form the ingredients into a dough as well as its color and consistency. Doughs in which the protein powder was at least 50% HWPI, formed more readily than those with 0% HWPI (i.e., 100% WPI). They required less mixing time, were more cream colored, and more flexible and taffy like. When the protein powder contained 25% HWPI, it took longer to mix the ingredients into a homogeneous mass and for the dough to form, which apparently resulted in overmixing of the dough. It has been observed during bar manufacture that if doughs containing HWPI are mixed for too long this negates their ability to maintain bar softness. Extensive overmixing of such bars can even cause them to harden during manufacture. So while such HWPI protein powders have been developed and used to prevent hardening during storage of HPN bars, bar formulas containing HWPI require more precise mixing (with regard to extent of mixing) during the manufacturing process (S. Paulsen, Glanbia Nutritionals, Inc., personal communication, March 14, 2008).

Immediately following manufacture the bars made with 100% WPI had a shorter, less flexible texture and were white in color. As HWPI was added into the formulation, the bars became more flexible but darker in color such that bars with 75 and 100% HWPI were taffy-like in texture and cream colored. The bars made using 100% HWPI were very soft, sticky, and did not hold their shape perfectly, while those bars containing only WPI were soft, dry, and maintained their shape.

### **Change in Color During Storage**

Bar color significantly changed during storage with increasing  $L^*$ ,  $a^*$  and  $b^*$  values that were influenced by use of WPI or HWPI ( $P < 0.001$ ), use of HFCS or SS ( $P < 0.001$ ), and storage time ( $P < 0.001$ ). All of the two-way interactions were also statistically significant ( $P < 0.001$ ), except that there was no protein-saccharide/polyol interaction for  $a^*$  color values. Extent of color change was related to amount of Maillard browning reactants (aldehydes and amines) in the bars. The HFCS consists of primarily of glucose and fructose which are both reducing sugars while sorbitol is a non-reducing sugar alcohol without any aldehyde groups. Hydrolyzing whey proteins produces amino acids and peptides, so HWPI has more  $\alpha$ -amino groups than WPI, which consists mainly of intact proteins. In the experiment using different levels of HWPI in combination with HFCS, only visual observations of color were made and the bars became darker during storage as the proportion of HWPI used was increased.

Differences in bar color were detected even after just 1 d of manufacture depending on whether none, one or two of the bar components had a high level of Maillard browning reactants. Extent of browning (measured by increase in yellow color, i.e.,  $b^*$  value), was HWPI/HFCS > WPI/HFCS or HWPI/SS > WPI/SS bars (Table 1). During storage all bars became darker but with different rate and extent of color change. The WPI/SS bar had statistically significant but relatively small increase in  $b^*$  value, and after 34 d at 32 °C was still a cream color with a  $b^*$  value the same as the initial  $b^*$  value for the WPI/HFCS and HWPI/SS bars (Table 1). The HWPI/HFCS bars underwent the most color change and after 34 d were black in color while the WPI/HFCS and HWPI/SS bars had an intermediate (caramel) color (Figure 1). By d 20, the  $b^*$  value for the HWPI/HFCS bars decreased but at the same time the bar samples changed from brown to black and  $L^*$  value decreased.

### **Influence of HWPI on Hardness**

When different levels of HWPI were used in conjunction with HFCS, storage time, HWPI level, and the time  $\times$  HWPI level interaction all significantly ( $P < 0.001$ ) influenced bar hardness. After manufacture (i.e., d 2), all bars had similar hardness (peak force during penetration) of  $3.4 \pm 0.4$  N (Figure 2). Within 7 d of 32 °C storage, significant differences in hardness between bar formulations were observed. Bars with 0% or 25% HWPI were much harder than the other bars, and not significantly different from each other with average hardness values of 17 N. Bars with 100% HWPI were the softest with an average hardness value of 5.2 N. These trends continued throughout storage with all the bars increasing in hardness over time (Figure 2). This inhibition of bar hardening with increasing amounts of HWPI was expected because HWPI is used commercially for this purpose (Gottschalk 2006). □

By the end of the study, bars without any HWPI had reached a hardness of 59 N. Those bars made with 25% HWPI were even harder, presumably because of overmixing of the dough, while the hardness of those bars made with 100% HWPI were 15 N (which is about what the 0% HWPI bars were after only 7 d).

## ***Influence of Saccharide/Polyol on Hardness***

When bars were made from combinations of WPI or HWPI with HFCS or SS, both the protein and saccharide/polyol significantly ( $P < 0.001$ ) influenced bar hardness but there was not a significant ( $P = 0.15$ ) interaction effect. Hardness increased during storage ( $P < 0.001$ ) and the interactions of storage time with protein ( $P < 0.001$ ) and saccharide/polyol ( $P = 0.03$ ) were also significant. Use of HWPI had a greater influence on reducing bar hardness than did use of sorbitol (Figure 3). At manufacture (d 1), all bars containing HWPI had similar hardness values in the range of 1.8 to 3.0 N, while bars containing WPI were harder (15 to 24 N). After 7 d storage at 32 °C, significant differences in bar hardness were observed with the WPI/HFCS bar significantly hardest at  $49.3 \pm 3.2$  N followed by the WPI/SS bar at  $29.3 \pm 1.3$  N. The bars made using HWPI remained soft (3 to 5 N) through 27 d of storage at 32 °C after which the HWPI/HFCS bar started to harden (Figure 3).

## ***Water Activity***

Water activity was significantly affected by use of HWPI ( $P < 0.001$ ), saccharide/polyol ( $P < 0.001$ ) and their interaction ( $P = 0.004$ ). At d 1, the WPI bars had significantly ( $P < 0.05$ ) lower  $a_w$  than those containing HWPI, and there was a trend in the bars made with SS having a slightly lower  $a_w$  (Table 2). Storage time ( $P < 0.001$ ) as well as the two-way interaction of time with protein ( $P = 0.02$ ) and saccharide/polyol ( $P = 0.01$ ) also influenced  $a_w$  of the HPN bars. During storage,  $a_w$  increased with the largest changes occurring with bars that contained both WPI and HFCS. The WPI/HFCS bars went from having an initial  $a_w$  of 0.63 to 0.69 after 34 d storage at 32 °C. The  $a_w$  of the HWPI/SS bar did not significantly change during storage and remained in the range of  $a_w = 0.59$  to 0.62.

## ***Microstructure***

Initial microstructure of the HPN bars was as expected for a tertiary mixture of dry protein powder mixed with a sugar (or polyol) syrup and oil. As shown in Figure 4 for bars made using WPI or HWPI with HFCS, there were three interspersed phases that was indicative of incomplete mixing (mixing was only performed for the minimum time required to form the dough). There was a lipid phase (red) and two non-lipid phases that either contained protein (green) or lacked protein (black). Given the limit of resolution of LSCM, and that the intensity of fluorescence depends upon the amount of fluorophore in that location as well as the material the fluorescent light passes through from the focal plane to the objective lens, it is not possible to determine whether this dispersion occurred at the molecular level. Because the water content of the doughs was only about 15%, and the relatively short mixing time used, it would be very unlikely that sufficient water exists to completely hydrate the proteins.

With the extent of mixing that was used in making the doughs, it appeared that most of the protein powder had been dispersed throughout the HFCS with a few exceptions. Differences in intensity of fluorescence from FTIC (which appears in the micrograph as light green (high intensity) and darker green (low intensity) areas) suggests that the level of protein varies within the combined HFCS/protein phase. Some large black areas (about 50 to 200  $\mu\text{m}$  in size) appear to be HFCS droplets that had been coated with a 15 to 30  $\mu\text{m}$  layer of shortening during the mixing process, which prevented any mixing with the protein powder. There are also numerous small HFCS droplets (black in color and about 10 to 20  $\mu\text{m}$  in size) that are dispersed throughout the non-lipid phase. These are not covered with lipid and probably represent the size of droplets that the HFCS is reduced to as a result of mechanical shearing during the mixing process when the doughs were being manufactured. Some of the protein powder also appears to become covered with shortening that prevents their further dispersion and these appear as small (5 to 20  $\mu\text{m}$ ) green areas with a red annular ring around them. All of these microstructural elements are indicative of material in which the three ingredients are dispersed together with some fat, sugar syrup, and protein still being in particulate form, as well as formation of a combined aqueous protein-sugar phase.

In our first experiment, we observed that immediately (d 2) after manufacture, all bars regardless of the amount of HWPI in the formulation, were similar in hardness and microstructure. This was attributed to the dry protein particles only being dispersed throughout the bar matrix and the system not having reached an equilibrated state between the proteins, the carbohydrates, and the water contained in the sugar syrup. Bars formulated using SS rather than HFCS had the same type of initial structure (data not shown).

Within the first week of storage (at 32°C) the microstructure of the bars in which the protein powder was 100% HWPI, had changed to being less particulate in appearance to a two-phase system with continuous lipid and non-lipid phases (Figure 5). This change takes place as water migrates from the higher  $a_w$  HFCS syrup to the lower  $a_w$  powders (Li and others, 2008). There were still some small droplets (about 5 to 60  $\mu\text{m}$  size) that were black (i.e.,

lacked protein and lipid) but these were not as prevalent as observed in the bars at 2 d after manufacture (Figure 4). This structure remained throughout 29 d storage, and then at d 36 some larger black areas were observed that were about 200 nm in size.

In contrast, the bar made with only WPI (i.e., 0% HWPI) consisted by d 7 of a continuous fat phase with what appeared to be a coarse stranded protein phase and separate large syrup regions (200 to 1000 nm) that were devoid of lipid and protein (i.e., they had no fluorescence from Nile Red or FITC (Figure 5)). This structure persisted throughout storage with the lipid phase often being present between the protein-rich (green) and protein-depleted (black) aqueous phases. For the 25% HWPI bars, the presence of the three-phase system with large areas devoid of protein and fat was apparent by d 29 of storage (Figures 5 and 6). There was possibly the beginning of formation of such a carbohydrate-only phase (small black regions) in the 50% and 75% HWPI bars by d 36. These bars would have to be examined over a longer time period for this to be confirmed.

In our second experiment in which bars were made with either WPI or HWPI and HFCS or SS, the same two classes of structure were observed at the end of storage (Figure 6). A stable two-phase system was observed in bars made using HWPI while the three-phase system with large aqueous regions containing only sugar or polyol were observed in the WPI bars.

### **Image Analysis**

Using the image analysis software to quantify the number of red, green and black pixels in the micrographs there were distinct differences in bar microstructure as a function of proportion of HWPI in the bars ( $P < 0.0001$ ) and storage time ( $P < 0.005$ ). Bars containing 0% and 100% HWPI were significantly different ( $P < 0.05$ ), but statistical significance was not achieved when comparing the intermediate bars (25, 50 and 75% HWPI) probably because only 2 images of each replicate of these bars were acquired instead of the 12 images obtained of the 0% and 100% HWPI bars.

In the images captured on d 2 of storage, the percentages of each color were similar regardless of bar formulation (Table 3). When comparing bars with 0% or 100% HWPI (as shown in Figure 4) it was observed that red made up approximately 20% of all the color in the image, green made up 68%, and black made up 10%. On a weight basis, the bar formulas contained 19% of shortening, 38% protein powder and 43% HFCS, which confirms that the lipid phase is being imaged correctly, and that the protein powder is dispersed with most of the carbohydrate syrup, with about 20% to 25% of the syrup being devoid of protein as described above. During storage the percentage of red pixels randomly varied but remained within the range of 16% to 31%. There were significant changes in the percentage of green and black in the 0% HWPI bars during storage. The percentage of green decreased while the percentage of black increased from ~9% at d 2 up to 24% to 40% (Table 3). In contrast, the microstructural images of the 100% HWPI bars showed very little change in red, green, and black percentages with values of  $25 \pm 6\%$ ,  $63 \pm 5\%$ , and  $9 \pm 5\%$ , respectively, which is probably within experimental variation. The total of red, green, and black pixels was slightly <100% as the software was unable to assign some pixels to a specific color. A low amount of black pixels ( $9 \pm 5\%$ ) was interpreted as the sugars being evenly distributed with the protein throughout the non-lipid phase, while a high percentage of black pixels ( $32 \pm 8\%$ ), was interpreted as the sugars and the protein having separated into 2 distinct aqueous phases.

## **DISCUSSION**

### **Browning and Hardness**

When these various observations of various physical changes during storage (i.e., hardness, color, water activity and microstructure) of HPN bars are considered together, the only changes consistent with hardening were an increase in water activity and the phase separation of the protein from the sugar/polyol. For any individual HPN bar, there was both an increase in browning and hardening during storage but this was not so when comparing bars of different composition. Bars made using HWPI remained soft throughout storage yet had excessive browning and became black in color, when HFCS was used as the sugar/polyol syrup. Similarly, browning could be prevented by using SS as the carbohydrate syrup but such bars made with WPI became excessively hard during storage. We therefore concluded that while Maillard browning can occur during storage of HPN bars, it is not the mechanism by which bar hardening occurs.



## **Water Activity**

Changing the sugar/polyol syrup from HFCS to SS did not influence initial water activity of HPN bars (Table 2) even though SS has a higher moisture content (30%) compared to HFCS (23%), and a higher water activity, 0.69 versus 0.60 for HFCS. There was, however, a lowering of water activity of HPN bars from 0.64 to 0.60, when HWPI was used instead of WPI. This would be expected given the increased hydrophilicity of the peptides and amino acids produced during the production of HWPI and its lower water activity (0.18 compared to 0.21 for WPI). The increase in water activity in bars exhibiting hardening (e.g., from 0.63 to 0.69 in WPI/HFCS bars) agrees with Li and others (2008) who found that bars made using a polyol syrup increased from an initial water activity of 0.52 to 0.56 after 42 d storage at 32 °C. Normally an increase in water activity (such as by increasing the total moisture content of the e bars) would be expected to make the bars softer but in this case it appears while there is an apparent increase in the e amount of bulk water in the bars, that some water molecules are losing their ability to act as a plasticizer around the e protein particles (Li and others 2008). In HPN bars, water can be considered to exist in one of three states: bound water, free (or intermediate) water, and bulk water (Zhou and others 2008b) with exchange between them occurring at t the hydration layer around the protein particle surfaces.

Bound water molecules are those that have strong water-ion, and possibly hydrogen-ion and water-dipole interactions, between the water and protein and sugar/polyol molecules (Kuntz and Kauzmann 1974; Zhou and others 2008b). Intermediate water molecules have weak interactions with the protein surface and are part of the local domain of water molecules in the region of solution immediately surrounding the protein molecules (McClements 200002). Zhou and others (2008a) observed hardening of protein buffer solutions (40% moisture) during storage at 34 °C and an increase in bound water from 64% to 68% of the total water. There was a concomitant increase in bulk water from 0.5% to 1.7%, such that intermediate water decreased from 36% to 30% over the same time. In our study of F HFCS versus SS, water activity stayed constant for the first week of storage and had increased in the WPI bars by the e second week indicating an increase in bulk water coinciding with hardening of the bars.

## **Cosolvation**

During and after mixing of the sugar/polyol syrup and protein powder there is a transfer of water molecules from the syrup to the protein powder. The appearance of less particulate material in LSCM images of the 2-d old HWWPI bars (Fig 4), suggests that the proteins in the these bars are more solvated and incorporated into the HPNS than the proteins in the WPI bars. There is insufficient water in HPN bars for the proteins to be fully solvated, even with the higher water affinity of hydrolyzed proteins. However, water is not the only component of HPN bars that can solvate the whey proteins. Sugars and polyols can function as weakly interacting cosolvents (McClements 2002) and d have a stabilizing effect on protein structure (Crowe and others 1987).

Changes in structure of HPN bars during the first week of storage can be explained as the protein particles becoming more solvated through interactions with both water and sugar/polyol molecules. This allows formation of a more homogeneous phase containing protein, sugar/polyol, water, and any minerals present in WPI/HWPI powders. Stability of this system depends on molecular size of the cosolvent and the extent of cosolvent interactions with the protein molecules. Larger cosolvent molecules will be preferentially excluded from the local domain surrounding the protein (unless they exhibit a strong specific interaction with the proteins). Thus, the cosolvent concentration will generally be lower in the local domain around the protein than in the bulk solution (McClements 20001).

When comparing the influence of different polyols on bar hardening, Liu and others (2009) reported that glycerol was better at preventing hardness than sorbitol or maltitol. Glycerol has the lowest glass transition temperature of these polyols, which they proposed would allow it to act as a better plasticizer than sorbitol or maltitol. Glycerol (a 3-carbon polyol) also has a lower molecular size than sorbitol (a 6-carbon polyol) and maltitol (a 12-carbon polyol) so that it would be less preferentially excluded in the local domain around the protein. Chaanasatru and others (2007) found that the steric exclusion of glycerol from b-lactoglobulin was compensated by a differential interaction with the protein such that it has a neutral impact on unfolding of b-lactoglobulin, and bar r hardening. In contrast, propylene glycol (also a 3-carbon polyol but with only two hydroxyl groups) has lower molecular weight and glass transition temperature than glycerol, but causes rapid hardening (Liu and others 2009). This occurs because it interacts hydrophobically with proteins and is preferentially accumulated in the local domain around the whey protein particles.

## **Protein-Cosolvent Compatibility**

Li and others (2008) compared six different protein powders and observed that there are some synergistic effects on preventing bar hardness between different proteins but the mechanism for this is unclear. Also, some proteins produced bars that hardened rapidly when used with polyol syrups but not when used with sugar syrups, and some had the opposite effect. This can be understood when considered in terms of how the sugar/polyol molecules interact with the protein particles. Steric exclusion of cosolvent molecules from the local domain around the protein depends on the relative size of the solvent (water) and cosolvent (sugar/polyol) molecules, while differential interactions depend on differences in strength of molecular interactions between the cosolvent and solvent molecules and the protein surface (Chanasattru and others 2008). The surface of a globular protein is highly heterogeneous, consisting of functional groups of differing polarity, shape and size. Each of these groups interacts differently with cosolvent and solvent molecules, depending on their molecular characteristics, and influences the preferential exclusion or accumulation of cosolvent in the local domain. The stability of the protein-water-sugar/polyol system will include the net effects of free energy changes based on protein conformation and protein-protein interactions, the protein-water interface and the protein-cosolvent interface, as well as free energy changes as the cosolvent increases in concentration in the bulk solution, or as the protein becomes more concentrated.

Glycerol can act as a better plasticizer than larger polyols (Liu and others 2009) because it is smaller and less excluded from the protein surface and presumably less excluded from hydrophobic regions on the protein surface (Chanasattru and others 2008). However, glycerol acts less favorably with such hydrophobic regions than water. This is because water can still rearrange in the presence of a nonpolar group and form 4 hydrogen bonds with other water molecules (McClements 2001). In contrast, such rearrangement of glycerol requires the hydrogen bonds between its alcohol groups to be broken. Excluding the cosolvent from the local domain requires increasing its concentration in the remaining bulk solution. This can be thermodynamically unfavorable, and so protein conformation may change to minimize the entire protein-solvent/cosolvent interface (Liu and others 2009). In some cases, such as with glycerol, this stabilizes the protein against unfolding but when the polyol can interact strongly with the protein and is preferentially accumulated in the local domain, such as with propylene glycol, protein aggregation can be induced. Likewise, while sorbitol more effectively stabilizes b-lactoglobulin in its native state than glycerol (because of a larger gain in free energy if the protein unfolds), sorbitol can promote self-association of b-lactoglobulin into large insoluble aggregates (Chanasattru and others 2008).

## **Phase Separation**

Phase separation is a major determinant of a food's texture, mechanical stability, consistency and, ultimately, appearance and taste (Semenova 2007). Such phase separation can result from the presence of multiple polymers in solution, which reduces the number of possible configurations, and decreases the chance that one polymer will fit into the domain of the other (Rogers and others 2006). By separating into a protein-rich phase and a polysaccharide-rich phase the system gains entropy (with a resultant decrease in free energy). Usually one of the biopolymer-rich phases forms a continuous phase with the other being dispersed throughout (Tolstoguzov 1998).

In HPN bars, the proteins are the only polymers present as there are usually no large molecular weight polysaccharides included in the bar formulation. Phase separation in systems containing simple sugars and proteins has been observed when under high stress such as high humidity or temperature because of sugar crystallization (Suikko and others 2005). However, we did not observe any sugar/polyol crystals in LSCM images of any of the HPN bars we examined. Nor were crystals detected when we examined HPN bars using polarized light microscopy or using X-ray powder crystallography by the method of Suikko and others (2005) (data not shown). Thus, the increase in water activity and hardening during storage of the HPN bars was not attributed to crystallization causing sugars to lose their ability to bind water.

We had also tested HPN bars using differential scanning calorimetry to see if the sugars had formed into a glassy state as their concentration in the bulk solution increased as water was transferred to the protein local domain. A 50:50 fructose/glucose syrup (which approximates HFCS) has a glass transition temperature calculated at 24 °C (Arvanitoyannis and others 1993) so it is conceivable that it could form a glassy state during storage of HPN bars at room temperature and bring about a phase separation of the sugar glass from the proteins. It is unlikely that this would occur with SS since it has a lower glass transition temperature of -2 °C (Lui and others 2009) yet the WP/SS bars also exhibited phase separation and hardening while the HWPI/SS bars did not. When

bars were heated from room temperature (~23 °C) to 100 °C at a rate of 5 °C/min there was no stepwise shift in specific heat capacity (data not shown) that would suggest devitrification of a glassy state had occurred. Thus, there was a lack of evidence to support formation of a sugar glass as being the cause of the changes we observed in microstructure of HPN bars that hardened during storage.

The remaining alternative for the cause of phase separation in HPN bars into protein-rich (carbohydrate-depleted) and protein-depleted (carbohydrate-rich) phases, is preferential exclusion of sugar/polyol cosolvent from within the local domain surrounding the protein particles. As described above, this can have unfavorable entropic effects on the overall free energy of the aqueous system, so there will be a tendency to minimize the protein-sugar/polyol interface. Apparently, the drop in free energy brought about by partitioning the protein away from the sugar/polyol syrup is greater than the increased free energy as the sugar/polyol concentration increases in the protein-depleted phase.

One consequence of formation of 2 aqueous phases in HPN undergoing hardening is that a portion of the water molecules will become bulk water in the protein-rich phase. Since this phase is also carbohydrate-depleted, the low concentration of sugars/polyols in the bulk water is apparently observed as an overall increase in water activity of the HPN bars. Such an increase in water activity in WPI bars (containing HFCS or SS) was observed by d 14 (Table 2) suggesting that phase separation had commenced by then and increased during the next 20 d of storage.

After phase separation has occurred, protein molecules are within closer proximity to each other and the influence of the sugar/polyol cosolvent on maintaining native structure of the proteins (especially b-lactoglobulin) is diminished. Both of these factors would increase the likelihood of protein-protein interactions and protein-moisture interactions. Moisture-induced protein aggregation can then occur along with the formation of covalent and noncovalent bonds between proteins as described by Zhou and others (2008a, 2008b) and Lui and others (2009).

When considering the compatibility of a protein with a sugar/polyol syrup (Li and others 2008) the transfer free energy of the protein from water into the sugar/polyol solution could provide information useful for determining the ability of a protein to have a stabilizing effect in HPN bars. It gives a measure of the preference of the functional groups on the protein surface for interactions with the sugar/polyol cosolvent species relative to its preference for interaction with water (Venkatesu and others 2006). Too much interaction between the protein and the cosolvent can result in very rapid aggregation of the proteins, destabilization of the system, and hardening of HPN bars, as occurs when propylene glycol is used (Liu and others 2009). If the cosolvent has a high preferential exclusion from the local domain around the protein (which is assumed to be the case for WPI in HFCS or SS) then a phase separation can occur with an increased potential for protein aggregation and possibly glassy state formation.

Use of partially hydrolyzed proteins along with intact proteins (such as in the HWPI powder used in our experiments) produces a protein-sugar/polyol-water system that is stable and remains in HPN bars as a single phase (Figures 5 and 6). Hydrolyzing proteins increases the number of protein/peptide molecules (in a given weight of protein powder), such that the protein-cosolvent interfacial area is greater in HWPI bars than WPI bars. Since the protein and sugar/polyol in the HWPI bars remained as a single phase throughout storage, there was apparently no entropic driving force (because of cosolvent depletion in the local domain around the proteins) acting to minimize the protein-cosolvent interface. This can be attributed to (1) increased hydrophilicity brought about by hydrolysis of peptide bonds allowing sugar/polyol cosolvent molecules to interact more with the protein particles, (2) decreased hydrophobicity so that cosolvent molecules are less preferentially excluded from the local domain, and (3) less entropy gain brought about by protein-protein interactions that shield hydrophobic groups and reduce protein-cosolvent interactions.

## CONCLUSIONS

High protein nutrition bars made using protein powder, lipid (in the form of vegetable shortening) and a sugar or polyol syrup underwent changes during accelerated storage at 32 °C that were dependent on the type of protein and carbohydrate syrup we used. The bars darkened in color based on the presence of Maillard browning reactants present in the formulation. Extent of browning was HWPI/HFCS bars > WPI/HFCS bars > HWPI/SS bars > WPI/SS bars. The bars became harder during storage with bars made using WPI the hardest, and those made with partially hydrolyzed protein powders remained soft, especially when the carbohydrate was sorbitol rather than the glucose and fructose in HFCS.

The most notable difference in microstructure between bars that rapidly hardened during storage and those that remained relatively soft, was a separation between the proteins and the carbohydrate into two distinct phases. If the carbohydrate (whether sugar or polyol) remained in a single homogeneous non-lipid phase then the bars

remained soft. Phase separation into large protein-rich and protein-depleted aqueous regions is proposed as being the mechanism that initiates bar hardening and increased protein-protein interactions. Water activity of the bars was slightly lower when HWP1 was used and stayed constant during storage, while  $a_w$  of the WPI bars had increased after 2 wk suggesting an increase in bulk water in the protein-rich phase and as associated loss of ability of solvent molecules to maintain protein flexibility. Successful formulation of HPN bars depends on cosolvent properties of the polyol/sugar towards the proteins and their preferential exclusion from the solvation layer surrounding the proteins. Using HWP1 in the bars maintained the compatibility of the protein and carbohydrate components.

## ACKNOWLEDGMENTS

Author S. Adams was supported through a Center-Industry Partnership research grant from Glanbia Nutritional Inc. and the Western Dairy Center. This research was also supported by the Utah Agricultural Experiment Station, Utah State University, and approved as journal paper number 8028.

## REFERENCES

- Arvanitoyannis I, Blanshard JMV, Ablett S, Izzard MJ, Lillford PJ. 1993. Calorimetric study of the glass transition occurring in aqueous glucose:fructose solutions. *J Sci Food Agric* 63(2):177-188.
- Chanasatru W, Decker EA, McClements DJ. 2007. Physicochemical basis for cosolvent modulation of  $\beta$ -lactoglobulin functionality: interfacial tension study. *Food Res Int* 40(8):1098-1105.
- Chanasatru W, Decker EA, McClements DJ. 2008. Impact of cosolvents (polyols) on globular protein functionality: Ultrasonic velocity, density, surface tension and solubility. *Food Hydrocolloids* 22(8):1475-1484.
- Crowe JH, Crowe LM, Carpenter JF, Wistrom CA. 1987. Stabilization of dry phospholipid bilayers and proteins by sugars. *Biochem J* 242(1):1-10.
- Gautam A, Garcia AC, Hander RJ, inventors; Unilever Intellectual Property Group, Slim-Fast Foods Company, Division of Conopco, Inc. 2006 June 1. Nutrition bar or other food product and process of making. U.S. patent application 20060115554.
- Gottschalk L. 2006. Winning wheys in bars and beverages. *Prepared Foods* 175(8):71-72, 75.
- Halliday J. 2005. Glanbia solves whey protein problems with R&D. *NutraIngredientsUSA*, <http://www.nutraingredients-usa.com/Industry/Glanbia-solves-whey-protein-problems-with-R-D>, Accessed Oct 24, 2008.
- Kuntz ID, Kauzmann W. 1974. Hydration of proteins and polypeptides. *Adv Protein Chem* 28:239-345.
- Labuza T. 2008. Influence of moisture on aggregation of protein during storage. 5th Conference on water in food, Nürtingen, Germany, <http://www.efw2008.de/Abstracts%20Labuza%20Oral%20A5.pdf>, Accessed Mar 15, 2009.
- Li Y, Szlachetka K, Chen P, Lin X, Ruan R. 2008. Ingredient characterization and hardening of high-protein food bars: an NMR state diagram approach. *Cereal Chem* 85(6):780-786.
- Libaek HM, Broe ML, Hoier E, Fatum TM, Ipsen R, Sorensen NK. 2006. Improving the yield of mozzarella cheese by phospholipase treatment of milk. *J Dairy Sci* 89(11):4114-4125.
- Liu X, Zhou P, Tran A, Labuza TP. 2009. Effects of polyols on the stability of whey proteins in intermediate-moisture food model systems. *J Agric Food Chem* 57(6):2239-2345.
- McClements DJ. 2001. Estimation of steric exclusion and differential interaction contributions to protein transfer free energies in aqueous cosolvent solutions. *Food Hydrocolloids* 15(4-6):355-363.
- McClements DJ. 2002. Modulation of globular protein functionality by weakly interacting cosolvents. *Crit Rev Food Sci Nutr* 42(5):417-471.
- Rogers MA, Roos YH, Goff HD. 2006. Structural heterogeneity and its effect on the glass transition in sucrose solutions containing protein and polysaccharide. *Food Hydrocolloids* 20(6):774-779.
- Semenova MG. 2007. Thermodynamic analysis of the impact of molecular interaction on the functionality of food biopolymers in solution and in colloidal systems. *Food Hydrocolloids* 21(1):23-45.
- Suihko EJ, Forbes RT, Apperley DC. 2005. A solid-state NMR study of molecular mobility and phase separation in co-spray-dried protein-sugar particles. *Eur J Pharm Sci* 25(1):105-112.
- Stefan J. 2003. Made to last. *Prepared Foods*, Sept., <http://www.preparedfoods.com/CDA/Archives?issue=65117>, Accessed Oct 24, 2008.
- Tolstoguzov VB. 1998. Functional properties of protein-polysaccharide mixtures. In: Hill SE, Ledward DA, Mitchell JR, editors. *Functional properties of food macromolecules*. Gaithersburg, MD: Aspen Publishers, Inc. p 252-277.

- Venkatesu P, Lee M-J, Lin H-M. 2006. Transfer free energies of peptide backbone unit from water to aqueous electrolyte solutions at 298.15K. *Biochem Eng J* 32(3):157-170
- Vissa A, Cornforth DP. 2006. Comparison of milk mineral, sodium tripolyphosphate, and Vitamin E as antioxidants in ground beef in 80% oxygen modified atmosphere packaging. *J Food Sci* 71(2):C65-C68.
- Wilkinson C, Dijksterhuis GB, Minekus M. 2000. From food structure to texture. *Trends Food Sci Technol* 11(12):4442-450.
- Wright R. 20008. Nutrition bar update. *Nutraceutical World*, Feb., <http://www.nutraceuticalsworld.com/articles/2008/01/nutrition-bar-update>, Accessed March 10, 2009.
- Zhou P, Liu XX, Labuza TP. 2008a Moisture-induced aggregation of whey proteins in a protein/buffer model system. *J Agric Food Chem* 56(6):2048-2054.
- Zhou P, Liu XX, Labuza TP. 2008b. Effects of moisture-induced whey protein aggregation on protein conformation, the state of water molecules, and the microstructure and texture of high-protein-containing matrix. *J.Agric Food Chem* 56(12):4534-4540.

**Table 1-** Meann b\* values of high protein nutrition bars formulated with either whey protein isolate (WPI) or partially hydrolyzed whey protein isolate (HWPI) and sweetened with either high fructose corn syrup (HFCS) or sorbitol syrup (SS) during 34 d accelerated storage at 32 °C

Storage (d)	b*			
	WPI/HFCS	WPI/SS	HWPI/HFCS	HWPI/SS
1	14.2 <sup>az</sup>	10.0 <sup>bz</sup>	21.6 <sup>cz</sup>	13.8 <sup>az</sup>
7	24.9 <sup>ay</sup>	12.2 <sup>az</sup>	36.6 <sup>by</sup>	16.6 <sup>bz</sup>
14	32.8 <sup>ax</sup>	13.4 <sup>az</sup>	40.5 <sup>by</sup>	18.5 <sup>bz</sup>
21	34.5 <sup>ax</sup>	14.2 <sup>az</sup>	32.7 <sup>ay</sup>	20.1 <sup>by</sup>
27	36.0 <sup>ax</sup>	14.6 <sup>ay</sup>	31.4 <sup>az</sup>	22.4 <sup>by</sup>
34	39.0 <sup>ax</sup>	14.4 <sup>ay</sup>	28.1 <sup>bz</sup>	25.3 <sup>bx</sup>

<sup>abc</sup>Means within a row without a common letter differ (a=0.05).

<sup>wxyz</sup>Means within a column without a common letter differ (a=0.05).

**Table 2-** Meann water activity of high protein nutrition bars formulated with either whey protein isolate (WPI) or partially hydrolyzed whey protein isolate (HWPI) and sweetened with either high fructose corn syrup (HFCS) or sorbitol syrup (SS) over 34 d storage at 32 °C.

Storage time(d)	Water Activity			
	WPI/HFCS	WPI/SS	HWPI/HFCS	HWPI/SS
1	0.63 <sup>az</sup>	0.64 <sup>az</sup>	0.60 <sup>bz</sup>	0.59 <sup>bz</sup>
7	0.63 <sup>az</sup>	0.63 <sup>az</sup>	0.60 <sup>bz</sup>	0.60 <sup>bz</sup>
14	0.67 <sup>ay</sup>	0.66 <sup>ay</sup>	0.63 <sup>bz</sup>	0.61 <sup>bz</sup>
21	0.69 <sup>ay</sup>	0.68 <sup>ay</sup>	0.66 <sup>ay</sup>	0.61 <sup>bz</sup>
27	0.68 <sup>ay</sup>	0.66 <sup>ay</sup>	0.66 <sup>ay</sup>	0.62 <sup>bz</sup>
34	0.69 <sup>ay</sup>	0.68 <sup>ay</sup>	0.63 <sup>bz</sup>	0.61 <sup>bz</sup>

<sup>ab</sup>Means within a column without a common letter differ (a = 0.05).

<sup>xyz</sup>Means within a row without a common letter differ (a = 0.05).

**Table 3-** Percentages of red, green, and black pixels in micrographs of high protein nutrition bars formulated so that the protein portion consists of either whey protein isolate (WPI) powder or partially hydrolyzed WPI (HWPI) over 29 d storage at 32 °C.

Storage Time (d)	Pixels (%)					
	WPI			HWPI		
	Red	Green	Black	Red	Green	Black
2	20 <sup>az</sup>	68 <sup>az</sup>	9 <sup>az</sup>	19 <sup>az</sup>	68 <sup>az</sup>	10 <sup>az</sup>
8	25 <sup>az</sup>	33 <sup>ay</sup>	40 <sup>ay</sup>	31 <sup>az</sup>	64 <sup>az</sup>	4 <sup>bz</sup>
15	19 <sup>az</sup>	35 <sup>ay</sup>	40 <sup>ay</sup>	27 <sup>az</sup>	59 <sup>az</sup>	8 <sup>bz</sup>
22	16 <sup>az</sup>	44 <sup>ay</sup>	35 <sup>ay</sup>	23 <sup>az</sup>	60 <sup>az</sup>	14 <sup>by</sup>
29	18 <sup>az</sup>	53 <sup>az</sup>	24 <sup>az</sup>	25 <sup>az</sup>	65 <sup>az</sup>	7 <sup>bz</sup>

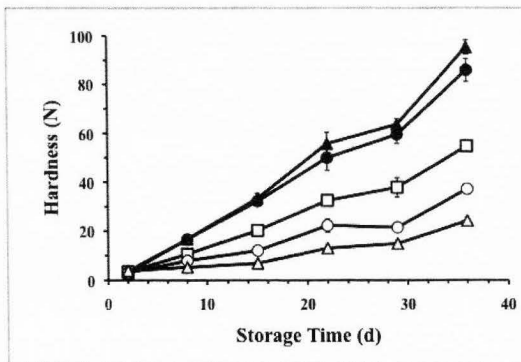
<sup>abc</sup>Means for the same color within the same row with no common superscripts differ (a = 0.05).

<sup>xyz</sup>Means within the same column with no common superscript differ (a = 0.05).

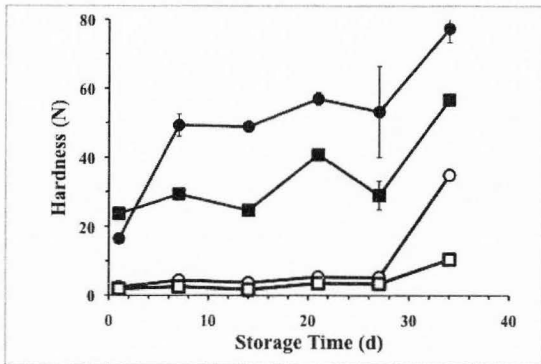
**Figure 1-** High protein nutrition bars after 34 d storage at 32 °C, formulated using (from left to right) whey protein isolate (WPI) and high fructose corn syrup (HFCS), WPI and sorbitol syrup (SS), hydrolyzed WPI (HWPI) and HFCS, or HWPI and SS.



**Figure 2-** Penetration hardness of high protein nutrition bars formulated such that the protein portion consists of 0% (■), 25% (●), 50% (□), 75% (○), or 100% (△) of a partially hydrolyzed whey protein isolate during 36 d storage at 32 °C.



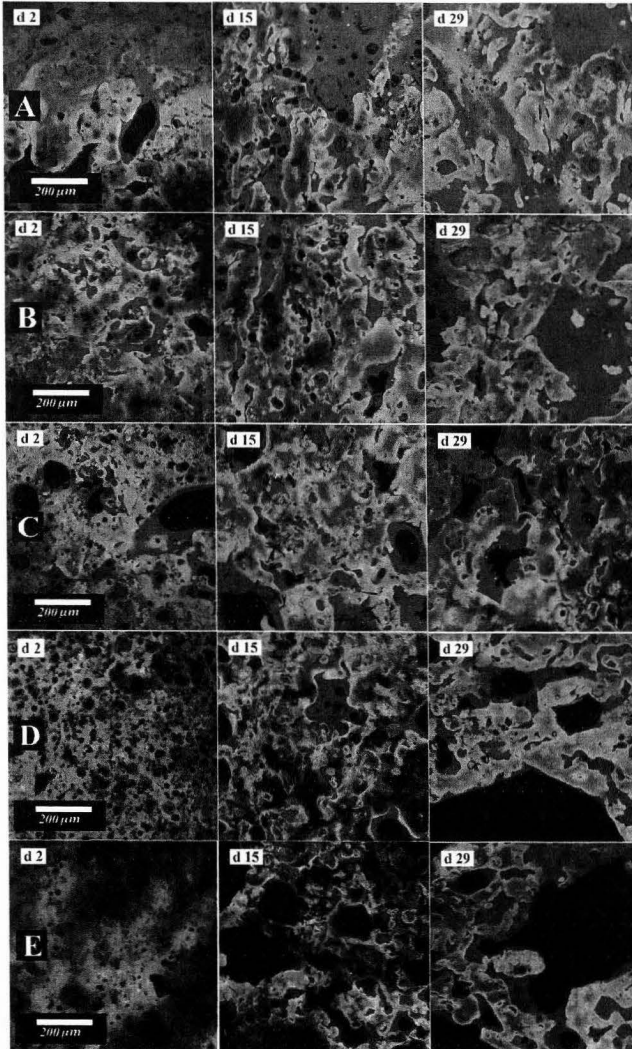
**Figure 3-** Penetration hardness of high protein nutrition bars formulated with whey protein isolate (WPI) or partially hydrolyzed whey protein isolate (HWPI) and sweetened with high fructose corn syrup (HFCS) or sorbitol sssyrup (SS) in combinations of WPI/HFCS (●), WPI/SS (■), HWPI/HFCS (○), or HWPI/SS (□) during 34 d storage at 32 °C.



**Figure 4-** Confocal laser scanning micrographs of high protein nutrition bars manufactured using (A) whey protein isolate (WPI) or (B) partially hydrolyzed WPI, mixed with high fructose corn syrup and vegetable oil shortening and after 2 d of storage. Red corresponds with fluorescence from Nile Red in the presence of lipid material, green corresponds with fluorescence from fluorescein isothiocyanate associated with protein, and black areas were indicative of regions devoid of both lipid and protein and assumed to be the carbohydrate syrup that had not mixed with the protein.

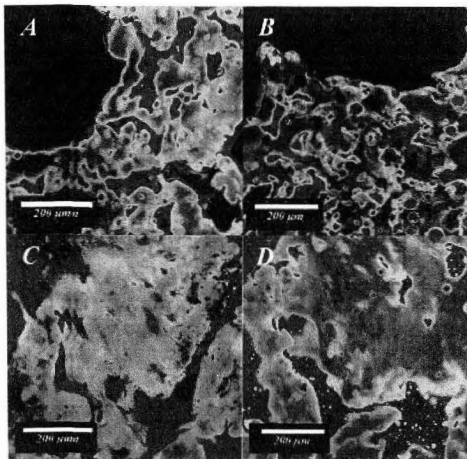


**Figure 5-** Confocal laser scanning micrographs of high protein nutrition bars after 2, 15 and 29 d storage at 3: °C in which red and green represent the presence of fat and protein respectively, and black represents their absence. Bars were formulated such that the protein portion consisted of (A) 100%, (B) 75%, (C) 51%, (D) 25%, or (E) 0% of a partially hydrolyzed whey protein isolate.





**Figure 6-** Confocal laser scanning micrographs of high protein nutrition bars formulated with whey protein isolate (WPI) or partially hydrolyzed whey protein isolate (HWPI) and sweetened with high fructose corn syrup (HFCS) or sorbitol syrup (SS) in combinations of WPI/SS (A), WPI/HFCS (B), HWPI/SS (C), and HWPI/HFCS (D) or during 34 d storage at 32 °C.



# Production of High Protein Cheddar Cheese with an Improved Extrusion-Modified Texture

MARIE WALSH: Utah State University  
DONALD MCMAHON: Utah State University

## Objectives:

- The extruder physiochemical (formulation, temperature and pressure) and configuration parameters (screw and paddle sequences) will be optimized for the extrusion of cheddar Cheese. Optimization will be initiated first and continue until product characteristics are improved.
- The texture of high protein (low fat) Cheddar cheese can be improved by the extrusion of blends of aged, full fat cheddar cheese and nonfat cheddar cheese. This will be initiated at the start of the project and continue over a 4-6 month shelf life.
- The effects of fat replacers on the texture of extruded Cheddar cheese will be determined.

## ABSTRACT

This project will investigate the use of extrusion technology to improve the texture of high protein (low fat) Cheddar cheese. High protein Cheddar cheese has a composition of approximately 54% moisture, 6% fat and 34% protein. This composition results in a cheese that has flavor defects and a rubbery texture. Therefore, several blends of Cheddar cheeses (full fat aged, and nonfat) will be grated and blended prior to extrusion in a twin-screw extruder. Extruder conditions of low temperatures (40 to 60 C), screw and paddle configurations of medium shear, and moderate pressures (~350 psi) will be explored to improve the cheese texture by disruption of the casein matrix and evenly distributing fat, moisture and air cells. Additionally, the use of fat replacers (Temp Pro WPC, Novagel, Simplesse, Vitacel MCG and Vitacel Plus) to improve the texture of the extruded cheese will be investigated. All cheeses will be analyzed for protein, moisture and fat content and the texture will be determined and compared to full fat aged and nonfat Cheddar cheeses. The goals of this project are to optimize the extruder configurations (screw and paddle sequences) and the physiochemical parameters (formulation, temperature and pressure) to allow the extrusion modification of Cheddar cheese texture and to investigate the influence of fat replacers on the texture of the extruded cheese. The use of full fat, aged cheeses blended with nonfat cheese in conjunction with extrusion may yield a product with enhanced flavor and texture compared to currently available high protein Cheddar cheeses.

## BACKGROUND INFORMATION

Low fat cheese (Table 1) approaches the definition of a high protein product with approximately 9.5 g protein in a 1.28 oz serving, while nonfat cheese has over 10 g protein per serving. Ten grams of protein per serving is classified as high (20% or more of RDI of a nutrient) based on DRV (daily reference value) of 50 g considering 2,000 calories/day. The higher protein content of low and nonfat Cheddar cheese is a result of the fat reduction. Unfortunately, the lower fat with higher moisture results in a product with textural and flavor deficiencies compared to the full or reduced fat Cheese products.

Table 1. Approximate composition of Cheddar cheeses produced at the Western Dairy Center.

Cheese	Moisture (%)	Fat (%)	Protein (%)	g fat/28 g	g protein/28 g
Full fat, aged	37	32	25	9	7
Aggiano	32	35.7	28.6	10	8
Reduced fat, aged	48.5	16	30	4.5	8.4
Low fat	54	6	34	1.68	9.52
Non fat	60	0.3	38	0.1	10.65

#### Low fat cheeses properties

Reducing the fat content in Cheddar cheese results in a product that is high in protein. Fat plays a critical role in the flavor, texture and appearance of Cheddar cheese and low and nonfat cheeses are usually identified as bland, firm, rubbery, and defective in color. The higher moisture in low-fat cheeses results in a lower salt content in the moisture phase. This change in the microenvironment is related to the changes in the sensory characteristics as well as in the microbiology and chemistry of the cheeses (Mistry, 2001).

Flavor defects arise from the lack of butterfat and the development of bitterness. Recent research (McMahon, unpublished data) shows that reduced fat Cheddar cheese has less milkfat flavor and higher sulfur, brothy and bitter flavors.

Texture profile and sensory analysis of low fat cheese show that lowfat cheese exhibits more springiness, firmness, and cohesiveness with a higher fracturability (McMahon unpublished data). This profile results in a rubbery texture to consumers. The structure of low fat cheese as determined by confocal microscopy is different from full or reduced fat. The fat in the cheese forms cavities and channels that help give the cheese an open structure. Typically, in full and reduced fat cheeses, the protein matrix is open, with spaces occupied by fat globules. In low fat cheese, the matrix is compact (Rahimi et al, 2007; Aryana and Haque, 2001; McMahon, 1996). The low fat cheese, without fat cavities and channels, is a dense aggregation of casein, hence the rubbery texture. In addition, there is limited breakdown of  $\alpha_1$ -casein during ripening which contributes to the firm texture (Mistry, 2001.)

To overcome these deficiencies, three different categories of modifications have been investigated. The categories include cheese-make process modifications, starter and adjunct culture use, and the use of fat replacers. Increasing the moisture content has been suggested to improve the properties of low-fat cheeses (Rodriguez, 1998), yet others have suggested that it is necessary to maintain the same moisture in nonfat as found in full fat (Mistry, 2001). One popular approach is the use of fat replacers to maintain the same moisture:solids ratio.

#### **Use of Fat Replacers in Low Fat Cheeses**

The definition of fat-free is less than 0.5 g fat per serving size (approx 1.8 % fat in cheese). Low fat is 3 g fat per serving size (approx 10.7% fat in cheese) (21 CFR 101.62). Therefore, the high protein cheeses that will be produced in this project fits into the low fat claim category. According to the CRF (21 CFR 130.10) "deviations from noningredient provisions of the standard identity are permitted in order that the substitute food possesses performance characteristics similar to those of the standardized food". Other ingredients are also allowed "to improve texture, add flavor, prevent syneresis, extend shelf life, improve appearance, or add sweetness so that the product is not inferior in performance characteristics to the standardized food ...". The fat replacers proposed in this project do have defined functionalities which may improve the texture, prevent syneresis, and improve appearance of low fat/high protein cheese.

The use of fat replacers has been extensively explored to improve the texture and appearance of low and nonfat cheeses (Rahimi et al., 2007). Fat replacers based on microparticulated protein or carbohydrates have been recommended for use in cheese products (Romeih et al., 2002). They can mechanically entrap water giving the sense of lubricity and creaminess, but cannot positively impact the flavor defects. Table 2 lists some of the recent publications that have investigated the use of fat replacers in cheese. The fat replacers were added to the milk (or blended with cream) prior to cheese making. Only one reference estimated the amount of fat replacer retained in the curd (McMahon, 1996). This reference assumed 70% retention. Therefore, the use levels given in Table 2 can be reduced by 30% as the starting concentration since no loss is expected if the fat replacers are added to the cheese blends prior to extrusion.

Table 2. Some studies that have investigated the use of fat replacers in low fat cheeses

Cheese type	Fat replacers used individually	Functional changes compared to low or reduced fat	Reference
Low-fat white-brined cheese	0.7 or 1.4% Oat beta-glucan	Improved texture but lower flavor and color	Volikakis 2004
Low-fat fresh Kashar	1% Simplesse 100 or 1% Dairy-Lo or 5% Raftiline HP	Simplesse and Raftiline improved the texture and sensory properties up to 60 days	Koka 2004
Imitation Mozzarella cheese	8-43 % dry basis Novelose 240 (fiber)	Decreased hardness	Noronha 2007
Low-fat Iranian White Cheese	0.75 % Gum Tragacanth	Improved texture, water binding, decreased hardness	Rahimi 2007
Low-fat Cheddar	Beta-glucan Nutrim	Decreased hardness and sensory scores	Konuklar 2004
Low-fat white pickled cheese	0.5% Simplesse 100 or 0.5% Dairy-Lo or 0.5% Perfectamyl or 0.4% Satiagel	Dairy-Lo and Satiagel were similar in texture to low-fat sample	Kavas 2004
Low-fat white brined cheese	1% Simplesse 100 or 0.125 % Novagel NC200	Improved texture, Simplesse also showed improved appearance	Romeih 2002
Low-fat Cheddar cheese	1% Dairy Lo or 1.5% Simplesse or 1.2% Stellar, or 0.2% Novagel	Simplesse and Novagel imparted discontinuity to the casein matrix	Aryana 2001
Low-fat Mozzarella	0.6% Simplesse or 0.6% Stellar or 2.5% Dairy-Lo or 2.5% Novagel	Cheeses with Stellar and Simplesse showed greater initial meltability but all cheeses showed the same meltability after 21 days.	McMahon, 1996

The microstructure of low-fat cheese made with different fat replacers (Simplesse, Dairy Lo, Stellar, and Novagel) was compared to a full fat and a low-fat Cheddar cheese (Aryana and Haque, 2001). The electron micrographs of cheeses in this study containing full fat showed numerous holes and had a smooth surface. The holes were from the delipidation of the samples. The low-fat cheese showed less holes and a rippled surface. The addition of protein based fat replacers (Simplesse and Dairy-Lo) showed fewer ripples, and the authors correlated the amount of ripples to the hardness of the cheeses, in this case, the low-fat cheeses made with protein based fat replacers were less hard than the low-fat cheese without fat replacers. The Simplesse particles (0.75 micron) were visible in the matrix, interrupting the casein network. The surfaces of low-fat cheeses made with carbohydrate fat replacers were undulated and rough. The cheese made with Novagel showed large particles (50 micron) that were fewer in quantity than the Simplesse, but also interrupted the network. Their conclusions were that Simplesse and Novagel softened low-fat Cheddar cheese by imparting discontinuity to the casein matrix. McMahon et al. (1996) investigated the microstructure of low-fat Mozzarella cheese and low-fat Mozzarella made with two protein based (Simplesse and Dairy-Lo) and two carbohydrate based (Stellar and Novagel) fat replacers. The only fat replacer that increased the openness of the cheese structure was Novagel because it was too large to be embedded in the protein matrix; instead it created large serum channels in the cheese. The Novagel particles were 30-300 micron compared to 0.5 microns for Stellar while the protein based fat replacers contained particles of 5-10 and 0.5 to 1.0 micron for Dairy-Lo and Simplesse respectively.

## **Applications of Extruders in Food Processing**

Extruders can be configured for low, medium, or high shear by the sequence of the screws and paddles in the barrel. The screws promote conveyance and the paddles interrupt the flow and create shear and back pressure. Twin-screw extruders are more functional than single screw extruders and they can be used at higher moisture levels (>40 %) as compared to single screw extruders (<35) (Walsh and Carpenter, 2008). Extruders are used in the food industry for the production of direct expanded (high shear), low density snacks, flat breads, and breakfast cereals; medium shear products such as animal feeds and textured vegetable protein; and low-shear products including pasta.

A recent patent (Mueller 2005, USP 6942888) describes the use of a twin-screw extruder for producing pieces of cheese from blocks of compressed aged or unaged cheese curds. This process is to produce pieces of cheese that have an exact weight from blocks of compressed curds with an overall goal to eliminate storage and aging of the large cheese blocks for later cutting into consumer-sized pieces of cheese. This process uses temperatures of 30 C in the extruder and a cooling section at 7.2 C and the extruder is configured with intermeshing feed screws, which increase in thickness from the input to the cheese output end. This arrangement is to increase pressure in the system to compress the cheese pieces into a uniform, homogenous cheese flow. The cheese then enters a cooling device at 7.2 C where the cheese solidifies so a properly shaped end product results. In the process described in this proposal, a cooling device was not included but may be added to the extruder if needed. We have previously used a cooling device for the production of textured whey protein for use as a meat extender. Additionally, we may need to press the extruded product to remove excessive air.

The majority of extruded snacks on the market fall into the direct expanded category and the most popular are the corn based such as corn curls, balls and rings. Other corn-based snacks such as Doritos are also extruded products along with potato snacks including Pringles, Baked Lays and Munchos. Seafood based snacks are produced from shrimp and fish mixed with a starch to form a dough that is expanded via extrusion. Co-extruded snacks have an extrusion produced outer shell with a filling such as peanut or cheese filled pretzels or fruit filled cereal based products. Examples of breakfast cereals that are derived from extrusion include crisp rice, sugarcoated fruit flavored rings, cornflakes, cinnamon and sugar graham shapes, coco balls, bran/wheat flakes, frosted stars and flakes.

Textured vegetable protein is an example of medium shear and it can be formed into meat-free (vegetarian) hot dogs, hamburgers, chicken patties/shapes, hams, and sausage, pepperoni, and bacon. Lose meat products are incorporated into tacos, canned chili and spaghetti sauces. Textured vegetable protein is currently produced from soy flour, sesame flour, wheat gluten and canola and rapeseed concentrates.

In conjunction with the screw and paddle sequence, the speed of the co-rotating screws and the temperature during extrusion significantly influence the texture of the final product. High temperatures will melt (denature the proteins) to allow for new protein-protein interactions. The rate of speed will influence the amount of input shear, residence time in the barrel, and also denature the proteins. For this project, we are looking to produce a product using medium shear conditions at moderate temperatures with the goal to change the protein network and evenly distribute the fat, moisture, protein and air to positively impact the Cheddar texture.

## **References**

- Aryana, KJ, ZU Haque. 2001. Effect of commercial fat replacers on the microstructure of low-fat Cheddar cheese. *Int J Food Sci Technol* 36:169-177.
- Kavas, G., G Oysun, O Kink, H Uysal. 2004. Effect of some fat replacers on chemical, physical and sensory attributes of low-fat white pickled cheese. *Food Chem.* 88:381-388.
- Koca, N. and M Metin. 2003. Textural, melting and sensory properties of low-fat fresh kashar cheeses produced by using fat replacers. *Int Dairy J.* 14:365-373.
- Konuklar, G, GE Inglett, K Warner, CJ Carriere. 2004. Use of a beta-glucan hydrocolloidal suspension in the manufacture of low-fat Cheddar cheeses: textural properties by instrumental methods and sensory panels. *Food Hydrocolloids* 18:535-545.
- McMahon, DJ, MC Alleyne, RL Fife, CJ Oberg. 1996. Use of fat replacers in low fat Mozzarella cheese. *J Dairy Sci* 79:1911-1921
- Mistry, VV. 2001. Low fat cheese technology. *Int. Dairy J.* 11:413-422.
- Noronha, N. ED O'Riordan, M O'Sullivan. 2007. Replacement of fat with functional fibre in imitation cheese. *Int Dairy J.* 17:1073-1082.
- Rahimi, J. A Khosrowshahi, A Madadou, S. Aziznia. 2007. Texture of low-fat Iranian white cheese as influenced by

- gum trigacanth as a fat replacer. *J Dairy Sci* 90-4058-4070.
- Rodriguez, J., T. Requena, H. Goudebranché, M. Juárez. 1999. Effect of different membrane separation technologies (ultrafiltration and microfiltration) on the texture and microstructure of semihard low-fat cheese. *J Ag Food chem.* 47:558-565.
- Romeih, E.A., A. Michaelidou, C.G. Biliaderis, G.K. Zerfiridis. 2002. Low-fat white-brined cheese made from bovine milk and two commercial fat mimetics: chemical, physical and sensory attributes. *Int Dairy J.* 12:525-540
- Volkakis, I., C.G. Biliaderis, C. Vamvakas, G.K. Zerfiridis. 2004. Effects of a commercial oat-beta-glucan concentrate on the chemical, physico-chemical and sensory attributes of a low-fat white-brined cheese product. *Food Res Int* 37:83-94.
- Walsh, M.K. and C.E. Carpenter. 2008. Creating whey protein-based meat analogs using twin-screw extrusion. In *Whey: Processing, Functional Properties, and Health Benefits*. Dr. Peter Huth, Charles Onwulata, Ph.D. Eds. Blackwell Publishing, Ames, IA, USA.

## MATERIALS AND METHODS

Full fat aged and nonfat cheeses will be ground to determine the suitable size (0.5 to 2 mm) for extrusion using a Hobart mixer/grinder. Cheeses will be added to the Hobart mixer/grinder based on the composition of each cheese as stated in Table 1 and below in Table 3.

Table 3. Blends of cheeses to achieve 10g protein per 28g serving size

Number	Full fat, aged Old Juniper (%)	Full fat, aged Aggiano (%)	Amt Low fat (%)	Amt nonfat (%)	g fat/28 g	g protein /28 g
1	15			85	1.4	10.1
2	10			90	1	10.27
3		15		85	1.6	10.25
4		10		90	1.1	10.37

Values will vary slightly (increase in protein and fat) due to the moisture content of the blends. There is room for an additional 1% of non-protein ingredient before protein concentrations fall below 10 g/serving. This can also be offset by the use of Temp Pro WPC 80 in combination with the non-protein ingredients.

The full fat, aged Cheddar cheeses can get pasty if they are ground in the Hobart alone, therefore, the full fat aged and nonfat cheeses will be added together into the Hobart mixer/grinder to reduce the pastiness of the cheese. We believe the smallest particle size (0.5 mm) will be optimum as a starting material for the extruder if the texture is not pasty. Otherwise, we may need to increase the cheese particle size to reduce the pastiness.

The extruder we will be using is an APV Baker MPF19 twin-screw extruder with a length/diameter barrel of 25. The temperature zones (5) along the barrel are controlled with a CAL3200 Autotune temperature controller. The sample is introduced into the barrel via a KTron volumetric dispenser. A new twin auger will need to be purchased for the dispenser based on the particle size of the cheese as the starting material. Based on the size of the cheese particles, a double or single screw auger will be purchased to replace the current dry feed auger. The extruder also has a liquid feed inlet that dispenses in the first barrel zone.

The blended cheeses will be extruded at various extruder screw and paddle configurations using a twin screw extruder at temperatures between 40 and 60 C to determine the optimum extruder configurations that yield a cohesive product with evenly incorporated air pockets. The screw and paddle configurations will be sequenced to provide a moderate amount of shear. Generally, one screw and paddle sequence can be tried per day per cheese blend.

Initially, the cheeses will be extruded and screw speed and temperature varied to visually inspect the changes in the cheese matrix. The cheeses will also be analyzed for texture as stated below in the methods to determine the influence of screw speed, screw configuration and temperature on the final product texture. We will start with a medium shear configuration and temperature and screw speed settings to allow product flow through the extruder. We want to limit the temperature to approximately 60 C to eliminate any volatile flavor loss and extensive protein:protein crosslinking. We may need to press the cheese after extrusion if there is excess air in the product.

Fat replacers that we will investigate as texture modifiers are given below in Table 4. The fat replacers will be added to the cheeses while they are in the Hobart mixer/grinder to ensure even distribution. Initially we will use 30% of the lowest use level of each fat replacer. We will also use double that amount to investigate the influence of the fat replacer on the cheese texture.

Additionally, we can use both the Temp Pro™ heat stable whey in conjunction with the other fat replacers to increase the amount of protein if necessary in the sample. We also may need to add liquid to the sample during extrusion if hydration of the fat replacer is necessary. The liquid addition can be set to the microgram volume and increased to hydrate the fat replacer to ensure the cheese flows through the extruder.

Table 4. Texture modifying ingredients

Product name and manufacturer use level for cheese	Composition	Functionality
<sup>1</sup> Temp Pro™ Use level is 0.4 to 4%	80% WPC	Heat stable whey protein, stays fluid at retort temperatures, will not interact with casein, matrix interruption
<sup>2</sup> Novagel NC 4230 cellulose gel Use level is 0.125-2%	Microcrystalline cellulose	Fat replacer  Gel particles interrupt casein structure by reacting with kappa casein to form a curd that can entrap moisture
<sup>3</sup> Simplex 100 use level 0.5-1%	54 % whey protein	Microparticulated whey provides matrix interruption, creaminess, UHT and retort stable, provides for uniform moisture distribution
<sup>4</sup> Vitacel MCG 611F Use level 0.4-2 %	Insoluble microcrystalline cellulose	Matrix interruption, fat imitation
<sup>4</sup> Vitacel Plus HF Use level 0.4-2 %	Insoluble and soluble fibers	Matrix interruption, fat imitation

<sup>1</sup>Leprino Foods

<sup>2</sup>FMC BioPolymer, Can be blended in 0.4% Temp Pro™ and dispersed into ground cheese

<sup>3</sup>CP Kelco Co

<sup>4</sup>JR Rettenmaier

All extruded Cheddar cheese samples will be analyzed for texture using the Texture Profile Analysis assay. Additionally, the moisture, protein and fat concentrations and color of each cheese will be determined.

All analysis will be done in triplicate for each cheese sample that shows improved texture compared to nonfat and reduced fat Cheddar cheese over a shelf life of 4-6 months. AOVA will be used to determine the significant differences in texture based on the treatments (fat replacers, amount, extrusion conditions etc).

All cheeses used in this study will be manufactured in the USU Dairy Products Laboratory under the direction of Don McMahon.

The protein concentration will be determined by the Kjeldahl method and converting the nitrogen measurement to protein content by multiplying by 6.38. The texture will be analyzed using a Texture Analyzer (TA-XT) equipped with a flat plunger as described by Volikakis et al., 2004 and McMahon (unpublished data). The color of cheese at days will be determined with a Hunter Lab system to generate L, a and b values. The moisture will be determined by drying in an oven and the fat content will be determined by the babcock or Leco methods.

All analysis will be done in triplicate for each cheese sample that shows improved texture compared to nonfat and reduced fat Cheddar cheese over a shelf life of 4-6 months. All data will be analyzed by ANOVA to determine the significant differences among treatments.

## RESULTS AND DISCUSSION

Objective 1: Various USU Cheddar cheeses (USU Aged Old Juniper, Aged Aggiano, and nonfat Cheddar) will be ground to yield particle sizes of 0.5, 1 and 2 mm to determine the appropriate size that would be suitable to use as a starting material for twin-screw extrusion.

We have initiated this objective and we have grounded various cheeses (Aggiano, nonfat Cheddar and Aged Old Juniper cheese) to a particle size of about 2 mm. The nonfat cheese is facile to grind, while the full fat cheeses are sticky. We plan to blend the cheeses prior to grinding to minimize the stickyness of the grounded cheeses. We have also changed the configuration of our extruder to be able to feed the cheeses through the hopper into the extruder.



# Effect of High Intensity Ultrasound (HIU) of Functional Properties of Whey Proteins

SILVANA MARTINI: Utah State University

## ABSTRACT/SUMMARY

This project will test the hypothesis that high intensity ultrasound (HIU) can improve the functional properties of whey proteins. HIU will be applied to whey protein solutions and the effect of acoustic waves on functional properties crucial to the manufacture of beverages will be evaluated. The functional properties that will be analyzed are: heat stability, clarity/turbidity, and flavor. Different HIU settings (power, duration of the signal) will be tested in combination with several environmental conditions such as protein concentration (to simulate the concentrations found in the whey protein production process) and temperature. HIU treated whey will be analyzed for flavor and exposed to different temperatures to reproduce a hot filling, pasteurized and UHT processing at three pH values and analyzed for clarity/turbidity and solubility. The presence of off-flavors such as overall aroma intensity, cooked, diacetyl, sourness, cucumber, brothy, cardboardy, soapy, bitterness and astringency will be quantified among the treatments and compared to a control whey protein solution (non-HIU treated). The acoustic setting that results in the greater improvement on heat stability and turbidity will be chosen to evaluate the effect of protein concentration on these properties.

## BACKGROUND

Ultrasound techniques use sound waves of frequencies higher than those perceived by the human hearing (> 18 kHz). Acoustic waves have been used in medical, mechanical and food applications. Specifically in the food industry, ultrasound has been used to monitor (Saggin and Coupland, 2004; McClements and Povey, 1992, McClements *et al.*, 1990; Martini *et al.*, 2005a-c, Ueno *et al.*, 2002, 2003) and induce (Higaki *et al.*, 2001; Martini *et al.*, 2008) lipid crystallization, to induce the crystallization of sugars and ice (Chow *et al.*, 2003, 2005), to evaluate the rheology of food materials (Mert and Campanella, 2007; Maleky *et al.*, 2007), and to reduce the size of carbohydrate molecules (Kjartansson *et al.*, 2006a,b; Baxter *et al.*, 2005). Other food applications of ultrasound include pasteurization, emulsification, de-foaming, and de-gassing of soft food materials. Acoustic waves can be applied to materials in the form of low intensity waves to passively monitor (no disruption of molecular entities) physical changes in the material caused by non-acoustic sources; or as high intensity waves, where disruption of molecular entities or changes in the physicochemical characteristics of the materials are originated by the acoustic waves.

The velocity of sound in liquids is approximately 1,500 m/s resulting in acoustic wavelengths of 7.5 to 0.015 cm for frequencies between 20 kHz and 10 MHz. The higher the frequency the smaller the wavelength. Clearly, no direct coupling of the acoustic field with chemical species on a molecular level can account for the effects of acoustic waves on the properties of the material. Instead, the effects of ultrasound derive from the generation of cavitation or bubbles that oscillate and eventually collapse originating several physical mechanisms, such as an increase in temperature, shear forces and pressures. The presence and intensity of these mechanisms is dependant on the nature of the material under study and on the environmental conditions that surround that material. High intensity ultrasound (HIU) has been commercially used in different food science applications (<http://www.foodscience.csiro.au/ultrasonics.htm>). Emulsification, dispersion of solids, crystallization, de-gassing, and extraction

are some of the current commercial uses of acoustic waves. The advantages of using ultrasound over other technologies are (Patist and Bates, 2008):

- i) low cost,
- ii) reproducibility and reliability,
- iii) capability of measuring in a continuous process (on-line, real-time measurements),
- iv) non invasive,
- v) ability to measure materials properties through opaque media such as cheese and shortenings.

Several researches demonstrate that high intensity ultrasound (HIU) can be used to cleave polymers and to change the conformation of proteins. Wu *et al.* (2008) evaluated the degradation of chitosan using HIU. They found that the degradation rate is proportional to the  $M_w^{-3}$  (where  $M_w$  is the molecular weight of the polymer) and to the ultrasonic environments and parameters such as acoustic intensity, temperature, polymer concentration, and ionic strength. Similar results were found by Kasaai *et al.* (2008).

HIU has also been used to tenderize meat (Jayasooriya *et al.*, 2007). Ultrasound was used to disrupt cell membranes and increase meat tenderness. Other mechanisms proposed include the physical weakening of muscle structure, or the activation of proteolysis by releasing cathepsins from lysosomes and/or  $Ca^{++}$  ions from intracellular stores activating calpains (Dolatowski *et al.* 2007). Some studies show increased tenderness with low frequency ultrasound (22–40 kHz) treatment (Dickens *et al.*, 1991, Dolatowski, 1988, 1989). Zayas and Gorbatow (1978) also reported improvement of the tenderness of meat immersed in brine, with a sonifying frequency of 22 kHz and 1.5-3 W.  $cm^{-2}$ .

HIU has also found application in the dairy industry. In 2002, Sanchez *et al.* reported an acceleration of the proteolysis of Mahon cheese caused by ultrasound. They showed how cheeses that have been acoustical brined exhibited higher concentrations of free amino acids.

Several studies showed the effect of HIU on the functional properties of whey proteins. Kresic *et al.* (2008) showed an increase in water solubility of whey proteins treated with HIU. They suggested that HIU enhanced protein solubility by changing protein conformation and by decreasing its molecular weight. Jambrak *et al.* (2008) evaluated the effect of ultrasound on the solubility and foaming properties of whey protein suspensions. They found that both functional properties were improved when HIU was used. However, the results were dependant on the acoustic frequency used. Higher frequencies (40 kHz) were not as efficient as lower ones (20 kHz).

Stathopoulos *et al.* (2004), and Villamiel and de Jong (2000) reported conformational changes in HIU treated proteins. The first group of researchers reported the formation of aggregates with high  $\beta$ -content in non-dairy proteins such as myoglobin and lysozyme. The second group of researchers found denaturation of whey proteins when ultrasound, in combination to heat was applied to milk.

Summarizing the literature review, HIU can be used to modify the functional properties of proteins by either:

1. changing the conformation (enzyme inactivation)
2. inducing proteolysis
3. inducing cleavage

Even though some of the research described above indicates that HIU improves the functional properties of proteins, there is no research showing its effect on the flavor of whey proteins. We propose to apply HIU to whey proteins and evaluate the effect of the acoustic waves on their functional properties such as heat stability (solubility) and clarity/turbidity, for application in beverages. We would like to couple these experiments with the analysis of the effect of HIU on the flavor of the whey protein solutions.

The solubility of proteins is of primary importance since it influences other functional properties such as foam formation, emulsification, gelation, and whipping. The solubility of proteins relates to the hydrophobic (protein-protein) and hydrophilic (protein-solvent) interactions. Conditions which favor protein-solvent interactions, favor protein solubility whereas conditions which favor protein-protein interactions lead to protein aggregation and precipitation (Scopes, 1993).

The major environmental factors that influence protein solubility include pH, temperature and ionic strength and their interactions. Generally, proteins have less solubility at their isoelectric point (pI) due to increased protein-protein interactions from the lack of charges on the proteins. At pH values higher and lower than the pI, the proteins are highly charged and interact with the solvent and repel each other, leading to high solubility. This pH induced change in solubility is reversible.

In general, protein solubility increases with an increase in temperature from 0 to 50°C. Higher temperatures (closer to the protein denaturation temperature) lead to protein unfolding due to loss of secondary and tertiary structure. Unfolded proteins are likely to aggregate via exposed hydrophobic interactions, which may not be reversible.

The amount of salt in the solution influences the solubility. Physiological ionic strength is approximately 0.15 M and at salt concentrations lower and higher than this value, proteins may aggregate and precipitate. At high salt concentrations, the salt solvates the protein-bound water exposing hydrophobic patches on the proteins which leads to an aggregation and precipitation. Optimum salt concentrations results in protein-solvent interactions with the salt ions decreasing the electrostatic interactions between protein molecules. At low salt concentrations, the protein-protein interactions are favored due to reduced electrostatic repulsion.

A recent study by Pelegrine and Gasparetto (2005) investigated the influence of temperature and pH on the solubility of whey protein (Figure 1). The pI of whey protein collectively was determined to be at pH 4.5. This decrease in solubility was further lowered with an increase in temperature up to 60 °C. There was also limited solubility at neutral pH (6.8) with an increase in temperature, presumably due to denaturation at this temperature. A pI value of 5.65 resulted in the highest solubility, even at the highest temperature tested (60 °C). In general, the immunoglobulin fraction of whey protein is the most heat sensitive, followed by serum albumin, beta-lactoglobulin, alpha-lactalbumin and the proteose peptone fraction.

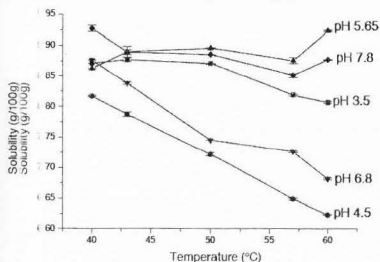


Fig. 1. Effect of pH and temperature on the whey protein solubility.

The solubility of whey protein is also influenced by processing conditions (Schmidt *et al.*, 1984) as well as concentration. Generally, the solubility of commercially available whey protein in the form of a concentrate (40-90 % protein) is in the range of 25-80%, but a clear solution can only be formed at low protein concentrations (3-4%). Whey protein can be modified using limited proteolysis to increase the solubility over a range of pH values by modifying the collective pI. Recent work by Foegeding (Davis *et al.*, 2005) on the influence of protease type and extent of hydrolysis showed that partial hydrolysis of whey proteins improves their thermal stability and functionality, but excess hydrolysis reduces heat stability and functionality. They concluded that the controlled hydrolysis of whey protein is necessary to provide the desired thermal stability and functional properties.

Enzymatically hydrolyzed whey protein is available commercially (e.g. Hilmar Ingredients and Grande Cheese Ingredients) for use in nutritional, sports and medical applications. These products generally have differing degrees of hydrolysis.

Very closely related to the solubility of a protein is its heat stability. This functional property is especially important for the use of whey proteins in clear beverages since a process of extreme heating is applied to these products before leaving the production plant. These heating processes may include hot filling conditions (85°C for 1 min), pasteurization (75°C for 20 seconds) and UHT conditions (135°C for 2 seconds). When heated, proteins might aggregate or denature, causing the appearance of turbidity which limits the use of whey proteins in some beverage applications. Improving the heat stability of whey proteins will broaden their use for clear beverage applications (Vardhanabhuti and Foegeding, 2008).

Independently of the functional properties of whey proteins, their use for food applications is limited to the presence of off-flavors such as brothy, diacetyl, sour bitter and astringent (Carunchia Whetstone *et al.*, 2005). Dr. Drahnke's group characterized the flavor profile of seven WPC and eight WPI from different cheese manufacturers. They found that, depending on the manufacturer, these proteins exhibited sweet aromatic, cardboard/wet paper, animal/wet dog, soapy, brothy, cucumber and cooked/milky flavors. These off-flavors might be originated by lipid oxidation (Swaisgood, 1996) and/or proteolysis (Holmes *et al.*, 1977; Amundson, 1984) occurring in the whey. Beecher *et al.* (2008) also reported the presence of astringency in whey protein beverages. They evaluated the effect of viscosity and pH values on the presence and intensity of this attribute. They reported that viscosity did not have an effect on the astringency level of the proteins, however, they showed that at pH 6.8 the whey beverage reached a minimum level of astringency, while at pH 3.4, the astringency level was maximum. These authors suggested that the presence of astringency was a consequence of protein aggregation and they proposed that interactions

between positively charged proteins and negatively charged saliva might be related to the generation of astringency. It is clear from these results that the presence of astringency is a direct consequence of the protein structure and conformation.

Depending on the effect of HIU on whey proteins we expect to improve their functional properties in different forms and manners than previously investigated. If proteins are cleaved or a change in the conformation is generated by HIU application, we expect to increase the solubility of whey proteins in water, decrease turbidity and improve their heat stability. These improvements will find direct applications in the beverage industry. The capability of HIU to randomly cleave proteins will improve the flavor of the proteins by inhibiting off-flavors formation and by generating a pool of flavorless amino acids. Although some studies (Chemat *et al.*, 2004) reported that HIU can be used to generate hydroperoxides and can therefore increase lipid oxidation, we expect to use relatively low ultrasound power levels and we do not expect any lipid oxidation in our samples. The different HIU settings included in the project design of this proposal, will allow us to identify and optimize the best HIU conditions to improve the functional properties of whey proteins.

## References

- Amundson, C.H. 1984. Residual milk clotting enzymes. Pages 2-10 in Proc. 1984. Whey Products conf., Chicago, IL.
- Baxter S. R., Zivanovic S., Weiss J. 2005. Molecular weight and degree of acetylation of high-intensity ultrasonicated chitosan. *Food Hydrocolloids*. 19(5):821-830.**
- Beecher, J.W., Drake, M.A., Luck, P.J., and Foegeding, E.A. 2008. Factors regulating astringency of whey protein beverages. *J. Dairy Sci.* 91:2553-2560.
- Carunchia Whetstone, M.E., Croissant, A.E. and Drake, M.A. 2005. Characterization of dried whey protein concentrate and isolate flavor. *J. Dairy Sci.* 88:3826-3839.
- Chemat, F., Grondin, I., Costes, P., Moutoussamy, L., Shum Cheong Sing, A., and Smadja, J. 2004. High power ultrasound effects on lipid oxidation of refined sunflower oil. *Ultrasonics Sonochemistry* 11, 281-285. <http://www.sciencedirect.com>
- Chow, R., Blindt, R., Chivers, R., and Povey, M. 2005. A study on the primary and secondary nucleation of ice by power ultrasound. *Ultrasonics*, 43:227-230.
- Chow, R., Blindt, R., Chivers, R., and Povey, M. 2003. The sonocrystallization of ice in sucrose solutions: primary and secondary nucleation. *Ultrasonics* 41:595-604.
- Dickens, J.A., Lyon, C.E., Wilson, R.L. 1991. Effect of ultrasonic radiation on some physical characteristics of broiler breast muscle and cooked meat. *Poultry Sci.* 70:389-396.
- Davis, J.P., Doucet, D., and Foegeding, E.A. 2005. Foaming and interfacial properties of hydrolyzed  $\beta$ -lactoglobulin. *J. Colloid. Inter. Sci.* 288:412-422.
- Dolatowski, Z.J. 1988. Ultrasonics 2. Influence of ultrasonics on the ultrastructure of muscle tissue during curing. *Fleischwirtschaft* 68:1301-1303.
- Dolatowski, Z.J. 1989. Ultrasonics 3. Influence of ultrasonics on the production technology and quality of cooked ham. *Fleischwirtschaft* 69:106-111.
- Dolatowski, Z.J., Stadnik, J., and Stasiak, D. 2007. Applications of ultrasound in food technology. *Acta Sci. Pol., Technol. Aliment.* 6:89-99.
- Higaki, K., Ueno, A. Koyano, T. and Sato, K. 2001. Effects of Ultrasonic Irradiation on Crystallization Behavior of Tripalmitoylglycerol and Cocoa Butter. *J. Am. Oil Chem. Soc.*, 78:513-518.
- Holmes, D.G., Duersch, J.W. and Ernstrom, C.A. 1977. Distribution of milk clotting enzymes between curd and whey and their survival during Cheddar cheese making. *J. Dairy Sci.* 60:862-869.
- Jayasooriya, S.D., Torley, P.J. D'Arcy B.R., Bhandari, B.R. 2007. Effect of high power ultrasound and ageing on the physical properties of bovine Semitendinosus and Longissimus muscles. *Meat Sci.* 75:628-639.
- Jambrak, A.R., Mason, T.J., Lelas, V., Herceg, Z. and Herceg, I.L. 2008. Effect of ultrasound treatment on solubility and foaming properties of whey protein suspensions. *J. Food Eng.* 86:281-287.
- Kasaai, MR, Arul, J, and Charlet, G. 2008. Fragmentation of chitosan by ultrasonic irradiation. *Ultrasonic Sonochemistry*. 15:1001-1008.
- Kjartansson G.T., Zivanovic S., Kristbergsson K., Weiss J. 2006a. Sonication-assisted extraction of chitin from shells of fresh water prawns (Macrobrachium rosenbergii) *J. Agri. Food Chem.* 54(9):3317-3323.

- Kjarraansson G.T., Zivanovic S., Kristbergsson K., Weiss J. 2006b. Sonication-assisted extraction of chitin from North Atlantic shrimps (*Pandalus borealis*) *J. Agri. Food Chem.* 54(16); 5894-5902.
- Kressic G., Lelas V., Jambrak AR, Herceg Z, and Brncic SR. 2008. Influence of novel processing technologies on the rheological and thermophysical properties of whey proteins, *J. Food Engineering* 87:64-73.
- Mallicky, F., Campos R. and Marangoni, A.G. 2007. Structural and Mechanical Properties of Fats Quantified by Ultrasonics *J. Amer. Oil Chem. Soc.* 84:331-338.
- Maruttiini, S., Suzuki, A.H. and Hartel, R.W. 2008. Effect of high intensity ultrasound on crystallization behavior of anhydrous milk fat *J. Am. Oil Chem. Soc.* 85:621-628 (DOI 10.1007/s11746-008-1247-5).
- Maruttiini, S., Herrera, M.L. and Marangoni, A.G. 2005a. New Technologies to Determine Solid Fat Content on-line *J. Am. Oil Chem. Soc.* 82:313-317.
- Maruttiini, S., Bertoli, C., Herrera, M.L., Neeson, I. and Marangoni, A.G. 2005b. Attenuation of Ultrasonic Waves: Influence of Microstructure and Solid Fat Content *J. Am. Oil Chem. Soc.* 82:319-328.
- Maruttiini, S., Bertoli, C., Herrera, M.L., Neeson, I. and Marangoni, A.G. 2005c. In-situ monitoring of Solid Fat Content by Means of p-NMR and Ultrasonics *J. Am. Oil Chem. Soc.* 82:305-312.
- McClements, D. J., and Povey, M. J. W. 1992. Ultrasonic analysis of edible fats and oils *Ultrasonics* 30, 383-388. <http://www.sciencedirect.com>
- McClements, D. J., Povey, M. J. W., Jury, M., and Betsanis, E. 1990. Ultrasonic characterization of a food emulsion *Ultrasonics* 28, 266-272. <http://www.sciencedirect.com>
- Mertr, B. and Campanella, O.H. 2007. Monitoring the rheological properties and solid content of selected food materials contained in cylindrical cans using audio frequency sound waves *J. of Food Eng.* 79 (2007) 546-552.
- Nalith, S. 2004. Complexation behavior of proteins with polyelectrolytes and random acrylic polyampholytes using turbidimetric titration. *J Chem Technol & Biotechnol.* 62(3):295-300
- Pelegri, D.H.G., and CA Gasparetto. 2005. Whey protein solubility as function of temperature and pH. *Lebensm.-Wis. U-Technol.* 38 (77-80).
- Saggiini, R. Coupland, J.N. 2004. Shear and Longitudinal Ultrasonic Measurements of Solid Fat Dispersions *J. Am. Oil Chem. Soc.* 81, 27-32.
- Sanchez, E.S., Simal, S., Femenia, A. Llull, P., and Rossello, C. 2001. Proteolysis of Mahon cheese as affected by acoustic-assisted brining. *Eur. Food Res. Technol.* 212:147-152.
- Schmidt, R.H., VS Packard, and HA Morris. 1984. Effect of processing on whey protein functionality. *J. Dairy Sci.* 67:2723-2733.
- Scopes, R.K. 1993. Protein Purification, Principles and Practice, 3<sup>rd</sup> editin. Springer, New York. USA.
- Statthopoulos PB, Scholz GA, Hwang YM, Rumfeldt JAO, Lepock JR and Meiring EM. 2004. Sonication of proteins causes formation of aggregates that resembles amyloid. *Protein Sci.* 13:3017-3027.
- Swaaisgood, H.E. 1996. Characteristics of milk. Pages 841-876 in Food chemistry. 3<sup>rd</sup> Ed. O.R. Fennema, ed. Marcel Dekker, New York, NY.
- Uenno, S., Ristic, R., Higaki, K. and Sato, K. 2003. In Situ Studies of Ultrasound-Stimulated Fat Crystallization Using Synchrotron Radiation *J. Phys. Chem. B*, 107 (21), 4927-4935.
- Uenno, S., Sakata, J., Takeuchi, M and Sato, K. 2002. Study for Ultrasonic stimulation effect on promotion of crystallization of triacylglycerols *Photon Factory Activity Report* pp 182.
- Varadhanabhuti, B. and Foegeding, E. A. 2008. Effects of dextran sulfate, NaCl, and initial protein concentration on thermal stability of  $\beta$ -lactoglobulin and  $\alpha$ -lactalbumin at neutral pH. *Food Hydrocolloids.* 22:752-762.
- Villilamiel, M., and de Jong, P. 2000. Influence of high intensity ultrasound and heat treatment in continuous flow on fat, proteins and native enzymes of milk. *J. Agric. Food Chem.* 48:472-478.
- Wu J T., Zivanovic S., Hayes D.G., Weiss J. 2008. Efficient reduction of chitosan molecular weight by high-intensity ultrasound: underlying mechanism and effect of process parameters. *J Agri Food Chem.* 56:5112-5119.
- Zayyas, J.F. and Gorbato, W.M. 1978. Use of ultrasonics in meat technology. *Fleischwirtschaft.* 6:1009-1012.

## MATERIALS AND METHODS

### Application of HIU

Two different protein concentrations will be used in this project. Concentrations of 3 and 45% of protein will be chosen to represent the protein content in whey before and after concentration prior to spray drying. Each protein solution will be sonicated using a 20 kHz acoustic wave. In addition, different acoustic setting will be tested. Two

power levels (50 and 150 W/cm<sup>2</sup>), two different signal durations (5 and 15 min), and two different application temperatures (25 and 70°C - just before the average denaturation temperature of whey proteins). The objective of this experimental design is to evaluate the effect of different acoustic (power, signal duration) and environmental conditions (temperature) on the functional properties of whey proteins. Therefore, the number of treatments are: 2 (protein concentrations) x 2 (acoustic power) x 2 (signal durations) x 2 (temperatures) = 16 treatments.

### **Measurement of Functional Properties**

After sonication, the functional properties of the 16 solutions obtained from part (a) will be measured. To standardize the measurement of functional properties, heat stability and turbidity will be measured in 3% protein solutions while flavor will be measured in 10% protein solutions. To achieve these concentrations, solutions of 45% protein from part (a) will be diluted to 3% protein for heat stability and turbidity measurements and to 10% for flavor determinations. Three percent solutions from part (a) will be used directly for heat stability and turbidity. These same solutions will be freeze dried and reconstituted to obtain a 10% solution for flavor measurement.

1. Heat stability: The 3% of protein solutions will be adjusted to pH values of 3.5, 4.5 and 6.8 (Pelegrine and Gasparetto, 2005). One milliliter of each solution will be placed in a vial, covered with a lid and placed in an oil bath that will be kept at 85°C (hot fill conditions), 72°C (HTST) and 135°C (UHT conditions) for 1 minute, 20 and 2 seconds, respectively. After the corresponding time at high temperatures, samples will be taken off the oil bath, placed in a water bath at room temperature until their temperature is stabilized. Details of heat stability measurements are presented in the materials and methods section. The number of total treatments for the measurement of heat stability is: 16 (from part (a)) x 1 (protein concentration) x 3 (pH values) x 3 (heating treatments) = 144
2. Clarity/turbidity (solubility): The same solutions used for heat stability will be used to measure the turbidity generated by the whey proteins. Turbidity will be measured as described in the materials and methods section (see below). The number of treatments for turbidity measurements as described for heat stability is (144 treatments).
3. After heat stability and turbidity are measured, HIU conditions that result in an increased heat stability and decreased turbidity will be chosen. This condition will be used to repeat the heat stability and turbidity measurements of the protein, but now using different protein concentrations, from 3% to 10% (4, 6, 8 and 10%) at different pH values and at different temperatures. The number of treatments for this activity is 1 (HIU condition) x 3 (pH values) x 3 (Temperatures) x 4 (protein concentrations) = 36 treatments.
4. Flavor: Ten percent solutions of protein in distilled water will be prepared to evaluate the flavor profile of HIU treated whey proteins. Details of the methodology are presented in the Materials and Methods section. The total number of treatments for flavor determination is 16.

### **Starting Materials**

Whey protein concentrate (WPC80) will be used in these experiments. WPC80 will be purchased from Grande and two solutions of 1 and 45% of proteins will be prepared. The adequate amount of WPC will be weighted, dissolved in distilled water and stirred overnight to allow complete dissolution. The solution will be filter through Whatman #1 paper to eliminate any remaining impurities in the solution.

### **High Intensity Ultrasound**

Ultrasound will be applied using a Misonix S3000 present in Dr. Martini's lab. Acoustic waves of 20 kHz of frequency will be applied (Kresic *et al.*, 2008) using 2 power levels (50 and 150 W/cm<sup>2</sup>), 2 signal duration (5 and 15 min) and at two different temperatures (25 and 70°C). Whey protein solutions will be placed in a thermostated cell that will allow temperature control. Temperature in the solution will be measured with a thermocouple and recorded every minute to evaluate the increase in temperature during sonication. Acoustic waves will be applied at the specified times and if a temperature increase is detected, the sample will be kept in the thermostated cell until the temperature is stabilized. After sonication, samples will be placed in a freezer set at -80°C and kept at this temperature until the functional properties are determined.

## Functional Properties

Heat stability, turbidity, and flavor will be evaluated for the proteins with and without the application of HIU. Three percent protein solutions will be used for heat stability and turbidity measurements while 10% protein will be used for flavor quantification. Each solution will be prepared in triplicate; one replicate will be set at pH 3.5, the second and third at pH 4.5 and the third at pH 6.8.

For heat stability, solutions will be placed in assay tubes and placed in a water bath at 72, 85 and 135°C for a total of 20 seconds, 1 minute (Nunmer, Food Science Extension specialist) and 2 seconds, respectively to simulate the processing of beverages under pasteurized, hot filling and UHT conditions. After heat treatment, samples will be placed in a water bath set at 25°C until they reach room temperature. After this procedure is completed, heat stability will be quantified as the amount of soluble protein present in these solutions. To measure protein solubility after heating, samples will be centrifuged at 13,000 rpm for 20-30 min to precipitate the insoluble fraction. The supernatant will then be measured for protein content using a spectrophotometric assay (BCA Pierce Chemical Co.) (Taylor et al., 2006) or a Kjeldahl nitrogen method (Pelegrine and Gasparetto, 2005).

The turbidity of the protein solutions after heating will be determined using transmission at a wavelength of 420 nm on samples diluted to the microgram range (Nath, 2004).

The flavor of the sonicated samples reconstituted as mentioned before (10% of protein in distilled water) will be evaluated using the lexicon developed by Carunchia Whestine et al. (2005). Some of the attributes to be included are: overall aroma intensity, cooked, diacetyl, sourness, cucumber, brothy, cardboardy, soapy, bitterness and astringency. Special attention will be given to the attributes "bitter" and "astringent" since these are the most common off-flavors found in whey. The flavor profile will be determined by the descriptive sensory panel trained at Utah State University using a 15-point intensity scale. At least 10 hours of training on whey protein identification and quantification will be provided to the panelists. References for training will be used as reported by Carunchia Whestine et al. (2005). Panel performance will be evaluated using PanelCheck software. Additional training will be given to the panelists if needed. After training, samples will be presented to the panelists in groups of four in a random and balanced manner. The 16 samples will be evaluated in the same sitting with a 15 min break between each set of 4 samples. The total duration of the tasting is estimated to be 1:30 h. A whey protein control (without HIU treatment) will be included in each set of 4 samples to follow the panelists' performance during the tasting.

## Analysis of Results

The HIU application and the measurement of functional properties will be performed in duplicate. A control consisting of whey protein solutions without the application of HIU will be included in all the determinations. Appropriate statistical analysis will be used to interpret the data. Statistical tools such as one- and two-way ANOVA and PCA plots will be used. SIMS2000 will be used for sensory data collection and training and SAS 9.1.3 will be used for the analysis.

## References

- Carunchia Whetstine, M.E., Croissant, A.E. and Drake, M.A. 2005. Characterization of dried whey protein concentrate and isolate flavor. *J. Dairy Sci.* 88:3826-3839.
- Kresic G, Lelas V, Jambrak AR, Herceg Z, and Brncic SR. 2008. Influence of novel processing technologies on the rheological and thermophysical properties of whey proteins. *J. Food Engineering* 87:64-73.
- Nath, S. 2000. Complexation behavior of proteins with polyelectrolytes and random acrylic polyampholytes using turbidimetric titration. *J. Chem Technol & Biotechnol.* 62(3):295-300
- Pelegrine, D.H.G., and CA Gasparetto. 2005. Whey protein solubility as function of temperature and pH. *Lebensm.-Wis. UJ-Technol.* 38 (77-80).
- Taylor, D.P., CE Carpenter, MK Walsh. 2006. Influence of sulfonation on the properties of expanded extrudates containing 32% whey protein. *J. Food Sci.* 71(2):E17-24.

## RESULTS AND DISCUSSION

### Objective 1: To apply HIU to whey protein solutions

WPC80 solutions were prepared at a final concentration of 6 and 26% solids. HIU was applied as described in the materials and methods section. Samples were immediately frozen and freeze dried. These samples were used to

perform the experiments in Objective 1. No significant problems were encountered in this step. No visual changes were observed in these solutions during sonication. When sonication was applied without temperature control, the sample temperature increased from 20 – 40 °C approximately.

**Objective 3: To evaluate the clarity/turbidity of HIU whey proteins treated at different temperatures and pH values**

A calibration curve was obtained for the turbidity measurement. Dilutions of the 6% WPC80 were performed to obtain the following concentrations: 0.0638, 0.306, 0.585, 3.19, 6.38 mg/ml. The solutions were prepared in duplicate and their transmittance (T%) was measured at 420 nm. A negative exponential relationship ( $R^2 = 0.9975$ ) was found between the transmittance measured and the solution concentration (Figure 2). The higher the concentration in the solution, the lower the transmittance, since fewer solids are dissolved and less light is transmitted through the spectrophotometer cell, indicating a higher turbidity. This relationship shows that the transmittance of light measured at 420 nm can be used as a measurement of the turbidity of the solution. This is an inversely proportional relationship where a high transmittance is associated with a low turbidity in the solution or dispersion. It is important to note here that in this report concentrations are expressed in terms of solid % or solid content and NOT in terms of protein content.

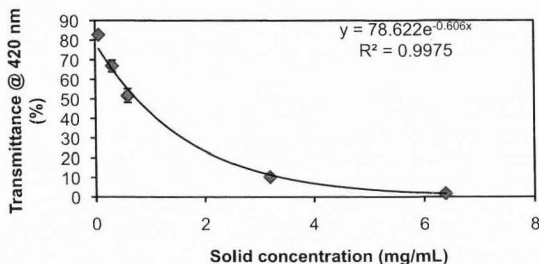


Figure 2: Calibration curve for WPC80 solutions

After validating the use of transmittance (T%) as a tool to measure turbidity in a solution, the T% of the sonicated WPC80 solutions was measured. Controls (WPC80 solutions without sonication) were also used. Each sonication treatment was performed once and, for each treatment, T% was measured in triplicate. Results are shown in Tables 1-6.

Table 1: T% values for the 6% WPC80 solutions sonicated at 20 °C

6% solids-20 °C Application time (min)	HIU power (W)		
	0	3	15
5	41.36a ± 3.0	44.83a ± 5.03	46.10a ± 3.72
15	41.36a ± 3.0	48.98b ± 1.91	49.75b ± 3.96

No significant differences between application time (rows)

For the same row, values with same letter are not significantly different



**Table 2:** T% values for the 6% WPC80 solutions sonicated at 60 °C

6% solids-60 °C		HIU power (W)		
Application time (min)	0	3	15	
5	44.86 ± 3.14	41.70 ± 2.21	39.34 ± 4.04	
15	37.67 ± 3.41	35.21 ± 4.55	39.44 ± 2.15	

No significant differences between application time (rows) or HIU power (columns)

**Table 3:** T% values for the 26% WPC80 solutions sonicated at 20 °C

26% solids-20 °C		HIU power (W)		
Application time (min)	0	3	15	
5	47.85 ± 2.06	42.89 ± 2.44	44.72 ± 1.50	
15	47.85 ± 2.06	45.10 ± 2.93	48.57 ± 3.20	

No significant differences between application time (rows) or power (columns)

**Table 4:** T% values for the 26% WPC80 solutions sonicated at 60 °C

26% solids-60 °C		HIU power (W)		
Application time (min)	0	3	15	
5	42.58a ± 3.58	44.02a ± 0.99	46.73a ± 2.22	
15	38.82a ± 2.44	46.98b ± 0.95	42.38ab ± 3.28	

No significant differences between application time (rows)

For the same row, values with same letter are not significantly different

**Table 5:** T% values for the 6% WPC80 solutions sonicated with no temperature control

6% solids-no temp control		HIU power (W)		
Application time (min)	0	3	15	
5	41.36a ± 3.00	49.52b ± 2.42	48.23b ± 0.96	
15	41.36a ± 3.00	49.52b ± 1.99	55.07c ± 3.41	

No significant differences between application time (rows)

For the same row, values with same letter are not significantly different

**Table 6:** T% values for the 26% WPC80 solutions sonicated with no temperature control

26% solids-no temp control		HIU power (W)		
Application time (min)	0	3	15	
5	47.85 ± 2.06	49.37 ± 2.33	51.31 ± 3.35	
15	47.85 ± 2.06	50.49 ± 5.31	53.58 ± 1.87	

No significant differences between application time (rows) or power (columns)

From Tables 1-6 we can observe that HIU application time (5 or 15 min) does not affect significantly the T% measurements. However, in some of the WPC80 solutions when higher power settings were used, an increase ( $p < 0.05$ ) in the T% of the sample was observed, indicating a lower turbidity. This is the case of 6% WPC80 sonicated at 20 °C using power 3 and 15W for 15 min (Table 1), 26% WPC80 solution sonicated at 60 °C for 15 min using 3W of power and for the 6% WPC80 solution sonicated without any temperature control. In this case, the temperature in the sample increased from 20 °C to approximately 40 °C during sonication. Changes in these samples were the most evident ones, showing an increase in the T% from approximately 41% in the control to 55% in the sample sonicated for 15 min using 15W of power.

To evaluate the effect of pH values on the T% measurements, samples were prepared as described before and the pH adjusted to 3.5, 4.5, and 6.8. The T% of these samples was measured and the results are presented in Tables 7-12, 13-18, and 19-24, respectively.

**Table 7:** T% values for the 6% WPC80 solutions sonicated at 20 °C (pH = 3.5)

6% solids-20 °C	HIU power (W)		
Application time (min)	0	3	15
5	24.03 ± 2.79	27.09 ± 3.63	28.07A ± 1.48
15	24.03a ± 2.79	29.85b ± 2.74	36.44cB ± 0.69

Capital letters: differences between rows

**Table 8:** T% values for the 6% WPC80 solutions sonicated at 60 °C (pH = 3.5)

6% solids-60 °C	HIU power (W)		
Application time (min)	0	3	15
5	29.90aA ± 0.35	24.45b ± 1.62	26.03abA ± 1.30
15	35.35aB ± 3.49	28.58b ± 2.96	32.44abB ± 1.85

Capital letters: differences between rows

**Table 9:** T% values for the 26% WPC80 solutions sonicated at 20 °C (pH = 3.5)

26% solids-20 °C	HIU power (W)		
Application time (min)	0	3	15
5	25.96a ± 2.25	28.31ab ± 0.79	35.07bA ± 2.42
15	25.96a ± 2.25	30.65a ± 4.60	43.69bB ± 5.32

Capital letters: differences between rows

**Table 10:** T% values for the 26% WPC80 solutions sonicated at 60 °C (pH = 3.5)

26% solids-60 °C	HIU power (W)		
Application time (min)	0	3	15
5	32.29 ± 1.56	26.07 ± 2.32	27.85 ± 1.11
15	30.72 ± 7.42	28.57 ± 3.72	30.13 ± 1.36

No significant differences between application time (rows) or columns (power)

**Table 11:** T% values for the 6 6% WPC80 solutions sonicated with no temperature control (pH = 3.5)

Application time (min)	HIU power (W)		
	0	3	15
5	24.03a ± 2.79	31.29b ± 2.39	34.12b ± 2.14
15	24.03a ± 2.79	36.44b ± 1.38	39.75b ± 3.37

No significant differences between application time (rows)

**Table 12:** T% values for the 2 26% WPC80 solutions sonicated with no temperature control (pH = 3.5)

Application time (min)	HIU power (W)		
	0	3	15
5	30.85ab ± 5.45	28.49a ± 0.86	35.70ab ± 1.31
15	30.85a ± 5.45	34.47a ± 1.42	41.89b ± 2.48

No significant differences between application time (rows)

For the T% measured at pH 3.5 (Tables 7-13) a significant difference ( $\alpha = 0.05$ ) between the application time and ultrasound power is observed, especially for higher powers (15 W and longer times 15 min) for the 6 and 26% WPC80 solution sonicated at 20 °C. Interestingly, for these same solutions but sonicated at 60 °C the T% trend was to decrease with acoustic power (the higher the power used, the lower the T% observed). A significant increase ( $\alpha = 0.05$ ) in the T% with acoustic power was also observed in the WPC80 samples that were sonicated without temperature control.

**Table 13:** T% values for the 6% WPC80 solutions sonicated at 20 °C (pH = 4.5)

Application time (min)	HIU power (W)		
	0	3	15
5	31.21 ± 2.70	26.44 ± 5.38	20.93 ± 2.25
15	31.21 ± 2.70	27.95 ± 8.94	29.92 ± 5.67

No significant differences

**Table 14:** T% values for the 6% WPC80 solutions sonicated at 60 °C (pH = 4.5)

Application time (min)	HIU power (W)		
	0	3	15
5	30.48 ± 5.52	23.39 ± 6.58	31.12 ± 5.26
15	23.94 ± 2.87	27.71 ± 5.35	29.29 ± 2.30

No significant differences

**Table 15:** T% values for the 26% WPC80 solutions sonicated at 20 °C (pH = 4.5)

26% solids-20 °C		HIU power (W)		
Application time (min)	0	3	15	
5	24.28 ± 5.19	26.69 ± 6.87	23.67 ± 7.11	
15	24.28 ± 5.19	24.95 ± 5.87	26.00 ± 9.81	

No significant differences

**Table 16:** T% values for the 26% WPC80 solutions sonicated at 60 °C (pH = 4.5)

26% solids-60 °C		HIU power (W)		
Application time (min)	0	3	15	
5	24.30 ± 8.23	21.13 ± 1.72	25.11 ± 4.22	
15	23.80 ± 0.38	15.02 ± 2.65	19.23 ± 7.24	

No significant differences

**Table 17:** T% values for the 6% WPC80 solutions sonicated with no temperature control (pH = 4.5)

6% solids-no temp control		HIU power (W)		
Application time (min)	0	3	15	
5	31.21 ± 2.70	30.82 ± 4.94	30.45 ± 4.70	
15	31.21 ± 2.70	29.04 ± 5.23	29.43 ± 2.97	

No significant differences

**Table 18:** T% values for the 26% WPC80 solutions sonicated with no temperature control (pH = 4.5)

26% solids-no temp control		HIU power (W)		
Application time (min)	0	3	15	
5	21.13a ± 6.01	25.31ab ±	31.58b ±	
		2.53	4.63	
15	21.13a ± 6.01	30.55ab ±	31.01b ±	
		3.53	4.44	

Tables 13-18 show the T% of the sonicated samples when these were measured at pH = 4.5. As expected, the T% values were lower than the ones measured at neutral pH and at pH = 3.5. This is due to the lower solubility of the whey proteins and pH = 4.5 (pH close to the isoelectric point). Also, no differences were found between the different acoustic conditions. The only exception was the 26% WPC80 solution sonicated without temperature control, where a slight increase in T% was observed at high acoustic powers.

**Table 19:** T% values for the 6% WPC80 solutions sonicated at 20 °C (pH = 6.8)

6% solids-20 °C		HIU power (W)		
Application time (min)	0	3	15	
5	48.75a ±	55.89bc ±	54.91ac ± 4.62	
	3.06	3.11		
15	48.75a ±	55.88bc ±	53.18ac ± 2.94	
	3.06	1.65		

No significant differences between time (rows)

**Table 20:** T% values for the 6% WPC80 solutions sonicated at 60 °C (pH = 6.8)

6% solids-60 °C		HIU power (W)		
Application time (min)	0	3	15	
5	54.00Aa ± 1.64	43.76Abc ± 5.14	46.05Aac ± 2.39	
15	40.12Ba ± 4.61	49.02Ab ± 4.75	50.11Ab ± 4.88	

**Table 21:** T% values for the 26% WPC80 solutions sonicated at 20 °C (pH = 6.8)

26% solids-20 °C		HIU power (W)		
Application time (min)	0	3	15	
5	48.51a ± 2.10	52.22a ± 2.44	50.30a ± 0.68	
15	48.51a ± 2.10	53.80ab ± 6.12	57.27b ± 3.52	

No significant differences between time (rows)

**Table 22:** T% values for the 26% WPC80 solutions sonicated at 60 °C (pH = 6.8)

26% solids-60 °C		HIU power (W)		
Application time (min)	0	3	15	
5	45.92a ± 3.50	48.60a ± 3.47	44.50a ± 2.09	
15	43.72a ± 2.82	54.14b ± 6.43	43.05a ± 5.52	

No significant differences between time (rows)

**Table 23:** T% values for the 6% WPC80 solutions sonicated with no temperature control (pH = 6.8)

6% solids-no temp control		HIU power (W)		
Application time (min)	0	3	15	
5	48.75a ± 3.06	52.89a ± 3.67	56.52a ± 5.87	
15	48.75a ± 3.06	55.37ab ± 3.33	56.70b ± 2.88	

No significant differences

**Table 24:** T% values for the 26% WPC80 solutions sonicated with no temperature control (pH = 6.8)

26% solids-no temp control		HIU power (W)		
Application time (min)	0	3	15	
5	48.51a ± 2.10	53.93b ± 1.11	59.29c ± 4.05	
15	48.51a ± 2.10	56.03b ± 1.06	58.05b ± 1.42	

Tables 19-24 shows the same results but for solutions at pH = 6.8. It can be seen that these are very similar to the values observed in Tables 1-6, with no differences between application times and differences ( $\alpha = 0.05$ ) with acoustic power.

#### **CONCLUSIONS/NEXT STEPS**

These results show that the measurement of T% at 420 nm is an appropriate tool to evaluate the turbidity of whey protein solutions. We also proved that differences in protein solubility can be detected by this measurement. The technique was optimized and is ready to be used for the next period of this grant, where HIU will be applied to the samples provided by Glanbia.

# Antioxidant Mechanism of Milk Mineral—High-Affinity Iron Binding

K. ALLENEN AND D. CORNFORTH

**ABSTRACT:** Milk mineral (MM), a by-product of whey processing, is an effective antioxidant in meat systems, but the antioxidant mechanism has not been established. MM has been postulated to chelate iron and prevent iron-catalyzed lipid oxidation. The objective of this research was to examine this putative mechanism. MM was compared to sodium tripolyphosphate (STPP), calcium phosphate monobasic (CPM), and calcium pyrophosphate (CPP) to determine iron-binding capacity, sample solubility, and eluate soluble phosphorus after treating samples with a ferrous chloride standard. Scanning electron microscopy with energy-dispersive X-ray analysis was used to localize minerals on iron-treated MM particle surfaces. Histochemical staining for calcium was performed on raw and cooked ground beef samples with added MM. MM bound more iron per gram ( $P < 0.05$ ) than the other compounds, and was much less soluble ( $P < 0.05$ ) than either STPP or CPM. Mineral localization showed an even distribution of calcium, phosphorus, oxygen, and iron across the MM particle surface, directly demonstrating iron binding to MM particles. Unlike other common chelating agents, such as STPP and citrate, histochemical staining demonstrated that MM remained insoluble in ground beef, even after cooking. The ability of MM to bind iron and remain insoluble may enhance its antioxidant effect by removing iron ions from solution. However, MM particles must be small and well distributed in order to adequately bind iron throughout the food system.

**Keywords:** antioxidant, chelator, iron, milk mineral

## Introduction

Many consumers equate freshness of raw meats with red color and often base their purchase decisions on this characteristic (Issanchou 1996). Indeed, studies suggest the presence of brown colors in raw meats result in reduced sales (Hood 1980), with industry estimates as on the subsequent financial losses in the 1990s ranging from \$7000 million to \$1 billion (Hoffman-La Roche Inc. 1993; Liu and others 1995). The preservation of red color in raw beef products could reduce such losses. High-oxygen modified atmosphere packaging has been shown to delay the onset of browning in fresh beef products (Jakobsen and Bertelsen 2000; Ho and others 2003). However, while red color may be preserved, the high-oxygen atmosphere appears to increase lipid oxidation, leading to the development of rancid flavors and odors (Jayasingh and others 2002). Warmed over flavor, an indicator of lipid oxidation, is also the limiting factor for consumer acceptance of uncured, precooked meats (for example, cooked ground beef patties, pizza toppings, and cooked ground beef crumbs).

Much attention has therefore been focused on antioxidants, which can be used to decrease lipid oxidation. Several studies have focused on the use of milk mineral (MM) in cooked meat systems (Cornforth and West 2002; Jayasingh and Cornforth 2003; Vasavada and Cornforth 2005) and raw meats (Vissa and Cornforth 2006). These studies have found MM to be a potent antioxidant, rivaling sodium tripolyphosphate (STPP) in its effectiveness. It has been suggested that the calcium phosphate fraction of MM may function as a Type I (iron-binding) antioxidant, as does STPP by binding iron and effectively preventing it from catalyzing lipid oxidation (Cornforth and West 2002). However, this has not been definitively demonstrated.

Milk mineral is a by-product of the production of whey protein concentrates. It is a fine, slightly gritty white powder (particle size  $< 7 \mu\text{m}$ ; Figure 1) obtained by purifying and drying the ultrafiltration permeate of whey. The major component of MM is the mineral fraction, predominately calcium and phosphorus, though it contains some lactose and protein and trace amounts of fat (Table 1). The mineral fraction is derived from the colloidal inorganic and protein-associated minerals present in the casein micelle and the free ions in equilibrium with these forms. The composition of the colloidal fraction in milk has been studied extensively (see Gaucheron 2005 for a review), and it is known to contain calcium, phosphate, magnesium, and citrate. The inorganic form of calcium phosphate present has been suggested to be predominately brushite ( $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ ; Holt and Sawyer 1988; Holt and others 1996) or a mixture of amorphous and crystalline hydroxyapatite ( $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ ; Thomsen and others 1995; Bak and others 2001). However, little is known about the nature of the calcium phosphate present in MM. The objective of this research was to study the mode of action by which MM prevents lipid oxidation by (1) comparing its iron-binding capacity to STPP and other calcium phosphate forms; (2) examining its solubility in raw and cooked beef; and (3) localizing minerals across particle surfaces using scanning electron microscopy (SEM) to examine the possibility that a physical association exists between MM calcium phosphate and added iron.

## Materials and Methods

### Iron-binding column preparation

Columns were prepared using small (14.5-cm length) disposable borosilicate Pasteur-type pipettes (Scientific Products, McGraw Park, Ill., U.S.A.). Columns were plugged with glass wool, then filled with test material to a depth of 2.5 cm. The amount of test material added to each column was determined by weight difference. MM (Glanbia, Monroe, Wis., U.S.A.), STPP (Fisher, Fairlawn, N.J., U.S.A.),

MS 2006/04/26 Submitted 8/1/2006, Accepted 9/22/2006. Authors are with Nutrition and Food Sciences, Utah State Univ., Logan, UT 84322-8700, U.S.A. Direct inquiries to author Allen (E-mail: sln2k@cc.usu.edu).

calcium phosphate monobasic (CPM; JT Baker, Phillipsburg, N.J., U.S.A.), and calcium pyrophosphate (CPP; Aldrich, St. Louis, Mo., U.S.A.) were used as test materials (Figure 2). Columns were pre-wetted with 1 mL of distilled water (DI), then either 0.5 mL of 1 mg/mL iron standard (ferrous chloride, JT Baker) and 0.5 mL DI, or 1 mL of 5 mg/mL iron standard was added. Eight additional 1 mL DI rinses were added, for a total wash volume of 10 mL. At least 9.75 mL of filtrate was recovered in all cases. Ten replicates ( $n = 10$ ) were performed for each test material.

#### Percent packing loss

Spent iron-binding columns were dried overnight at 90 °C, then cooled in a desiccator. Columns were weighed to determine the amount of packing solubilized. Results were expressed as a percent of the original packing weight for each column.

#### Iron retention

Total iron content of the filtrates was determined using the Ferrozine assay (Carter 1971). Samples were read at 562 nm using a Shimadzu UV2100U spectrophotometer (Columbia, Md., U.S.A.) as a measure of total iron concentration. Each filtrate was assayed in duplicate. Iron retention in mg iron/g packing compound was calculated based on a target value of either 0.05 mg iron/mL or 0.5 mg iron/mL filtrate, the expected concentration if no iron is retained by the column.

#### Percent soluble phosphorus

Total orthophosphate content of the filtrates was determined using the ammonium molybdate/antimony potassium tartrate

method (Environmental Protection Agency Method 365.3). Samples were read at 650 nm using the Shimadzu UV2100U spectrophotometer. Only soluble orthophosphates were detected; pyro- and polyphosphates will produce the typical blue chromagen only when some degree of hydrolysis has occurred. Each filtrate was assayed in duplicate. Percent soluble phosphorus was calculated based on the weight of the original packing material and the percent phosphorus (Table 1, Figure 2) in each compound.

#### SEM

After drying, 1 MM iron retention column was broken and the contents were used for mineral localization by SEM with energy-dispersive X-ray spectrometry (EDS). A Hitachi S3000-N (Pleasanton, Calif., U.S.A.) SEM was used, with the following operating conditions: sample distance, 15.4 mm; accelerating voltage, 15.0 kV; and tilt angle, 0.0°. Examinations were made at 1200× magnification. A qualitative elemental scan was performed to identify the major constituents of MM.

#### Light microscopy

Lean ground beef (90%) was obtained from the Utah State Univ. (USU) Meat Lab. Samples for calcium staining were prepared by adding MM or STPP at 0.75% and 1.5% levels to 50 g of meat. Samples were mixed thoroughly (kneading 25 times), wrapped in plastic film, then placed in resealable sandwich bags and held under refrigeration for 3 d. Cooked samples were shaped, after mixing into 5 × 0.5 cm patties, heated on both sides on a flat grill to an internal temperature of 75 °C, then cooled to room temperature before wrapping and refrigerating.

Samples for iron staining were prepared by thoroughly mixing 0.3252 g MM with 0.5 mL 1 mg/mL iron standard, which was allowed to sit overnight until dry. The MM/iron mixture was then added to 49.68 g of meat (equivalent to 0.75% MM). Raw sample was mixed and stored as described above.

Samples were prepared for light microscopy by dehydrating, embedding in paraffin, sectioning, and rehydrating. Von Kossa and Perl's staining (Sheehan and Hrapchak 1980) was performed on the rehydrated sections to test for the presence of undissolved calcium and undissolved iron, respectively. Slides were viewed at 40× magnification.

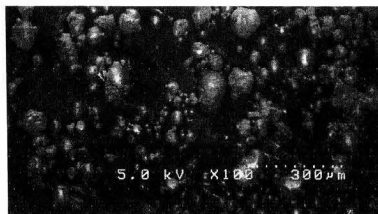
#### Data analysis

Statistical analysis was performed for percent packing loss, iron retention, and soluble phosphorus values by analysis of variance using the proc GLM function in SAS version 9.0 (SAS Institute Inc., Cary, N.C., U.S.A.). Statistical significance was identified at the 95% confidence level. Least significant difference (LSD) tests were used to separate means of treatments (packing compound type).

## Results and Discussion

### Iron-binding capacity, soluble phosphorus, and packing loss

The type of packing compound used significantly affects the amount of iron bound by the columns ( $P < 0.0001$ ), the percent soluble phosphorus ( $P < 0.0001$ ), and the percent of the packing that was solubilized ( $P < 0.0001$ ; Table 2). MM was found to bind more iron per gram than any of the other 3 compounds and was less soluble than either STPP or CPM. CPM filtrates contained more soluble phosphorus than the others, but less than what may be expected based on its high solubility. This was likely due to the presence of high levels of nonchelated iron in these filtrates, as iron can interfere with the initial binding of orthophosphate to form the molybdate



**Figure 1**—SEM micrograph of milk mineral showing particle size distribution (100× magnification; scale on picture = 300  $\mu$ m)

**Table 1**—Compositional data for milk mineral as provided by the manufacturer, and mineral content as determined by ICP analysis. Values are given as a percent by weight.

Constituent	Manufacturer specifications	ICP analysis
Total mineral (%)	80.2	—
Inorganic mineral (ash) (%)	71.2	—
Organic mineral (citrate) (%)	9.0	—
Calcium (%)	24.0	21.6
Phosphorus (%)	13.5	10.8
Magnesium (%)	1.5	1.5
Iron (%)	0.09	0.06
Lactose (%)	10.0	—
Protein (%)	5.0	—
Free moisture (%)	4.0	—
Fat (%)	0.5	—

n/a = specs not available.



complex and can consume the reducing agent (ascorbic acid) required to intensify the blue color. Absence of testable soluble phosphorus in STPP was due to the presence of phosphates in the poly (compared with ortho) form.

**Mineral localization**

EDS elemental localizations (Figure 3) indicate an even distribution of calcium (Figure 3b), phosphorus (Figure 3c), and oxygen (Figure 3d) across the MM particle surface. Considerable amounts of magnesium (Figure 3e) were also present. Approximately 0.02% iron by weight was present in the MM sample (obtained from a used iron-binding column), and it was likewise evenly dispersed across

the surface of the particle (Figure 3f). The qualitative elemental scan (Figure 4) reveals the presence of low levels of carbon, a possible indication of the presence of citrate in the mineral particles. The EDS procedure can identify elements in the sample, since each element generates X-rays of a characteristic energy level when exposed to the electron beam during the SEM procedure. Due to limitations of the EDS procedure, however, the peak heights provide only qualitative information regarding elemental concentrations. For the iron-treated MM sample (Figure 4), iron is present at low levels relative to phosphorus, oxygen, and calcium, but no conclusions may be drawn regarding the relative concentrations of oxygen, phosphorus, and calcium.

**Microscopic examination for undissolved calcium and iron**

The presence of insoluble calcium in ground beef + MM samples was verified by the black spots seen in Figure 5. Even after cooking, insoluble calcium-based particles were observed. (It is important to note the black color associated with the calcium in Figure 5 as a result of the histological stain used and would not normally be visible in meat treated with MM.) The absence of such spots in the ground beef + STPP samples was not surprising, as no calcium was added.

However, evidence supporting the solubility of STPP can also be seen. Compared to the control, the 0.75% STPP sample shows much less muscle fiber shrinkage (white gaps between red muscle fibers). In the 1.5% STPP sample, this difference was even more pronounced. In addition to iron, soluble phosphates will bind water. This series of slides shows the effect of increasing levels of STPP: as the percentage increased, so did the water binding capacity, and less cell shrinkage was observed.

Slides of Perl's staining for iron showed no evidence of iron particulate, despite the addition of a significant amount of iron to the MM. This indicates that any iron present was dispersed in relatively fine "particles" throughout the meat sample and was not present in the form of larger, insoluble ferric clusters.

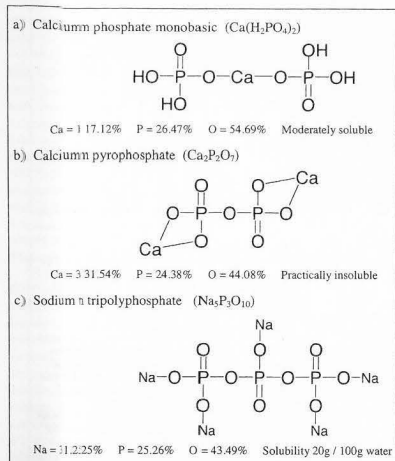
**Similarities between MM and other calcium phosphates**

On average, 20% of the MM column packing was lost. MM contains approximately 80% mineral, 10% lactose, and 4% to 5% protein and moisture; it is likely the majority of packing lost from the column was solubilized lactose and protein, though some small fraction of the finer mineral particles may have been lost as well. After correcting for mineral content, the solubility of MM was closer to that of CPP, suggesting that the form of calcium phosphate present in MM was more similar to CPP than CPM. Likewise, soluble phosphorus results showed that, of the compounds tested, MM was most similar to CPP.

Other types of calcium phosphate compounds are insoluble or sparingly soluble in water, and may be present in MM. It has been suggested that after heat treatment of milk, the forms of calcium phosphate present shift to hydroxyapatite (Ca<sub>10</sub>(PO<sub>4</sub>)<sub>6</sub>(OH)<sub>2</sub>; Visser and others 1986) or β-tricalcium phosphate (Ca<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub>; Nelson and others 1989), both of which are insoluble. In considering other possible calcium phosphate forms, it is necessary to consider their potential to bind iron. In this study, neither of the calcium phosphate compounds examined bound iron as effectively as MM. It is reasonable to predict that compounds such as β-tricalcium phosphate, in which the majority of the phosphate oxygen groups are bound to calcium in an insoluble complex, would not bind iron as efficiently, since there is no negative charge present to interact with positively charged iron ions. In addition to being insoluble, the

**Table 2.—Iron-binding capacity, % solubility of packing material, and % soluble phosphorus for column experiment. Iron-binding capacity is given in mg iron bound per g packing material. % soluble phosphorus is calculated against the total phosphorus content of the packing material, and includes only soluble orthophosphates. All values represent the mean ± standard deviation. Values sharing letters within columns are not significantly different (P >> 0.05). P values are given for effect of packing material.**

	Iron-binding capacity	% Solubility	% Soluble phosphorus
Milk mineral II	14.9 ± 1.1a	20.7 ± 0.6b	3.46 ± 1.64b
Sodium tripolyphosphate	11.0 ± 0.9b	69.4 ± 25.3a	0.00 ± 0.00c
Calcium pyrophosphate	1.12 ± 0.12c	1.13 ± 0.38c	2.56 ± 1.39b
Calcium phosphate monobasic	0.43 ± 0.46d	75.3 ± 9.4a	17.5 ± 7.2a
	P < 0.0001 LSD = 0.34	P < 0.0001 LSD = 6.12	P < 0.0001 LSD = 1.71



**Figure 2.—Structures, composition, and solubility data for additional column packing compounds**

Food Chemistry & Technology

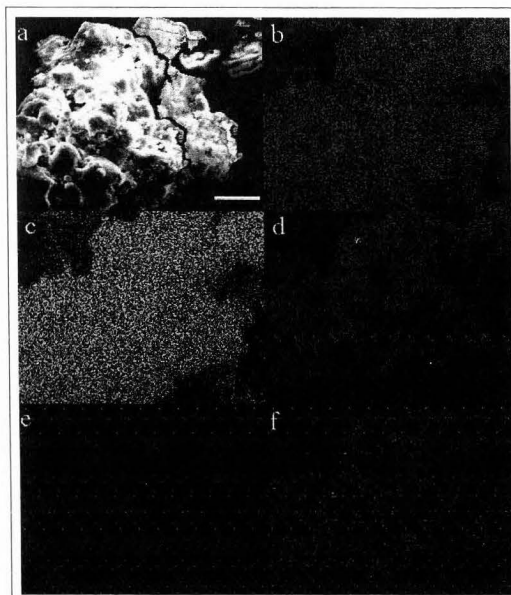
calcium phosphate form(s) suggested to be in MM must be capable of binding relatively large amounts of iron to fit the results presented here.

Recently, Bak and others (2001) used  $^{31}\text{P}$  solid-state magic angle spinning nuclear magnetic resonance to examine both the inorganic calcium phosphate and protein-associated calcium phosphate in casein micelles. Lu and others (2000) used both X-ray photoelectron spectroscopy and time-of-flight secondary mass spectrometry to determine the forms of calcium phosphate present on the surface of

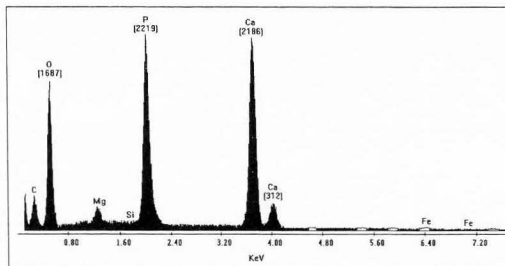
a bone sample. In both studies, characteristic spectra were obtained for multiple calcium phosphate forms, which were then compared to sample spectra to determine the ratios of each form present. Such analyses have yet to be applied to MM, but would provide invaluable information on the nature of its calcium phosphate.

### Iron-binding potential

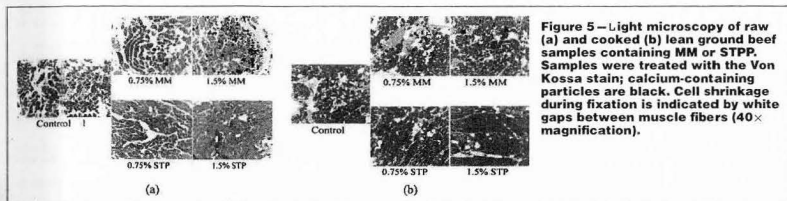
The iron-binding values for STPP, CPM, and CPP represent maximums where quantifiable levels of iron were observed in the filtrate.



**Figure 3**—SEM micrograph of milk mineral particle (a) and mineral localization by SEM EDs of calcium (b), phosphorus (c), oxygen (d), magnesium (e), and iron (f). Milk mineral was from spent iron-binding column, and contains approximately 0.02% iron by weight. Large particle size is due to clumping during column rinsing and drying. Black "cracks" on micrograph are artifacts of microscopy. (1200 $\times$  magnification; bar = 10  $\mu\text{M}$ ).



**Figure 4**—Qualitative elemental scan of milk mineral from SEM EDs. Horizontal axis is energy in KeV (thousand electron volts). Vertical axis is detected X-ray count (arbitrary units). The energy (horizontal axis) of the X-rays emitted after electron bombardment by SEM is characteristic of a given element. The intensity of detected X-rays (peak height on vertical axis) provides a qualitative measure of the concentration of a given element at the sample surface. Peak heights (in brackets) are shown for O, P, and Ca for reference only.



**Figure 5—Light microscopy of raw (a) and cooked (b) lean ground beef samples containing MM or STPP. Samples were treated with the Von Kossa stain; calcium-containing particles are black. Cell shrinkage during fixation is indicated by white gaps between muscle fibers (40 $\times$  magnification).**

Both STPP or MM columns were loaded with 10 $\times$  the iron as CPM and CPP. No iron was detectable in the MM filtrates even at this level. It was not possible to load more iron onto the columns than this without at decreasing the subsequent rinse volume, a factor that could confound results. Therefore, iron-binding values were calculated based on the fact that none of the iron added was found in the MM filtrate. *e. venosus*, MM bound significantly more iron than any of the other compounds tested. It is possible that the true binding potential for MM is higher than indicated in this study.

At this point, it is necessary to address the potential contribution of other nonphosphate components to the iron-binding potential. There is residual protein present in MM, but according to manufacturer's specifications, it consists primarily of the whey proteins ( $\alpha$ -lactalbumin and  $\beta$ -lactoglobulin), though it is possible some trace amount of ferritin was also present. Additionally, ferritin can bind only 2 iron molecules per peptide (Steijns 2001), so its contribution to the overall binding potential would be minimal. Likewise, citrate may chelate iron, but based on the manufacturer's specifications and the SEM data presented here, its contribution was probably minimal.

SEM results clearly indicate the potential for a physical association between calcium phosphate and iron. Since SEM images typically represent the top 1 to 2  $\mu$ m at the surface of an object, this association can be characterized as a surface phenomenon (though interdiffusion is between phosphates and iron in the interior of a somewhat porous particle cannot be excluded). Considered in conjunction with column solubility data and the Von Kossa staining results, a unique relationship is seen: MM interacts with iron, removing it from solution, and therefore the insoluble complex would be less subject to subsequent dissociation of iron. Govindarajan and Hultin (1977) likewise suggested the presence of an iron-oxalate precipitate to account for the effectiveness of 1% oxalate as an inhibitor of color and lipid oxidation in ground beef. This is in sharp contrast to STPP, which is highly soluble. The observed differences in iron-binding potential between MM and STPP may be due, at least in part, to dissociation of iron from STPP in solution.

US Dept. of Agriculture regulations allow the addition of 0.5% STPP as a preservative of final product weight, to various cooked meats. Assuming this binding capacity obtained in this study (approximately 15 mg Fe/g MM) is a true maximum, and the average non-heme iron concentration in cooked ground beef is 8.5  $\mu$ g/g (Carpenter and Clark 1995), MM added at 0.05% by weight could theoretically be expected to control iron-catalyzed oxidation. However, because this binding potential was obtained by passing a liquid through a column, contact between iron and binding sites was optimized. In a relatively solid, nonhomogeneous matrix such as ground meat, it is reasonable to expect that a higher than theoretical level must be added to contact and bind iron. Cornforth and West (2002) found that the optimum level of MM to prevent lipid oxidation was 2% for cooked ground beef and pork and 1% for ground turkey. However, in

all cases, addition of 0.5% MM significantly reduced thiobarbituric acid reactive substance values as compared to controls. Vasavada and Cornforth (2005) found 1.5% MM sufficient to prevent lipid oxidation in cooked beef meatballs. MM was found to decrease oxidation of both lipid and oxymyoglobin (thus preserving red color) in raw ground beef at only 0.75% addition (Vissa and Cornforth 2006).

### Conclusion

MM has been demonstrated to be a Type II, iron-chelating antioxidant. It was found to bind significantly more iron per gram than STPP. The ability of MM to bind iron and remain insoluble may enhance its antioxidant effect. Thus, MM particles must be small and well distributed in order to adequately bind iron throughout the food system. Neither of the calcium phosphate forms examined in this study can account for the iron-binding behavior of MM, though its solubility was similar to CPP. The nature of calcium phosphate present in MM is of interest, as there is an ongoing debate over whether certain forms decrease iron absorption (Lynch 2000; Grinder 2004; Roughead 2005). The characterization of MM calcium phosphate by nuclear magnetic resonance, X-ray photoelectron spectroscopy, or time-of-flight secondary mass spectrometry, in conjunction with iron absorption studies, would be valuable to ascertain the possible negative effects of MM addition on iron bioavailability.

### Acknowledgments

The authors thank Drs. Ramona Skriptunas and Jeff Hall, both from the Veterinary Diagnostic Laboratory, Utah State Univ., for their assistance with histological and ICP analyses, respectively, and Paul Slusser of the Materials Characterization Facility, Univ. of Utah, for his assistance with SEM analysis. We also thank the USU Agricultural Experiment Station and Dairy Management Inc., for financial support of this work, and Glanbia Nutritionals, Twin Falls, Idaho, for the milk mineral samples used in this study (JAES journal paper nr 7814).

### References

- Bak M, Rasmussen LK, Petersen TE, Nielsen NC. 2001. Colloidal calcium phosphates in casein micelles studied by slow-speed spinning  $^{31}$ P magic angle spinning solid-state nuclear magnetic resonance. *J Dairy Sci* 84:1310-9.
- Carpenter CE, Clark E. 1995. Evaluation of methods used in meat iron analysis and iron content of raw and cooked meats. *J Agric Food Chem* 43:1824-7.
- Carter P. 1971. Spectrophotometric determination of serum iron at the submicrogram level with a new reagent (ferrozine). *Anal Biochem* 40:450-8.
- Cornforth DP, West EM. 2002. Evaluation of the antioxidant effects of dried milk mineral in cooked beef, pork, and poultry. *J Food Sci* 67:615-8.
- Gaucheron F. 2005. The minerals of milk. *Reprod Nutr Dev* 45: 473-83.
- Govindarajan S, Hultin HO. 1977. Myoglobin oxidation in ground beef: mechanistic studies. *J Food Sci* 42:71-7, 862.
- Grinder-Pedersen I, Bukhave K, Jensen M, Hojgaard L, Hansen M. 2004. Calcium from milk or calcium-fortified foods does not inhibit nonheme-iron absorption from a whole diet consumed over a 4-d period. *Am J Clin Nutr* 80:404-9.
- Ho CP, Huang NY, McMillin KW. 2003. Microflora and color of ground beef in gas exchange modified atmosphere packaging with abusive display temperatures. *J Food Sci* 68(S1):1771-6.

- Hoffman-La Roche Inc. 1993. Vitamin E: better beef color and better retail profits. Nutley, N.J.: Roche Animal Nutrition and Health Fact Sheet.
- Holt C, Sawyer L. 1988. Primary and predicted secondary structures of the caseins in relation to their biological function. *Protein Eng* 2:251-9.
- Holt C, Wahlgren NM, Drakenberg T. 1996. Ability of a  $\beta$ -casein phosphopeptide to modulate the precipitation of calcium phosphate by forming amorphous dicalcium phosphate nanoclusters. *Biochem J* 314:1035-9.
- Hood DE. 1980. Factors affecting the rate of metmyoglobin accumulation in pre-packaged beef. *Meat Sci* 4:247-65.
- Issanchou S. 1996. Consumer expectations and perceptions of meat and meat products. *Meat Sci* 43:55-19.
- Jakobsen M, Bertelsen G. 2000. Color stability and lipid oxidation of fresh beef. Development of a response surface model for predicting the effects of temperature, storage time, and modified atmosphere composition. *Meat Sci* 54:49-57.
- Jayasingh P, Cornforth DP. 2003. Comparison of antioxidant effects of milk mineral, butylated hydroxytoluene and sodium tripolyphosphate in raw and cooked ground pork. *Meat Sci* 66:83-9.
- Jayasingh P, Cornforth DP, Brennan CP, Carpenter CE, Whittier DR. 2002. Sensory evaluation of ground beef stored in high-oxygen modified atmosphere packaging. *J Food Sci* 67:2493-6.
- Liu Q, Lamari MC, Schaefer DM. 1995. A review of dietary vitamin E supplementation for improvement of beef quality. *J Animal Sci* 73:3131-40.
- Lu HB, Campbell CT, Graham DJ, Ratner BD. 2000. Surface characterization of hydroxyapatite and related calcium phosphates by XPS and TOF SIMS. *Anal Chem* 72:2896-94.
- Lynch SR. 2000. The effect of calcium on iron absorption. *Nutr Res Rev* 13:141-58.
- Nelson LS, Holt C, Hutkins DWL. 1989. The EXAFS spectra of poorly crystalline calcium phosphate preparations from heated milk. *Physica B* 158:103-4.
- Roughhead ZK, Zito CA, Hunt JR. 2005. Inhibitory effects of dietary calcium on the initial uptake and subsequent retention of heme and nonheme iron in humans: comparisons using an intestinal lavage method. *Am J Clin Nutr* 82:589-97.
- Sheehan DC, Hrapchak BB. 1980. *Theory and practice of histotechnology*, 2nd ed. Columbus, Ohio: Battelle Press.
- Stejns JM. 2001. Milk ingredients as nutraceuticals. *Int J Dairy Tech* 54:81-8.
- Thomsen JK, Jakobsen HJ, Nielsen NC, Petersen TE, Rasmussen LK. 1993. Solid-state magic-angle spinning  $^{31}\text{P}$ -NMR studies of native casein micelles. *Eur J Biochem* 230:454-9.
- Vasavada MN, Cornforth DP. 2005. Evaluation of milk mineral antioxidant activity in beef meatballs and nitrite-cured sausage. *J Food Sci* 70:C250-3.
- Vissa A, Cornforth D. 2006. Comparison of milk mineral, sodium tripolyphosphate, and vitamin E as antioxidants in ground beef in 80% oxygen modified atmosphere packaging. *J Food Sci* 71:C65-8.
- Visser J, Minnhan A, Smits P, Tjani SB, Heertje I. 1986. Effects of pH and temperature on the milk salt system. *Neth Milk Dairy J* 40:351-68.

Myoglobin Oxidation in a Model System as Affected by  
Nonheme Iron and Iron Chelating Agents

KARIN E. ALLEN\* AND DAREN P. CORNFORTH

Department of Nutrition and Food Sciences, Utah State University, 8700 Old Main Hill, Logan, Utah  
84322

A model system was used to study the effect of nonheme iron on myoglobin oxidation at pH 5.6 and pH 7.2 at 23 °C. The addition of ferrous iron significantly ( $p < 0.05$ ) increased the rate of myoglobin oxidation in the absence of lipid, demonstrating that iron promoted myoglobin oxidation independent of the effect of lipid oxidation. The addition of the type II, iron chelating antioxidants sodium tripolyphosphate (at pH 7.2) or milk mineral (at pH 5.6) negated the effect of added iron, slowing oxidation of myoglobin. A clear concentration dependence was seen for iron-stimulated myoglobin oxidation, based on both spectral and visual evidence. Further investigation is needed to determine the possible role for nonheme ferrous iron on myoglobin oxidation in vivo or in meat.

**KEYWORDS:** Myoglobin oxidation; nonheme iron; milk mineral

## INTRODUCTION

Several studies have demonstrated a marked color-stabilizing effect of iron chelators in raw ground beef or beef model systems. Oxalate (1), phytic acid (2), and sodium pyro- and polyphosphate (3) were all found to inhibit metmyoglobin (MetMb) formation and lipid oxidation during storage. More recently, milk mineral (MM; 4), a whey-derived compound consisting primarily of colloidal calcium phosphate particles, was also found to preserve red color and prevent lipid oxidation in fresh ground beef. It was further demonstrated that MM has strong iron chelating properties (5, 6). The mechanism by which color is preserved has not been determined, but it is possible that the observed effect is a function of the removal of "free" iron from the system.

It is well-established that iron indirectly stimulates myoglobin (Mb) oxidation via lipid oxidation catalyzed by redox active iron (7–9). Current work regarding the effect of lipid oxidation on Mb stability follows two lines of thinking. (i) It has been proposed that aldehydes, byproducts from lipid oxidation, are capable of affecting the Mb molecule itself (8, 10). Binding sites for 4-hydroxy-2-nonenal were recently identified by Alderton et al. (11). It was suggested that the resulting change to the tertiary structure of Mb opens the heme cleft, allowing oxidizing species easier access. (ii) Monahan et al. (12), however, suggested that the effect of lipid oxidation on Mb results in part from the consumption of O<sub>2</sub> and the concomitant lowering of its partial pressure (pO<sub>2</sub>). This would agree with previous observations regarding the protective effect of high and very low pO<sub>2</sub> against Mb oxidation (13).

Several different pathways for Mb oxidation in the absence of lipid have been proposed. These include the dissociation of

superoxide (O<sub>2</sub><sup>•-</sup>) from MbO<sub>2</sub> (14), anion-mediated electron transfer (15), nucleophilic displacement of oxygen from MbO<sub>2</sub> (16, 17), oxidation of Mb by oxygen-derived species such as H<sub>2</sub>O<sub>2</sub> and hydroxyl radicals (OH<sup>•</sup>) (18, 19), or a combination of two or more of the above factors (9, 20). Most proposed models for Mb oxidation do not include a defined role for nonheme iron, although a few have attempted to provide for some contribution, suggesting that some oxidation of MbO<sub>2</sub> to MetMb is caused by Fe<sup>3+</sup>. Gorelik and Kanner (9) included this pathway as one of several contributing to the overall oxidation of Mb but questioned the extent of its contribution.

The aim of this study was to demonstrate that ferrous iron can stimulate Mb oxidation, independent of the effect of lipid oxidation and its byproducts. To examine the effect of iron removal, two phosphate type iron chelators [sodium tripolyphosphate (STPP) and MM] were chosen. Two pH levels were used, representative of physiological (pH 7.2) and postmortem muscle (pH 5.6), since Mb stability is known to decrease with increasing acidity (15, 16, 21, 22).

## MATERIALS AND METHODS

**Materials.** Ferrous chloride (FeCl<sub>2</sub>) was obtained from JT Baker (Phillipsburg, NJ). STPP, Tris(hydroxymethyl)aminomethane HCl (Tris), and 2-(4-morpholine)ethanesulfonic acid (MES) were obtained from Fisher Scientific (Fairlawn, NJ). Lyophilized horse skeletal Mb (95–100% purity), sodium dithionite, and bathophenanthroline were obtained from Sigma Scientific (St. Louis, MO). Sephadex G-25 Fine beads were obtained from Pharmacia Fine Chemicals, Inc. (Piscataway, NJ). Soy phosphatidylcholine (SPC) was obtained from Avanti Polar Lipids (Alabaster, AL). MM was obtained from Glanbia Nutritional (Twin Falls, ID).

**Reagent and Buffer Preparation.** FeCl<sub>2</sub> solution was prepared at a concentration of 0.1 mg/mL in 0.1 N HCl. Bathophenanthroline solution was prepared at a concentration of 0.13 mg/mL in a 5% ethanol/95% hexane mixture. To reduce potential competition for iron between

\* To whom correspondence should be addressed. Tel: 435-797-1768. Fax: 435-797-2379. E-mail: karin.allen@usu.edu.

**Table 1.** Model System Formulations<sup>a</sup>

model system	component				SPC <sup>d</sup> (mg)
	buffer <sup>b</sup> (mL)	MbO <sub>2</sub> (mL)	FeCl <sub>2</sub> ( $\mu$ L)	chelator <sup>c</sup> (mg)	
1-control	1.25	1.25			(5)
2-control + Fe	1.20	1.25	50		(5)
3-MM	1.25	1.25		5	(5)
4-MM + Fe	1.20	1.25	50	5	(5)
5-STPP	1.25	1.25		5	(5)
6-STPP + Fe	1.20	1.25	50	5	(5)

<sup>a</sup> Lipid-free and lipid-containing systems were formulated identically, with the exception of the lipid component. <sup>b</sup> Buffer used was either 0.04 M 2-(4-morpholine)ethanesulfonic acid at pH 5.6 or 0.1 M Tris(hydroxymethyl)aminomethane HCl at pH 7.2. <sup>c</sup> Chelator used was either MM or STPP, as designated in the model system name. <sup>d</sup> SPC was used only in lipid-containing systems. MbO<sub>2</sub> = 0.1 mM MbO<sub>2</sub>; FeCl<sub>2</sub> = 0.1 mg/mL in 0.1 N HCl.

**Table 2.** Means Pooled Over Time for %MbO<sub>2</sub> Remaining in MS and LS Model Systems at pH 5.6<sup>a</sup>

pH 5.6	%MbO <sub>2</sub> remaining in MS (lipid-free)	%MbO <sub>2</sub> remaining in LS (2 mg lipid/mL)	<i>p</i> value (MS vs LS)
1-control	61.7 ± 20.8 B,C,D	50.7 ± 18.3 A,B	NS
2-control + Fe	45.4 ± 16.2 D	42.2 ± 14.6 B	NS
3-MM	82.1 ± 17.2 A	53.5 ± 19.5 A	<i>p</i> = 0.0003
4-MM + Fe	54.2 ± 18.1 C,D	46.1 ± 19.2 A,B	NS
5-STPP	73.6 ± 16.5 A,B	45.4 ± 15.9 A,B	<i>p</i> = 0.0003
6-STPP + Fe	63.3 ± 17.6 B,C	49.5 ± 16.9 A,B	NS

<sup>a</sup> Values represent means ± standard deviations. Values sharing letters within (but not between) columns are not significantly different (*p* > 0.05). *p* values are shown for lipid-free vs lipid-containing systems with the same formulation. NS, no significant difference.

buffers and test compounds, nitrogen-based buffers (Tris and MES) were used. These buffers also slowed the rate of iron autooxidation (and the subsequent formation of oxidizing species such as hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>)) as compared to oxygen-based buffers (23). MES buffer solution (0.04 M) was prepared in distilled water and adjusted to a final pH of 5.6 using a concentrated sodium hydroxide solution. Two portions of Tris buffer (0.1 M) were prepared as described for MES, with final pH values of 7.2 and 8.0. Residual iron was then removed from buffers using a bathophenanthroline extraction (24). Briefly, 100 mL of buffer was extracted three times with 10 mL aliquots of bathophenanthroline solution, using a separatory funnel. The extracted buffer was heated to approximately 95 °C while being stirred rapidly to remove residual ethanol. The buffer was cooled, and the final volume adjusted back to 100 mL with distilled, deionized water (DDI; <1 mL).

**Iron Content of Mb and Phospholipid.** Because Mb and lipid could not be purified of contaminating iron using the bathophenanthroline extraction, their total iron content was determined. Samples were dry ashed in triplicate and then assayed for iron using the Ferrozine procedure (25). The ratio of moles of Mb to moles of iron was 1:0.994, which was very close to the expected ratio of 1:1. Therefore, it was concluded that the horse skeletal Mb was not contaminated with extraneous iron. However, the SPC contained 12  $\mu$ g iron/g. This contamination was considered in terms of model system design and subsequent interpretation of results.

**Generation of Oxymyoglobin (MbO<sub>2</sub>) Stock Solutions.** A concentrated solution of horse skeletal Mb (~20 mg/mL) was prepared in DDI water (21). Because precipitation was observed when sodium dithionite crystals were added directly, the Mb was reduced by adding 100  $\mu$ L of a 40 mg/mL dithionite solution (26). The Mb solution was agitated gently until the characteristic purplish color of deoxymyoglobin (dMb) was observed. To remove excess dithionite, the dMb solution was passed through a 4 cm Sephadex G25 column and eluted with Tris buffer (pH 8.0). dMb was converted to oxymyoglobin (MbO<sub>2</sub>) by swirling and gently bubbling air through the solution with a Pasteur

**Table 3.** Means Pooled over Time for %MbO<sub>2</sub> Remaining in MS and LS Model Systems at pH 7.2<sup>a</sup>

pH 7.2	%MbO <sub>2</sub> remaining in MS (lipid-free)	%MbO <sub>2</sub> remaining in LS (2 mg lipid/mL)	<i>p</i> value (MS vs LS)
1-control	77.7 ± 13.6 C	66.5 ± 23.8 A,B	<i>p</i> = 0.01
2-control + Fe	64.3 ± 16.7 D	58.7 ± 20.4 B	NS
3-MM	87.5 ± 12.9 A	68.6 ± 22.7 A,B	<i>p</i> < 0.0001
4-MM + Fe	80.3 ± 11.4 B,C	63.5 ± 21.2 A,B	<i>p</i> < 0.0001
5-STPP	91.1 ± 7.6 A	70.2 ± 23.6 A	<i>p</i> < 0.0001
6-STPP + Fe	86.1 ± 7.7 A,B	65.2 ± 21.5 A,B	<i>p</i> < 0.0001

<sup>a</sup> Values represent means ± standard deviations. Values sharing letters within (but not between) columns are not significantly different (*p* > 0.05). *p* values are shown for lipid-free vs lipid-containing systems with the same formulation. NS, no significant difference.

pipet. Conversion to MbO<sub>2</sub> was confirmed spectrophotometrically, based on the presence of the characteristic MbO<sub>2</sub> peaks at 545 and 580 nm (27). The concentration of the MbO<sub>2</sub> stock solution was adjusted to ~0.1 mM with the appropriate buffer (MES, pH 5.6, or Tris, pH 7.2). Fresh MbO<sub>2</sub> stock was generated for each experiment and replicate. The total Mb concentration (MbO<sub>2</sub> + dMb + MetMb) of each individual stock was determined using a millimolar extinction coefficient of 7.6 at the isobestic point of 525 nm (27). The actual concentration of each model system solution was calculated as one-half of the corresponding stock concentration, because the MbO<sub>2</sub> stock was diluted 1:1 in all cases (Table 1).

**Preparation of Mb Model System Samples.** Model systems (MS) were prepared in the appropriate buffer (MES, pH 5.6, or Tris, pH 7.2) using a combination of MbO<sub>2</sub>, FeCl<sub>2</sub>, and/or antioxidant (Table 1) to observe the effect of nonheme iron on the conversion of MbO<sub>2</sub> to MetMb in the absence of lipid. Lipid-containing model systems (LS) were prepared in the appropriate buffer (MES, pH 5.6, or Tris, pH 7.2) using a combination of MbO<sub>2</sub>, SPC, FeCl<sub>2</sub>, and/or antioxidant to examine the effect in the presence of lipid. Systems were designed to provide the following concentrations: MbO<sub>2</sub>, 50  $\mu$ M; Fe, 35  $\mu$ M (where the ratio of Mb to added iron was equal to reported concentrations of Mb and Fe in beef muscle; 21, 28); SP, 2 mg/mL; and antioxidant (MM or STPP), 2 mg/mL. Systems were prepared in disposable 3.5 mL spectrophotometer cuvettes, covered with disposable cuvette caps, and inverted 10 times prior to running the initial spectral scan (400–650 nm), using a Shimadzu UV2100U spectrophotometer (Shimadzu Corporation, Columbia, MD). Scans were repeated for each system at 15, 30, 45, and 60 min and at 1 and 2 days. Samples were held at room temperature (23 °C), as previous reports indicated that the effect of temperature on Mb oxidation was minimal between 10 and 20 °C (22). Five complete replicates were performed at each pH.

**Preparation of Samples for Visual and Spectral Examination.** Samples and control were prepared using the same formulation as for model systems 1–4 (Table 1) at pH 5.6 only, using 0.2 mM MbO<sub>2</sub> stock. Representative spectra (400–650 nm) were obtained at 0.5 min, 1 h, and 1 day. Digital photographs of the cuvettes were taken at 1 h and 1 day. Samples were held at room temperature (23 °C).

**Preparation of Samples for Measurement of Iron Concentration Effects.** Samples were prepared as for model system 2 (Table 1) at pH 5.6 only, but the iron concentration was varied from 35, 180, and 350  $\mu$ M added iron. Control (0  $\mu$ M added iron) consisted of equal parts MbO<sub>2</sub> and MES. Additional control samples were prepared containing 2 mg/mL MM or STPP, with no added iron. Representative spectra (400–650 nm) were obtained every 5 min for 1 h, for a total of 13 scans per sample.

**Calculation of %MbO<sub>2</sub> Remaining.** Absorbance values were corrected to account for turbidity in samples resulting from the addition of MM and/or SPC. The total Mb concentration (MbO<sub>2</sub> + dMb + MetMb) can be determined using a millimolar extinction coefficient of 7.6 at the isobestic point of 525 nm (27). Because the concentration of Mb added to each model system was known (KC), a turbidity correction factor was calculated based upon the measured absorbance at 525 nm. The KC was used to estimate what the absorbance would be in the absence of turbidity (where  $KC \times 7.6 = A_{525}$ ). The calculated

**Table 4.** Means for %MbO<sub>2</sub> Remaining at Selected Time Points in Model Systems at pH 5.6<sup>a</sup>

samples at pH 5.6	%MbO <sub>2</sub> remaining			
	initial (0.5 min)	15 min	60 min	1 day
	lipid-free model systems			
1-control	81.2 ± 7.6 A,B	73.5 ± 10.9 A,B,C	69.3 ± 10.7 A,B	36.4 ± 4.4 <sup>*</sup> B,C
2-control + Fe	73.3 ± 5.9 A,B	54.0 ± 4.6 <sup>*</sup> C	41.7 ± 6.8 C	27.1 ± 9.0 C
3-MM	93.9 ± 12.9 A	89.7 ± 10.6 A	89.1 ± 12.6 A	65.8 ± 7.7 <sup>*</sup> A
4-MM + Fe	66.7 ± 18.5 B	61.3 ± 16.9 B,C	59.6 ± 16.5 B,C	38.3 ± 5.5 <sup>*</sup> B,C
5-STP	84.5 ± 12.1 A,B	82.4 ± 12.1 A,B	80.0 ± 11.5 A,B	57.9 ± 6.5 <sup>*</sup> A,B
6-STP + Fe	79.5 ± 12.1 A,B	72.8 ± 10.7 A,B,C	68.1 ± 7.9 A,B	44.3 ± 8.9 <sup>*</sup> A,B,C
	lipid-containing model systems			
1-control	69.5 ± 10.6 NS	61.9 ± 8.9 NS	55.7 ± 8.8 A,B	24.9 ± 6.7 <sup>*</sup> NS
2-control + Fe	62.4 ± 12.6 NS	51.7 ± 8.8 NS	37.3 ± 9.5 B	25.1 ± 4.7 <sup>*</sup> NS
3-MM	72.8 ± 15.7 NS	62.4 ± 10.6 NS	58.0 ± 9.9 <sup>*</sup> A	27.9 ± 7.3 NS
4-MM + Fe	59.2 ± 22.5 NS	54.8 ± 18.5 NS	50.1 ± 15.2 A,B	28.0 ± 6.2 <sup>*</sup> NS
5-STP	55.5 ± 19.2 NS	50.9 ± 12.2 NS	50.8 ± 14.2 A,B	29.6 ± 10.3 <sup>*</sup> NS
6-STP + Fe	63.4 ± 16.1 NS	55.7 ± 11.2 NS	55.3 ± 13.9 A,B	28.6 ± 6.2 <sup>*</sup> NS

<sup>a</sup> Values represent means ± standard deviations. Values sharing letters within (but not between) column groupings are not significantly different ( $p > 0.05$ ). Asterisk (\*) denotes first significantly different value ( $p < 0.05$ ) vs initial reading ( $t = 0.5$ ) for the given system (within rows). NS, no significant difference.

**Table 5.** Means for %MbO<sub>2</sub> Remaining at Selected Time Points in Model Systems at pH 7.2<sup>a</sup>

samples at pH 7.2	%MbO <sub>2</sub> remaining			
	initial (0.5 min)	15 min	60 min	1 day
	lipid-free model systems			
1-control	97.6 ± 4.9 A	85.5 ± 3.7 <sup>*</sup> A,B	79.3 ± 3.8 B	64.4 ± 3.1 B,C
2-control + Fe	86.2 ± 3.9 B	72.9 ± 4.2 <sup>*</sup> C	67.6 ± 3.9 B	46.4 ± 12.3 D
3-MM	105.7 ± 10.3 A	94.0 ± 2.9 <sup>*</sup> A,B	88.9 ± 2.3 A	77.3 ± 4.1 A,B
4-MM + Fe	96.3 ± 7.0 A,B	84.1 ± 1.2 <sup>*</sup> B,C	82.8 ± 3.6 A	69.5 ± 3.4 B,C
5-STP	99.1 ± 5.3 A	95.9 ± 4.7 A	93.6 ± 4.7 A	84.8 ± 6.0 <sup>*</sup> A
6-STP + Fe	94.7 ± 8.2 A,B	90.6 ± 4.3 A,B	86.5 ± 4.8 A	79.7 ± 4.7 <sup>*</sup> A,B
	lipid-containing model systems			
1-control	87.5 ± 12.9 NS	79.9 ± 8.7 NS	77.0 ± 5.4 NS	33.2 ± 14.0 <sup>*</sup> NS
2-control + Fe	81.5 ± 16.2 NS	70.1 ± 8.8 NS	67.7 ± 7.4 NS	30.2 ± 11.6 <sup>*</sup> NS
3-MM	90.0 ± 10.8 NS	81.1 ± 11.4 NS	75.2 ± 12.9 NS	39.2 ± 10.9 <sup>*</sup> NS
4-MM + Fe	84.0 ± 14.0 NS	72.9 ± 11.5 NS	70.4 ± 8.5 NS	35.4 ± 9.3 <sup>*</sup> NS
5-STP	84.9 ± 14.0 NS	87.0 ± 12.0 NS	78.3 ± 15.4 NS	37.7 ± 7.3 <sup>*</sup> NS
6-STP + Fe	83.9 ± 13.2 NS	76.3 ± 9.8 NS	71.5 ± 10.9 NS	34.4 ± 9.7 <sup>*</sup> NS

<sup>a</sup> Values represent means ± standard deviations. Values sharing letters within (but not between) column groupings are not significantly different ( $p > 0.05$ ). Asterisk (\*) denotes first significantly different value ( $p < 0.05$ ) vs initial reading ( $t = 0.5$ ) for the given system (within rows). NS, no significant difference.

$A_{525}$  was subtracted from the measured  $A_{525}$ , and the difference was used to correct spectra for turbidity.

Because only relative loss of MbO<sub>2</sub> was of interest during the time course of the experiment, actual MbO<sub>2</sub> concentrations were not calculated. The ratio of the sum of absorbance at 545 and 580 nm (characteristic features of MbO<sub>2</sub>) to the absorbance at 505 nm [a characteristic feature of MetMb (29)] was monitored in each model system and compared to the initial reading of the corresponding MbO<sub>2</sub> stock (to account for background absorbance at 505 nm):

$$\text{MbO}_2\text{:met ratio} = \frac{A_{545} + A_{580}}{A_{505}}$$

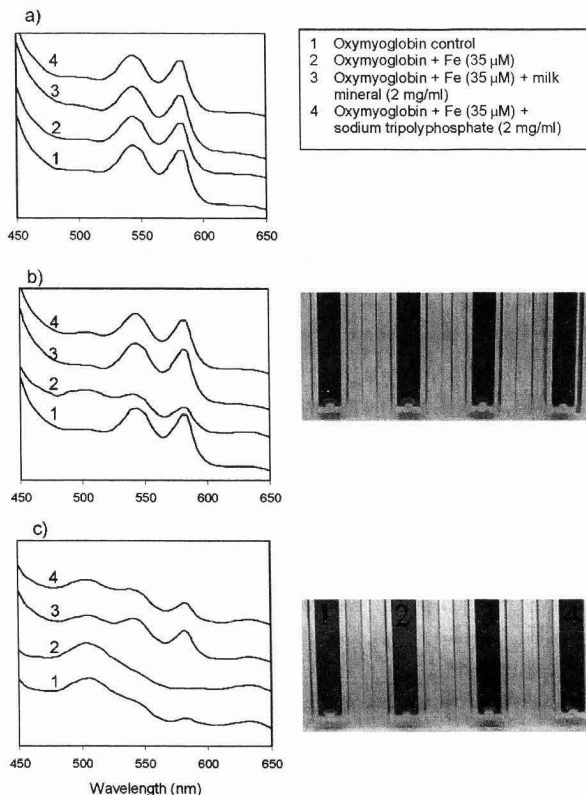
$$\% \text{ MbO}_2 \text{ remaining} = \frac{\text{sample MbO}_2\text{:met ratio}}{\text{stock MbO}_2\text{:met ratio}} \times 100$$

**1 Data Analysis.** For model system values, %MbO<sub>2</sub> remaining was evaluated as a repeated measures design. The Proc mixed function in SAAS version 9.0 (SAS Institute, Inc., Cary, NC) was used with an autoregressive moving average structure. Statistical significance was identified at the 95% confidence level, and posthoc means comparisons were made based on  $p$  values obtained using the Tukey–Kramer adjustment. For data obtained from the effect of iron concentration, %MbO<sub>2</sub> remaining was plotted vs time for each iron concentration.

## RESULTS

**Model Systems.** For lipid-free systems at pH 5.6, significantly more MbO<sub>2</sub> was oxidized to MetMb over the 2 day time course of the experiment in the iron-added system (system 2) than in the systems containing an iron chelator (systems 3 and 5; **Table 2**). At pH 7.2, the difference was even more pronounced, since the iron-added system exhibited more MbO<sub>2</sub> oxidation than any other system, including the control (**Table 3**). Thus, addition of an iron chelator minimized conversion of MbO<sub>2</sub> to MetMb, regardless of pH. While the differences were not as pronounced in lipid systems, MM minimized Mb oxidation at pH 5.6 (system 3; **Table 2**), and STPP minimized Mb oxidation at pH 7.2 (system 5; **Table 3**). At pH 7.2, the addition of iron without a chelator promoted Mb oxidation to the same degree as lipid alone (**Table 3**).

At pH 5.6 (**Table 4**), significant oxidation occurred in the iron-added sample after 15 min, as compared to its initial reading (time = 0.5 min). In other model system formulations, Mb oxidation was not significantly increased until 1 day incubation time. By 15 min, the iron-added system (system 2) had significantly more MbO<sub>2</sub> oxidation than either system containing a chelator (systems 3 and 5). This difference became more pronounced as time passed. After 1 day, the MM sample (system



**Figure 1.** Spectral and visual evidence of Mb oxidation by nonheme iron after (a) 0.5 min, (b) 1 h, and (c) 1 day. Samples were held at room temperature (23 °C) at pH 5.6.

3) had more MbO<sub>2</sub> remaining than both the control (system 1) and the iron-added sample (system 2).

In lipid systems at pH 5.6 (Table 4), the MM system (system 3) exhibited a significant conversion of MbO<sub>2</sub> to MetMb by 60 min, while other systems did not change significantly in MbO<sub>2</sub> content until day one. However, the MM sample was less oxidized at 60 min than the iron-added sample.

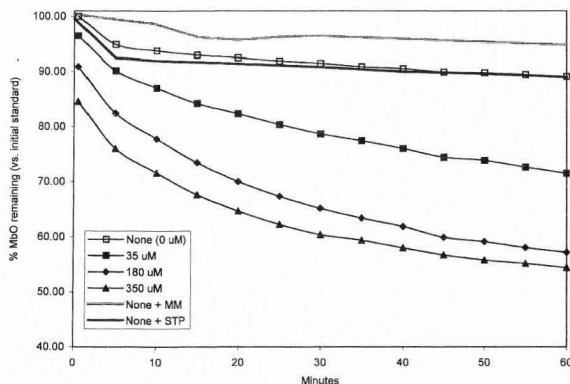
At pH 7.2, the addition of STPP (with or without additional iron) delayed the onset of Mb oxidation (Table 5). Although other samples differed significantly from the initial readings by 15 min, samples containing STPP did not differ until 1 day. No significant difference existed, however, between samples containing only MM or STPP at any time point. The addition of either chelator preserved MbO<sub>2</sub> vs the control and the iron-added samples after 60 min; the effect was more pronounced after 1 day. In lipid systems, MbO<sub>2</sub> values did not differ from initial readings until 1 day had passed (Table 5).

#### Spectral and Visual Changes as Affected by Added Iron.

Initially (0.5 min), spectra for all samples show distinct peaks at 545 and 580 nm, characteristic of MbO<sub>2</sub> (Figure 1). After 1 h, the sample containing 50  $\mu$ M added iron (without chelator) was noticeably more brown, with lower absorbance values at 545 and 580 nm. As absorbance values at 545 and 580 nm decreased, new peaks appeared at 505 and 630 nm, indicative of a conversion from MbO<sub>2</sub> (bright red) to MetMb (brown). After 1 day, spectra indicated a shift to MetMb in all samples. This shift was more prominent, however, in the control and iron-added samples. Visually, the samples with added MM or STPP retained more red color than the control or iron-added samples. Distinct MbO<sub>2</sub> peaks were still visible in the spectra of both samples containing an iron chelator (MM or STPP).

**Effect of Iron Concentration.** MbO<sub>2</sub> oxidation appears to occur more rapidly with the addition of iron, in a concentration-dependent manner (Figure 2). After 60 min, in the MM sample





**Figure 2.** Effect of added iron concentration on Mb oxidation at pH 5.6. %MbO<sub>2</sub> remaining was calculated as the ratio of the MbO<sub>2</sub> peaks to the MetMb peak divided by the same ratio in the initial stock solution: %MbO<sub>2</sub> remaining = [(A<sub>645</sub> + A<sub>580</sub>)/(A<sub>505</sub>) in sample]/[(A<sub>645</sub> + A<sub>580</sub>)/(A<sub>505</sub>) in stock solution].

only, 5% of the initial MbO<sub>2</sub> had oxidized, while in both the control and the STPP samples, 11% was oxidized. The oxidation of MbO<sub>2</sub> in the control sample suggests that a trace level of unbound iron may have been present in the Mb stock, since this loss was not seen in either the MM or the STPP control. In samples with 35, 180, or 350 μM added iron, 18, 43, and 46% of the MbO<sub>2</sub> originally present, respectively, was converted to MetMb.

## DISCUSSION

The addition of unchelated, nonheme ferrous iron had a significant effect on the rate at which MbO<sub>2</sub> was converted to MetMb in this experiment. In some systems containing an iron chelator, MbO<sub>2</sub> oxidation was prevented for over 1 h. However, all samples eventually exhibited some degree of Mb oxidation. The source of this background oxidation was not discernible based on these experiments. In lipid systems, the oxidation of MbO<sub>2</sub> was most likely due to the presence of lipid oxidation byproducts. Nonenal, a product of lipid oxidation, has been shown to bind to certain histidine residues in Mb, altering tertiary structure and potentially opening the heme cleft (11). This conformational change increases the accessibility of oxidant species to the heme iron.

The observed stimulatory effect of iron on Mb oxidation in the absence of lipid is in disagreement with the findings of Gorelik and Kanner (9). Ferric iron was used in their study, and no difference in oxidation was seen between the Mb control and the sample with added ferric iron in lipid free systems. It has been suggested that for iron to exhibit a catalytic effect on the oxidation of biological molecules, it must be redox active (i.e., both ferric and ferrous forms must be present; 38). In the case of ferrous iron, both species would be present in aerobic systems as a result of autooxidation. Ferric iron, however, requires the addition of a compound capable of reducing it to its ferrous form. In the Gorelik and Kanner study (9), ascorbic acid was used as the reductant. Ascorbic acid minimized Mb oxidation in their study, both with and without the addition of iron. This result was most likely due to the addition of ascorbic acid in excess, reducing all ferric iron to the ferrous form, thus preventing redox cycling.

Although the exact manner in which ferrous iron promoted Mb oxidation in this study cannot be determined based on the current results, several possibilities exist. Castro (30) reported that, in anaerobic systems, ferric iron did not react with MbO<sub>2</sub>, while ferrous iron accelerated the rate of dissociation of O<sub>2</sub>. Because it is generally accepted that Mb is the oxidized species (15, 21), nonheme ferrous iron may hasten Mb oxidation through this action, although the extent to which Castro's results apply to aerobic systems is not known.

Ferrous iron is also known to participate in various reactions resulting in the generation of oxidizing species. For example, H<sub>2</sub>O<sub>2</sub> reacts with Fe<sup>2+</sup> to produce OH<sup>-</sup> through the Fenton reaction. H<sub>2</sub>O<sub>2</sub> may also be generated as a byproduct of iron autooxidation. Although these species are potent oxidants, the extent of their contribution to Mb oxidation has been questioned. Wazawa et al. (18) reported the rate at which H<sub>2</sub>O<sub>2</sub> reacts with dMb was only 1 order of magnitude higher than that for its reaction with MetMb (1.3 × 10<sup>7</sup> and 1.6 × 10<sup>6</sup> h<sup>-1</sup> M<sup>-1</sup>, respectively). In contrast, OH<sup>-</sup> reacts with biomolecules rapidly and relatively indiscriminately (31, 32). Although it is undoubtedly capable of oxidizing the heme iron of Mb, its overall contribution may be limited due to the logistics of transporting such a reactive species from its generation site.

While the ability of H<sub>2</sub>O<sub>2</sub> and OH<sup>-</sup> to initiate biological oxidations cannot be denied, it has been suggested that a more important initiator is an iron-oxygen complex, such as the ferryl (Fe<sup>2+</sup>-O) or ferryl (Fe<sup>2+</sup>-O<sub>2</sub>) ion (33-35). In a spin trapping study using dimethyl sulfoxide, ethanol, or glucose as substrates, Qian and Buettner (36) demonstrated that iron-oxygen complexes, formed through "Fe<sup>2+</sup> + O<sub>2</sub> chemistry", were the primary initiators of biological oxidation reactions. Evidence for the existence of such a complex in aqueous solution has also been suggested by Hochstein (37). This complex could participate in the formation of an Fe<sup>2+</sup>-OO-Fe<sup>2+</sup> bridge, which has been proposed as an intermediate step in Fe<sup>2+</sup> autooxidation (38) and was detected in free porphyrin ring oxidation (39). Additional studies are needed to determine the possible existence of iron-oxygen intermediate species in the oxidation of aerobic Mb model systems.

In conclusion, oxygen-based chelators such as phosphate

stabilize iron in the ferric state, while nitrogen-based chelators stabilize ferrous iron (40). In the absence of an efficient reducing system, any phosphate-chelated iron would remain in the nonreactive ferric state, preventing its participation in any of the above-mentioned mechanisms. This explains the effectiveness of the type II iron-binding antioxidants used in this and previous studies (1–4). Regardless of the mechanism by which nonheme iron stimulates Mb oxidation, the effect has been demonstrated in this study and should be considered when discussing Mb oxidation pathways. Further investigation is needed to determine the possible role for nonheme ferrous iron on Mb oxidation in vivo or in meat.

#### ABBREVIATIONS USED

MetMb, metmyoglobin; MM, milk mineral; Mb, myoglobin; STP, partial pressure of oxygen; STPP, sodium tripolyphosphate; Tris, Tris(hydroxymethyl) aminomethane HCl buffer; MES, 2-(4-morpholine)ethanesulfonic acid buffer; SPC, soy phosphatidylcholine; DDI, distilled, deionized water; dMb, deoxymyoglobin; MbO<sub>2</sub>, oxymyoglobin; MS, myoglobin model system; LS, lipid model system; O<sub>2</sub><sup>•-</sup>, superoxide radical; OH<sup>•</sup>, hydroxyl radical.

#### LITERATURE CITED

- Govindarajan, S.; Hultin, H. O. Myoglobin oxidation in ground beef: mechanistic studies. *J. Food Sci.* **1977**, *42*, 571–577, 582.
- Lee, B. J.; Hendricks, D. G.; Cornforth, D. P. Antioxidant effects of carnosine and phytic acid in a model beef system. *J. Food Sci.* **1998**, *63*, 394–398.
- Lee, B. J.; Hendricks, D. G.; Cornforth, D. P. Effect of sodium phytate, sodium pyrophosphate and sodium tripolyphosphate on physico-chemical characteristics of restructured beef. *Meat Sci.* **1998**, *50*, 273–283.
- Vissa, A.; Cornforth, D. Comparison of milk mineral, sodium tripolyphosphate and vitamin E as antioxidants in ground beef in 80% oxygen modified atmosphere packaging. *J. Food Sci.* **2006**, *71*, C65–C68.
- Allen, K. E.; Cornforth, D. P. Mode of action of milk mineral in preventing oxidative rancidity in cooked meat systems. *Proc. 51st Int. Congr. Meat Sci. Technol.* **2005**, paper F10.
- Allen, K. E.; Cornforth, D. P. Antioxidant mechanism of milk mineral—high affinity iron binding. *J. Food Sci.*, in press.
- Kanner, J.; Shegalovich, I.; Harel, S.; Hazan, B. Muscle lipid peroxidation dependent on oxygen and free metal ions. *J. Agric. Food Chem.* **1988**, *36*, 409–412.
- Lynch, M. P.; Faustman, C. Effect of aldehyde lipid oxidation products on myoglobin. *J. Agric. Food Chem.* **2000**, *48*, 600–604.
- Kanner, J.; Gorelik, S. Oxymyoglobin oxidation and membranial lipid peroxidation initiated by iron redox cycle. *J. Agric. Food Chem.* **2001**, *49*, 5939–5944.
- Faustman, C.; Liebler, D. C.; McClure, T. D.; Sun, Q.  $\alpha\beta$ -Unsaturated aldehydes accelerate oxymyoglobin oxidation. *J. Agric. Food Chem.* **1999**, *47*, 3140–3144.
- Alderton, A. L.; Faustman, C.; Liebler, D. C.; Hill, D. W. Induction of redox instability of bovine myoglobin by adduction with 4-hydroxy-2-nonenal. *Biochemistry* **2003**, *42*, 4398–4405.
- Monahan, F. J.; Skibsted, H. S.; Andersen, M. L. Mechanism of oxymyoglobin oxidation in the presence of oxidizing lipids in bovine muscle. *J. Agric. Food Chem.* **2005**, *53*, 5734–5738.
- George, P.; Stratman, C. J. The oxidation of myoglobin to metmyoglobin by oxygen. 2. The relation between the first order rate constant and the partial pressure of oxygen. *Biochem. J.* **1952**, *51*, 418–425.
- Weiss, J. J. Nature of the iron-oxygen bond in oxyhaemoglobin. *Nature* **1964**, *202*, 83–84.
- Wallace, W. J.; Houtchens, R. A.; Maxwell, J. C.; Caughey, W. S. Mechanism of autooxidation for hemoglobins and myoglobins. *J. Biol. Chem.* **1982**, *257*, 4966–4977.
- Brantley, R. E.; Smerdon, S. J.; Wilkinson, A. J.; Singleton, E. W.; Olson, J. S. The mechanism of autooxidation of myoglobin. *J. Biol. Chem.* **1993**, *268*, 6995–7010.
- Satoh, Y.; Shikama, K. Autooxidation of oxymyoglobin: A nucleophilic displacement mechanism. *J. Biol. Chem.* **1981**, *256*, 10272–10275.
- Wazawa, T.; Matsuoka, A.; Tajima, G.; Sugawara, Y.; Nakamura K.; Shikama, K. Hydrogen peroxide plays a key role in the oxidation reaction of myoglobin by molecular oxygen. *Biophys J.* **1992**, *63*, 544–550.
- Goldstein, S.; Samuni, A. Intra- and intermolecular oxidation of oxymyoglobin and oxyhemoglobin induced by hydroxyl and carbonate radicals. *Free Radical Biol. Med.* **2005**, *39*, 511–519.
- Tajima, G.; Shikama, K. Autooxidation of oxymyoglobin: An overall stoichiometry including subsequent side reactions. *J. Biol. Chem.* **1987**, *262*, 12603–12606.
- Brown, W. D.; Mebine, L. B. Autooxidation of oxymyoglobins. *J. Biol. Chem.* **1969**, *244*, 6696–6701.
- Yin, M. C.; Faustman, C. Influence of temperature, pH, and phospholipid composition upon the stability of myoglobin and phospholipid: A liposome model. *J. Agric. Food Chem.* **1993**, *41*, 853–857.
- Welch, K. D.; Davis, T. Z.; Aust, S. D. Iron autooxidation and free radical generation: Effects of buffers, ligands, and chelators. *Arch. Biochem. Biophys.* **2002**, *397*, 360–369.
- Schlit, A. A. *Analytical Applications of 1,10-Phenanthroline and Related Compounds*, 1st ed.; Pergamon Press: Oxford, New York, 1969.
- Carter, P. Spectrophotometric determination of serum iron at the submicrogram level with a new reagent (ferrozine). *Anal. Biochem. J.* **1971**, *40*, 450–458.
- Sage, J. T.; Morikis, D.; Champson, P. M. Spectroscopic studies of myoglobin at low pH: Heme structure and ligation. *Biochemistry* **1991**, *30*, 1227–1237.
- Bowen, W. J. The absorption spectra and extinction coefficients of myoglobin. *J. Biol. Chem.* **1949**, *179*, 235.
- Schricker, B. R.; Miller, D. D. Effects of cooking and chemical treatment on heme and nonheme iron in meat. *J. Food Sci.* **1983**, *48*, 1340–1343, 1349.
- Brill, A. S.; Williams, R. J. P. The absorption spectra, magnetic moments and the binding of iron in some haemoproteins. *Biochem. J.* **1961**, *78*, 246–253.
- Castro, C. E.; Wade, R. S.; Belsner, N. O. Conversion of oxymyoglobin to methemoglobin by organic and inorganic reductants. *Biochemistry* **1978**, *17*, 225–231.
- Graff, E.; Mahoney, J. R.; Bryant, R. G.; Eaton, J. W. Iron-catalyzed hydroxyl radical formation. *J. Biol. Chem.* **1984**, *259*, 3620–3623.
- Halliwell, B.; Gutteridge, J. M. C. Oxygen toxicity, oxygen radicals, transition metals and disease. *Biochem. J.* **1984**, *219*, 1–14.
- Koppenol, W. H.; Liebman, J. F. The oxidizing nature of the hydroxyl radical: A comparison with the ferryl ion. *J. Phys. Chem.* **1984**, *88*, 99–101.
- Bielski, B. J. H. Generation of iron (IV) and iron (V) complexes in aqueous solutions. *Methods Enzymol.* **1990**, *186*, 108–113.
- Yin, D.; Lingner, H.; Ekstrand, B.; Brunk, U. T. Fenton reagents may not initiate lipid peroxidation in an emulsified linoleic acid model system. *Free Radical Biol. Med.* **1992**, *13*, 543–556.
- Qian, S. Y.; Buettner, G. R. Iron and dioxygen chemistry is an important route to initiation of biological free radical oxidations: An electron paramagnetic resonance spin trapping study. *Free Radical Biol. Med.* **1999**, *26*, 1447–1456.
- Hochstein, P.; Nordenbrand, K.; Ernster, L. Evidence for the involvement of iron in the ADP-activated peroxidation of lipids in microsome and membranes. *Biochem. Biophys. Res. Commun.* **1964**, *14*, 323–328.

- (38) F€eigeig, A. L.; Lippard, S. J. Reactions of non-heme iron(II) centers with dioxygen in biology and chemistry. *Chem. Rev.* **1994**, *94*, 7759-805.
- (39) CChihin, D.; Del Gaudio, J.; La Mar, G. N.; Balch, A. L. Detection and characterization of the long-postulated Fe-O-O-Fe intermediate in the autoxidation of ferrous porphyrins. *J. Am. Chem. Soc.* **1977**, *99*, 5486-5488.
- (40) MMiller, D. M.; Buettner, G. R.; Aust, S. D. Transition metals as cocatalysts of "autoxidation" reactions. *Free Radical Biol. Med.*

1990, 8, 95-108.

---

Received for review August 11, 2006. Revised manuscript received October 18, 2006. Accepted October 20, 2006. We thank Glanbia Nutritionals (Twin Falls, Idaho), Dairy Management, Incorporated (Chicago, Illinois), and the Utah CURI Grant Program for their financial consideration. This is Utah Agricultural Experiment Station paper #7757.

JF0623182