12-1989

# Evaluation procedures to predict the keeping quality of ultra-high temperature (UHT) processed skim milk

Athanas AJ Ndomba

To the Graduate Council:

I am submitting herewith a thesis written by Athanas AJ Ndomba entitled "Evaluation procedures to predict the keeping quality of ultra-high temperature (UHT) processed skim milk." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Food Science and Technology.

Genevieve L. Christen, Major Professor

We have read this thesis and recommend its acceptance:

R. Biswal, B. Demott

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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and recommend its acceptance:

B. J. Dennott

R. N. Biswal

Accepted for the Council:

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Vice Provost
and Dean of The Graduate School

# EVALUATION OF PROCEDURES TO PREDICT THE KEEPING QUALITY OF ULTRA-HIGH TEMPERATURE (UHT) PROCESSED SKIM MILK

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Athanas A. J. Ndomba

December 1989

## DEDICATION

I dedicate this work to my lovely mother Gonsalba Ndomba, my brothers, Chrisostoms, Aidan, Hadrian, Fulko, my sisters, Valeria, and Singsberta for their tireless prayers and for missing me for a long time.

## ACKNOWLEDGMENTS

I am grateful for the cooperation, encouragement, and assistance given me by many individuals during the period of my graduate study. It is impossible to record my gratitude and appreciation to all; however, I would like to single out some whose part was particularly important. I benefited greatly from the advising of Dr. Genevieve L. Christen. Dr. Christen not only served as my academic advisor, but offered financial assistantship in my first year of study, and gave generously her encouragement and valuable insights, for which I am especially grateful. I am also grateful to Dr. R. Biswal, and Dr. B. Demott for their assistance, helpful suggestions, and for serving on my graduate committee. My appreciation goes to Dr. Jack Reese, former Chancellor and Dr. Jim Gehlhar, acting director, Center for International Education for offering financial support. Indeed, I am deeply indebted to them. Especially significant is the friendship, support, and advise given me by Dr. George Legall, of Statistics department. Thanks are due to people at the creamery, especially Joe and Tommy, who took time from their busy schedules and gave me a hand. Thanks are due to Dr. M. Penfield for helping me in sensory evaluation. Thanks also to all Food Tech laboratory technicians, especially Chuck, for their friendship and assistance. For this, and other things, I am truly grateful. While I alone, of course, take responsibility for this study, I am sure that it is a better product as a result of the thinking of these many persons which they shared with me.

## ABSTRACT

This study evaluated the ability of an improved carbon-14 protease assay as a rapid procedure to screen raw milk and predict the keeping quality of UHT processed skim milk. Raw milk composition and microbiological quality were evaluated as possible parameters which could determine the keeping quality of UHT processed skim milk. Protein, total solids, fat, free amino groups, standard plate count, psychrotrophic bacteria count, proteolytic bacteria count, somatic cell count, and initial protease activity (measured by the radioisotope procedure) were determined on 51 raw milk samples pooled from area farms, University farm and University creamery storage tank, which were then skimmed and UHT processed and held for 60 days at 25 C. The samples were analyzed for change in free amino groups upon coagulation or at 60 days whichever occured first. Viscosity was measured on a weekly basis except on the first two weeks. The samples which lasted 60 days were analyzed for bitterness by a panel of experienced judges.

Shelf-life was determined as days to coagulation or 60 days, whichever was sooner. Shelf-life was weakly correlated to initial protease assay ($r=.26$; $p=.06$). Initial protease activity was inversely and weakly correlated to bitterness flavor score ($r=-.27$; $p=.08$). Shelf-life was also weakly and inversely correlated to somatic cell count($-.25$; $p=.08$). However, initial protease activity was positively correlated to psychrotrophic bacteria count but not with other bacteria counts. Bacterial counts were correlated to each other and to somatic cell count. Change in free amino groups were inversely correlated to shelf-life ($r=-.30$; $p=.03$). Viscosity was weakly correlated with bitterness flavor ($r=-.48$; $p=.05$). The relationship between initial protease activity and shelf-life was not

a dramatic one, thus a longer evaluating period is recommended. However, the positive correlation between initial protease activity and psychrotrophic bacteria count is an important finding and suggests the potential of radiolabeled protease assay for rapid enumeration of psychrotrophic bacteria.

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# CHAPTER I

## INTRODUCTION

Raw milk is an extremely heterogenous system which is not very stable due to changes such as creaming and microbiological deterioration. The widespread use of farm bulk tanks where raw milk may be stored for 2 or 3 days at 2-4 C, coupled with prolonged holding of milk at the plant (3-5 days at 5-7 C) prior to processing have further enhanced the risk of quality impairment of dairy products (35). During the 2-3 days or longer which may elapse between milking on the farm and processing in the factory, there is ample time for psychrotrophic microorganisms to grow in cooled milk and produce enzymes (35). The ability of these microorganisms to produce extracellular proteases which are hydrolytic in nature, has drawn attention in the dairy industry in recent years due to the heat resistance of the enzymes produced.

The presence of heat resistant enzymes is a major problem encountered in ultra-high temperature (UHT) pasteurized milk (2). Although heat treatment may destroy the microorganisms, frequently the enzymes survive (43) and they may remain active and cause deterioration of dairy products (65). The action of protease is a major cause of bitter flavor and age-gelation in UHT processed milk. Proteolysis is considered a major contributor to irreversible changes in the physical properties of raw milk (35). Since the marketability of UHT processed milk depends on its long shelf-life and "fresh" taste; proteolysis, a major factor in inhibiting keeping ability needs to be controlled (20,41,48).

Proteolysis does not require large amount of protease activity (48). However, low levels of protease are extremely difficult to measure with the current protease assay procedures (84). A rapid and more sensitive method for

quantification of the activity of partially purified proteases derived from psychrotrophic bacteria has been developed (25). Investigations to determine the ability of this test procedure to correlate with the growth of microorganisms or with subsequent keeping ability of UHT processed milk have not been as successful and more investigation is required.

The objectives of this study were: (a) to re-evaluate improved radioisotopic procedure (carbon-14 protease assay) for ability to screen raw milk and predict the keeping ability of skimmed milk after UHT processing; and (b) to evaluate milk composition and microbiological quality as parameters which could determine the keeping quality of UHT-processed skim milk.

# CHAPTER II

# LITERATURE REVIEW

## A. Ultra-high Temperature (UHT) Processing of Milk

"UHT-milk" is defined as milk that has been heated to at least 130 C for not less than 1 second and then aseptically packaged (70). Generally, UHT-milk processing involves temperatures of 130 C to 150 C and holding time of 1 second or more (22); 2-8 seconds is the general acceptable holding time (50). The bottom line in UHT processing is to achieve a product which is commercially sterile (47,67). The product is commercially sterile when it is heat treated to eliminate all pathogenic organisms and to reduce spoilage organisms to a level where they will not produce a health hazard or reduce the quality and acceptability of the product during its designated shelf-life. According to Burton (22), temperatures below 135 C are unlikely to be sufficient for a commercial process unless the holding times are made undesirably long; and above 150 C, the required holding time would be too short to be maintained effectively. The first recorded UHT-milk processing plant was pioneered by Jonas Nielsen in 1913 and later an aseptic canning system was developed. In late 1940, modern UHT-milk processing plants started using higher temperatures with correspondingly shorter holding times, to give products better bacteriological quality; which resulted in less change in color, flavor and nutritive value of the milk (22). The U.S. Standards of Identity and the Pasteurized Milk Ordinance stipulate that a product labeled "Ultra-pasteurized" must have been heated to

137.8 C or above for at least 2 sec (105) and stored at 4-7 C. Pasteurization standards for U.S. (105) and sterilization standards for other countries (96) have been established. UHT processing is classified according to types of heat exchangers either as direct or as indirect processng.

Direct UHT Processing

The direct UHT process relies on heating by injecting steam into the product or infusing the product into the steam, either method causes virtually instantaneous heating. After sterilization, the product is flash-cooled to remove an amount of water equivalent to the amount of steam condensed during heating (108). There is essentially no net change in the volume of the product. Direct heating systems are more complex than indirect methods; requiring more plant components and controls and as well as needing high quality steam (culinary steam) (21). For these reasons, direct contact heating is relatively expensive both in initial investment and running costs. The major advantage of direct systems are that there is a very rapid heating and cooling and consequently, a low total temperature load on the product. Other advantages include less scaling in the equipment as compared to indirect heating thus permiting longer production runs with less frequent cleaning cycles. Also, the final product contains less oxygen because expansion cooling does not only remove water but also removes dissolved air (oxygen) resulting in an oxygen content of final product (milk) of about or less than 0.1 ppm. A final advantage of the direct method is the ability of the systems to handle viscous products more readily than indirect systems, and giving longer running times (106). Recent modification of infusing milk-into-steam process has resulted in

development of a free-falling-film UHT system (11,61,104). Here the product falls through a steam chamber as a thin laminar, free-falling film. The final product is sterile and is reported to have flavor similar to pasteurized milk. Systems for producing sterile milk, ice cream and other dairy products by "direct" UHT processing have recently been developed (49,63).

Indirect UHT Processing

The indirect UHT process relies on heat transfer to and from the product through stainless steel interfaces such as in pressurized plate or tubular heat exchangers (108). The heat conducting barrier separates the heating agent from the product. The time required for heating the product to sterilization temperature and for cooling after are therefore considerable longer than those required in direct UHT processing. The largest disadvantage of indirect systems is their sensitivity to deposit formation on the heat exchanger surfaces. Deposit formation is a difficult and intractable problem, to which no solution has so far been found. The amount of deposit can vary markedly from one milk source to another (22). Aging milk before processing reduces the amount of deposit, but unfortunately this method causes an unacceptable flavor change (22). Plate heat exchangers are particularly affected by deposit, because of the small inter-plate spacings which rapidly become obstructed. Although tubular systems are more expensive per unit capacity than the plate systems, they are less affected by deposits. The much higher ratio of cross-sectional area to heat exchanger surface which allows more deposits to be accommodated explains part of this difference. The much higher internal pressure that can be tolerated allowing useful products flows to be maintained in partly obstructed flow passages explains another part of the difference (22). Burton (21), Westhoff

(105), Hsu (50) and Ashton (14) have summarized the UHT processes available. The use of electrical resistance (electric heaters) as a source of heat, and heat generated by friction by passing a thin film of milk across the surface of a high-speed rotor, have been described by Burton (22). Milk has also been sterilized by microwave energy, although not commercially (22).

There appears to be no scientific reason related to milk quality which governs the choice of the processing system (21). Practical and commercial reasons are used in considering the relative merits of direct or indirect processing.

## B. Nutritive Value of UHT-Treated Milk

The nutritional quality of UHT-treated milk and milk products is high. The effect of UHT treatment on the nutritive value of milk has to be considered in the light of the effect of processing stage itself and of the changes which occur during storage (108). Nutritive value of UHT is milk is lost because of the chemical structures of the nutrients (70).

### Processing Induced Changes

The effect of UHT processing differs for the various nutrients of milk (70). The essentially unaffected components are fat, fat-soluble vitamins, carbohydrates and minerals, whereas water-soluble vitamins and proteins are adversely affected (108).

UHT processing results in moderate denaturation of whey proteins (25-80% of total) depending on the process employed. However, the nutrititional value is not impaired as a result of denaturation (70). Processing temperatures above 150 C for 2-8 sec may cause considerable denaturation (up to 80%) of the serum proteins of milk, especially beta-lactoglobulin (22). Beta-lactoglobulin, on denaturation, complexes with casein (70). The size of the casein aggregates increases with UHT processing, resulting in changes in their composition (21). White and Sweetsur (106) were unable to show any significant changes in the rate of heat-induced (110-140 C) aggregation of casein by removal of serum proteins or colloidal calcium phosphate or by prevention of kappa-casein/beta-lactoglobulin interaction.

On heat treatment of milk above about 75 C, the sulphur-containing amino acids undergo reactions leading to the formation of volatile materials such as hydrogen sulphide and mercaptans, initiating the typical cooked flavor of sterilized milk (70). As a result of these reactions, the levels of cystine and methionine in UHT milk may be slightly reduced. Aboshama and Hansen (1) observed a 34% loss of cysteine and cystine (as cysteic acid), and methionine in skim milk during UHT processing versus only 5-10% during pasteurization. Lysine levels in milk are reduced by milk sterilization (70); however, methionine and tryptophan which also are heat labile are not much affected (100). No significant changes have been observed for the other amino acids during processing or storage of UHT milk (100).

Studies on test animals fed UHT milk have shown that, in general, the biological value, protein efficiency ratio and digestibility coefficient were not affected by UHT sterilization (21,82). A proportionate decrease in the growth-supporting value of milk protein for test animals occured when serum proteins

were denatured (21,82). A study (10) on 400 newborn infants in Holland, showed an average gain of 7 gm more weight per day to 200 children fed UHT milk compared for 200 who were fed pasteurized milk. The normal weight loss after birth was regained by UHT-milk fed infants. The UHT milk also caused fewer digestive problems.

UHT milk processing has little effect on the nutritional value of milk lipids. The loss of polyunsaturated fatty acids, in which milk is already poor, may be important. Heating milk at 130 C for 20 sec causes loss of 34% linoleic, 13% linolenic and 7% arachidonic acids (100).

UHT processing appears to have no effect on the nutritional value of mineral components (70). Pelet and Donath (77) tested the effect of uperization of "humanised" cow's milk with 8 newborn infants, and found nitrogen balance and phosphorus retention were not affected by uperization; however, the retention of calcium and potassium was higher in infants fed uperized milk than in those fed pasteurized milk (77)

Vitamins, according to several investigators (21,82,100), are more stable under UHT processing conditions than under pasteurization or other "low temperature" heat treatments. Fat-soluble vitamins A, D, E and carotene are little affected by UHT processing (21,82,100). Losses of up to 35% of vitamin A and carotene have been observed on prolonged sterilization of milk (100). Pantothentic acid, nicotinic acid and biotin are not affected (21,82,100). Losses of up to 20% were observed during different UHT treatments. Riboflavin, is heat stable, but is susceptible to light (100) and losses less than 10% have been observed during sterilization (21). Thiamin, biotin, niacin, vitamins B6 and B12 suffer little loss (70). Ascorbic acid (vitamin C) is heat-stable, but is converted to heat-labile dehydroascorbic acid in the presence of oxygen. Folic acid appears to be stable on processing, and is lost after ascorbic acid is eliminated (21,82).

Up to 20% of folic acid may be lost. Of the chemical forms of the vitamins C, dehydroascorbic acid is destroyed, while ascorbic acid is unaffected (about 75% of vitamin C in milk is in the form of ascorbic acid, the remainder in the form of dehydroascorbic acid, which has no vitamin C activity). The effect of initial oxygen content on the levels of folic and ascorbic acid in milk after processing is not clearly defined. To retain nutritional value, low oxygen content is required. However, to remove unacceptable flavor in the first few weeks of storage, high oxygen again is required to avoid the formation of more undesirable flavors (79).

Storage Induced Changes

During storage of UHT milk, casein aggregates increase proportionally with the time of storage (23). Maillard reactions continue during storage of UHT milk, with their rates dependent on storage conditions (70). As a result of these reactions, the nutritional quality of proteins is slowly reduced. Casein, for example becomes more resistant to enzymatic digestion. Up to 30% of lysine may become unavailable after extended storage at higher temperatures (6 months at 30-37 C). The rate of Maillard reaction is unaffected by light or oxygen.

Mineral fraction are unaffected in storage of UHT milk. Equally, the nutritional value of lipids are not altered.

Storage of UHT milk may have a great impact on loss of vitamins. Milk saturated with oxygen losses virtually all of the folic acid and ascorbic acid within a few weeks. Ascorbic acid surviving heat treatment may be lost in storage (21). If the amount of oxygen dissolved in milk is limited, ascorbic acid

losses are minimal (21,82). Oxygen availability is more critical than is the heat treatment in destroying ascorbic acid in milk. Only 1.5-2.0 mg/l of oxygen is required for oxidation of total vitamin C (milk saturated with oxygen contains 8 mg/l). Most indirect processes produce milk with high oxygen content. Vitamin B6 and B12 are lost progressively and continously down to 45% of initial after 90 days. Since UHT milk is not a major nutritional factor in most populations, the loss of these vitamins is not critical (82). However, if unfortified UHT milk is used for feeding of infants and children, these vitamins could be of importance.

## C. Quality Defects Associated with UHT Milk

### Microbiological Aspects

The life of UHT processed milk is limited by a number of factors. Spoilage is the overriding aspect of quality to consumers (22). An UHT product can spoil for a variety of reasons, of which the obvious fault of bacteriological spoilage is one (21,42). To justify the sale of the product as having effective sterility, the level of bacteriological spoilage should be sufficiently low. The definition of "effectively sterile" as with all sterilized products, is a difficult one. Although a small proportion of resistant spores of very high heat resistance, may be expected, Franklin (39) found that a strain of *Bacillus cereus* produced spores in which 1 in 100,000 spores would survive 135 C for 4 h. High counts are unlikely if plant cleaning and equipment pre-sterilization have been effective. Post-sterilization contamination is a great danger. This may be contributed by either inadequate plant cleaning and sterilization (for an organisms likely to be heat resistant) or through atmospheric contamination.

A sterilization process cannot produce absolute sterility (22). It can only reduce microorganisms in the incoming milk to a level that makes the proportion of spoilage in the final product acceptable, either from the public health standpoint or, as is the case most often with UHT products, from commercially acceptable standpoint (22). Perkin (79), suggested that a good commercial UHT process for milk should be based on a 2-log cycle reduction of spores of *Bacillus stearothermophillus* .

Sedimentation

Denaturation of milk proteins or precipitation of the salts in milk are a common phenomena occuring as a result of intensive heat during the UHT process (70). Denaturation of proteins or precipitation of salts causes sedimentation. Studies have shown that, the higher the sterilizing temperature, the more the sedimentation (70). Maximum sedimentation in UHT processing at 140, 145, and 150 C occurs with a 4 sec holding time (21). The calcium balance of the milk and addition of salts affect sedimentation (70). Sodium citrate or bicarbonate inhibit sediment formation, but calcium promotes sediment formation (87). Studies by Biryukova et al. (19) found that adding 0.025-0.10% sodium citrate or disodium phosphate increased the heat stability of milk against sedimentation by 50% to 100%. Preheating helps stabilize milk exposed to heat treatment according to some investigators (6). According to Biryukova et al. (19), milk used for indirect UHT processing should be preheated to 75 C for 20 sec and the precipitated protein removed by centrifugation. Suggestions to help alleviate the problem of milk-stone deposits on heating surfaces by preheating milk in a retarder vessel at 85 C for 6 min have been documented by Ball (17). Other proposed measures which

decrease sedimentation include homogenization of milk at a lower temperature than normal and homogenization after heating rather than before (50).

Sediment formation in UHT milk soon after processing may often be the result of too low a pH in the raw milk (107). A pH of 6.65 will lead to the formation of sediment in the product with substantial amounts of sediment forming if the pH is below 6.55 (107). Neutralization to pH values greater than 6.70 or by addition of phosphate ions to the system may prevent this defect (108). Claesson et al. (27) have suggested that deposit formation in UHT sterile milk could be prevented by adjusting the pH of the milk to 6.9, however, this increases the non-protein nitrogen (NPN) in the UHT milk. Prolonged storage apparently causes some of the sediment to return to solution thus reducing the total amount of sediment (21). Studies have shown that indirect heating causes more sediment than direct heating (21,22). Different views from investigation by Perkins et al. (78) indicate that when heat treatments of the same sporocidal effectiveness were given, directly heated UHT milk had twice as much sediment as indirectly heated milk. Sedimentation is common in whole milk, skim milk, recombined milk and recombined concentrated milk subjected to UHT processing.

Gelation

The formation of semi-solid gel during prolonged storage (age-gelation) of UHT milk limits the shelf life of the product (21). Gelation is therefore an important problem because it signifies the final limit of storage life (21). The mechanism for gelation has not been well established but is probably similar to that for clotting of milk during cheese-making (70). The primary difference is

that the former occurs naturally and the later is induced. A gel is softer than a clotted curd. The phenomenon may occur as quickly as three to four weeks after manufacture or can affect the product after more than six months. Generally, this defect occurs after a period of time which depends on both storage conditions and raw milk characteristics. Typically, the time required for this defect to occur ranges from 15 weeks at 30 C to more than 18 months at 2 C (108). Sensitivity to gelation is greater with UHT processing than with sterilization in a container (70). Milk does not gel for long period after being autoclaved in the bottle (87). No clear relationships have been established between sedimentation and gelation. Investigation by Andrews (7) based on molecular weight changes of casein component, enabled him to postulate that at least two processes takes place during storage of UHT milk. The first process is the result of physical forces of association such as hydrophobic bonding between casein and lactose, which leads to the formation of a gel. The second process is the Maillard reaction where formation of covalently-bonded polymers leads to browning and sediment formation.

Although recent evidence suggests proteolysis by native and/ or bacterial heat resistant proteases to be the cause of age gelation (21,22), other studies point to a mechanism involving purely physico-chemical processes (47). Harwalkar (45,46) has discussed various factors such as processing conditions, composition or quality of milk, additives and temperature of storage which may influence the onset of age gelation in UHT milk . Age gelation is not the result of bacterial action, rather, it is generally accepted as being due to the action of protease that survive UHT treatment (108). Protease is believed to be produced by psychrotrophic bacteria present in the raw milk.

Extensive protein breakdown of kappa-casein to para-k-casein (similar to rennet action) was observed in UHT milk that gelled due to protease (*Pseudomonas fluorescens* ) activity (64). Beta-casein was also broken down rapidly while alpha-casein was degraded slowly. Burton (21) has concluded that coagulation is caused by the slow action of proteolytic enzymes which ultimately destabilize casein. Conflicting studies by Samel et al. (87), found proteolysis not to be the cause of gelation in UHT milk. Suggestions that both coagulation and development of bitter flavor might be caused by protein changes have been documented (21,93). Some reports (75,87) have indicated that reactive sulfhydryl groups may contribute to instability of milk protein leading to gelation and/or deposit formation. An inverse relationship has been observed between the degree of protein decomposition and the time of the on set of gelation in UHT milk (87).

The prevention and control of age gelation in concentrated milk and unconcentrated UHT milk has attracted much research effort (59). A heat treatment at sub-pasteurization temperature immediately after UHT processing has been claimed to delay age gelation in UHT skim milk (103). The process known as low-temperature-inactivation (LTI) involves a heat treatment of milk for 60 min at 55 C. The retardation of the onset of age gelation by LTI has been shown to involve a unique inactivation (conformational change) of heat resistant proteolytic enzyme from psychrotrophic bacteria (18). This heat-induced change is then followed by aggregation of altered proteases with casein to form an enzyme-casein complex, which results in inactivation of the enzyme. LTI treatment results in at least a three-fold increase in shelf life of UHT skim milk without a deleterious effect on milk quality or flavor (58). LTI treatment is feasible before UHT processing as the extended period required to ensure inactivation of the enzyme may make it difficult to maintain sterility (58).

The use of additives to control age gelation may also have potential benefit (59). Corradini (29) determined that the resistance of UHT milk to gelation on storage can be increased in three ways: by reducing the flow-rate of milk through sterilizing equipment, by adding 0.01-0.15% disodium phosphate and by storing the product at 10 C. Varying heat treatment improves control of gelation; however, adding polyphosphates controls best (59). In the past, control or prevention of age gelation has relied on ensuring that raw milk is of as high a quality as possible. More recently, sodium hexametaphosphate (SMP) has been shown to control the development of age gelation in UHT milk (59). At a concentration of 0.5 g/kg, SMP added to raw or UHT milk resulted in at least seven-fold increase in the product shelf-life. The addition of disodium ethylenediaminetetraacetic acid (EDTA) at 1 or 3 g/kg to cold stored raw milk rather than to UHT milk eliminated problems associated with post-sterilization contamination. Much research has been directed toward determining the effects of additives in controlling age gelation (58).

## D. Milk Proteinases

### Proteases of Milk Origin

Two comprehensive literature reviews on milk proteinases have recently been published (52,83). According to Kiermer and Semper (57), the first publication on naturally occurring enzymes was published by Babcock and Russell in 1897. Shahani et al. (92) reported that milk protease was not a single enzyme as previously thought. Most recent studies have indicated protease system to consist of two serine proteases, a chymotrypsin-like protease (an acid protease which is associated with beta-casein) and a neutral

protease which has the specificity for alpha S2-casein. Characterization of aminopeptidase and endoprotease activities have been reported recently (8). Aminopeptidase activity is present in milk serum and the endoproteases are associated with casein micelles. Since most of the work on indigenous milk proteases has been done on the trypsin-like enzyme with the caseins, questions related to the origin of milk enzymes have centered on similarities between isolated trypsin-like enzymes and plasmin prepared from blood serum (38,55).

Plasmin as a Major Indigenous Milk Protease

Kaminogawa et al. (55) reported similarities between the major native milk protease and blood plasmin (E.C. 3.4.21.7.) as far as optimum pH, pH-stabilty, heat-stability, inhibitor susceptibility and molecular weight are concerned. Milk plasmin is the same as blood plasmin (38,52,86,95) and is thought to originate in blood plasma and reach the milk through blood transferred to the mammary gland (52). Studies comparing kinetics of highly purified and stabilized bovine plasmin/plasminogen and the serine protease in milk prepared from the same cow has shown that, one of the serine proteases in milk and plasmin are identical (83). Recently, Politis et al. (80) have developed a model which describes the plasminogen/plasmin system in bovine milk.

Plasmin has optimum activity at pH 8.0 in buffer solution, and is stable in the pH range of 6.0-8.0. This enzyme is relatively labile below pH 5.0 and above 9.0. The enzyme is heat labile, with decreasing activity after heating at 40 -70 C for 10 min. The enzyme is completely inactivated at 80 C (55).

Richardson and Pearce (86) and Korycka-Dahl et al. (60) indicated that high temperature short time pasteurization (HTST) resulted in 17% and 10% decrease in plasmin activity, respectively. Normal bulk raw milk and HTST pasteurized milk had a similar plasmin activity (33). Commercial UHT treatment reduced plasmin activity to undetectable levels and reduced plasminogen derived activity by about 90% (60).

Plasmin in milk hydrolyzes beta-casein to gamma-casein, proteose-peptone component 5 (PP5), proteose-peptone component 8-Fast (PP8F) and other unidentified fragments (10,44). Work by Andrews (8) using qualitative polyacrylamide gel electrophoresis (PAGE) found PP5 as an intermediate product subjected to further proteolysis, while PP8F was a stable end product. PP5 represents a mixture of N-terminal residues 1-105 and 107 of beta-casein, corresponding to the C-terminal matching portions of $\gamma$2- and $\gamma$3-casein (10). PP8F is the N-terminal phosphopeptide, residues 1-28, matching $\gamma$1-casein which is residual 29-209 (10). The central portion of $\beta$-casein, residual 29-105 and 29-107, were suggested by Eigel and Keenan to represent proteose-peptone component 8-slow (PP8S) (10). Plasmin hydrolyzes alpha s2-caseins to beta-casein but not kappa-casein (83). Lambda-casein has been identified by Aimutis and Eigel (4) as a plasmin-derived fragment of bovine alpha s2-casein.

Microbial Protease

Raw milk may inevitably become contaminated with psychrotrophic microorganisms during and/or after milking process if the equipment is not cleaned and sanitized properly. Prolonged storage of raw milk in the bulk tank

prior to processing may enable psychrotrophic microorganisms to grow and produce hydrolytic enzymes including proteases.

Numerous studies have been done on protein degradation and keeping quality of milk and dairy products especially by psychrotrophic bacteria or their proteolytic enzymes (30,31,32,53,73,88). The proteinases (proteases) of microbial origin have been found to survive UHT treatment and are implicated in storage gelation of UHT milk and evaporated milk (5,76,85). These enzymes appear to be stable to the UHT process and thus can survive to cause proteolysis and age gelation even when the parent microorganisms have been destroyed. The action of bacterial proteinases on individual proteins is different from that of indigenous proteinases. Bacterial proteinases appear to degrade kappa-casein preferentially (2,38,65) in contrast to indigenous proteinase. Although there is limited information regarding the specificity of bacterial proteinase, Mitchell and Marshall (71) have recently been able to determine the specificity of heat-stable proteases from four strains of *Pseudomonas fluorescens* on oxidized ribonuclease A. Because of the differences in mode of attack on individual proteins, the bacterial and native milk proteinases influence the storage behavior of UHT products to different extents. Observations by Snoeren et al. (94) revealed that a sample of milk which was deliberately contaminated with psychrotrophic bacteria before UHT treatment, gelled on storage sooner than aseptically drawn milk. The contaminated milk had the appearance of rennet curd in contrast to the uncontaminated milk

(containing only milk proteinases), which coagulated without typical gel formation. Although gelation of both samples was accompanied by extensive proteolysis, the rate was higher in the contaminated milk.

Protease produced by psychrotrophs may also cause off-flavors in other dairy products such as fluid milk, cottage cheese, butter, milk and yogurt; cause bitter flavor development and age gelation of UHT processed milk; influence the growth of lactic acid bacteria; and reduce the quality of certain cheeses and cheese yield. Although the growth and activity of bacteria are reduced at low temperatures, and refrigeration can extend the keeping quality of milk, psychrotrophs do cause spoilage of milk and dairy products. Studies by many researchers have concluded that a strong relationship exists between psychrotrophic contamination and keeping ability of dairy products.

Protease Associated with Somatic Cells

The protease activity associated with elevated somatic cells (SCC) has the potential for proteolytic damage to the raw milk supply upon normal refrigerated storage, in pasteurized fluid milks over shelf-life, and in milk during cheese making (89). Somatic cells (large body cells) in milk increases in late lactation as the mammary gland prepares to become nonfunctional or as a consequence of injury. Mastitis is a reaction of the udder to injury. According to Politis et al. (80), mastitic infection of the mammary gland results in increased proteolytic activities that is related to a higher concentration of plasmin. A model which describes the plasminogen/plasmin system in bovine milk and its relationship to SCC and other factors such as stage of lactation, season, etc. have been developed (80). Politis et al. (80) found that higher plasminogen

and plasmin concentration were associated with increasing SCC. Plasmin is the most significant protease in total activity (80). The influence of mastitis on breakdown of protein (proteolysis) is of great importance in the dairy industry (91). Changes in the synthetic mechanism of milk secretion causing alterations in the casein and whey protein fraction has been linked to mastitis (89). Changes in milk composition associated with elevated SCC include a decrease in total casein, lactose, fat, and total solids. In contrast, whey protein, chloride and sodium increases. The decreases in alpha $S_1$-, beta- and kappa-caseins are of particular interest and have been reported by several authors (2,3,4,6,9,37,89,91), who have also suggested that an increase in proteolytic activity due to inflammatory response may be responsible for the changes in protein fraction in milk. Murphy et al. (72) have also found an increase in protease activity associated with mastitic infection which caused SCC elevation.

## E. Determination of Protease Activity in Milk

Worldwide, numerous investigations have been conducted to study proteolysis in milk and dairy products. Despite the existence of the many procedures which are widely used in the dairy industry today, none have been shown to be completely satisfactory. Lack of sensitivity and rapidity are the major factors which make many of these procedures to be unsatisfactory. Even the rapid and more sensitive procedure recently developed (25) has shown problems with the stability of the substrate.

The Hull procedure is used frequently in the dairy industry, and is considered to be a traditional procedure since its development in 1947 by Hull (51). The procedure is based on measuring the amount of trichloroacetic acid (TCA)-soluble tyrosine and tryptophan-containing peptides released by the action of protease by reacting the supernatant fluid with Folin-Ciocalteau reagent and measuring the absorbance at 650 nm. Some of the limitations of this procedure include; its incapability to detect TCA-soluble peptides lacking in tyrosine and tryptophan, time consumption, and as studies by Juffs (54) indicated, counts greater than 1,000,000 cfu/ml were necessary to detect protease activity. However, this procedure can only confirm spoilage and not predict it (51). The lack in sensitivity has disqualified this procedure for detecting low levels of protease that are necessary for evaluating the predisposition of milk to proteolytic spoilage.

Like the Hull method, the Lowry procedure (68), which was developed in 1951, is cumbersome and lacks in sensitivity. A procedure which measure absorbance at 280 nm is well documented (66). Since amino acids tyrosine and trypotophan can absorb light in ultraviolet (UV) range, it is easy to detect them by measuring absorbance at 280 nm. According to Vakaleris and Price (99), cheese- ripening can be easily monitored by this procedure. This procedure have also been used by Richardson and TeWhaite (84) for partial characterization of the activity of heat stable proteases of psychrotrophic bacteria origin. Tyrosine and tryptophan-containing peptides are required in this procedure.

The use of non-specific amino acid reagent to follow the increase in TCA-soluble peptides resulting from proteolysis have been developed. The 2,4,6-trinitrobenzene-sulfonic acid (TNBS) has been used by McKellar (69) to determine proteolysis in milk. Polychroniadou (81) used TNBS procedure to assess proteolysis and determine ripening in Greek cheeses. The chemical reaction involves TNBS with amines resulting in spontaneous hydrolysis (40). The reaction comes to an end by neutralizing the nitrophenylated amino acids. Absorbance is measured at 420 nm. This procedure is time consuming. Equally, it does not give the required sensitivity and one has to make two reagents everyday. The reagents for blank are susceptible to contamination by picric acid most of the time (40).

Another procedure which involves reacting TCA-soluble peptides with fluorescamine, a non-specific amino acid reagent is described by Chism et al. (24). The assay is very sensitive, and has been used to determine protease activity in sterile milk.

Hide powder azure (HPA), a proteolytic substrate which releases a chromogen when hydrolyzed, has been used to measure protease activity of psychrotrophic bacteria common in milk. This procedure can measure proteolysis directly in milk. The substrate contains a covalently bound dye making it very susceptible to proteolytic activity (28). Although the procedure is considered to be reliable and sensitive compared to casein precipitation assays, some investigators have found that the procedure cannot detect psychrotrophic count below 1,000,000 cfu/ml, in most cases proteolytic activities caused by counts this high renders the milk unfit for UHT products manufacturing.

A statistical procedure was developed by Kwan et al. (62) to compare the Lowry procedure, absorbance at 280 nm, TNBS procedure, and fluorescamine procedure. According to their observations, the fluorescamine procedure was reported to be the most reliable and most sensitive. In interpreting these comparisons we have to take into account the different researchers goals for the studies they performed to best fit their purpose. Time, cost, and technical skills required for each procedure were different.

A procedure which does not utilize TCA precipitation prior to the reaction has been described by Church et al. (26). This assay is claimed to be simpler to perform and is as sensitive as fluorescamine. The procedure involves reacting alpha-amino groups released by proteolysis with the o-phthaldialdehyde and beta-mercarptoethanol, which then absorbs strongly at 340 nm. It is also considered to be more accurate than absorbance at 280 nm and Hull procedure. Its rapidity and ability to be read on spectrophotometer, makes it a promising procedure to be used in most laboratories. Nothing was found in the literature describing the use of this procedure to follow proteolysis by psychrotrophic bacteria.

A casein fluorescein isothiocynate (FITC) assay, which originally was developed for digestive proteases has been described (98). This assay involves casein, a protease substrate attached covalently with a fluorescent-emitting compound (dye). Following hydrolysis by protease, materials not precipitated by TCA are converted to a fluorescent compound by adjusting the pH and measuring the resulting adduct. The use of spectrofluorimeter limits the application of this procedure, because the equipment is not so common, however, the assay is simple to perform and it requires only one reagent casein, FITC (98).

Senica (90), compared three methods of detecting protease activity in milk. Results indicated that fluorescamine was more sensitive, statistically differentiating between four levels of enzyme activities, whereas the other two methods the casein, FITC-casein, Carbon-14 ($^{14}$C-casein) differentiated between only three levels of enzyme activity. The FITC and Fluorescamine procedures detected a level of 0.012 activity units (as defined by Hull) while $^{14}$C-casein procedure detected as low as 0.006 activity units.

Ninhydrin procedure primarily measures the free amino groups released after proteolysis has occured in milk. According to Hockney and Hockney (48), it is hard to note immediate changes because of the short time the assay takes, and it is impractical to prolong the reaction time in order to achieve detection point.

Today, radiolabeled casein is used to detect proteolytic enzymes (e.g. pepsin and trypsin) in the medical field (13). The proteolytic enzymes release small amounts of radiolabeled substrates which can then be detected using scintillation counting techniques. Welch (102) used *Bacillus amyloliquefaciens* protease and was able to determine two pH optima and an increased activity up to 65 C (which was the highest temperature investigated, with an optimum incubation time of 10 min). Radioisotopic methods are believed to be up to 100 times more sensitive than the traditional spectrophotometric procedures.

A procedure using $^{125}$Iodine-labeled human serum albumin as a substrate to detect low and very low proteolytic activities in foodstuffs including milk, has been described by Kas and Rauch (56). The major advantage of using the $^{14}$C-casein assay over traditional TCA-soluble methods is that it requires much smaller amounts of proteolytic enzyme (13). However, the major disadvantage is its need for sophisticated scintillation-counting equipment. Radiation levels which are used in the assay are below the regulated amount in

most states and are comparable to the amount of radioactivity used in the Charm antibiotic tests.

Christen (25) has developed a rapid and sensitive method for quantification of partially purified proteases derived from psychrotrophic bacteria. The method utilizes the radioisotopic counter which is part of the Charm antibiotic testing procedure and commercially available radiolabeled casein. The procedure requires 30 min for complete analysis and is approximately 10,000 times more sensitive than the traditional Hull method of measuring protease activity. The test is simple to perform and requires only one reagent in addition to the substrate and enzyme source. The main problem with this procedure appears to be the stability of substrate. Further studies are necessary to evaluate the usefulness of this procedure.

# CHAPTER III

# MATERIALS AND METHODS

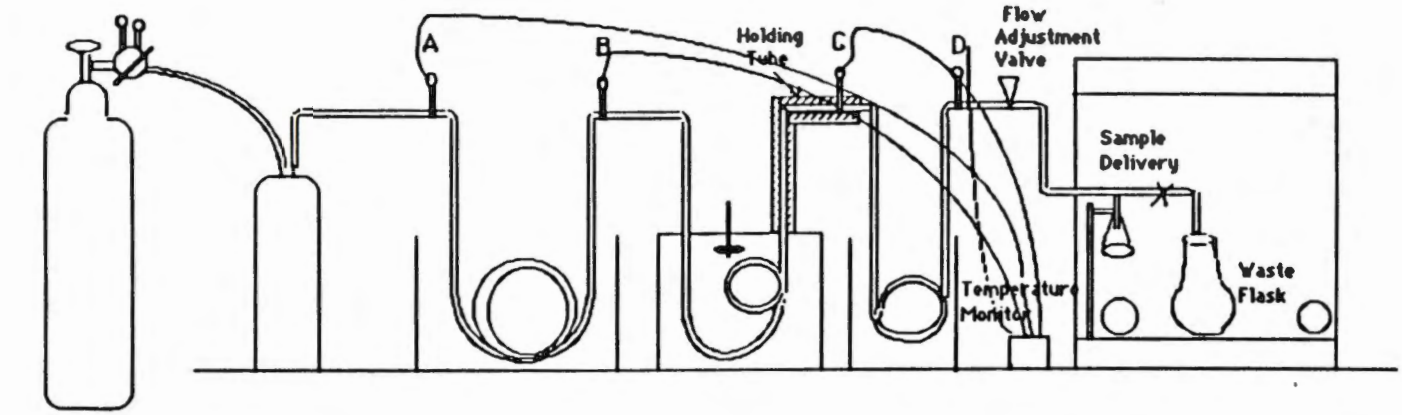
## A. Design and Construction of the UHT Milk Processor

A laboratory-scale, indirect, ultrahigh-temperature (UHT) milk processing system was constructed using about 11 meters of 6.35 mm OD stainless steel, thin walled, beverage tubing (Valley Pipe and Fittings Co., Knoxville, TN). The design was according to Wadsworth and Bassette (101) with modifications. The modified system involved propelling gas directly from the nitrogen cylinder to a milk tank without the use of valves. A 5-gal soft drink syrup tank was used to hold milk to be processed.

Two tanks were used interchangeably for holding the milk sample, cleaning solution, rinsing water, sanitizer, and steam for sterilization of the system. A water bath was used as a preheater, a plastic bucket as a cooling bath, and an oil bath (Fisher Hi-temp bath, Fisher Scientific, Atlanta, Ga.) as the sterilizing medium. Figure 1 shows a sketch of the UHT system constructed.

## B. Sample Collection

A total of 51 raw milk samples were aseptically collected in 1-gallon plastic jars over a four week period. Samples were cooled to 4 C and transported in an ice chest and kept cold until processed, with the exception of samples collected from the University creamery. Sixteen samples were collected from Friesian Holstein and Jersey cows at the University dairy farm.




Key: A, B, C, and D are temperature probes

Figure 1. Laboratory size indirect tubular UHT milk processing system.

Cows were grouped in five categories as excellent, good, acceptable, high, and exceptionally high depending on their somatic cell counts (SCC) as determined from the Dairy Herd Improvement (DHI) records. Table 1 shows the selected cows and part of their record. Somatic cell counts was used as a criteria in sampling. Cows grouped as excellent had a range of 0-71,000 SCC, good represented cows in the range of 72,000-565,000 SCC, acceptable was for cows in the range of 566,000-1,131,000 SCC, high were for cows in the range of 1,132,000-2,262,000 and exceptionally high represented cows in the range more 2,263,000-4,523,000 SCC. Nineteen samples were collected from farm bulk tanks at six area farms. Milk at five farms were from Friesian Holstein herds and one farm had Jersey herd. Fifteen milk samples were collected from the University creamery storage tank. Samples were subjected to temperature and storage abuse in order to achieve quality differences (Table 2).

## C. Pre-processing Preparations

### Skim Milk Production

At the start of this study, samples were processed three times a week, and one at a time. Later on, processing was performed on a daily basis with three samples a day. Two gallons of each batch were preheated to 45-55 C in a soft drink syrup tank, by immersing the tank in a bucket surrounded by steam and stirring continously to avoid burning the milk. The preheated milk samples were skimmed, using a De Laval laboratory milk separator (De Laval Corp; Sweden). Approximately 1 gal of skim milk (< 0.5% fat) was collected.

Table 1. Selected individual cows information from Dairy Herd Improvement Association records.

| Sample number | Cow I.D. | Breed | Somatic[a] cell counts | Days in lactation |
|---|---|---|---|---|
| C-1 | 0475 | Jersey | 4 | 234 |
| C-2 | 1702 | Holstein | 5 | 40 |
| C-3 | 0512 | Jersey | 6 | 115 |
| C-4 | 1455 | Holstein | 7 | 191 |
| C-5 | 0653 | Jersey | 2 | 152 |
| C-6 | 1382 | Holstein | 8 | 196 |
| C-7 | 1659 | Holstein | 7 | 129 |
| C-8 | 1661 | Holstein | 8 | 241 |
| C-9 | 1691 | Holstein | 7 | 117 |
| C-10 | 1206 | Holstein | 6 | 198 |
| C-11 | 1384 | Holstein | 6 | 245 |
| C-12 | 1691 | Holstein | 6 | 136 |
| C-13 | 0622 | Jersey | 0 | 54 |
| C-14 | 1260 | Holstein | 0 | 96 |
| C-15 | 0617 | Jersey | 3 | 159 |
| C-16 | 1150 | Holstein | 3 | 154 |
| C-17 | 0658 | Jersey | 1 | 161 |

| [a] SCCS | Counts(1,000) | SCCS | Counts(1,000) |
|---|---|---|---|
| 0 | 0-18 | 5 | 283-565 |
| 1 | 19-35 | 6 | 566-1,131 |
| 2 | 36-71 | 7 | 1,132-2,262 |
| 3 | 72-141 | 8 | 2,263-4,523 |
| 4 | 142-283 | 9 | 4,524-9,999 |

Table 2. Milk sample collected from university creamery bulk tank and treatments received prior to processing.

| Sample number | Storage temperature and period |
|---|---|
| A-1 | 7 C for 24 h |
| A-2 | No storage. Processed immediately |
| A-3 | Room temperature (25 C) for 2 h |
| A-4 | 4 C for 24 h |
| A-5 | 25 C for 1 h and then 4 C 15 h |
| A-6 | 4 C for 23 h |
| A-8 | 25 C for 1 h |
| A-9 | 4 C for 24 h |
| A-10 | 7 C for 12 h and then 4 C for 12 h |
| A-11 | No storage. Processed immediately |
| A-12 | No storage. Processed immediately |
| A-13 | 25 C for 1 h |
| A-14 | 4 C for 24 h |
| A-15 | 4 C for 72 h |
| A-16 | 4 C for 96 h |
| A-17 | 7 C for 72 h |

Sterilization of UHT Processor

Before milk processing, the system was cleaned by passing 5 gal of strong hot alkali (Dioklor, Diversey Wyandotte Corp, Wyandotte, MI; 1 oz. alkali/1 gal. water at 50 C) through the system at 75 psi. Two gallons of cold water were circulated to rinse the system. Five gallons of an acidic cleaning solution (2 oz /1 gal water of Diversey Wyandotte 3858 Low Foaming Acid, Wyandotte, MI) were circulated. The system was rinsed again with 2 gal of cold water. Three and a half gallons of 100 ppm chlorine sanitizer (Dibac Liquid Chlorine sanitizer, Diversey Wyandotte Corp., Wyandotte, MI; 0.5 oz./3.5 gal water) was circulated to flush out chlorine residue. All other cleaning and sterilizing procedures were performed as described by Wadsworth and Bassette (101).

## D. Sample Processing

Nitrogen gas (75 psi) propelled skim milk (50 C) from the soft drink syrup tank through the tubing system. The milk was preheated to 55-60 C in a hot water bath, brought to sterilization temperature of 132-145 C in an oil bath and held for 2-8 sec in a holding tube. The milk was cooled rapidly after leaving the holding tube to about 8 C in a stainless steel tubing immersed in a bucket containing ice water. The milk flow rate was controlled using a stainless steel valve (Swagelok nupro valve, Leirnart's Inc., Knoxville, TN), positioned after the cooling bucket. Calculations for the holding tube are shown in Appendix 12. The sterile milk was directed into a glove box (Plexiglass, Manostat, VWR Scientific, Marietta, GA) and collected in fourteen 120-ml and two 250-ml sterile amber glass bottles. Samples were stored at 25 C for 2 months.

## E. Microbial Analyses

### Media Composition and Preparation

Standard methods agar (SMA) (Difco, Detroit, MI) was used for microbial analyses. The medium was prepared by dissolving 23.5 g SMA in 1.0 l distilled water. The contents were mixed thoroughly and then heated with frequent agitation for 1 min until it boiled and completely dissolved. The solution was poured into 100 ml media bottles, autoclaved at 121 C for 15 min and then cooled and tempered at 45 C.

Skim milk agar was prepared in the same manner as SMA except that the medium was prepared immediately before pouring plates by mixing 100 ml of sterile skim milk (10%) with 1.0 L of melted SMA adjusted to 50 C.

### Sample Plating

Raw milk samples were analyzed for microbial contamination on each day of processing. Standard plate counts (SPC) were performed by plating apppropriately diluted samples (1:100, 1:1,000, 1:10,000 and 1:100,000) in SMA with incubation at 32 C for 48 h (97). Modified psychrotrophic plate counts (MPBC) were performed by plating appropriately diluted samples (1:10, 1:100, and 1:1000) in SMA with incubation at 21 C for 25 h (97). Proteolytic bacteria counts (PBC) were performed by plating appropriately diluted samples (1:10 000, 1:100,000, 1:1,000,000) in skim milk agar with incubation at 32 C for 48h (97). All counts were reported as colony forming units per ml (cfu/ml).

## F. Somatic Cell Counts

To enumerate somatic cells, an electronic somatic cell counting procedure using a Coulter counter located in the Department of Animal Science, was performed. Milk samples were brought to room temperature and mixed thoroughly. One drop Somafix was placed in a 13 x 100 screw-top test tubes, and while vortexing (vortex speed : 6-8), 1.0 ml of milk was added using a selectapette. The capped tubes were placed in a rotating water bath at 60 C for 10 min. The tubes were then removed from the water bath and placed in ice water for 4-6 min, cooled and placed in refrigerator until ready to run. The fixed samples were run within 2 days. Counts were reported as SCC/ml.

## G. Chemical Analyses

### Protein Content Determination

Protein was determined by the Kjeldhal procedure (74). Two grams of each milk samples were measured in duplicate and placed in Kjeldhal flasks. Kel-pak 5 (containing potassium sulfate mixed with mercuric oxide) was added to each flask. Twenty five ml concentrated sulfuric acid (sp. gr. 1.84, 95.8%) were added. Three boiling stones (zinc granules) were added to the flasks. The flasks were placed on the digestion apparatus for 1-2 h and occassionally shaken. As the liquid turned from clear to brown and back to clear again, heat was adjusted to low and high respectively. A blank containing all reagents except the sample was run concurrently. The flasks were then cooled and 300 ml of distilled water were added. Fifty milliters of 4% boric acid containing the

methyl red-methlene blue indicator were measured into 500 ml Erlenmeyer flask and placed under the condenser of distilling unit. Eighty milliliters of 50% NaOH containing sodium thiosulphate were added to the Kjeldhal flask. Fifteen to twenty grams of granular zinc were added and the flasks were connected to the condenser. Up to 300 ml of distillate were collected in the Erlenmeyer flask. The distillate was titrated with 0.1N HCl (Baker, Atlanta, Ga) until a grey endpoint appeared. The percentage nitrogen was obtained by subtracting ml HCl titrated for blank from ml HCl titrated for sample, and multiplying by normality of HCl (0.1N), molecular weight of nitrogen (14.01), and 100 and then dividing the results by the sample weight (mg). The % protein was computed by multiplying the % N by 6.38

Milkfat Determination

Fat in raw milk was determined by Babcock test for milkfat as described in the AOAC (74). Milk samples were tempered to 16-21 C and mixed thoroughly. Duplicate 17.6 ml samples were placed into Babcock bottles (8%). A 17.5 ml sulfuric acid (16-21 C) of 1.82-1.83 sp. gr. at 20 C were added and the contents were mixed thoroughly and gently until a chocolate color appeared. The bottles were then centrifuged in a heated centrifuge (65 C) for 5 min. Hot distilled water (57-67 C) was added until the level reached the neck of the bottles. The bottles were further centrifuged for 2 min. Additional hot distilled water was added until fat in the bottles was between 0% and 8% mark. A final centrifuge for 1 min was followed by placing the bottles in a water bath (57-60 C) for 3 min. The fat column was measured to the nearest 0.05% by measuring fat column from bottom of the meniscus to the top of the meniscus using a caliper.

Total Solids Determination

Total solids were determined as described by Atherton and Newlander (16). Empty solid dishes were heated in the vaccum oven at 100 C for 10 min and then cooled in the dessicator for 5 min. The dishes were then weighed on an analytical balance. Samples were thoroughly mixed and about 2 g were weighed into the prepared and weighed dishes in duplicate. Milk was spread over the bottom of the dish to form a thin film. The dishes were then placed on a hot plate (180 C), and pressed using a contact tool to ensure uniform evaporation and heated until brown traces appeared. The dishes were then transferred to a vacuum oven at a temperature of 100 C and 20 inches of vacuum and the samples evaporated to dryness. The sample was then cooled to room temperature in a dessicator for 5 min. Finally, the dishes and the contents were weighed and percentage of solids calculated by dividing dry sample weight by wet sample weight and multipling by 100.

Analysis of Free Amino (FA) Groups

Free amino groups were determined on processed sample at day 0 and day 60. Trinitrobenzene sulfonic acid (TNBS) methods of Fields as modified by Spadaro et al. and as reported by McKellar (69) was used. Duplicate 2 ml samples of each milk were treated with 4 ml of 0.72 N trichloroacetic acid (TCA) (117.648 g/L) and allowed to stand for 20 min at room temperature; then filtered through Whatman #1 filter paper. The resulting supernatant fluid (0.2 ml) was mixed with 2 ml of 1 M potassium borate buffer (pH 9.2) and 0.8 ml 1 mM TNBS (0.0085 g/25 ml). The mixture was incubated in the dark at room temperature for 30 min. Standard solution of glycine (2.5, 2.0, 1.5, 1.0, and 0.5 micromoles/ml) were run along with the sample.

After 30 min, 0.8 ml of 2 M (6.90 g/25 ml) monobasic sodium phosphate containing 18 mM sodium sulfite (0.0565 g/25 ml) was added and the absorbance at 420 nm was measured in Shimadzu double-beam spectrophotometer UV-190 (Shimadzu Seisakusho Ltd, Kyoto, Japan). Absorbances readings were converted to micromoles of free amino groups per milliliter of milk using a glycine standard curve.

## H. Protease Activity Determination

Protease activity was determined prior to raw milk sample processing using the procedure of Christen (25). Carbon-14 labeled casein (Casein, [methyl -$^{14}$C] methylated alpha) (New England Nuclear, Boston, MA) was used as a stock substrate. Five milliliters of the working substrate were prepared by diluting one microcurie (0.2 ml) of carbon-14 casein with 4.8 ml of 0.1 M phosphate buffer (pH 7.2). Fifty microliter portions of the substrate were dispensed into microcentrifuge tubes and stored frozen until use. Tubes were removed each day of testing, and preincubated at 37 C for 5 min. Duplicate 50 microliters of each milk sample were added to separate tubes. All tubes were incubated at 37 C for 30 minutes, after which 100 microliters of acetate solution added. A blank tube was prepared by adding in sequence 50 microliters of substrate, 50 microliters of raw milk sample and 100 microliters of acetate solution (50:50 mixture of 10% acetic acid and 1 N sodium acetate). The precipitates were centrifuged (13,000 x G for 5 min) and 100 microliters of supernatant fluid were removed and spread in a thin layer on metal planchets.

The spread fluid was allowed to air dry before reading the counts in the Charm Test Analyzer, model 160 (Penicillin Assays, Inc., Malden, MA). For each planchet, counts were read for 10 minutes. Before each testing day the Charm analyzer was tested for background interference by counting an empty planchet for 10 minutes. Initial radiation reading below 7.0 cpm for blank was considered to be reasonable. Results were reported as counts per 10 minutes (cpm).

## I. Viscosity Measurements

Viscosity of the processed milk samples was measured from the first day of storage (day 0), and then after 2 weeks (day 14). Thereafter, measurements were performed on a weekly basis. Viscosity measurements were determined using a Brookfield Synchro-lectric L.V. Viscometer (Brookfield Engineering Laboratories, Inc. Stoughton, MA) equipped with an ultra-low viscosity (U.L.) adaptor. The readings were taken over shear rates of 60 revolutions per minute (rpm) for 1 min, 2 min, and 3 min respectively (59). Viscosity for each sample was the average of the three readings. All readings were taken at room temperature (25 C). Viscosities were determined on the undisturbed samples and sampling was without replacement. Readings were converted to centipose (cP) and then to mPa. s. Samples were considered gelled when their viscosities exceeded 10 mPa. s. One mPa.s is equal to 0.01 poise equal to 1.0 cP. Viscosity was expressed as mPa.s.

## J. Flavor Evaluation

Thirteen people were screened for bitterness testing using eight levels of caffeic acid (0.005, 0.01, 0.02, 0.03, 0.04, 0.06, 0.08, and 0.10% respectively) in milk in a triangle threshold test. Eight panelists who were able to detect the caffeic acid at 0.04% (0.12g/300 ml milk) were selected for evaluation of stored samples. After 2 months of storage at 25 C, 15 ml of sample was dispensed in 50 ml plastic cups, coded with 3 digit random numbers, for each panelist and equilibrated to 15 C before serving. Control consisted of freshly processed sample with no caffeic acid and a duplicate to the reference sample. Panelists were seated in individual booths and testing was under white light between 2 p.m. and 4 p.m. Multiple comparison test were performed and each panelist was asked to identify the degree of bitterness of the sample in question as compared to the reference sample (0.04%). A nine-point rating scale as described in the ASTM (15), was used to determine degree of bitterness. The score for bitterness was the mean of all the panelists for each individual sample. An example of scorecard and the score scale sheet are included in the appendix.

## K. Statistical Analysis

Data were analyzed by calculating correlation coefficients between each variable measured in the study to determine if relationships between different variables existed. Descriptive statistics for mean, standard deviation, and range were performed. Analysis of variance (ANOVA) using General Linear Model (GLM) (SAS Institute, Cary, NC) was used to compare and test differences of means for each variable from three different sources of milk samples.

Separations of means was performed using Tukey's Studentized Range (HSD) test. Significance was pre-established at alpha = 0.05 level.

# CHAPTER IV

## RESULTS AND DISCUSSION

### A. Compositional Values for Raw Milk

The mean, range, and standard deviation for total solid, protein, and fat are presented in Table 3. All measurements fall within the standard gross compositional ranges for milk. Fat, total solids, and protein were all positively correlated. Initial protease activity, somatic cell count, and psychrotrophic bacteria count were not correlated to either fat, total solids or protein. This suggests that the gross composition of raw milk is not the determining factor of proteolysis. Somatic cell count was expected to correlate with the total solids, fat, and protein respectively. Murphy et al. (72) found that elevated somatic cell counts in milk which is associated with mastitis may lead to a decrease in total casein (protein), fat, and total solids.

### B. Protease Assay

The average initial protease content of milk sample analyzed by carbon-14 labeled casein assay was 40.1 counts per minute (CPM) (Table 3), but the range was wide. This variation in protease activity may be due to differences in the quality of milk samples analyzed.

Table 3. Descriptive statistics of the variables analyzed at the beginning, during or end of the study.

| Variables Measured | Mean | Standard Deviation | Range |
|---|---|---|---|
| Compositional Data | | | |
| Fat (%) | 3.9 | 0.74 | 2.9-6.3 |
| Total Solids (%) | 12.6 | 0.82 | 11.3-14.7 |
| Protein (%) | 3.1 | 0.28 | 2.6-4.0 |
| Free amino groups after processing (micromoles) | 0.5 | 0.17 | 0.21-0.89 |
| Microbiological Data (log 10) | | | |
| Standard Plate Count | 4.6 | 0.56 | 3.4-5.96 |
| Psychrotrophic Bacteria Count | 4.5 | 0.65 | 3.0-5.76 |
| Proteolytic Bacteria Count | 4.1 | 0.72 | 2.95-5.34 |
| Somatic Cell Count (log 10) | 5.7 | 0.57 | 4.46-7.25 |
| Protease Activity (CPM) | 40.1 | 7.98 | 21.3-64.0 |
| Storage Data | | | |
| Bitterness Flavor | 3.3 | 0.61 | 1-9 |
| Mean Change Free Amino Groups (micromoles) | 0.5 | 0.68 | -0.6 -2.20 |
| Shelf-life (days) | 33 | 57.4 | 33-73 |
| Viscosity (mPa.s) | | | |
| Day 0 | 1.5 | 0.46 | 1.2-3.8 |
| Day 14 | 2.2 | 0.86 | 1.2-4.0 |
| Day 21 | 2.7 | 0.68 | 1.5-3.8 |
| Day 28 | 2.7 | 0.69 | 1.5-4.5 |
| Day 35 | 2.8 | 0.92 | 1.0-4.6 |
| Day 42 | 3.2 | 0.83 | 1.5-6.0 |
| Day 49 | 3.1 | 0.88 | 1.25-5.0 |
| Day 56 | 3.2 | 0.75 | 1.25-4.5 |

There was significant corrrelation (r=.32; p = 0.02) between initial protease activity and psychrotrophic bacteria count (Table 4). Psychrotrophic bacteria may have contributed to the variation in the protease activity. These microorganisms are believed to produce extracellular thermostable enzymes, which even at very low levels may degrade casein. Several authors have attributed protease activity to protease of microbial origin especially psychrotrophic bacteria (2,4,8,30,32). There is evidence from this study that sanitation practices, cooling of milk and other milk handling procedures can encourage increased psychrotrophic bacteria growth. The relationship between initial protease activity and psychrotrophic bacteria count is shown in Figure 2. As low as 1,000 colony forming units per milliliter (CFU/ml) were able to produce enough proteolytic activity (approximately 22 CPM). Counts up to 100,000 CFU/ml were necessary to achieve a peak proteolytic activity (64 CPM) in this study. Large number of psychrotrophic bacteria are not required in order to have a significant amount of proteolysis (2).

Somatic cell influence on the proteolytic activity of milk is well documented (34,72,80). The lack of correlation (r =-.16; p = .25) between initial protease activity and somatic cell count (SCC) supports the observations of Andrews (8). In his study of the breakdown of caseins by proteases of bovine milks with high SCC of the protease activity originated from the somatic cells. Some of the samples collected in this study had exceptionally high somatic cell counts (up to $1.8 \times 10^{-7}$ SCC/ml), the contribution towards increased protease activity appeared to be minimal.

Table 4. Correlation coefficients and p-values for selected variables (N = 51).

| Variables Correlated | Correlation Coefficient | p-Value |
|---|---|---|
| Initial Protease Activity and Psychrotrophic Bacteria Count | 0.32 | 0.02 |
| Standard Plate Count and Psychrotrophic Bacteria Count | 0.79 | 0.0001 |
| Standard Plate Count and Proteolytic Bacteria Count | 0.72 | 0.001 |
| Psychrotrophic Bacteria Count and Proteolytic Bacteria Count | 0.72 | 0.0001 |
| Somatic Cell Count and Standard Plate Count | -0.25 | 0.0767 |
| Somatic Cell Count and Proteolytic Bacteria Count | -0.23 | 0.0985 |
| Somatic Cell Count and Change in Free Amino Groups | 0.27 | 0.0418 |
| Somatic Cell Count and Shelf-life | -0.25 | 0.0818 |
| Initial Protease Activity and Flavor | -0.27 | 0.0807 |
| Initial Protease Activity and Shelf-life | 0.26 | 0.0610 |
| Shelf-life and Change in Free Amino Groups | -0.30 | 0.0312 |

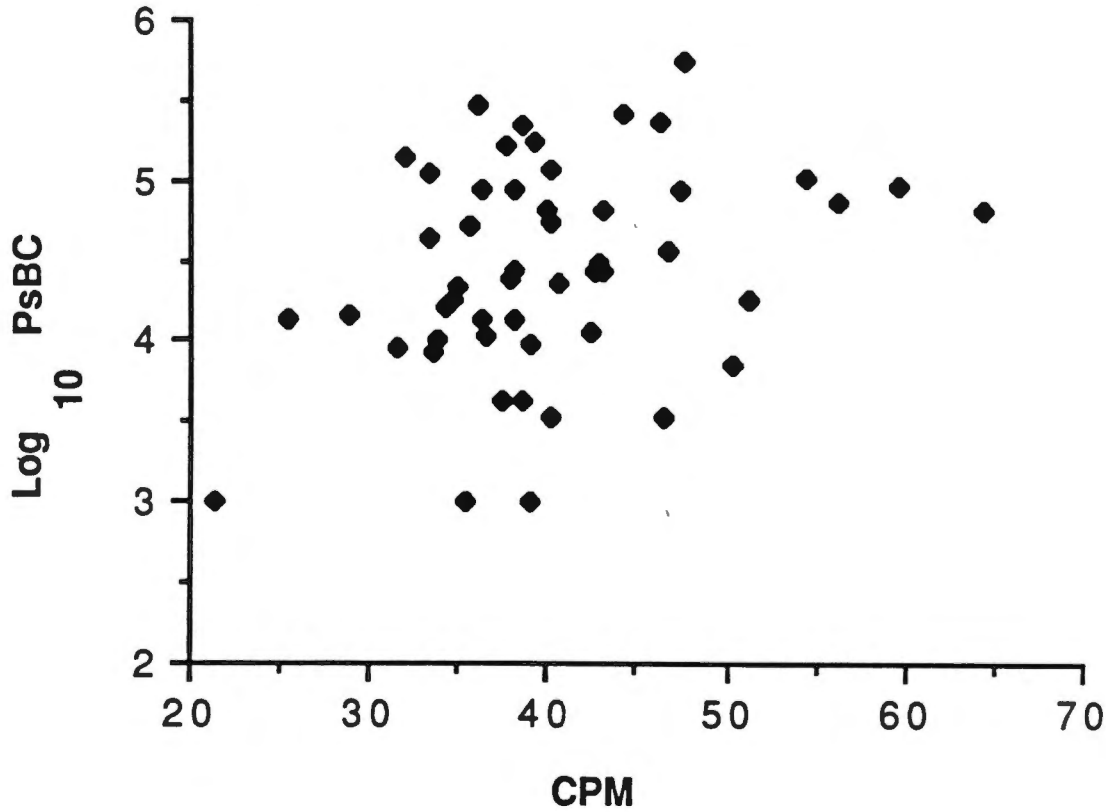



Figure 2. Relationship between initial protease activity and psychrotrophic bacteria count.

When samples were separated by source of milk several interesting correlations were discovered (Tables 5,6, and 7). Somatic cell count and flavor scores were negatively correlated for the creamery samples subjected to abuse (Table 5). However, the initial microbiological counts which were all highly positively correlated with flavor (Tables 5) indicated that the higher the initial microbial population the more bitter was the sample. High correlation between initial protease activity and standard plate count (SPC) were observed with individual cow milk samples (Table 7). SPC was positively correlated also with proteolytic bacteria count for source of milk. Figure 3 shows that the relationship is closely linear, suggesting that proteolytic bacteria make up a consistent proportion of the total bacterial population of these milks.

Comparison of the means for selected variables by source (Table 8) indicated that milk samples from the creamery and area farms were significantly different ($p < 0.05$) in initial protease activity. The means for psychrotrophic bacteria count between creamery milk and area farms milk were also significantly different. Samples taken from the creamery were subjected to different storage and temperature treatments (Table 2). These treatments were attempted to encourage psychrotrophic bacteria growth. Most of the milks collected from the farm were every other day pick and were lower in psychrotrophic bacteria.

Initial protease activity and somatic cell count were significantly different between these two sources as well probably because different herds were sampled in area farms and the same herd was sampled in the creamery abused samples. Storage of milk for 3-5 days at refrigeration temperature encourages the growth of bacteria which produces proteolytic enzymes. A test for proteolytic bacteria was used to identify practices during handling and storage

Table 5. Correlation coefficients and p-values for selected variables of milk samples from abused creamery milk (N = 16).

| Variables Correlated | Correlation Coefficient | p-Value |
|---|---|---|
| Somatic Cell Count and Standard Plate Count | -0.82 | 0.0001 |
| Somatic Cell Count and Psychrotrophic Bacteria Count | -0.51 | 0.0416 |
| Somatic Cell Count and Proteolytic Bacteria Count | -0.63 | 0.0096 |
| Standard Plate Count and Psychrotrophic Bacteria Count | 0.68 | 0.0036 |
| Standard Plate Count and Proteolytic Bacteria Count | 0.94 | 0.0001 |
| Proteolytic Bacteria Count and Psychrotrophic Bacteria Count | 0.68 | 0.0036 |
| Bitterness Flavor and Somatic Cell Count | -0.82 | 0.0001 |
| Bitterness Flavor and Psychrotrophic Bacteria Count | 0.56 | 0.0246 |
| Bitterness Flavor and Standard Plate Count | 0.66 | 0.0051 |
| Bitterness Flavor and Proteolytic Bacteria Count | 0.70 | 0.0023 |
| Bitterness Flavor and Slope[a] | -0.62 | 0.0106 |
| Bitterness Flavor and R-Square[b] | -0.48 | 0.0597 |

Table 5 (continued).

| Variables Correlated | Correlation Coefficient | p-Value |
|---|---|---|
| Slope[a] and Somatic Cell Count | 0.58 | 0.0185 |
| Proteolytic Bacteria Count and Free Amino Groups after Storage | 0.55 | 0.0257 |
| Standard Plate Count and Psychrotrophic Bacteria Count | 0.62 | 0.0101 |

[a] Change in viscosity as a function of storage time.

[b] Percentage of deviation explained by the variables in the model.

Table 6. Correlation coefficients and p-values for selected variables of milk collected from area farms (N =18).

| Variables Correlated | Correlation Coefficient | p-Value |
|---|---|---|
| Initial Protease Activity and Proteolytic Bacteria Count | -0.50 | 0.0361 |
| Somatic Cell Count and Standard Plate Count | 0.50 | 0.0359 |
| Somatic Cell Count and Proteolytic Bacteria Count | 0.54 | 0.0197 |
| Standard Plate Count and Proteolytic Bacteria Count | 0.84 | 0.0001 |
| Standard Plate Count and Psychrotrophic Bacteria Count | 0.87 | 0.0001 |
| Proteolytic Bacteria Count and Psychrotrophic Bacteria Count | 0.82 | 0.0001 |
| Bitterness Flavor and Somatic Cell Count | 0.62 | 0.0426 |
| R-Square[a] and Psychrotrophic Bacteria Count | -0.51 | 0.0310 |

[a] Percentage of deviation explained by the variables in the model.

Table 7. Correlation coefficients and p-values of selected variables of milk samples collected from individual cows (N=17).

| Variables Correlated | Correlation Coefficient | p-Value |
|---|---|---|
| Initial Protease Activity and Standard Plate Count | 0.61 | 0.0095 |
| Standard Plate Count and Psychrotrophic Bacteria Count | 0.90 | 0.0001 |
| Standard Plate Count and Proteolytic Bacteria Count | 0.65 | 0.0047 |
| Proteolytic Bacteria Count and Psychrotrophic Count | 0.59 | 0.0129 |
| Bitterness Flavor and Standard Plate Count | -0.57 | 0.0178 |
| Bitterness Flavor and Initial Protease Activity | 0.73 | 0.0008 |
| Bitterness Flavor and Psychrotrophic Bacteria Count | -0.57 | 0.0180 |

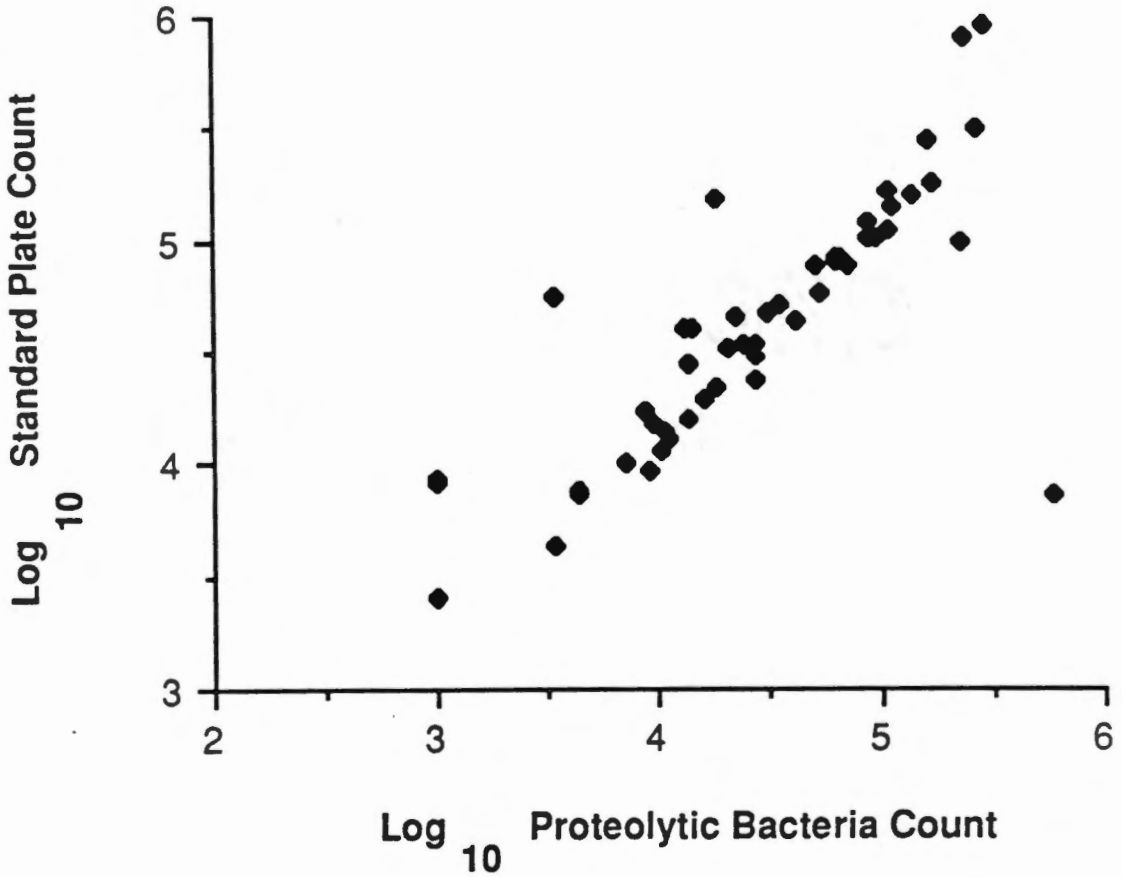



Figure 3. Relationship between standard plate count and proteolytic bacteria count.

Table 8. Means for selected variables of different milk sources.

| Variable | Abused Creamery Milk (N = 16) | Area Farms (N = 18) | Individual Cows (N=17) |
|---|---|---|---|
| Initial Protease Activity (CPM) | 44.1[a] | 37.4[b] | 39.2[a,b] |
| Somatic Cell Count (Log 10) | 5.18[a] | 6.00[b] | 5.73[b] |
| Psychrotrophic Bacteria Count (Log 10) | 4.78[a] | 4.24[b] | 4.38[b] |
| Proteolytic Bacteria Count (Log 10) | 4.44[a] | 4.00[a] | 3.88[a,b] |
| Shelf-life (Days) | 60.29[a] | 53.89[b] | 58.25[a,b] |
| Bitterness Flavor (1-9) | 3.09[a] | 3.57[a] | 3.20[a] |
| Change in Free Amino Groups (Micromoles) | 0.05[a] | 0.67[b] | 0.80[b] |

[a,b] Means in the same row with the same superscripts are not significantly different ($p < 0.05$).

of raw milk samples. Table 8 indicates that there was not a significant difference in proteolytic bacteria count between abused creamery milk and milk from the area farms. Tables 5,6, and 7 indicates high correlation between psychrotrophic bacteria count and proteolytic bacteria count. The high correlation may be because many of the same organisms were enumerated by both methods. Increased proteolytic bacteria count in raw milk, therefore, may have an influence on the proteolytic activity. An inverse relationship exists between initial protease activity and proteolytic bacteria count for milk samples collected from area farms (Table 6). This suggests that the lower the number of proteolytic bacteria the higher was the initial protease activity. This observation agrees with the study by Andrews and Alichanidis (10) who found that large populations of bacteria are not required for production of significant amount of protease activity.

C. Concentration of Free Amino Groups in Processed Stored UHT Skim Milk

The mean change in free amino groups as measured by the trinitrobenzene sulfonic acid (TNBS) was 0.5 micromoles per milliliter (Table 3). Surprisingly, a few stored samples had a negative change in free amino groups. This was probably no real change and due to the variability in the procedure. The majority of the stored samples had an increase in free amino groups. This suggests the presence of protease. The change in free amino groups was correlated to somatic cell count and shelf-life (Table 4) when all sources of milk were combined. Shelf-life and change in free amino groups were negatively correlated. This suggests that an increase in free amino

groups as a result of proteolysis may lead to a decrease in shelf-life. Senyk et al. (91) reported an increase in proteolysis (as measured by tyrosine value) was correlated to somatic cell counts. Means for change in free amino groups in abused creamery milk and milk from area farms, and between creamery and milk from individual cows were significantly different ($P < 0.05$) (Table 8).

Change in free amino groups did not correlate with proteolytic bacteria count ($r = -.01$, $p = .92$). Thus this source of protease may have a small effect on proteolysis. Additionally, Fox (41) and Eigel et. al. (38) have reported that plasmin, the natural milk protease has the most significant effect on total protease activity.

D. Effects of Storage of UHT Processed Skim Milk on Viscosity.

Age gelation or formation of semi-solid gel is a common phenomenon associated with prolonged storage of UHT milk (21). Since age gelation is an indication of quality deterioration, and the product viscosity changes as it gels, viscosity of the stored processed sample was used as an indicator of shelf-life. During the course of the study viscosity was measured on a weekly basis except during the first two weeks.

Slopes, intercepts, and R-square values were calculated for each of the viscosity measurements according to sources (Tables 9,10, and 11). Slope measured the change in viscosity with storage time, R-square measured the percentage deviation explained by the variable in the model, and intercept was the initial viscosity of the sample. Stored milk samples which had high R-square were selected and curves of apparent viscosities as a function of

Table 9. Relationship between R-Square, intercept and slope from viscosity measurements of UHT skim milk samples stored at 25 C[a].

| Sample I.D. | R-Square | Intercept | Slope |
|---|---|---|---|
| A-1 | 0.6467 | 1.3512 | 0.0429 |
| A-2 | 0.4076 | 1.7061 | 0.0167 |
| A-3 | 0.8628 | 0.9211 | 0.0414 |
| A-4 | 0.7109 | 0.8618 | 0.0544 |
| A-5 | 0.6169 | 1.3904 | 0.0393 |
| A-6 | 0.2751 | 1.5170 | 0.0234 |
| A-8 | 0.5269 | 0.6377 | 0.0655 |
| A-9 | 0.7876 | 1.2833 | 0.0340 |
| A-10 | 0.5803 | 1.4000 | 0.0469 |
| A-11 | 0.4758 | 1.2482 | 0.0484 |
| A-12 | 0.8504 | 1.5044 | 0.0294 |
| A-13 | 0.1203 | 2.4122 | 0.0131 |
| A-14 | 0.0037 | 2.5088 | -0.0023 |
| A-15 | 0.0081 | 1.9649 | 0.0042 |
| A-16 | 0.4463 | 1.5482 | 0.0229 |
| A-17 | 0.7703 | 1.3947 | 0.0432 |

[a] Abused UT creamery milk.

Table 10. Relationship between R-Square, intercept and slope from viscosity measurements of UHT skim milk samples stored at 25 C[b].

| Sample I.D. | R-Square | Intercept | Slope |
|---|---|---|---|
| B-1 | 0.6090 | 2.1974 | 0.0242 |
| B-2 | 0.6100 | 1.5877 | 0.0226 |
| B-3 | 0.2100 | 2.0965 | 0.0203 |
| B-4 | 0.7697 | 1.7061 | 0.0269 |
| B-5 | 0.5033 | 2.5702 | -0.0084 |
| B-6 | 0.7115 | 1.2500 | 0.0357 |
| B-7 | 0.0169 | 1.9054 | 0.0048 |
| B-8 | 0.6009 | 1.2365 | 0.0531 |
| B-9 | 0.7432 | 1.2095 | 0.0531 |
| B-11 | 0.3376 | 1.6468 | 0.0343 |
| B-12 | 0.3591 | 1.5614 | 0.0245 |
| B-13 | 0.5406 | 2.1621 | 0.0235 |
| B-14 | 0.8069 | 1.4167 | 0.0629 |
| B-15 | 0.6303 | 1.6140 | 0.0316 |
| B-16 | 0.9130 | 0.8711 | 0.0516 |
| B-17 | 0.8257 | 1.3707 | 0.0500 |
| B-18 | 0.5521 | 1.5526 | 0.0422 |
| B-19 | 0.6104 | 3.7416 | -0.0198 |

[b] Raw milk samples were from area farms.

Table 11. Relationship between R-Square, intercept and slope from viscosity measurements of UHT skim milk samples stored at 25 C[c].

| Sample I.D. | R-Square | Intercept | Slope |
|---|---|---|---|
| C-1 | 0.0839 | 2.4839 | 0.0094 |
| C-2 | 0.0446 | 3.0526 | -0.0087 |
| C-3 | 0.5526 | 1.5789 | 0.0301 |
| C-4 | 0.1814 | 1.9825 | 0.0200 |
| C-5 | 0.0144 | 2.7863 | 0.0055 |
| C-6 | 0.0614 | 2.4912 | 0.0125 |
| C-7 | 0.0021 | 2.7881 | 0.0021 |
| C-8 | 0.2230 | 3.0219 | 0.0197 |
| C-9 | 0.2272 | 2.5144 | 0.0215 |
| C-10 | 0.8445 | 1.7126 | 0.0354 |
| C-11 | 0.7400 | 1.1460 | 0.0598 |
| C-12 | 0.3016 | 1.7332 | 0.0349 |
| C-13 | 0.0284 | 2.9661 | 0.0088 |
| C-14 | 0.6077 | 1.5000 | 0.0510 |
| C-15 | 0.4358 | 2.3304 | 0.0295 |
| C-16 | 0.0024 | 1.8596 | 0.0015 |
| C-17 | 0.2959 | 1.9430 | 0.0355 |

[c] Raw milk samples were from individual cows.

storage time were established (Figures 4, 5, 6,7, and 8). R-square was inversely correlated to psychrotrophic bacteria count for samples from area farms (Table 6), but not with samples from individual cows. Samples processed from abused creamery milk showed an inverse correlation between bitterness and slope, as well as between bitterness and R-square (Table 5).

The changes in viscosity were characterized by a delay (Figures 4, 5, 6, 7, and 8) during which only slight changes occured. A sharp increase in apparent viscosity followed thereafter. Such changes, according to Kocak and Zadow (59) are typical of age-gelation viscosity curves. Inverse correlation between between bitterness flavor and change in viscosity (as measured by slope) for the abused samples indicates that proteolysis may have involved a thinning of the milk rather than a thickening. Microbial proteases are thought to be more active on beta-caseins by non-specific action which would lead to protein breakdown and possible age thinning rather than having chymosin-like activity which would lead to gelation through attack on kappa-casein (59). Kocak and Zadow (58) found that continued proteolytic attack during further storage of UHT milk resulted in a reduction in apparent viscosity of the product. Harwalkar (46) found no critical level of protein breakdown at which gelation occurred.

## E. Shelf-life of UHT Stored Samples

Initial bacteria population of the raw milk, processing conditions and storage conditions may play a role in determining the shelf life of UHT milk. The mean storage life of the UHT milk was 57 days . Four samples in the study were

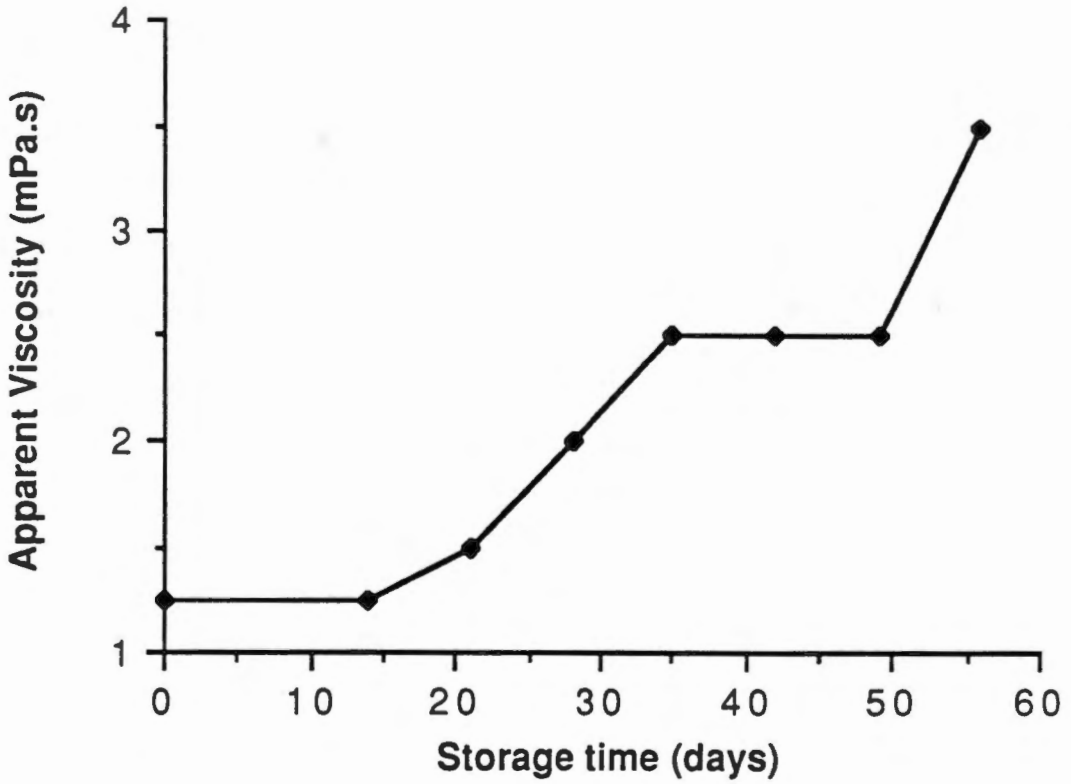



Figure 4. Relationship between apparent viscosity and storage time for abused creamery sample A-3 stored at 25 C. R-square= 0.86.

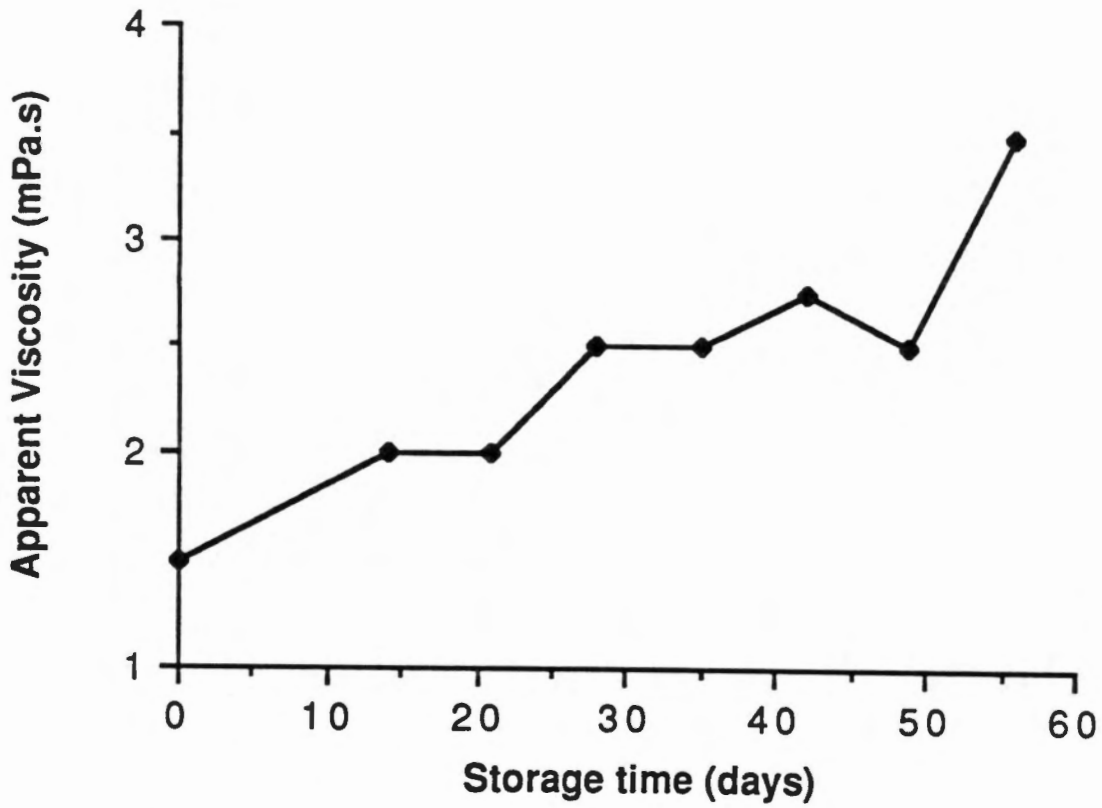



Figure 5. Relationship between apparent viscosity and storage time for abused creamery sample A-12 stored at 25 C. R-square= 0.85.

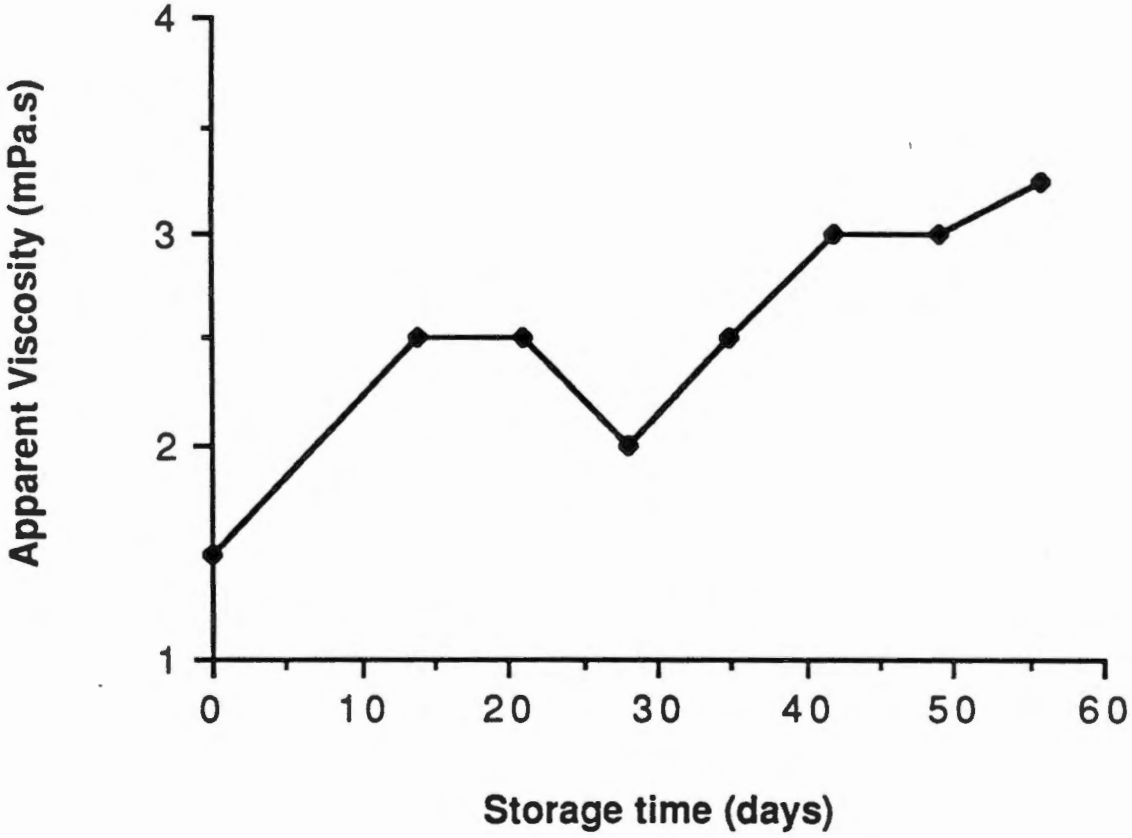



Figure 6. Relationship between apparent viscosity and storage time for area farm sample B-4 stored at 25 C. R-square= 0.77.

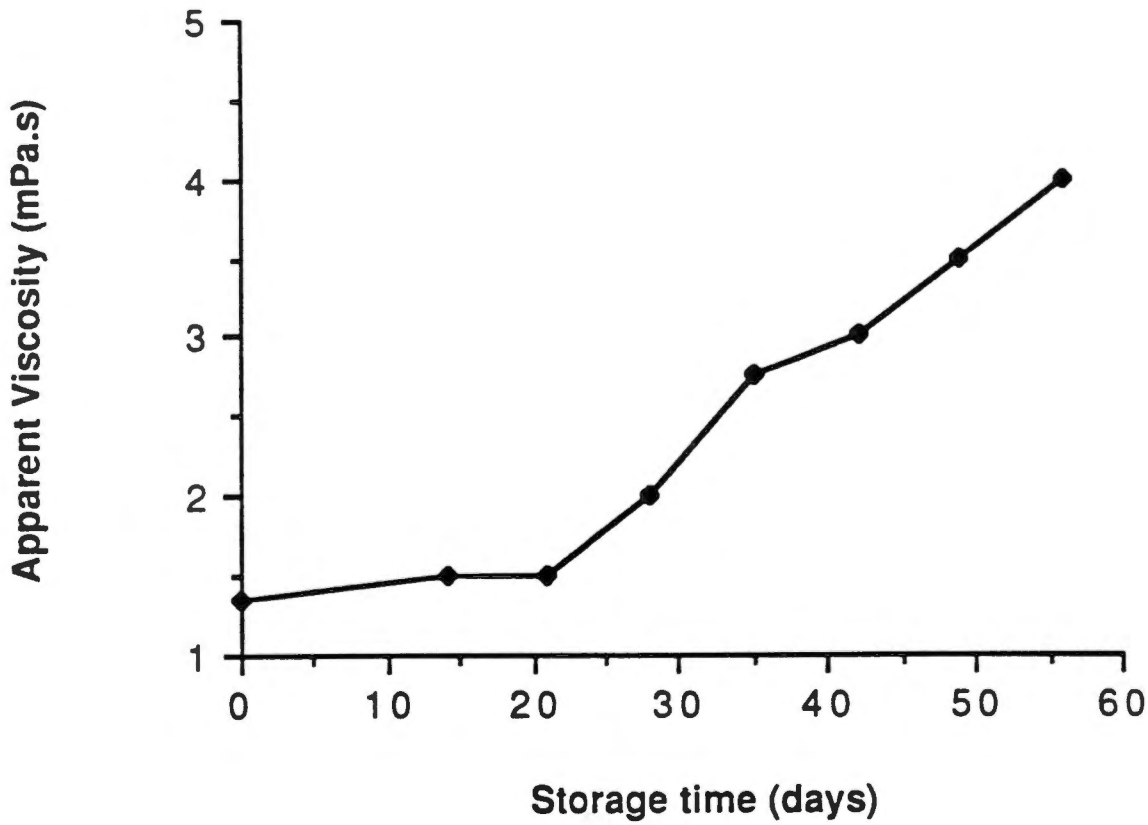



Figure 7. Relationship between apparent viscosity and storage time for area farm sample B-16 stored at 25 C. R-square= 0.91.

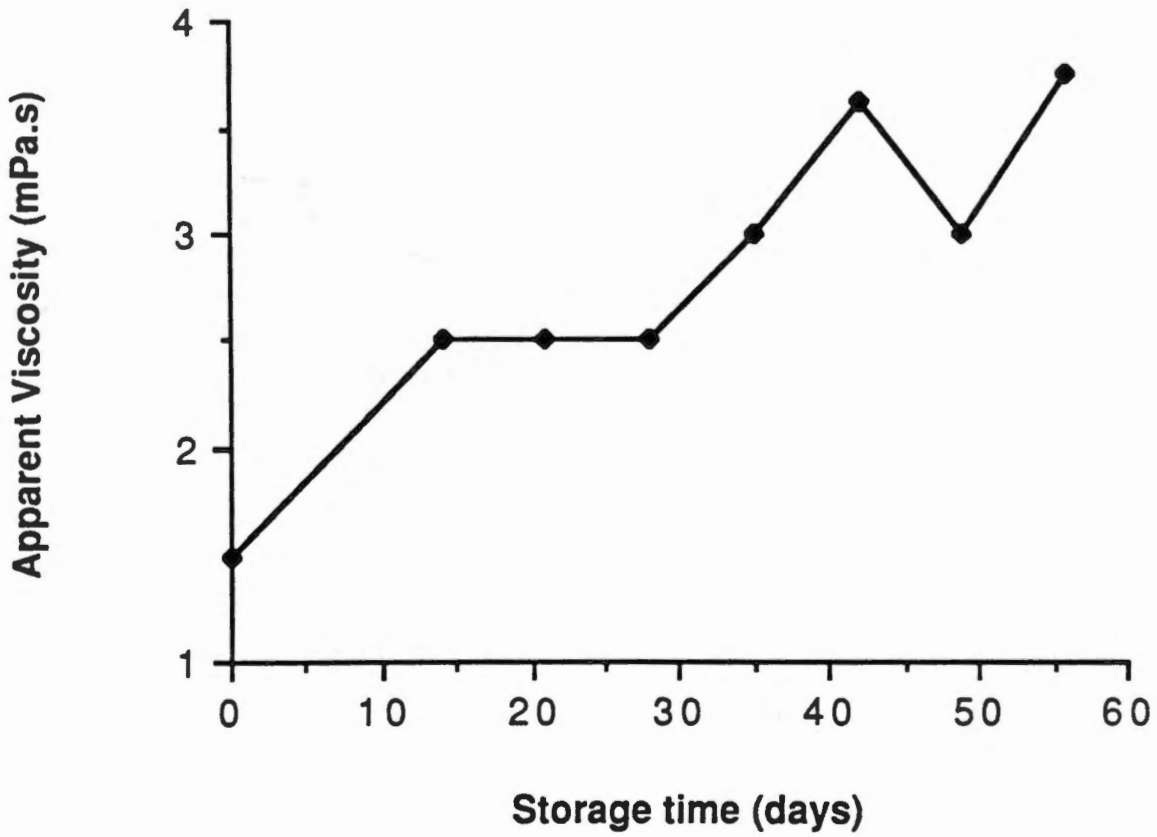



Figure 8. Relationship between apparent viscosity and storage time for individual cow sample C-10 stored at 25 C. R-square= 0.84.

gelled at day 44, and that was considered to be the end of the shelf life. Since samples were from one source, Analysis of Variance (ANOVA) was performed to compare those 4 samples with the rest in the group (samples from the area farms). There was a highly significant difference ($P < 0.05$) in somatic cell counts between the gelled samples and the non gelled samples (Table 12).

Shelf-life and somatic cell count for all samples were inversely correlated. Thus, high somatic cell count may lead to decrease in shelf-life of the product. Politis et al. (80) have indicated that elevated somatic cells is accompanied by an increase in plasmin which is responsible for hydrolysis of caseins and consequently decreased product shelf-life. Shelf-life was weakly correlated to initial protease activity. This lack of strong correlation, indicates that for practical purposes, estimation of shelf life of UHT milk by determination of the levels of protease in the samples is of limited application (58). This result does not agree with data of Janzen et al (53) who found correlation between initial protease activity and the keeping quality of skim and whole milk.

Means for shelf-life of abused creamery milk and milk from area farms were significantly different ($p < 0.05$) (Table 8). Figure 9 depicts the relationship between initial protease activity and shelf-life. Samples that had a shelf-life of more than 50 days had an initial protease activity of 55-73 CPM. Janzen et al. (53) have been able to relate the extent of proteolysis to shelf-life of skim and whole milk. Change in free amino groups and shelf-life were correlated and this was expected.

## F. Flavor Analyses

Sensory evaluation for bitterness flavor conducted by a panel of eight judges had a mean score of 3.3 on the scale of 1-9 (Table 3). Initial protease

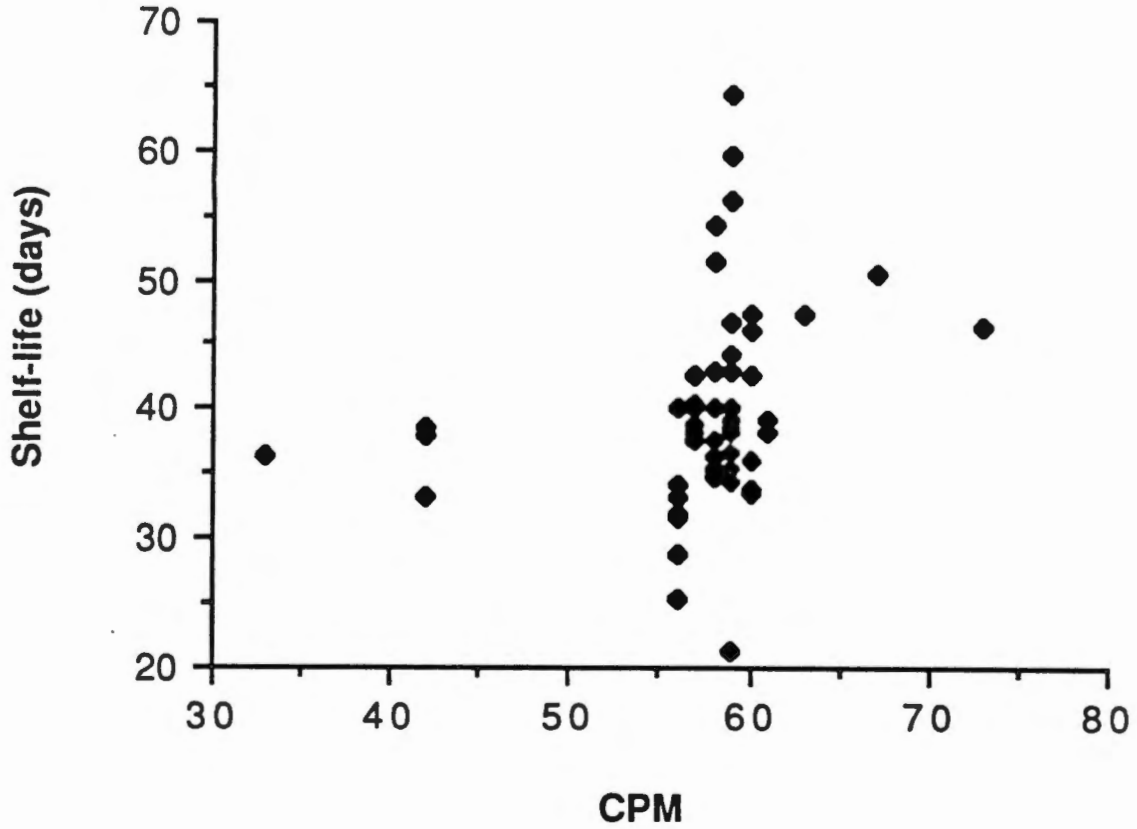



Figure 9. Relationship between initial protease activity and shelf-life.

Table 12. Mean somatic cell count for area farm samples which did or did not gel during storage at 25 C.

| Non gelled sample (N=14) | Gelled sample (N=4) |
|---|---|
| 5.99[a] | 6.05[b] |

[a,b] Means are significantly different ($p < 0.05$).

activity was weakly correlated with flavor score for the combined data (Table 4) However, the inverse relationship indicated that the higher the initial protease activity, the less bitter the sample. Figure 10 illustrates this relationship. This was unexpected. We did not measure residual protease activity after processing, protease may have been heat labile and did not affect the final sensory results. Correlation between two variables does not imply a cause and effect relationship. Figure 10 show the relationship between initial protease activity and flavor.

Interestingly, milk from individual cows had bitterness flavor scores which correlated strongly with initial protease activity (Table 7). Flavor was inversely correlated to psychrotrophic and standard plate counts. Abused creamery milk (Table 5) had bitterness flavor score which correlated well with all bacteriological counts. This suggests that initial bacteria counts in raw milk determines the final flavor of the manufactured product. No correlation between microbiological counts and bitterness was found for milks from the area farms (Table 6). This observation may suggest that not all bacteria in raw milk produce protease enzymes which can impart bitterness on the stored final product.

The lack of correlation between initial protease activity and change in free amino groups contradicts with the findings of McKellar (69). McKellar found that bitterness correlated very well with free amino groups (from as low as 0.4 micromoles/ml). Although it was not the case in this study, some correlation between shelf-life and flavor was expected. According to Hill (47), researchers have demonstrated that bitterness is an earlier signal of shelf-life termination than age gelation. Means for flavor between the three sources of milk were not significantly different (Table 8). This indicates that the source of milk did not play a major role in determining the final flavor of the processed product.

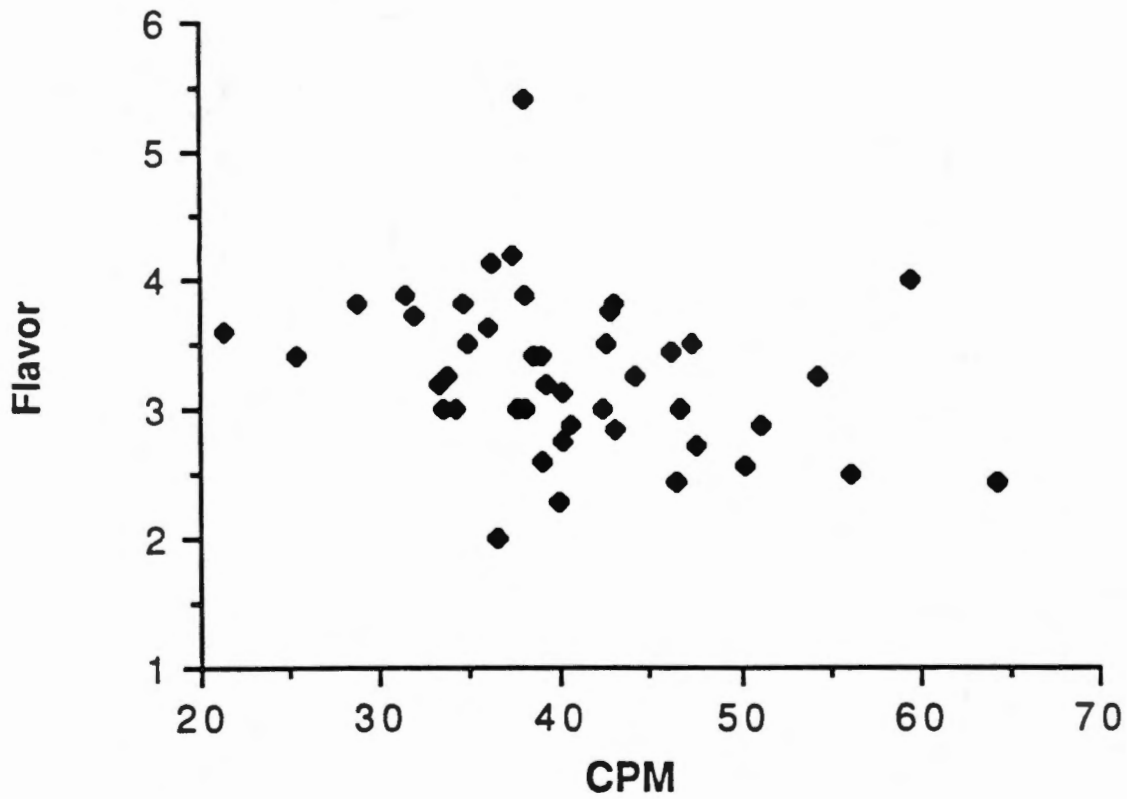



Figure 10. Relationship between initial protease activity and flavor.

# CHAPTER V

## SUMMARY AND CONCLUSIONS

For milk from all sources, the best predictor of shelf-life was somatic cell count of the raw milk. The higher the initial somatic cell count, the shorter was the shelf-life. Somatic cell count was also positively correlated with the change in free amino groups, an indicator of proteolysis. The $^{14}$C-casein protease assay was correlated with both flavor and shelf-life. However the relationship was the opposite of what had been anticipated; samples with higher initial protease activity had a longer shelf-life and were less bitter.

When correlations coefficients were calculated for the combined milks the $^{14}$C-casein protease assay was highly positively correlated with psychrotrophic bacteria count. Perhaps this assay may be useful as an indicator of the presence of psychrotrophic bacteria. This is an area worthy of further investigation.

Correlation coefficients were calculated between variables for each source of milk. The largest number of statistically significant ($p < 0.01$) values were found for the abused creamery milk samples. Each of the microbial assays were highly positively correlated with bitterness flavor score and proteolytic bacteria counts were highly positively correlated with change in free amino groups. These samples were intentionally abused to promote microbial growth. The indigenous microbes were apparently able to produce adequate proteolytic enzymes to affect the quality of UHT skim milk. These data support the use of high quality raw milk in UHT milk products.

Initial somatic cell counts for the abused creamery milk samples were correlated negatively with bitterness flavor and positively with change in

viscosity (slope). Since many of these samples were initially from the same raw milk but subjected to different storage conditions, many of the somatic cell count values were the same, which may account for these results. Alternatively, somatic cell proteases may break down milk proteins in such a way to increase viscosity without producing bitter peptides.

The correlation of practical significance found for milks from area farms was a positive relationship between somatic cell count and bitterness. This is the inverse of what was reported above, perhaps supporting the initial speculation that the data above may be skewed.

There was a highly significant positive correlation between the $^{14}C$-casein protease assay and bitterness flavor score for the milk from individual cows. An inverse relationship existed between standard plate count and bitterness flavor and between psychrotrophic bacterial count and bitterness.

Finally, further work is indicated to establish whether or not the $^{14}C$-casein assay can be used to predict the shelf-life of UHT milk. A study over a much longer storage time, possibly 6-9 months, would be more comparable to actual conditions of storage of UHT milk commercially. The procedure may also be useful in estimating bacteria present in raw milk prior to processing.

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# APPENDIXES

## APPENDIX 1

Analysis of Variance (ANOVA) comparing Fat by Source

| Source | df | Sum of Squares | Mean Square | F Value | PR>F |
|---|---|---|---|---|---|
| Model | 2 | 3.0076 | 1.5037 | 2.98 | 0.060 |
| Error | 48 | 24.1975 | 0.5041 | | |
| C.Total | 50 | 27.202051 | | | |

## APPENDIX 2

ANOVA comparing Total Solids by Source

| Source | df | Sum of Squares | Mean Square | F Value | PR>F |
|---|---|---|---|---|---|
| Model | 2 | 3.2176 | 1.6088 | 2.55 | 0.089 |
| Error | 48 | 30.3118 | 0.6315 | | |
| C.Total | 50 | 33.5295 | | | |

## APPENDIX 3

ANOVA comparing Protein by Source

| Source | df | Sum of Squares | Mean Square | F Value | PR>F |
|---|---|---|---|---|---|
| Model | 2 | 0.1532 | 0.766 | 0.95 | 0.396 |
| Error | 48 | 3.8886 | 0.810 | | |
| C.Total | 50 | 4.0418 | | | |

## APPENDIX 4

ANOVA comparing Initial Protease Activity (CPM) by Source

| Source | df | Sum of Squares | Mean Square | F Value | PR>F |
|---|---|---|---|---|---|
| Model | 2 | 37446.8828 | 18723.4414 | 3.20 | 0.050 |
| Error | 48 | 280931.1565 | 5852.7324 | | |
| C.Total | 50 | 318378.0392 | | | |

## APPENDIX 5

ANOVA comparing Somatic Cell Count (SCC) by Source

| Source | df | Sum of Squares | Mean Square | F Value | PR>F |
|---|---|---|---|---|---|
| Model | 2 | 4.1197 | 2.0599 | 8.32 | 0.001 |
| Error | 48 | 11.8903 | 0.2477 | | |
| C.Total | 50 | 16.0100 | | | |

## APPENDIX 6

ANOVA comparing Shelf-life by Source

| Source | df | Sum of Squares | Mean Square | F Value | PR>F |
|---|---|---|---|---|---|
| Model | 2 | 375.8497 | 187.9248 | 6.35 | 0.004 |
| Error | 48 | 1420.3072 | 29.5897 | | |
| C.Total | 50 | 1796.1569 | | | |

## APPENDIX 7

ANOVA comparing Standard Plate Count (SPC) by Source

| Source | df | Sum of Squares | Mean Square | F Value | PR>F |
|---|---|---|---|---|---|
| Model | 2 | 1.2462 | 0.6331 | 2.06 | 0.139 |
| Error | 48 | 14.5447 | 0.3030 | | |
| C.Total | 50 | 15.7909 | | | |

## APPENDIX 8

ANOVA comparing Psychrotrophic Bacteria Count (PsBC) by Source

| Source | df | Sum of Squares | Mean Square | F Value | PR>F |
|---|---|---|---|---|---|
| Model | 2 | 3.2223 | 1.6111 | 4.28 | 0.019 |
| Error | 48 | 18.0577 | 0.3762 | | |
| C.Total | 50 | 21.2800 | | | |

## APPENDIX 9

ANOVA comparing Proteolytic Bacteria Count (PtBC) by Source

| Source | df | Sum of Squares | Mean Square | F Value | PR>F |
|---|---|---|---|---|---|
| Model | 2 | 3.5603 | 1.7801 | 3.78 | 0.030 |
| Error | 48 | 22.5964 | 0.4708 | | |
| C.Total | 50 | 26.1567 | | | |

## APPENDIX 10

ANOVA comparing Changes in Free Amino Groups by Source

| Source | df | Sum of Squares | Mean Square | F Value | PR>F |
|---|---|---|---|---|---|
| Model | 2 | 5.4245 | 2.7122 | 7.27 | 0.002 |
| Error | 48 | 17.9169 | 0.3733 | | |
| C.Total | 50 | 23.3414 | | | |

## APPENDIX 11

ANOVA comparing Flavor by Source

| Source | df | Sum of Squares | Mean Square | F Value | PR>F |
|---|---|---|---|---|---|
| Model | 2 | 1.6147 | 0.8073 | 2.30 | 0.113 |
| Error | 41 | 14.4021 | 0.3513 | | |
| C.Total | 43 | 16.0167 | | | |

## APPENDIX 12

### Calculation of Flow Rate for the UHT Processor

Formula: Volume = 3.14 x (Radius )$^2$ x Length

Diameter of the Stainless Steel Tubing = 4.5 mm (ID) = 0.45 cm

Radius of the tubing (r) = Diameter/2 = 0.45/2 cm

Holding tube length = 46 cm

Therefore; Volume = 3.14 x (0.45/2)$^2$ cm$^2$ x 46 cm = 7.3123 C.C.

NB:1 Cubic centimetre (C.C) = 1 milliliter (ml)

Assuming that 7.3123 ml of milk flowed through 46 cm in 2 seconds

then, 1000 ml will flow for how long?

X sec = (1000 ml/7.3123) x 2 = 273.51 sec/6o = 4 min 59 sec.

Since the holding times were at the range of 2-8 sec ,the holding time of up to (273.51 x 4)/60 = 18 min 23 sec was considered safe.

Therefore, markers to indicate the flow rates of 1000 ml per 4.59 min and 1000 ml per 18.23 min were used, and these marked points indicated the minimum and maximum rates at which the valve could be opened. The flow was kept at that fixed rates throughout the duration of this study.

# APPENDIX 13

## Score card used by panelists.

You are receiving samples of milk to compare for bitterness. You have been given a reference sample, marked R, with which you are to compare each sample; determine whether it is more bitter than, comparable to, or less bitter than the reference. Then mark the amount of difference that exists.

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Sample number | ____ | ____ | ____ | ____ | ____ | ____ | ____ | ____ | ____ |
| More bitter than R | ____ | ____ | ____ | ____ | ____ | ____ | ____ | ____ | ____ |
| Equal to R | ____ | ____ | ____ | ____ | ____ | ____ | ____ | ____ | ____ |
| Less bitter than R | ____ | ____ | ____ | ____ | ____ | ____ | ____ | ____ | ____ |

AMOUNT OF DIFFERENCE:

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| None | ____ | ____ | ____ | ____ | ____ | ____ | ____ | ____ | ____ |
| Slight | ____ | ____ | ____ | ____ | ____ | ____ | ____ | ____ | ____ |
| Moderate | ____ | ____ | ____ | ____ | ____ | ____ | ____ | ____ | ____ |
| Much | ____ | ____ | ____ | ____ | ____ | ____ | ____ | ____ | ____ |
| Extreme | ____ | ____ | ____ | ____ | ____ | ____ | ____ | ____ | ____ |

Comments:

## APPENDIX 14

### Scale of 1-9 used to rank the degree of bitterness.

You are receiving samples of milk to compare for bitterness. You have been given a reference sample, marked R, with which you are to compare each sample; determine whether it is more bitter than, comparable to, or less bitter than the reference. Then mark the amount of difference that exists.

| Sample number | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| More bitter than R | | ✓ | ✓ | ✓ | ✓ | | | | |
| Equal to R | ✓ | | | | | | | | |
| Less bitter than R | | | | | | ✓ | ✓ | ✓ | ✓ |
| **AMOUNT OF DIFFERENCE:** | | | | | | | | | |
| None | | | | | 6 | 4 | | | |
| Slight | | | | 7 | | | 3 | | |
| Moderate | | | 8 | | | | | 2 | |
| Much | | 9 | | | | | | | 1 |
| Extreme | | | | | | | | | |

Comments: 5 ↑

| 1 | 2 | 3 | 4 | 5 | 6 ~ 9 | |
|---|---|---|---|---|---|---|
| EXTREMELY LESS THAN R | MUCH LESS | | SLIGHTLY LESS THAN R | EQUAL TO R | SLIGHTLY MORE THAN R | EXTREMELY MORE BITTER THAN R. |

# VITA

Athanas A. J. Ndomba was born on January 16, 1955 in Tanzania, East Africa. He came to the United States in 1983 to begin undergraduate studies in Animal Science at the University of Wisconsin-Riverfalls until Summer, 1984 when he changed major and transferred to the University of Georgia, Athens. He received a Bachelor of Science degree in Dairy Science-Manufacturing from Georgia in June, 1986. He started a Master's degree program in Fall, 1986 and successful received his M. S. degree in Food Technology and Science in December,1989.

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