COAGULATION OF UHT TREATED AND ASEPTICALLY PACKED WHOLE MILK

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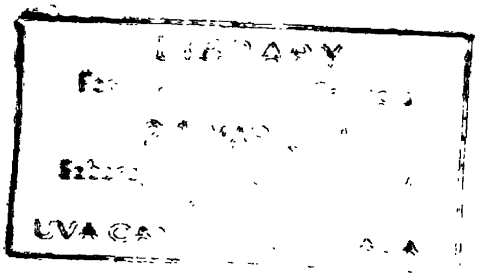

Declaration

I do hereby declare that the work reported in this thesis was exclusively carried out by me under the supervision of Dr. (Ms) Ajantha Horadagoda and Dr. Mahinda Wickramaratne. It describes the results of my owen independent research except where due references has been made in the text.

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AFFECTIONATLY DEDICATED TO MY

DEAR EVERLOVING BROTHER

DINESH

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ABSTRACT

The TROPI FRUT limited is the largest UHT treated milk producer in Sri Lanka. This company has lost millions of rupees recently owing to a milk coagulation problem. During this coagulation whey proteins separated from milk. In each occasion the whole batch was affected without any default in processing method. As there are heat resistant bacteria and bacterial enzymes, which can cause coagulation in milk. Firstly the presence of bacteria and secondly the enzymes that could present in the milk were assayed and finally the structural changes of the UHT milk was analyzed to study the changes that could have happen to casein.

Methylene blue dye reduction test and the Rezasurin test both showed no reduction activity in milk proving the absence of the bacteria in this UHT milk. Three enzymes lipase, urease and protease activity was tested, lipase and urease enzymes gave negative results. While the protease activity gave some positive results indicating the presence of heat stable bacterial protease in milk. Acid protease activity, which is used to detect plant protease by Anson (1939), was applied for the first time in milk to analyze the protease activity and at a pH range of (2-8) at room temperature. A protease enzyme, activity was detected between pH 2-8. The highest peak of this enzyme was between pH 3.5 to 4.5. This enzyme has very low molecular weight as it was detected at the dye front of a 12% acrylamide gel. However, this band was very faint therefore the concentration of the enzyme is minute. The results of UHT treated control milk samples indicated there were neither enzyme activity nor the bacterial activity. This study confirms the presence of enzymatic activity in UHT treated coagulated milk. These heat resistant enzymes may have released from psychrotrophic bacteria. These bacteria could have multiplied at the time of refrigeration during the bulk storage.

Finally the structural change of casein was studied using poly-acrylamide gel electrophoresis. It clearly showed in the coagulated UHT milk samples the molecular weight of the casein has increased when compared to the casein bands of the control UHT milk and the fresh cow milk sample. These conformational changes of casein molecules are due to many reasons. However, further studies are needed to isolate these enzymes and their enzymatic activity and also to reasons out the causes for the structural changes of casein

CONTENT

ABBRAVIATION

CMP- casein macro phosphate

GMP- glyco macro phosphate

Hb- heamoglobin

HTST- high temperature short time

I. D. F. - international dairy federation

LDPE- low density polyethylene

LTLT- low temperature long time

NPN- Non-Protein Nitrogen

PAGE- poly acrylamide gel electrophoresis

SDS- sodium dodesyle sulfate

TCA- trichloroacetic acid

UHT- Ultra High Temperature

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1. INTRODUCTION

Milk is the only food for young mammal during the first period of its life. The substances in milk provide both energy and the building materials for growth and responsible for the immunological protection when exposing to the new environment. Thus man alone, among mammals, has introduced the milk of other animals into his diet. Animal milks used in various part of the world, both in the general diet and as a food for young children, include milk of the buffalo, ass, sheep, goat, llama, reindeer and the yak, but cow milk is by far the most commonly employed. However, the composition of milk of different mammals varies considerably and all contain protein, lactose, fat, minerals and vitamins, and all are poor in iron.

Milk is a very perishable food and being a nutritious liquid it is a good source for the growth of micro-organisms either present in the milk or introduced during handling. Milk must therefore be handled with care to keep it safe for consumption. Thus bacteria can easily attract and can rapidly multiply. Hence in milk industry milk processing plays a major role. Processing means enhancement of keeping quality of milk. Like in the past if milk is consumed where it is produced with in a short duration sophisticated processing methods are not required. However, with the industrial revolution and the development of the cities people moved from villages to more urban areas. In these highly populated cities the demand for milk was rising. Thus the milk that is produced either in the farms or distance grasslands of the world needed to be transported to places where there is a demand. This journey of milk required pre-treatment of milk as freshly drawn milk can get contaminated while transporting for long hours at high ambient temperatures and in unhygienic utensils. Thus milk processing became very important to increase its keeping quality. Proper processing systems should have a minimum nutritional losses and maximum hygienic quality of the milk. To day the dairy industry is one of the largest industries in the world which have introduce a variety of products such as powdered milk, butter, skimmed milk, liquid milk products as Ultra High Temperature (UHT) treated milk and pasteurised milk.

When considering the milk products dried milk powder a solid form compound may be made with whole milk, or with skimmed milk, by employing spray drying or drum drying methods. This product has a very high shelf life and could be transported globally

Evaporated milk is another product, which has a very long shelf life. This product is also made by heating milk to about 95°C, for 10 min. and concentrated in vacuum pans at 50°C to 55°C, homogenized and canned. Pasteurisation of milk is undertaken to destroy only the common pathogenic organisms, which occur in raw milk, by heating to sufficiently high temperature for an appropriate length of time. Sterilized milk is also a liquid product which has a higher keeping quality than pasteurised milk. In this product all microbes are destroyed by heating milk to a temperature of 120°C, for a few seconds. Product is filled into bottles at 60 to 70 °C, the bottle hermetically sealed and heated at 120 °C for further 20 to 60 min to ensure that the temperature inside the bottle is at least 115 °C for 5 min. Similarly UHT treatment of milk is also destroy all microbes present in milk and the product is packed aseptically.

Currently in Sri Lanka these 'ready to drink' milk products are available and are becoming highly popular. Among these the UHT treated and aseptically packed liquid milk has achieved a high consumer preference. The UHT stands for ultra high temperature. The technique is also known as ultra-pasteurisation. Here the milk is heated in two stages first to 77°C for 15 seconds and then under pressure to about 142°C for 4 seconds. After this ultra high heat treatment milk is immediately cooled and aseptically packed. The intensity of UHT treatment is sufficient to destroy all heat resistant bacterial spores. UHT treated milk can therefore kept for longer duration without refrigerator facilities. The advantages of UHT milk is that it has a long shelf life greater then six months, high nutritive value due to the reduction in process time, cheaper packaging and the package size is independent of processing conditions.
UHT milk has a pleasant flavour. This may be due to the minimal chemical changes in milk than the other processing methods.

Even though UHT treatment inactivate the microbial activity and chemical reactions in milk followed by aseptic packaging, at storage some adverse reactions may arise due to the storage conditions improper packaging improper treatment or improper handling. One such major problem is the coagulation of milk. Milk coagulation is also experienced due to many kind of reasons, such as heat-induced coagulation, acid coagulation, rennet coagulation and age gelation. At normal conditions heat induced coagulation, acid coagulation, rennet coagulation are not performed (Dalgliesh, 1999) But age gelation is an aggregation phenomenon that effects shelf stable sterilized dairy

products such as UHT treated milk and concentrated milk. This happen after weeks to months of stores, there is a sudden sharp increase in viscosity accompanied by visible gelation and irreversible aggregation of the micelles forming a three-dimestional net work (Dalgliesh, 1999). There many be a possible cause which have lead direct and indirect relationship with the problem, such as proteiolytic brake down of casein, due to the native plasmin enzymes, or microbial enzymes, chemical reactions such as polymerisation of casein or formation of kappa-casein beta-lactogobulin complexes. Due to the enhancement of many other indirect possibilities this problem is taken place.

The problem of age gelation, which is common to the UHT treated milk (Fox, 1986) and it discards thousands of liters of production when arises at processing plants and it discards millions of rupees of the nation. The distortion not only relevant to the money but also to the business stability of a firm and other main criterion like depressed consumer preference, which decrease the market share for the UHT treated milk.

The TROPI FRÛT limited is the largest UHT treated milk producer in Sri Lanka. They too experienced a problem, which lead to coagulation of UHT, treated milks. Currently this has occurred thrice. During this coagulation whey proteins separated from milk at different intervals. In each occasion the whole batch was affected without any default in processing method. Also they were confident that the cause is not bacterial contamination instead may have a adverse effect on protein fraction.

OBJECTIVES

Thus the objective of this study is to identify the reasons for UHT milk coagulation. The aetiology of coagulation can improve the quality of UHT products and also thereby can increase the consumer acceptance.

Based on the objectives the following were needed to be studied.

First step is to check the milk samples for the presence of bacteria.

Secondly to test the enzymes present in UHT milk.

Thirdly to study the physical changes of the milk protein casein.

2. REVIEW OF LITERATURE

Milk is a secretion of the lacteal glands of female mammals. It is the first food for infant mammals. Thus the role of milk in nature is to nourish and provide immunological protection for the new-born. Bovine's milk is obtained by complete milking of healthy milch animals that are properly fed and kept. Its constituents are water, milk fat, protein, lactose, minerals, acids, enzymes, gases, vitamins, etc. Milk therefore is a very complex food with high nutritive value.

Bovine milk has been a food for humans since prehistoric times. It was mainly used as processed products or as boiled milk. Some ethnic groups consume, without any processing, as fresh milk because of their religious believes. However drinking of fresh milk is not advisable because of its unpleasant odour, flavour, and contamination of pathogenic bacteria in the environment or due to the diseased cows. When gradually people moved to the very sophisticated life in more urban areas. Milk processing also became an industry and producers tried to give their best to the consumer, overcoming different kind of processing problems to increase the shelf life of milk. In the increasement of shelf life milk can distribute to different geographical rejoins and can convert in to different food items. Long term availability is increased with different kind of products such as boiled milk, pasteurised milk, condensed milk, concentrated milk, powdered milk (reconstitute milk or dried milk), UHT milk, and milk based products such as cheese, butter and ghee, etc.

2.1. Raw milk

Fresh milk is highly nutritious thus is a very unstable food as microbes can survive and multiply rapidly, even before it is relieved for processing. When fresh milk is transported to long distance without proper cooling system microbial multiplication enhance, which have a great effect on changes in chemical and physical properties of the milk. Milk freshly drown from the cow is at a temperature around 38°C. To reduce the growth of contaminant bactena and to slow the rate of spoilage, the temperature of milk should be lowered to 4°C and 2°C, within 30 minutes of milking

2.2. Pasteurised milk

Pasteurisation is a heat treatment most commonly applied to liquid milks and creams. This is a process applied to destroy the pathogenic microorganisms associated with milk. This treatment is consistent with minimal chemical, physical and organoleptic changes to the product. In pasteurisation two methods are used, low temperature long time (LTLT) and high temperature short time (HTST). In LTLT method pasteurisation ensures a heat treatment of not less than 62.8°C and not more than 65.6°C for not less than 30 minutes. While HTST ensures at temperature at least 71.1°C for 15 seconds or equivalent conditions (Anson, 1995). However, time temperature combinations of 72°C to 75°C for 15 to 20 seconds are common, in U.S.A heat treatments of nearly 77°C for as long as 40 seconds are practice. Although pasteurisation is mild heat treatment the process has little effect on nutritive value of milk.

Most vegetative bacterial cells, yeast and mould in milk should be killed by pasteurisation. However, thermoduric bacteria and heat resistant spores of other bacterial flora will survive the pasteurisation and spoil the pasteurised milk.

2.3. UHT treated milk

While pasteurisation conditions effectively eliminate potential pathogenic microorganisms, it is not sufficient to inactivate the thermoresistant spores in milk. Without refrigerating it cannot store for long time, as development of thermoduric bacteria, spore stages and survival of some heat resistant enzymes cause post-production problems. This lead to processors to move to seek for other sophisticated more reliable processing methods such as UHT processing (Sterilisation). Although sterilisation as a means of preserving foods had been understood since 19th century, the technologies required to combine aseptic processing with aseptic packing, which did not develop adequately until 1950's. When ultra-high temperature processing become reality (Hostettler, 1981) As UHT processes have subsequently developed the temperatures and correct duration the application of the technique in many areas of food industry and notably in the dairy industry for the production of long life milk and cream. The term sterilisation refers to the complete elimination of all microorganisms. The food industry

uses the more realistic term "commercial sterilisation", a product is not necessarily free of all microorganisms, but those that survive the sterilisation process are unlikely to grow during storage and cause product spoilage.

Milk can be made commercially sterile by subjecting it to temperature in excess of 100°C, and packaging it in airtight containers. The milk may be packaged either before or after sterilisation. The basis of UHT (ultra-high temperature), is the sterilisation of food before packaging, then filling into pre-sterilized containers in a sterile atmosphere Milk that is processed in this way using temperatures exceeding 135°C, permits a decrease in the necessary holding time (2-5 sec) enabling a continues flow operation. However heat treatments of 140°C for 6.8 s or 145°C for 2 seconds are suitable to achieve to this and any combination within these value are recommended for milk (Perkins, 1985).

2.4. Milk quality and properties for UHT processing

2.4.1. Raw milk

The microbial contamination of raw milk during production in the farm can impose adverse effects in the production. Milk from uninfected cows will have somatic cell counts of below 200,000 cells per cm^3 and often below 100,000 cells per cm^3. But mastitis infection caused by major pathogens elevates the somatic cell count, while improperly cleaned udders and dirty teats may contribute between 100,000 and 500,000 bacteria per cm^3. (Fox, 1986; Early, 1997)

For UHT milk microbial quality should also be determined after bulk storage. The accepted microbial counts differ from producer to producer, but there is a great effect on post-production and pre-production deteriorations due to the elevated levels of cell counts.

2.4.2. Effects of time and temperature of storage

Products of bacterial metabolism

On those occasions where bacterial growth occurs to an extent exceeding 5 million per millilitre, there will be a considerable risk of noticeable abnormalities in the milk as a result of metabolic activity (Von Bockelmann, 1981). Similar changes may also be brought about in cooled milk due to contamination by psychrotrophic bacteria, even within the range of 2 to 4°C (Dommett and Baseby, 1978).

Thermo-resistant psychrotrophic bacteria

There are several known psychrotrophic bacteria, which are resistant to temperatures used in pasteurisation; some of them are spore forming. Milk contaminated with such organisms is liable to give rise to the production of faulty end products if growth has taken place to a marked extent before pasteurisation. Under practical conditions, the effects of thermo-resistant psychrotrophs are of little consequence in comparison with those brought about through post-pasteurisation contamination (Teuber and Busse, 1981). Undoubtedly when control measures enable post-pasteurisation to be reduced, much more attention will be directed to the effect of thermo-resistant psychrotrophic infection.

Thermo-stable bacterial enzymes

Several species of psychrotrophic bacteria produce significant amount of highly active thermo-resistant enzymes. Whilst most bacteria are killed by HTST pasteurisation, some bacterial lipases and proteases remain and unaffected; even after UHT treatment small residual amounts of these enzymes may remain and can be the cause of changes in organoleptic and physico-chemical characteristics depending on storage conditions. In pasteurised milk the effect of enzyme activity is usually not perceptible. These enzymes can also have undesirable effects in the maturing of cheese. At total counts higher than 1million per ml in milk, psychrotrophic organisms may have produced enzymes, which are thermo-stable in undesirable amounts (Teuber and Busse, 1981). Production of such enzymes has been reported at lower numbers of bacteria

Enzyme effects preceding heat treatment

Perhaps the known defect occurring in milk is that of the enzymatic effect of lipase on fat globules, which have been damaged superficially. Using modern milking equipment some damage to the fat globule must be expected. Ordinarily, provided the milk is cooled to below 4°C immediately after milking, the consequences (lipolysis) will remain within acceptable limits for several days. Apart from the lipolytic effects, changes can be induced by proteases. Unlike lipolysis which can have such pronounced effects on flavour, from proteases have certain effect on protein structure which by comparison are not very noticeable in practice, in pasteurised milk. In UHT milk, gelation may occur as a result of residual protease activity (Teuber and Busse, 1981).

Physico-chemical effects

Apart from the physico-chemical effects of damage to the fat globule, (exposure to lipolytic effect and effects of pre-treatment handling and storage) the storage of milk at low temperatures has some influence on the structure of protein fraction (Teuber and Busse, 1981). This will have some effects on cheese making; it may also effect the efficiency of machine separation of cooled milk.

Special attention should be paid to the part of the bacterial contamination that can result in an escalation of bad effects even in cooled milk. It is clear that bad hygiene in general, will have an adverse effect on end-product quality (Teuber and Busse, 1981).

2.4.3. Physico-Chemical properties

Milk is a complex colloidal dispersion containing fat globules, casein micelles and whey proteins in an aqueous solution of lactose, minerals and a few other minor compounds. Its physical and chemical properties depend on variety of compositional factors and processing factors.

The pH

Freshly drown milk is slightly acidic due to the presence of casein phosphates, albumin, carbon dioxide and citrate (Horadagoda, 1990). The pH of milk at25°C normally varies within relatively narrow range of 6.5 to 6.7(Dalgleish, 1999). The normal range pasteurised and boiled milk show same pH and acidity as raw milk while sterilisation show increased acidity but not the pH.

Acidity

Both pH and titratable acidity are used to measure milk acidity. The normal range of titratable acidity of herd milk is 12 to 20 mmol/L (Horadagoda, 1990). Because of the large inherent variation, the measure of titratable acidity has little practical value except to measure changes in acidity (Horadagoda, 1990).

Acid- base equilibria

Substances that resist pH change upon addition of acid or alkali are called pH buffers. This resistant called buffer action, the magnitude of which is measured by the amount of acid or base required to change the pH of solution by one unit. There are many components in milk, which provide a buffering action over a wide range of pH (Fox, 1997). The major buffering groups are caseins and phosphate.

Oxidation-Reduction equilibria

Oxidation is defined as the loss of electrons by a substance while reduction is the gain of electrons. The ascorbic acid content of fresh milk is about 11.2 - 17.2 mg/l, which is the major constituent relevant to this phenomenon. When milk drown from udder, all ascorbates are in reduced form, but reversible oxidation occurs to dehydroascorbate at a rate depend on temperature and on the concentrations of Cu, Fe, and O_2. The redox potential of milk is strongly influenced by heat treatment, bacterial activity, contamination with metal ions such as Cu^{+2}, concentration of O_2 and exposure to light. The redox potential of milk decreases during heating: this is largely related to the liberation of active sulphydryl groups of beta-lactoglobulin, some of which are oxidized by atmospheric oxygen. Reducing substances producing in milk as a result of the Maillard reaction

between lactose and protein also influence the redox potential of milk, particularly concentrated and dry milks (Harland et al., 1952). UHT treatment of milk results in lower redox values, and less oxidation of ascorbic acid and greater retention of disulphide-reducing substances (Fox, 1997).

2.4.4. Physical properties

Milk is an oil-in -water emulsion and its physical properties are, therefore, somewhat similar to those of water. Dispersion of solid component have, however, altered the physical properties of milk from water. When compared to chemical properties most of the physical properties have a smaller range of variation. Knowledge on physical properties of milk is useful to detect adulteration of milk. As buffalo milk is richer in fat than cows milk (kay, 1974) people can adulterate milk by diluting or by removing fat. This form of adulteration has been a problem throughout the history of the milk industry and countries to be a problem today.

Colour

The colour of milk is perceived by consumers to be indicative of purity and richness its white colour due to the scattering of reflected light by the inherent ultramicroscopic particles, fat globules, colloidal casein micelles, and calcium phosphate. The intensity of white colour is directly proportional to the size and number of particles in suspension. Homogenization increases the surface area of fat globules significantly as a result of the break-up of larger globules. Accordingly, homogenized milk and cream are whiter than their unhomogenized counter parts. Lack of fat globules gives skim milk a blue ting (Fox, 1997).

Cows milk contains the pigments carotene and xanthophyll, which tend to give a golden yellow colour to milk fat (Horadagoda, 1990).

Flavour

The flavour of milk is a property difficult to define, but there is no doubt that taste and aroma is critical to the assessment of milk. Flavour constitutes a critical criterion of quality for the consumer. It is a sensory property in which odour and taste interacts. The sweet taste of lactose is balanced against the salty taste of chloride, and both are somewhat moderated by proteins. This balance is maintained over a fairly wide range of milk composition even the chloride ion level is varies from 0.06 to 0.12%. Some workers attribute the characteristic rich flavour of dairy products to the lactose, methylketones, cretin aldehydes, dimehtyl sulphide, and certain short- chain fatty acids.

Although milk has a clean pleasantly sweet flavour, it is quite blend, and therefore any off-flavours are readily discernible. Off- flavours result when microbiological action, dairy farm or processing condition or chemical or biochemical actions alters the balance of flavuor compounds (Fox, 1997).

Foaming

The formation of stable foam depends upon two main factors. First the lowering of the surface tension allows gathering and spreading the surface-active components into thin films. Second, the films must be sufficiently elastic and stable to prevent the coalescence of the gas cells. Stable foam is thus formed when the surface tension of the liquid is not great enough to withdraw the film from between gas cells and when stabilizing agent has great internal viscosity.
Foaming of milk is at a minimum at 30 - 35°C. At 60°C, the foam volume is independent of the fat content. Below 20°C, for instance, during churning skim milk products slightly more stable foam above 30°C than whole milk or light cream.

Surface tension

Forces causing a reduction in surface area, which is a characteristic property of a liquid Surface properties are involved in adsorption phenomena and the formation and stability of emulsions. They are relevant to creaming, fat globule membrane function, forming and emulsifier use in dairy products. Normal cow's milk has an inherent surface

activity. Its surface tension approximates 70% of that of water. The surface tension of whole milk usually about 50-52 mN/m at 20°C. For cream, it is approximatly 46-47mN/m. Casein, along with the proteolysis products protease-peptones, is largely responsible for the surface activity. Whey proteins make little contribution. Fat reduces surface tension by physical effect. Lactose and most of the salts tend to rise it when they are present in the true solution. Surface tension decreases as temperature rises. Processing treatments such as heating, homogenization tend to increase surface tension (Fox, 1997).

Viscosity

Resistance to flow. A measure of the friction between molecules as they slide past one another. Whole milk and skim milk display viscosity of 2.0-2.1 and 1.5-1.8 cP at 20°C,respectively.whey has a viscosity of 1.2 cP. The viscosity of milk and cream creates the impression of 'richness' to the consumer. From an organoleptic standpoint, viscosity contributes to mouth feel and flavour release. The casein micelles of milk contribute more to the viscosity of milk than any other constituent (Dalgleish, 1999). Viscosity varies not only with changes in the physical nature of fat but also with the hydration of proteins. Alteration in the size of any dispersed constituents result in viscosity changes. The fat contributes less casein but more than whey proteins. When fat globules are greatly subdivided by an increase in viscosity is observed. The viscosity of skim milk decreases on heating to 62°C, after which it increases, apparently due to changes in H-bonding. An increase of temperature causes a marked reduction of viscosity (Fox, 1997).

Viscosity of milk is important to determining the rate of creaming, rates of mass and heat transfer, the flow conditions of dairy processes in dairy industry. Milk and skim milk, excepting cooled raw milk, exhibit Newtonian behaviour, in which, the viscosity is independent of the rate of shear.

Density

Density is defined as mass per unit volume. It has the units kgm^{-3}, density of whole milk is about 1030 kgm^{-3} and usually range from 1027 to 1033 kgm^{-3} at 20°C. This depend on temperature at time of measurement (Dalgleish, 1999).

Specific gravity

The ratio of the density of a product and the density of water at the same temperature. The average specific gravity of milk is 1.030 (Dalgleish, 1999).

Specific heat

Number of calories required to rise the temperature of 1 g of substance by .1°C. The specific heat of milk product is a function of their composition. The values of whole milk and skim milk at 15°C are 0.93, 0.95 BTU/lbF respectively (Fox, 1997).

Freezing point

Freezing point is a *colligative property* which is determined by the molarity of solutes than by the percentage by weight or volume, it is generally expressed as degrees Hovet (H). In the dairy industry, freezing point is mainly used to determine added water but it can also been used to determine lactose content in milk, estimate whey powder content in skim milk powder, and to determine the water activity of cheese. The freezing point of milk is usually in the range of -0.512 to 0.550°C with average of -0.522°C.

Lactose and chloride are the major milk constituents responsible for 70 - 80% of the overall depression in the freezing point of milk. Correct interpretation of freezing point data with respect to added water depends on good understanding of the factors effecting *freezing point depression*. With respect to interpretation of freezing points for added water determination, the most significant variables are the nutritional status of the herd and the access to water. Under feeding causes increased freezing points. Large temporary increases in freezing point occur after consumption of large volume of water because milk is iso-osmotic with blood. The primary sources of non-intentional added water in milk are residual rinse water and condensation in the milking system.

Boiling point

A solution boils at higher temperatures than does the pure solvent, according to the concentration of the dissolved substance. The boiling point of milk is 100.17°C The

milk constituents in true solution are mainly responsible for the elevation of boiling point above 100°C. Elevation of boiling point is based on the same principles as depression in freezing point. However, for detecting added water, the freezing point method is far superior on the grounds of accuracy and convenience (Fox, 1997).

Electrical conductivity

The reciprocal of electrical resistance exhibited by a 1cm cube of conductor (solution containing electrolytes). Current passes through the milk by virtue of its ionic mineral constituents, of which the chloride ions carry 60 - 68% of current. There is therefore a close correlation between the electrical conductivity of milk and its chloride content. The electrical conductivity of normal milk corresponds to that of approximately 0.25% sodium chloride solution (w/w). And conductivity of milk is the basis of the new ohmic process for sterilising milk (Fox, 1997).

2.4.5. Optical properties

When an electromagnetic radiation encounters chemical species (atoms, ions, or molecules) it can absorb, scattered, or it can excite fluorescence. Light scattering by fat globules and casein micelles causes milk to appear turbid and opaque. Light scattering occurs when the wavelength of light is near the same magnitude of particle. Thus, smaller particle scatters shorter wavelengths of visible light (blue) more than the red (Fox, 1997). The carotenoid precursor of vitamin A, β-carotene, contained in milk is responsible for creamy colour of milk. The refractive index of milk is 1.3440 to 1.3485 at 20°C. Optical properties provide the basis for many rapid, indirect methods of analysis such as proximate analysis by infrared absorbency or light scattering. Optical properties also determine the appearance of milk and milk products (Dalgleish, 1999).

It must be emphasized that the influence of these raw milk quality parameters on the quality of the end products depends very much on the treatment afterwards during processing, storage and distribution.

2.5. Methods for UHT processing

There are two principal methods of UHT processing:

1. Direct heating
2. Indirect heating

2.5.1. Direct heating systems

The product is heated by direct contact with steam of potable or culinary quality. The main advantage of direct heating is that the product is held at the elevated temperature for a shorter period of time. For a heat-sensitive product such as milk, this means less damage. There are two methods of direct heating; those are Injection and Infusion (Dalgleish, 1999).

2.5.2. Indirect heating systems

The heating medium and product are not in direct contact, but separated by equipment contact surfaces. Several types of heat exchangers are applicable: those are plate, tubular, scraped surface, and double-cone.

Plate heat exchangers

Similar to that used in HTST but operating pressures are limited by gaskets. Liquid velocities are low which cold lead to un-even heating and burn-on. This method is economical in floor space, easily inspected, and allows for potential regeneration.

Tubular heat exchangers

There are several types of this kind, that are shell and tube, shell and coil, double tube, and triple type. All of these tubular heat exchanges have fewer seals involved than with plates. This allows for higher pressures, thus higher flow rates and higher temperatures. The heating is more uniform but difficult to inspect (Dalgleish, 1999).

2.6. UHT processing

2.6.1. Milk clarification

The first step in the production. The aqueous phase of milk contains the milk proteins, lactose, water-soluble vitamins and minerals. The milk fat is dispersed through the aqueous phase as globules between 1μm and 18μm in diameter, stabilized by the milkfat globule membrane which is comprised of proteins and lipids existing in an ordered state or as discrete lipoproteins (Brunner, 1974). Raw milk also contains somatic cells and particles of soil and detritus, which enter the milk during milking operations. If milk is left to stand milkfat globules will gradually aggregate and rise through the aqueous phase of milk, to the surface, to form a cream layer. The insoluble materials will sink to form sediment. The differences in density between the aqueous phase of milk, the milkfat globules and the insoluble materials which may be incorporated into milk, from the basis of the centrifugal clarification and the centrifugal separation of cream (Early, 1997).

2.6.2. Processing

Processes based on plate or tubular heat exchangers typically have the following processing steps,

Standardized milk at 2-4°C is fed to the UHT plant balance tank; from the balance tank the milk is pumped through the regeneration section of the heat exchanger and the raw milk temperature is raised to 70- 80°C; the milk passes through a homogenizer operating at 18-25Mpa, where the fat globule size is reduced to 1- 2μm; in the heating section of the heat exchanger milk is heated to 140-145°C and then held at this temperature for 2-5 seconds, as it passes through the holding tube; on leaving the holding tube the milk is passed to the regeneration sections where it first gives up heat to cool end of the hot water circuit and then to incoming raw milk; from the regeneration sections the milk enters an aseptic tank to await aseptic packaging.

UHT processes incorporating tubular heat exchangers instead of plate heat exchangers operate in similar stages, although some differences exist The pre-heating

temperature used to induce whey protein gelation and stabilize the milk proteins is somewhat higher at around 95°C. The UHT temperature is also higher, in the range 140-150°C. As with a pasteurisation plant, UHT plants must be equipped with flow-diversion capability. In the event of a temperature drop during processing the under heated milk is diverted to a storage tank to await disposal as it cannot subsequently be re-processed as UHT milk (Early, 1997).

2.7. Aseptic packaging

The most important point to remember that it must be sterile. All handling of product post-process must be within the sterile environment. The technique of manufacturing sterile milk in the continuous flow heating processed only reached its full importance when it became possible to achieve filling into sales packs while excluding all possibilities contamination.

There are several different type of package forms that are cans, paperboard/plastic/foil/plastic laminates, thermoformed plastic containers, flow-moulded containers, bag-in- box, and bulk totes.

The aseptic packaging of milk consists of a sequence of operations designed to prevent product recontamination following heat treatment. The operations can be described broadly as:
Pre-sterilisation of packaging material; package forming; package filling; package sealing to produce a hermetic package.

Ronkilde poulsen (1982) recognizes the need to pack UHT milk in materials which are a barrier to the ingress of microbes that may cause product deterioration and which are also impermeable to odours and /or gases, resist the passage of light and provide rigidity there by resisting fracture or breakage. One of the most common package formats used for the packaging of UHT milk is Tetra Brik aseptic carton. The carton material is laminated (Tetra Pak, 1994) and consists of:

An external layer of low density polyethylene which serves to protect the carton from environmental moister and printed artwork from abrasion; a paper board layer which provides the carton with strength and rigidity and which support the printwork; an LDPE layer which binds the aluminium layer to the paper board layer; a layer of aluminium which acts as a gas barrier and which also light proofs the carton to prevent light induced auto-oxidation of the milkfat and extend the shelf-life of the milk; an internal LDPE layer which prevents the milk from coming into contact with the aluminium layer and which also serves to form the heat seals through which the package shape is fixed.

The aseptic packaging process is described as a chemo-thermal system (Tetra Pak, 1990). It is carried out within an aseptic packaging machine, which can be maintained as a commercially sterile environment. Chemical pre-sterilisation of packaging machinery and a supply of pressurized filtered air, which prevents dust and bacteria from being drawn o the packaging environment during operation, ensure that the risk of product contamination is minimized. A form-fill-seal process in which laminated carton material is fed to the packaging machine from a reel produces tetra paks of UHT milk. The material is sterilized by passing through a deep bath of heated hydrogen peroxide, which is subsequently removed by squeezer rollers and evaporation induced by a blast of hot air through nozzles. As the material is fed to the sterilisation bath a strip of plastic is applied to one edge to allow subsequent formation of the pack's longitudinal seal. The sterilized material is formed into a carton around a filler pipe by a series of mechanical operations. The first seal is the longitudinal seal, which ensures the carton material forms a tube surrounding the filler pipe. Clearly the second seal must close the base of the carton. UHT milk from an aseptic tank is filled into the carton through the filler tube and carton is closed with a seal formed below the level of milk. The operation, which closes the top of the carton also, forms the seal at the base of the next carton: hence the term form-fill-seal. Inductive heating is used to produce the seal and machine jaws shape the top of the carton are folded and sealed into place. Completed cartons are then discharged from the aseptic packaging machine to be packed in product outer packaging and staked on pallets to await distribution (Early, 1997).

2.8. Advantages of UHT processing

High quality:

The D and Z valves are higher for quality factors than microorganisms. The reduction in process time due to higher temperature and the minimal come-up and cool-down time lead to a higher quality product.

Long shelf life:

Greater than 6 months, without refrigeration, can be expected.

Packaging size:

Processing conditions are independent of container size, thus allowing for the filling of large containers for food service or sale to food manufactures.

Cheaper packaging:

Both cost of package and storage and transportation costs; laminated packaging allows for use of extensive graphics.

2.9. Disadvantages of UHT processing

Sterility:

Complexity of equipment and plant are needed to maintain sterile atmosphere between processing and packaging by higher skill operators, sterility must be maintained through aseptic packaging.

Particle size:

With larger particulate there is a danger of overcooking of surfaces and needs to transport material-both limits particle size

Equipment:

There is a lack of equipment for particulate sterilisation, due especially to settling of solids and thus over processing

Keeping quality:

Heat stable lipases or proteases can lead to flavour deterioration, *age gelation* of milk over time- nothing lasts forever. There is also a more pronounced cooked flavour to UHT milk.

2.10. Physical and chemical changes resulting from UHT treatment and storage

Although various processing and storage conditions are known to effect the gelation of UHT milk during storage and particularly gelation, the micelle structure changes still further. The storage temperature (Andrews et al., 1977) and the method of heat treatment (Blanc et al., 1980) both play a role in this change. In comparison to directly processed milk, the micelles of indirectly processed milk seem to change less. Probably because the heat treatment is more subsequently, the casein/ whey protein complexes produced are more irreversible (Hostettler, 1972). The differences become obvious when the storage temperature varies and also observed that there were structural differences between the various layers of packaged milk. In the upper layers with their higher fat content, fat globules linked by "casein bridges" were found, as is also case with UHT cream. Whereas the casein particles in the fresh coagulum ferment, by a process of simple adsorption, a three dimensional reticulation, in UHT gels fibrous "casein bridges" have been observed between the micelles and the fat globules. Before gelation, the aggregates formed from fat and casein particles produced the "sandy" taste (Hostettler and Imhof, 1963) the structural deteriorations observed mostly account for the rheological behaviour of UHT milk following processing and during storage. Immediately after processing, the viscosity of the milk increases by approximately 0.1 to 0.2 mP (cp) as a result of homogenization and heating (Blanc et al., 1980). The change in viscosity during storage depends mainly on the temperature.

Table 3.1

Physical and chemical changes of UHT treated milk

Property	Changes
Physical	
Stability to ethanol	Decrease
Stability to calcium ions	Decrease
Rennet coagulation time	Increase
Aggregation of proteins	Increase
Non-sedimentable casein	Increase
Ca/N ratio of the sediment	Increase
P/N ratio of the sediment	Increase
Viscosity	Increase
Chemical	
pH	Un-change
Millard-type reactions (Browning)	Increase
Sulphydryl groups	Decrease
Protein breakdown	Decrease
Casein-Nitrogen	Increase
Non-casein-Nitrogen	Increase
Non-protein-Nitrogen	Increase
N-acetyl neuraminic acid	Increase
Dephosphorylation (Pi)	Un-change

(Source. Samel et al., 1971)

2.11. Quality Problems of UHT treated milk

These are in 2 groups, that of during production and post-production problem Problems during production are not specific and can be minimized by proper maintenance, but post-production problems are usually more varied and more numerous may be assed in 3 groups

2.11.1. Microbial problems

Micro-organisms occurring in UHT treated milk will almost invariably create some type of spoilage and if they are present will either be survivors of the heat treatment or will have gained entry to the product subsequent to heat treatment. Although there is a little evident of survival of spores, in commercial practice surviving spores appear to be a fairly rare case of spoilage.

Faults due to microorganisms may show up as "blowing", which is the development of gas or by coagulation and proteolysis, or by bitterness and off flavours. In majority of cases, the organism has originated in the packaging system or subsequently due to faulty sealing or by extensive damage to the pack.

2.11.2. Physical problems

These include leakage of product due to faulty sealing or subsequent damage .If the homogenization has not been fully efficient and coalescence and aggregation occurs, the rise in fat globules may be excessive and the aggregated fat partly churned, giving the product and unsightly appearance. Other physical characteristics, which may develop on storage, are appearance of sediment from the milk constituents and thickening or gelation often accompanied by bitterness and even digestion of the coagulum. It must be stressed that these defects when they occur in the absence of any microorganism are not microbial, at least directly, in origin.

Earlier discussion was that the gelation occurs as result of physico-chemical process but it now appears that it is enzymatic due either to survival of a natural heat resistant proteiolytic enzyme in the milk.

2.11.3. Chemical or flavour problem

Due to higher temperature employed UHT treated milk is having a slightly cooked flavour, immediately after production and it possesses a strong cabbage flavour due to sulphydryls. However this flavour disappears in few days During storage milk will gradually develop flavours due to oxidation of the fat, generally, development of these

flavours are accelerated due to higher storage temperatures and hence delayed at lower temperatures. The catalogue shows the flavour and odour defects from different sources.

Table 3.2

Post-production quality problems

Cause	Description
Heat induced	Cooked (cabbagey), Heated, Caramelized or Scorched
Light induced	Light, Sun light, or Activated
Lipolyzed	Rancid, Butyric, Bitter, or Goat's milk
Microbial	Acid, Bitter, Fruity, Malted, Tainted, or Impure
Oxidized	Paper, Cardboard, Metallic, Oily, or Fishy
Transmitted	Fodder, Grass, Cowy or Byre
Miscellaneous	Astringent, Bitter, Chalky, Chemical, Flat, Foreign, Not fresh (stale) or Salty

Source (I. D. F., 1981)

This catalogue can be used as guide to identify post-production quality problems in UHT treated milk.

Although there are many kind of problems in UHT treated milk as shown in above, most of them are due to the milk protein, which is one of the major constituent of milk.

2.12. Milk proteins

The primary structure of proteins consists of polypeptide chain and amino acid residues joint together by peptide linkages, which may also be cross-linked by disulphide bridges. Amino acids contain both a weakly basic amino group, and a weakly acid carboxyl group both connected to a hydrocarbon chain, which is unique to different amino acids. The three dimensional organization of proteins or conformation, also

involve secondary, tertiary, and quaternary structures. The secondary structure refers to the spatial arrangement of amino acid residues that are near one another in the linear sequence. The tertiary structure refers to the spatial arrangement of amino acid residues that are far apart in the linear sequence, giving rise to further coiling and folding. If protein is tightly coiled and folded into a somewhat spherical shape, it is called a globular protein. If the protein consist of long polypeptide chains, which are intermolecularly linked, they are called fibrous proteins. Quaternary structure occurs when proteins with two or more polypeptide chains are associated (Dalgleish, 1999).

Milk contains several proteins including casein, lactalbiumin, lactoglobiulin and serum albumin and immunoglobulins. Of these, the first three are derived from blood. The levels of serum protein and immunoglobulins in normal milk are low, although high levels are found in the first milk, colostrum.

2.12.1. Milk protein fractionation

The nitrogen content of milk is distributed among caseins (76%), whey proteins (18%), and non-protein nitrogen (NPN) (6%). This does not include the minor proteins, which are associated with the FGM. Precipitation at pH 4.6 separates caseins from whey protein, while sodium acetate and acetic acid (pH 5.0) separate total proteins from whey NPN.

Table 3.3

The protein concentration of milk

	g/l	% of total protein
Total protein	33	100
Total caseins	26	79.5
Alpha casein s_1	10	30.6
Alpha casein s_2	2.6	8.0
Beta casein	9.3	28.4
Kappa-casein	3.3	10.1
Total whey proteins	6.3	19.3
Alpha lactalbumin	1.2	3.7
Beta lactoglobulin	3.2	9.8
BSA	0.4	1.2
Immunoglobulins	0.7	2.1
Proteose peptone	0.8	2.4

Source (Dalgleish, 1999)

2.12.2. The Caseins

The casein content of milk represents about 80% of milk proteins. The principal casein fraction are α-(s_1) and α-(s_2) caseins, β-casein, κ-casein the distinguishing property of all caseins is their low solubility at pH 4.6. The common compositional factor is that caseins are conjugated proteins, most with phosphate groups esterified to serine residues these phosphate groups are important to the structure of the casein micelle Calcium binding by the individual caseins is proportional to the phosphate content.

The conformation of casein is much like that of denatured globular proteins. The higher number of proline residues in caseins causes particular bending of the protein chain and inhibits the formation of close-packed, ordered secondary structures As well, the lack of tertiary structure accounts for the stability of caseins against heat denaturation because there is very little to unfold. Without a tertiary structure there is

considerable exposure of hydrophobic residues. This results in strong association reaction of caseins and rendered them insoluble in water (Dalgleish, 1999). Within the group of caseins, there are several distinguishing features based on their charge distribution and sensitivity to calcium precipitation:

Alpha (s_1)-casein:

Two hydrophobic regions, containing all the proline residues, separated by a polar region, which contain all but one of eight phosphate groups. It can be precipitated at very low levels of calcium.

Alpha (s_2)-casein:

Concentrated negative charges near N-terminus and positive charges near C-terminus. It can be precipitated at very low levels of calcium.

Beta-casein:

Highly charged N-terminal regions and a hydrophobic C-terminal region are present. Very amphiphilic protein acts like a detergent molecule. Self-association is temperature defended and forms a large polymer at 20°C but not at 4°C. Less sensitive to calcium precipitation (Dalgleish, 1999).

Kappa-casein:

Very resistant to calcium precipitation, stabilizing other caseins. Rennet cleavage at the Phe105-Met106 bond eliminates the stabilizing ability, leaving a hydrophobic portion, para-kappa-casein, and hydrophobic portion called kappa-casein glycomacropeptide (GMP), or more accurately, *caseinmacropeptide* (CMP) (Dalgleish, 1999).

2.12.3. Structure of casein micelle

Almost casein proteins are exist in a colloidal particle known as the casein micelle. Micelle contains calcium, phosphate, citrate, minor ions, lipase and plasmin

enzymes and entrapped milk serum. The casein sub-micelle model has been introduced to describe the properties of casein. But there is no universal acceptance for this model,

In the sub-micelle model, it is through out that there are small aggregates of whole casein, containing 10 to 100 casein molecules, called *submicelles*. There are two different kinds of sub-micelles; with and without kappa-casein. These sub-micelles contain a hydrophobic core and a hydrophilic coat, which is at least partly comprised of the polar moieties of kappa-casein. The hydrophilic CMP of the kappa-casein exists as a flexible hair.

The colloidal calcium phosphate acts as cement between the hundred and even thousands of submicelles that from the casein micelle. Binding may be covalent or electrostatic. Submicelles rich in kappa-casein occupy a surface position, whereas that with less are buried in the interior. The resulting hairy layr, at least 7nm thick, acts to prohibit further aggregation of sub micelles by steric repulsion. the casein micelles are not static; there are three dynamic equilibria between the micelle and its surroundings: That are the free casein molecules and submicelles, the free submicelle and micelles, and the dissolve colloidal calcium and phosphate(Dalgleish, 1999).

2.12.4. Factors effecting casein micelle stability

Ca^{++}

More than 90% of the calcium content of skim milk is associated in some ways or another with the casein micelle. The removal of calcium ions leads to reversible dissociation of beta-casein with out micelluar disintegration. The addition of Ca^{++} leads to aggregation.

H-bonds

Some occurs between the individual caseins in the micelle but not much because there is no secondary structure in casein proteins.

Disulphide bonds

Alpha (s1) and beta caseins do not have any cysteine residues. If any S-S bonds occur within the micelle, they are not the driving forces for stabilization.

Hydrophobic interactions.

Caseins are among the most hydrophobic proteins and there is some evidence to suggest they play a role in the stability of the micelle. It must be remember that hydrophobic interactions are very temperature sensitive.

Electostatic interactions

Some of the sub unit interactions may be the results of ionic bonding, but the overall micellar structure is very loose and open.

Van der waals forces

No success in resulting these forces to micellar stability.

Steric stabilization

As already noted, the hairy layer interferes with inter particle approach.

Salt content

Effect the Calcium activity in the serum and calcium phosphate content of the micelles

pH

Lowering the pH leads to dissolution of calcium phosphate until, at isoelectric point (pH 4.6), all phosphate is dissolved and the casein precipitate.

Temperature

At 4°C, beta-casein begins to dissociate from the micelle at 0°C, there is no micellar aggregation; freezing produces a precipitate called cryo-casein.

Heat treatment

Whey proteins become adsorbed, altering the behaviour of the micelle

Dehydration

Leads to aggregation of the micelles.

Source: (Dalgleish, 1999)

2.12.5. Casein micelle aggregation

Caseins are able to aggregate if the surface of the micelle is reactive. Although casein micelle is fairly stable, there four major ways in which aggregation can be induced.

1. chymosin
2. acid
3. heat
4. age gelation

Enzymatic coagulation

Chymosin, or rennet, is most often used for enzyme coagulation. During primary stage, rennet cleaves the Phe (105)-Met (106) linkage of kappa-casein resulting in the formation of the soluble CMP which diffuses away from the micelle and para-kappa-casein, a distinctly hydrophobic peptide that remains on the micelle. The patch or reactive site, which is left on the micelles after enzymatic cleavage, is necessary before aggregation of the para-casein micelles can begin.

During the secondary stage, the micelles aggregate. This is due to the loss of stearic repulsion of the kappa-casein as well as the loss of electrostatic repulsion due to the decrease in pH. As the pH approaches its isoelectric point (pH 4.6), caseins aggregate. The casein micelles also have a strong tendency to aggregate because of hydrophobic interactions. Calcium assists coagulation is very important to both the primary stage and secondary stages. With an increase in temperature up to 40°C, the rate of the rennet reaction increases. During the secondary stage, increased temperatures increase the hydrophobic reaction. The tertiary stage of coagulation involves the rearrangement of micelles after a gel has formed. There is a loss of para-casein identity as the milk curd firms and syneresis begins (Dalgleish, 1999)

Acid coagulation

Acidification causes the casein micelles to destabilize or aggregate by decreasing their electric charge to that of the iso-electric point. At the same time, the acidity of the medium increases the solubility of minerals. So that organic calcium and phosphorus contained in the micelle gradually become soluble in the aqueous phase. Casein micelles disintegrate and casein precipitates. Aggregation occurs as a result of entropically driven hydrophobic interactions (Dalgleish, 1999).

Heat induced coagulation

The heat-induced coagulation of milk is one of the few major classical problems in the dairy industry. Thus heat coagulation on the other hand, is the result of a complex series of simultaneous reactions some of which are probably complementary while at least some antagononistic reactions also occur. It is likely that these heat-induced changes will attack increasing attention in an effort to identify modifications to the caseinate system likely to lead to coagulation. At temperatures above the boiling point casein micelles will irreversibly aggregate (Fox, 1986). On heating the buffer capacity of milk salt change, carbon dioxide is released, organic acids are produced, and try calcium phosphate and casein phosphate may be precipitated with the release of hydrogen ions. These are due to decrease in pH, precipitation of calcium phosphate, denaturation of whey proteins and interaction with casein, Millard browning, modification of casein, (dephosphorilation, hydrolysis of kappa-casein, general hydrolysis,) changes in micelle structure (Dalgleish, 1999) (zeta-potential, hydrogen changes, association-dissociation).

Age gelation

Age gelation is an aggregation phenomenon that effect shelf stable dairy products such as UHT treated milk and concentrated milk. After weeks to months of storage there is a sudden sharp increase in viscosity accompanied by visible gelation and irreversible aggregation of micelles into long chains forming a three-dimensional network. The problem of gelation in UHT treated milk is less critical than with UHT treated concentrated milk. However, there are many factors, which are having negative and positive influences for the problem (Dalgleish, 1999)

1. Processing conditions

   Fore warming, time and temperature of sterilisation, direct or indirect UHT treatment, sequence of operation

2. Composition of milk

   Total solids, mineral balance, protein composition, and seasonal variation

3. Quality of milk
4. Additives

Mono-and di-sodium phosphate, citrate, carbonate, polyphosphate, polyhydric alcohols, minerals,oxidizing or reducing agents, H_2O_2, antioxidants, phosphatides, sulphydral reagents.

5. Temperature of storage.

Source (Harwlkar and Fox, 1986).

Although the actual case and mechanism is not yet clear, However some theories are exist:

Proteiolytic breakdown of casein:

Bacterial or native plasmin enzymes that are resistant to heat treatment may lead the formation of a gel.

Chemical reactions:

Polymerization of casein and whey proteins due to Millard type or other chemical reactions.

Formation of kappa–casein–beta–lactoglobulin complexes.

Source (Dalgleish, 1999)

There are many other possible causes, but that are not directly effected to problem may have some indirect relationships, that are

Dissociation or binding of Calcium ions.

Conformational changes of casein molecules.

General break down of micelle structure.

Interactions between beta-lactoglobulin and kappa–casein

S-S Exchange reactions.

pH change

Dephophorilation of the casein

Interactions between kappa–casein and carbohydrates.

Survival of bacterial spores.

Source (I. D. F., 1981)

Protease Hypothesis.

Storage gelation at UHT sterilized milk is accompanied by an increased level of products and by decreased stability to calcium ions (Samel et al, 1971). These changes, also characteristic of rennet coagulation of milk, have led many investigators (Snoeren et al, 1979) to suggest that the gelation of aged UHT milk takes place by a mechanism analogous to that of rennet coagulation in which an enzymetiggened primary protein degradation step is followed by a secondary aggregation reaction resulting in gelation. On the basis of extensive theoretical treatment Payens (1978) considers that the phenomenon of the initial-thinning, followed by an explosive growth of the average particle weight, results from the action of a clotting protease and rules out non enzymatic coagulation would be accompanied by a linear increase in weight-average molecular weight with time. Although proteases have been isolated from some UHT sterilized milk (Corradini and Pecis, 1979), it has not been possible to demonstrate their presence in other sterilized products.

Physico-Chemical hypothesis

The protease hypothesis has wide appeal because it provides readily the 'cause' and 'effect' aspects of gelation but the failure to detect a protease and to correlate the rate and extend of proteolysis to gelation in some sterilized milk products has led to the suggestion that gelation results from a modification of the surface properties of casein micelles by non-enzymatic physico-chemical processes (Hostettler, 1972), observed that extensive proteolytic breakdown accompanies the relation of UHT milk but were unable to observe such proteolysis during the gelation of UHT treated concentrated milk (Carroll et al, 1971), They suggest that concentrated product was affected by the nature and extent of heat induced complex formation between the whey proteins and caseins. In milk subjected to a severe heat treatment.

A chemical modification of micelles by Millard type (Andrews, 1975) reactions or by disulphide bonding (Green and Morant, 1981) has been implicated in the mechanism of gelation. Andrews and Cheeseman (1975) suggest that the gelation of UHT milk is linked with the polymerization of casein and whey proteins through Millard type reaction which are promoted as the temperature of storage is increased. However the failure to observe the gelation during storage of UHT milk at temperature above 35C is not consistent with their suggestion. Modification of the surface properties of the casein micelles may also be cased by partial dissociation of the micelles during storage. This process of dissociation or "Shedding" could expose regions on the surface of casein micelles that promote interaction between casein micelles however the forces that lead to the shedding of sub-units are not known.

Face energy hypothesis

Another hypothesis of this regard is, changes in face energy (Graf and Bauer, 1976). The micelles in UHT milk are perceived as being in a meta stable state with high potential during storage, a progressive, spontaneous transformation of the micelles from a "high" potential to move stable micelles with lower surface potential occurs by random potential difference which promotes aggregation of the micelles, depending upon the probability of contacts and the number of low-potential micelles; both these increase with time. Initially the aggregation is insignificant but when the average surface potential of the micelles attain a critical level, aggregation becomes evident as a pronounced increased in viscosity (Graf and Bauer, 1976) the stabilizing influence of an intense heat treatment and of additives are believed to result from a condition of the micelles in which changes in the surface potential and racemization of the micelles are reduced. The factors that cause stabilization against gelation are believed to enhance such changes However such a hypothesis based on potential energy changes is highly speculative, but no much merits still.

2.12.6. Microbiological aspect of casein degradation at storage

The survival of heat-stable enzymes is determined not only by the severity of heat treatment but also by the nature and quantity of the enzymes present Poor qua ty

milk, which contains increased levels of proteases of bacterial origin, shows extensive degradation of protein and a short gelation time during storage (Snoeren et al., 1979, Driessen and Van der waals, 1978). There is wide variation in the heat stability of proteiolytic enzymes of bacterial origin. Whereas the milk's natural lipases are destroyed by heating, the microbial lipases are not rendered completely inactivated by this process and bacterial proteases are obviously much more resistant to heat than lipases from the same source.

Numerous studies have indicated that the proteases of microbiological origin survive UHT treatment (law et al, 1977). The action of bacterial proteases on individual proteases from that of indigenous proteases in contrast to indigenous milk protease, bacterial proteases appear to degrade kappa-casein (Law et al, 1977) preferentially. The bacterial and milk proteases influence the storage behaviour of UTH products to different extents because they differ in their mode of attack on individual proteins. An observation shows that, a sample of milk that was deliberately contaminated with psychrotropic bacteria before UTH treatment gelled on storage sooner than aseptically drown milk.

Table 3.4

Heat resistance of proteases and lipases from psychrotropic bacteria

Reference	Microorganism	T(C)	D (min)	Z(C)	Incomplete Inactivation
Proteases					
BNGTSSON et al. (1973)	*Pseudomonas*	120	4	20	150 C, 2.4 s
MAYERHOFER et .al.	*P. flourescens*	149	1.5	32.5	
(1973)	*Pseudomonas*	150	1.7	32	
ADEMS et al. (1975)	*Pseudomonas*	149	0.4		
KISHONTI (1975)	*Pseudomonas*	150	0.5	32.5	
BARACH et al. (1976 a)	*Pseudomonas*	130	11	34.5	
BARACH et al. (1976 b)	*P. flourescens*				
DRIESSEN (1976)	*P. flourescens*				132 C, 7 min
MALIK (1976)	*P. flourescens*	150	27	28	
ANDREWS et al. (1977)	*P. flourescens*				140 C, 0.05 min
LAW et al. (1977)	*B. cerus*	150	0.016		
NIROUMAND (1977)					
Lipases					
DIRESSEN et al. (1973)	*P. flourescens*	130	16		
KISHONTI (1975)	*Pseudomonas*	150	1.7	25	
HEDLUND (1976)	*Pseudomonas*	160	1.25	37	
	Micrococcus	160	1	63	

Source (I. D. F., 1981)

T-temperature
D- decimal reduction time
Z- temperature coefficient

2.12.7. Whey proteins

The proteins appearing in the supernatant of milk after precipitation at pH 4.6 are collectively called whey proteins. These globular proteins are more water-soluble than caseins and are subject to heat denaturation. Native whey proteins have good gelling and whipping properties. Denaturation increases their water holding capacity. The principle fractions are beta-lactoglobulins, alpha- lactalbumins, bovine serum albumin (BSA), and immunoglobulin (Ig) (Dalgleish, 1999).

Beta-lactaglobulin

This group (MW-18000 Da), including eight genetic variations, comprises approximately half the total whey proteins. It has two internal disulfide bonds and one free thyol group. The conformation includes considerable secondary structures and exists naturally as a non-covalent dimmer. At the isoelectric point (pH 2.5-5.2), the dimmers are further associated to octamers but pH below 3.4, they are dissociate to monomers (Dalgleish, 1999).

Alpha-lactalbumin

These proteins (MW-1400 Da) contain eight cysteine groups, all involved in internal disulfide bonds, and for tryptophan residues. It a highly ordered secondary structure, and a compact, spherical tertiary structure (Dalgleish, 1999).

Enzymes

Enzymes are a group of protein that has the ability to catalyze the chemical reactions and the speed of such reactions. The action of enzyme is very specific Milk contains both indigenous and exogenous enzymes. Exogenous enzyme mainly consists of heat-stable enzymes produce by psychrotrophic bacteria lipases and proteases Indigenous enzymes are plasmins some of them are very heat stable while others fairly stable (Dalgleish, 1999).

Indigenous enzymes

There are many indigenous enzymes that have been isolated from milk. The most significant group is the hydrolases as lipoprotein lipase, plasmin, alkaline phosphatase (Dalgleish, 1999).

Lipoprotein lipase (LPL)

A lipase enzyme splits fats into glycerol and free fatty acids. This enzyme is found mainly in the plasma association with casein micelles. The milk fat is protected from its action by the FGM. If the FGM has been damage, or certain co-factors (blood serum lipo-proteins) are present, the LPL is able to attack the lipo-proteins of the FGM. Lipolysis may be cased in this way (Dalgleish, 1999).

Plasmin

Plasmin is a proteolytic enzyme; it splits proteins. Plasmin attacks both beta casein and alpha (s2)-casein. It is very heat stable and responsible for the development of bitterness in pasteurised milk and UHT processed milk it may also play a role in the ripening and flavour development of certain cheeses (Dalgleish, 1999).

Alkaline phosphatase

Phosphatase enzymes are able to split specific phosphoric acid esters into phosphoric acid and related alcohols. Unlike most enzymes, it has a pH and temperature optima differing from physiological values; pH of 9.8. The enzyme is destroyed by minimum pasteurisation temperatures (Dalgleish, 1999).

Exogenous enzymes

There are many exogenous enzymes in milk of bacterial origin Most of them are heat resistant lipases and proteases excrete by psychrotrophic bacteria, which grow in storage conditions.

3. MATERIALS AND METHODS

3.1. Materials

Samples

Coagulated milk samples (UHT treated) of one liter packs and normal milk (UHT treated) samples were obtained from TROPIFRÜT limited, Pirivena Road, Molligoda, Wadduwa, Sri Lanka. Normal raw milk was obtained from University farm shop, Faculty of Agriculture, University of Peradeniya, Peradeniya, Sri Lanka. UHT treated milk cartons were stored at room temperature and raw milk samples were collected freshly and were stored under refrigerated conditions untill use.

Chemicals and reagents

Bovine heamoglobin was purchased from Sigma Chemicals (St. Louis, MO 63178, U.S.A), Tris-HCl, Sodium dodecyl sulfate, Acrylamide, Ammonium persulfate, tetramethylethylenediamide were from Bio-Rad (U.S.A) and all other reagents used from British Drug House, Pool, England and Sigma Chemicals U.S.A were of analytical grade.

Apparatus

Vertical slab mini gel electrophoresis system (Mini-PROTEIN® Bio-Rad, Hercules, Califomia, 94547, U.S.A), UV. Visible spectrophotometer, 1ml quarts cuvette (Shimadzu, Japan), 20µl to 1ml micro pipettes, 1.5ml micro centrifuge tubes (Pharmacia, Sweden), Vortex Mixer (Barnstead | Thermolyne, Iowa, U.S.A), Dialysis tube (Pharmacia, Sweden), Centrifuge (capacity about 100ml), Refrigerated micro centrifuge with high speed (up to 15,000 rpm) (Pharmacia, Sweden), Water bath (Griffin and George, U K).

3.2. Preparation of samples

Coagulated sample was shaken well before opening. Samples were taken out from the package under sterile condition and mixed well to homogenize. Samples were centrifuged for ten minutes at 8,000rpm to separate the supernatant and coagulant.

Samples were graded as:

A - Supernatant (UHT spoiled)
B - Coagulant (UHT spoiled)
C - Whole sample (UHT spoiled)
D - Control (UHT normal)
E - Raw milk (Fresh cow milk)
F - Standard (Molecular markers)

3.3. Preparation of stock solutions

Preparation of Tris-buffer (1.5M Tris pH 8.8)

181.71g of Tris-HCl was dissolved in 950ml of distilled water, pH was rearranged into 8.8 by adding conc. HCl drop wise, volume was made up to 1 liter with distilled water.

Preparation of Tris-buffer (0.5M Tris pH 6.8)

60.57g of Tris/HCl was dissolved in 950ml of distilled water, pH was rearranged into 6.8 by adding conc. HCl drop wise, Volume was made up to 1 litter with distilled water.

Preparation of Sodium dodecyl sulphate (SDS) solution

10g of SDS was dissolved in 95ml of distilled water, volume was made up to 100ml with distilled water.

Sodium dodecyl sulphate (SDS) contains
Dodecyl sodium sulphate, Sodium lauryl sulphate, Lauryl sulphate

Preparation of Acrylamide solution (30%)

Acrylamide monomer 29.8g and bis-acrylamide (N,N' methylene bis-acrylamide) 0.8g were dissolved in 100ml of distilled water and stored at 4 °C cooled atmosphere.

Preparation of Ammonium persulfate (10%)

One gram of Ammonium persulfate was dissolved in 10ml of distilled water and stored at 4 °C.

Preparation of sample buffer (SDS-PAGE)

0.5M Tris-HCl pH 6.8 1ml, 10% SDS 2ml, glycerol 1ml with Bromophenol blue 20mg and 2-Mercapto ethanol 0.5ml was added into 5ml of distilled water, vortexed thoroughly, volume made up to ten milliliter and was vortexed well.

Preparation of sample buffer (Native-PAGE)

One milliliter of glycerol and 0.1mg of Bromophenol blue, volume made up to 2ml with 0.5M Tris-HCl pH 6.8 buffer.

Preparation of Electrode buffer (Native-PAGE)

7.206 gram of Glycine (0.192M) and 1.51g of Tris (0.25M) was dissolved in distilled water and volume made up to 500ml to obtain the electrode buffer.

Preparation of Electrode buffer (SDS-PAGE)

The weight of7.206 gram of Glycine (0.192M) and 1.51g of Tris (0.25M) and 5ml of 10% SDS solution was dissolved in distilled water and volume made up to 500ml to obtain the electrode buffer.

Preparation of Coomassie Blue stain solution

One gram of Coomassie blue was dissolved in 40ml of Methanol and 10ml of Acetic acid. Volume made up to 100ml with distilled water, stirred under magnetic stirrer and was filtered.

Preparation of Amido Black stain solution

0.2 gram of Amido black (Sigma, A 8181) was dissolved in 14ml of Glacial acetic acid and volume made up to 200ml with distilled water, was stirred under magnetic stirrer.

Preparation of de-staining solution (SDS-PAGE)

To a 400ml of methanol add 100ml of glacial acetic acid and made it up to 1 liter with distilled water.

Preparation of de-staining solution (Native-PAGE)

Seventy milliliter of glacial acetic acid, volume up to 1000ml with distilled water.

Preparation of haemoglobin substrate solution for Native-PAGE (1% Hb, pH 3.5 - 4.5)

One g of bovine haemoglobin dissolved in 100 ml of distilled water and mixed it under magnetic stirrer for about 1hour. pH was checked by using a pH paper (pH 3 - 6) and pH was adjusted by adding conc. HCl drops.

3.4. Preparation of haemoglobin for measurement of Acid protease activity

Twenty-one g of Bovine haemoglobin (Sigma, H-2500) was dissolved in 300ml of distilled water (7.5%) and mixed for about four hours under magnetic stirrer. Added into a dialyze bag (8 Mr) length of 40" and dialyzed extensively against distilled water to obtain denatured haemoglobin (six times in three days at 4 °C). The volume made up to 900ml with distilled water (2.5% haemoglobin). Approximately 2% denatured haemoglobin was obtained by centrifuging the mixture at 7,700rpm for 1hour at 4 °C Supernatant (as the substrate for acid proteases) was kept frozen in aliquots until use

3.5. Preparation of casein for measurement of Neutral protease activity

Five g of casein (BDH 44016 4R) was dissolved in 500ml of Tris buffer (pH 8.0) under magnetic stirrer and was incubated in a water bath for 1hour at 85 °C to 95 °C. Solution was filtered through filter paper and diluted with distilled water to obtain a 1% casein solution.

3.6. Preparation of 2.5% haemoglobin solution series

Perprepared 7.5% haemoglobin solution was diluted with 0.1M HCl and 0.1M NaCl to have pH series of haemoglobin solution. After mixing of different aliquots pH was measured accurately and pH was adjusted when deviated from needed value.

Table 3.1

2.5% haemoglobin solution series

0.1M HCl	0.1NaCl	Resulted pH
0.80	31.20	6.08
1.28	30.72	5.55
1.76	30.24	5.01
2.40	29.60	4.59
4.00	28.00	4.00
5.92	26.08	3.46
7.04	24.96	2.95
8.32	23.68	2.52
11.20	20.80	2.00

3.7. Determination of Microbial activity

3.7.1. Methylene blue dye reduction time test

Methylene blue tablet was dissolved in 90ml of distilled water under magnetic stirrer and volume made up of to 100ml. 10ml each of two aliquots from coagulated milk sample (C) and another two aliquots from normal UHT milk sample (D) were taken out. One ml of methylene blue aliquots was added to all samples. Reaction mixture was vortexed and incubated at 37 °C for 6 hours. After 10 minuets and at hourly intervals was checked the samples for the visual changes.

3.7.2. Resazurin test

Resazurin tablet was dissolved in 40ml of distilled water under magnetic stirrer and volume made up of to 50ml. 10ml two aliquots from coagulated milk sample (C) and another two aliquots from normal UHT milk sample (D) and 10ml of two aliquots of raw milk samples were taken out. One ml of Rezasurin aliquots was added to all samples. Reaction mixture was vortexed and incubated at 37 °C for 6 hours. After 10 minuets and every 2 hours interval was checked for the visual changes.

3.8. Determination of Enzyme activity

3.8.1. Determination of lipase activity

Two aliquots (5ml) from coagulated milk sample(C) and similarly another two aliquots from normal UHT milk sample (D) were taken out. Two drops of phenol red were added to all samples. 0.05M Sodium carbonate was added drop by drop until the phenol red is just red. Reaction mixture was vortexed and incubated at 37 °C for10 minuets After 10 minuets samples were checked for the visual changes

3.8.2. Determination of Urease activity

1.3g of urea (Sigma, U 5128) ($(NH_2)_2$ CO) was dissolved in 240ml of distilled water under magnetic stirrer, and volume made up to 250ml. Two aliquots (5ml) from coagulated milk sample(C) and similarly another two aliquots (5ml) from normal UHT milk sample (D) were taken out. Five ml of urea was added to all samples. Three drops of phenolphthalein was added. Reaction mixture was vortexed and incubated at 40 °C for10 minuets. After 10 minuets samples were checked for the visual changes.

3.8.3. Determination of acid protease activity

Acid protease activity was determined using the method of Anson with modifications (Anson, 1939) i.e., 100µl of milk sample (A, B, C, D) were added into the Eppendorf tubes (T: test) and mixed with 400µl of substrate solution (2% denatured haemoglobin at different pH values). Reaction mixture was vortexed and incubated at37 °C for 2 hours. The reaction was terminated by addition of 800µl of 5% (w/v) trichloroacetic acid (TCA) followed by mixing. The mixture allowed to stand for at least 10 minutes at room temperature, was centrifuged at 12,000rpm for twenty minutes, and the optical density of the supernatant at 280nm was measured. A blank (B) tube was prepared by adding the milk sample to the above reaction mixture after the trichloroacetic acid precipitation. All assays were performed with tests and blanks in duplicate. Activity was expressed as the difference between OD 280 values of T and B, (T-B)/1hour of incubation period/ml of enzyme solution. One unit (U) of proteolytic activity was defined as the increase in absorbence at 1.0 at 280nm per hour per milliliter of enzyme solution.

$$\text{Activity} = \frac{\text{T-B} * 1000}{100 * 2} \text{ U/ml}$$

(When 100ml of milk sample is used for 2 hours incubation period)

3.8.4. Determination of neutral protease activity

Neutral protease activity was determined using the method of Anson with modifications (Anson, 1939) i.e., 100µl of milk sample (A,B,C,D) were added into the Eppendorf tubes (T: test) and mixed with 400µl of substrate solution (1% denatured casein in Tris buffer at pH 8.0). Reaction mixture was vortexed and incubated at37 °C for 4 hours. The reaction was terminated by addition of 800µl of 5% (w/v) trichloroacetic acid (TCA) followed by mixing. The mixture allowed to stand for at least 10 minutes at room temperature and was centrifuged at 12,000rpm for twenty minutes, and the optical density of the supernatant at 280nm was measured. A blank (B) tube was prepared by adding the milk sample to the above reaction mixture after the trichloroacetic acid precipitation. All assays were performed with tests and blanks in duplicate. Activity was expressed as the difference between OD 280 values of T and B, activity was measured as the acid protease activity.

3.8.5. Poly acrylamide gel electrophoresis under non-denaturing conditions

Electrophoresis conducted on 12% gel in the absence of SDS to identify the protease activity on the gel itself, according to the method of Furihata, et al. (1972). Stacking gel was prepared in the same way as described above in the absence of SDS.

Samples were prepared by mixing 150 of sample and 50 of sample buffer (sample A was specially prepared 5:1 dilution with sample buffer (similar to SDS sample buffer, except that it did not contain SDS) and applied to the wells. A 10mA current was applied for stacking gel, 20mA for the resolving gel and performed the experiment at 4°C. Electrode buffer prepared freshly without adding SDS. Electrophoresis was followed by dipping the gel in 1% haemoglobin at pH 3.5 - 4.5 for 15 minutes in a shaker at room temperature and incubation followed for 4, 8, 12 hours at 37°C in a humid chamber after discarding haemoglobin. Area of reaction was taken place was visualized using 1% staining of Amido black 10B in 7% acetic acid

Table 3.3
Components of the 12% gel for Non- denaturing-PAGE

Component	Volume/resolving gel	Volume/stacking gel
Distilled water	3.35ml	3.05ml
1.5M Tris/HCl pH 8.8	2.5ml	-
0.5M Tris/HCl pH 6.8	-	1.25ml
Acrylamide (30%)/ Bis-acrylamide (0.8%)	4.0ml	650µl
10%Ammonium persulfate	50µl	25µl
Tetramethyl ethylenediamide	5µl	5µl

Adapted from current protocols in molecular biology, vol 1, Ausubel, F.M.R., Brent, R.E, Kingston, D.D., Moor, J.G., Seidman, J.A., Smith and K., Struh (eds) 1991.

3.8.6. SDS-Poly acrylamide gel electrophoresis

Sodium dodecyl sulfate (SDS) Poly accrylamide gel (10% gel) electrophoresis was used (Luemmli, 1970) to check the protein banding pattern of the coagulated UHT treated, normal UHT treated and raw milk to compare the differences. Molecular weight of the protein bands was determined by SDS-PAGE according to the method described by Weber and Osborn (1969). Commercial molecular marker proteins for molecular weight determination (Sigma - U.S.A) were used as standards. 100V and 200V voltages were applied for stacking gel and resolving gel (8.5 * 6cm) respectively. Protein bands were visualized by staining with 0.1% Coomassie brilliant blue R-250.

Table 3.2

Components of the 10% gel for SDS-PAGE

Component	Volume /resolving gel	Volume/stacking gel
Distilled water	1.775ml	3.05ml
1.5M Tris/HCl pH 8.8	1.25ml	-
0.5M Tris/HCl pH 6.8	-	1.25ml
10% SDS	50µl	50µl
Acrylamide (30%)/ Bis-acrylamide (0.8%)	1.9ml	650µl
10%Ammonium persulfate	25µl	25µl
Tetramethyl ethylenediamide	2.5µl	5µl

Adapted from current protocols in molecular biology, vol 1, Ausubel, F.M.R., Brent, R.E, Kingston, D.D., Moor, J.G., Seidman, J.A., Smith and K., Struh (eds) 1991.

Samples to be applied were prepared by boiling 10µl of samples, which was diluted earlier with Tris/HCl pH 6.8 480µl with 20µl of original sample (Sample concentration was increased from 1:100 to 1:50 at later stages, sample A was specially prepared directly with sample buffer 2:1 dilution), with 30µl SDS-sample buffer for two minutes.

4. Result and discussion

4.1. Methylene blue dye reduction time test

4.1.1. Results:

No colour change was observed in sample C and D, blue colour was retained during the experiment.

4.1.2. Discussion:

Usually bacteria and somatic cells in milk change the redox potential of medium using dissolved oxygen in milk. Therefore are reducing power is developed and it can reduce the dyes such as methylene blue and Resazurin. Some enzymes such as dehydrogenase and flavine enzymes in bacteria also can reduce these dyes. These enzymes transfer Hydrogen from substrate to biological acceptors. Methylene blue is blue in colour, due to the bacterial activity the colour is reduced from blue to white. So colour change can be used as an indication of bacterial activity. The dye reduction time in the medium is inversely proportional to the bacterial count.

However, when milk leaves a healthy udder it is relatively free from bacteria, if cow was suspended for any systemic disease milk may contain high count. But after the UHT treatment all the micro-organisms were destroyed and at normal conditions there are no spores contain of, if there is no other contamination is found from the environment.

Samples from UHT treated normal (D) and UHT treated coagulated milk (C) samples showed that, colour of the methylene blue was not changed in any sample, therefore both the coagulated UHT or normal UHT treated milk confirmed the absence of bacteria, and also they are not growing in the medium.

4.2. Resazurin test

4.2.1. Result:

No colour change was observed in C and D samples during 6 hours duration. However, within 10 minutes a quick colour change was observed in the raw milk sample (E), from white to pink colour.

4.2.2. Discussion:

Resaszrin is also a dye, which can get reduced due to the action of bacteria. Bacteria change the redox-potential in milk by utilizing dissolved oxygen in the milk. Thus they can reduce the dye Resazurin. Samples of UHT treated coagulated and normal milk samples showed no colour changes while raw milk sample (E) showed a change in colour very rapidly due to the microbial activity.

The result of methylene blue test also showed similar results, the UHT treated milk was not contain bacteria, in this test it confirmed the absence of microbes in UHT treated milk.

4.3. Determination of lipase activity

4.3.1. Result:

When phenol red was added medium was initially red and appeared to pink colour when added sodium carbonate.

After the incubation there were no colour changes in the medium.

4.3.2. Discussion:

Lipases are enzymes, which hydrolyse lipids into fatty acids and glycerol due to its specific activity. The pH of the medium will change when the reaction is taken place due to the release of glycerol and fatty acids Indicator colour is depended on the pH of the medium. Thus colour change is appeared in the media if the reaction was taken place.

Due to the result no colour change was observed in the UHT treated coagulated and normal milk samples due to the inactivation of lipases during the UHT treatment.

4.4 Determination of Urease activity

4.4.1. Result:

No colour change was observed after the incubation.

4.4.2. Discussion:

Urease is an enzyme, which convert the urea to carbon dioxide and ammonia. When the reaction was taken place carbon dioxide and ammonia dissolved in the medium. Carbon dioxide is a weak acid and ammonia is a weak base. But bicarbonate ions are unstable than ammonium ions. Thus pH of the medium is increased. Due to this reaction indicator (phenolphthaline) changed its colour ranging from pH 8 to 10 when the pH is increased, the pink colour of the medium should change to colourless. But the observation showed that no colour changes in the samples C and D, that might be due to the absence of Urease enzymes in the medium.

4.5 Determination of protease activity

4.5.1.Results:

Detectable proteiolytic activity at different pHs (range of 2-8) were observed in supernatant (A) coagulant (B) and the whole sample (C) of UHT treated coagulated milk samples.

No activity was observed with the UHT treated normal milk sample in same conditions.

Activity was observed in acid proteinase assays and neutral proteinase assays. But neutral proteinase assay showed low activity in even four-hour incubation While acid proteinase assay showed higher activity

4.5.2. Discussion:

Method of Anson (1939) was used successfully to detect the presence of proteases in UHT treated coagulated milk. Use of haemoglobin as a substrate in this method is particularly useful as it gives produces that absorb at 280nm, thus giving measurable readings in an assay (Kunitz, 1947). Particular care should be taken to remove the extraneous proteins using common, the correct concentration of trichloroacetic acid will effect the final reading giving lower values due to precipitation of relatively high molecular weight products of protease action (Anson, 1937; Anson, 1939). Although positive controls should be used in assay, it was not possible to use in this assay as the type of protease or proteases present were not known.

Since the haemoglobin assay has been utilized with age-thickened milk's protease for the first time, it was necessary to optimize the assay condition for the best performance. In the preliminary assays an incubation period of two hour, pH 3.5 to 4.5 were found to be best for the reaction, the reduction in rate of reaction and the activity calculated during varying incubation periods showed that the proteases found in all samples followed a general pattern. Reduction of substrate availability in the reaction mixture, autocatalysis of proteases and accumulation of products in the reaction mixture that might be inhibitory on proteases may have contributed to this reduction.

The observation of protease activity in A, B, C, samples and absent in D sample may due to the presence of proteases originated from bacteria or native plasmin enzymes which are resistant to the UHT treatment in milk. Absence of protease activity in D sample showed that the native plasmin enzymes which are resistant to the heat has destroyed at this UHT treatment (142°C, 3-4 seconds) and that may due to the presence of bacterial proteases in the raw milk. The survival of heat-stable enzymes is determined not only by the severity of heat treatment but also by the nature and quantity of the enzymes present Poor quality milk, which contains increased levels of proteases of bacterial origin, shows extensive degradation of protein and a short gelation time during storage (Snoeren et al., 1979, Driessen and Van der waals,1978) The survival of heat-stable enzymes is determined not only by the severity of heat treatment but also by the nature and quantity of the enzymes present Poor quality milk, which contains increased levels of proteases of bacterial origin, shows extensive degradation of protein and a short gelation time during storage (Snoeren et al., 1979, Driessen and Van der waals 1978).

The determination showed that there is a wide variation in protease activity in the sample ranging from pH 2 to 8. The acid protease and neutral protease activity resulted as high amount of activity in acid protease assay and low amount of activity in neutral protease assay. This is due to the pH stability of the enzyme and it can activate in wide variation of pH in the medium. But it shows its higher activity in the range of pH 3.5 to 4.5 and less activity in extreme values.

4.6. Effect of pH on acid protease activity

4.6.1.Results:

The protease activity measured in the pH range 2-8 showed the optimum pH lie between 3.5 to 4.5 for A, B, C. The activity in the supernatant and coagulant appeared similar.

This proteases have an ability to active in wide range of pH.

The highest activity at pH 4 in whole sample.

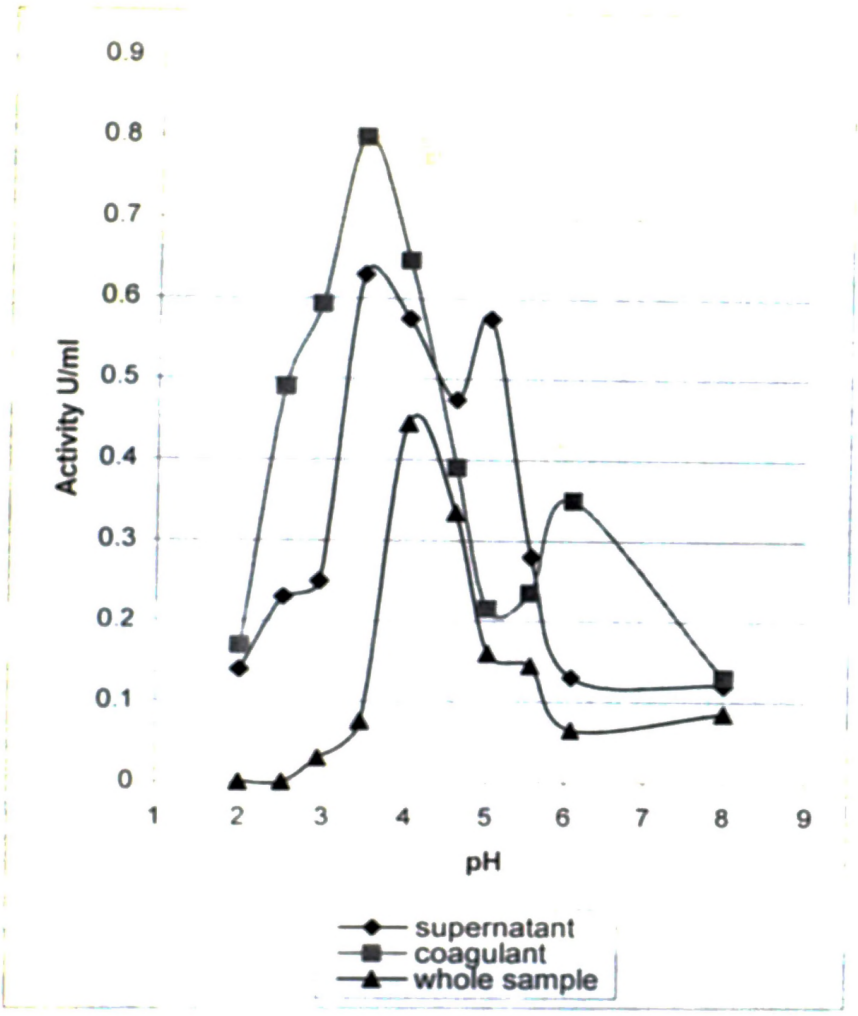

Figure 4.1

pH dependence of the protease

4.6.2. Discussion:

Haemoglobin (2%) at pH 2-6 and casein (1%) at pH 8was used with the appropriate buffers. Action of protease is found in the different pH. Therefore these proteases might be activated, when incubated or with the increasement of ambient temperature after the UHT treatment with little different changes in pH. Thus best activity of this proteases can achieved at pH 3.5 to 4.5.

4.7. PAGE-under non-denaturing conditions

4.7.1. Results:

Percentage of gel used was 12% as the bands observed on their 10%gel acid. Protease activity was observed on after activity staining as clean areas. It is at the lower part or bottom of the gel.

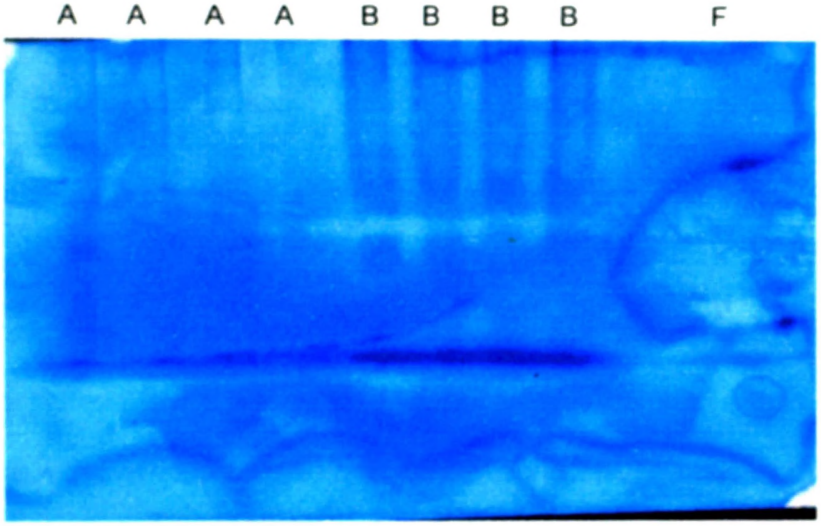

Plate 4.1
Poly acrylamide gel electrophoresis under non-denaturing conditions
(4 hours incubation)

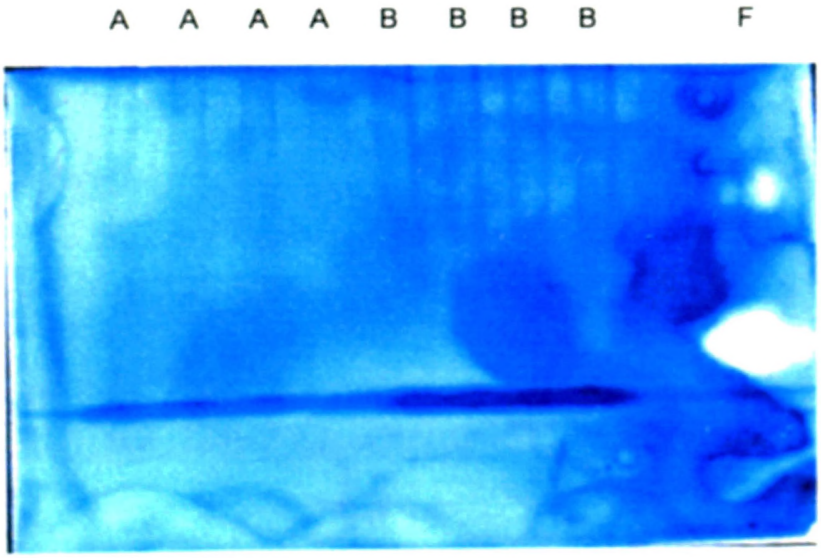

Plate 4.2
Poly acrylamide gel electrophoresis under non-denaturing conditions

(8 hours incubation)

Bands observed on PAGE under non-denaturing condition of coagulated milk samples.
A-supernatant B-coagulant F-standard

F E D D C C B B A A

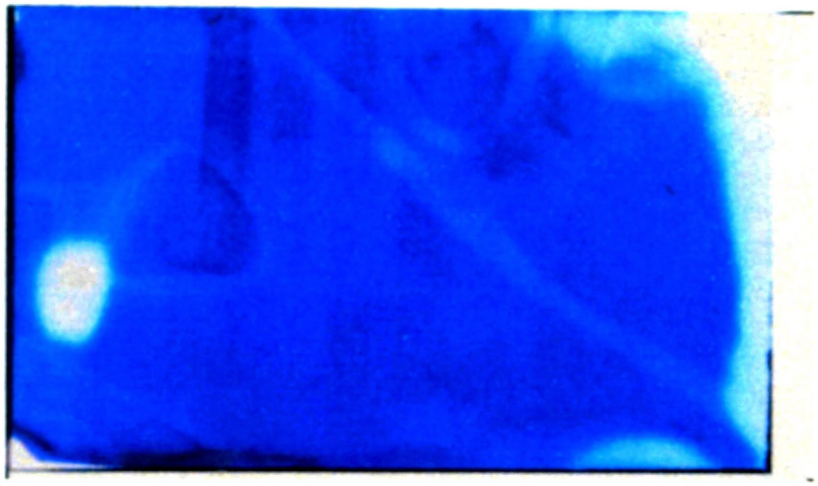

Plate 4.3

Poly acrylamide gel electrophoresis under non-denaturing conditions

(12 hours incubation)

A A B B C C D D

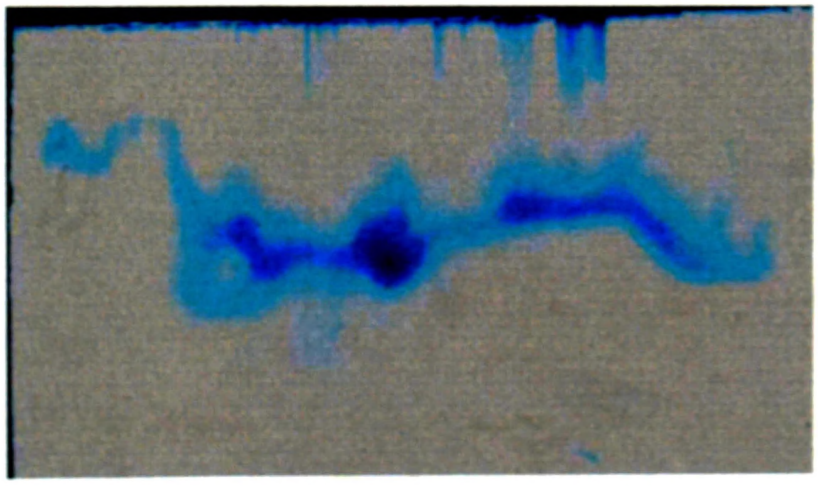

Plate 4.4

Poly acrylamide gel electrophoresis under non-denaturing conditions

(12 hours incubation)

Bands observed on PAGE under non-denaturing condition of coagulated milk samples.
A-supernatant B-coagulant C-whole sample D-control F-standard

4.7.2. Discussion:

PAGE under non-denaturing condition was performed with A, B, C, D samples with respect to A and B, to identify the bands responsible for acid proteases that are separated based on their charge and size. Activity was observed on PAGE under non-denaturing conditions, addition to the protease assay procedure. It may be a low molecular weight protease and negatively charged molecules with vary low in concentration. Both investigations showed that there were proteins, which May responsible for the storage gelation of UHT, treated milk. The survival of heat stable enzymes are determined not only of heat treatment but also by the nature and quantity of enzyme present, poor quality milk, which contains increased level of proteases of bacterial origin, shows extensive degradation of protein and short gelation time during storage (Snoeren et al., 1979; Zadow and chithula, 1975)

4.8. SDS-poly-acrylamide gel electrophoresis

4.8.1. Results:

Aliquots of A, B, C, D, E samples were subjected to SDS-PAGE, followed by coomassie brilliant blue staining to investigate the whether there were any differences in protein fractions. This was performed using 10% gel. Sample A was specially prepared to maintain a higher concentration and all samples applied in same amounts.

A, B, C, D, E showed many protein bands and some of them are thin while some of them border, two bands resulted two distinct casein bands, both bands of all samples remained at a particular level except sample B and C in the gel electrophoresis. Two bands in sample B and C was observed with low migration of casein bands than the bands of other two samples (D, E). Therefore protein bands of sample B remain above the other casein bands of the gel (figure 4.2).

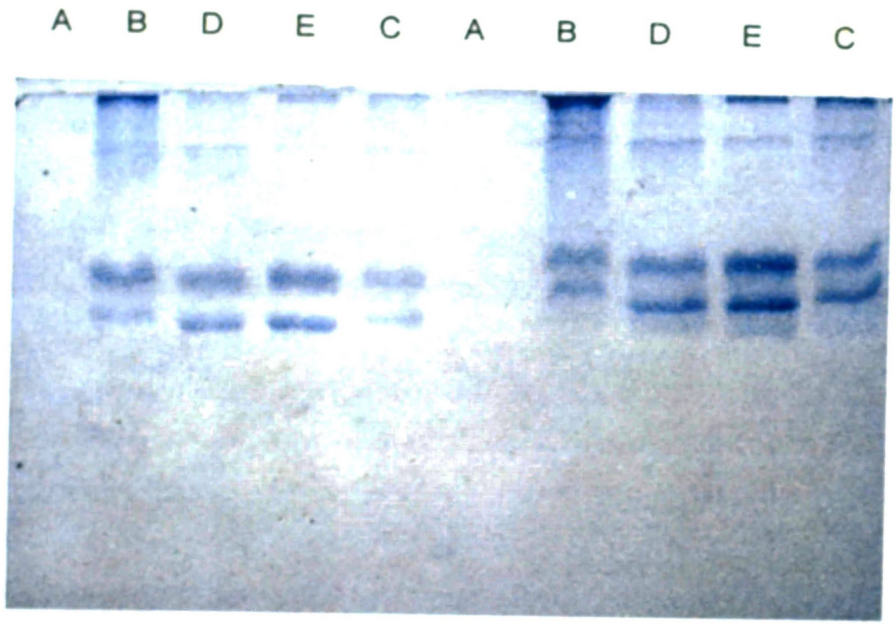

Plate 4.5

SDS-Poly acrylamide gel electrophoresis.

Protein bands observed on SDS-PAGE of milk samples with coomassie brilliant blue R-250 staining.

A-supernatant, B- coagulant, C-whole sample, D- control sample (UHT treated), E-raw milk sample.

4.8.2. Discussion:

SDS-PAGE analysis was used to see the protein band pattern of coagulated and normal UHT treated milk and to compair the differences between them whenever the gelation was taken place. Molecular weight of both bands in sample B has increase than the others that may be due to the polymerization of casein in UHT treated coagulated milk sample. Gelation of coagulated UHT milk takes place by a mechanism analogous to that of rennet coagulation. In which an enzymetiggened primary protein degradation step is followed by a secondary aggregation reaction resulting the gelation. On the basis of extensive theoretical treatment Payens (1978) considers that the phenomenon of the initial–thinning is followed by an explosive growth of the average particle weight. The results of the action of clotting protease rules out non-enzymatic coagulation would be accompanied by a linear increase in weight-average molecular weight with time.

Normal milk sample did not show any differences in the molecular weight, when comparing with raw milk sample (raw milk is used as the standard for this experiment) while coagulant (B) and whole sample showed the increased molecular weight. This increasmant may relevant to the polymerization of casein due to Millard type or other chemical reactions such as formation of kappa-casein-beta-lactoglobuulin complexes, which is still not clear But it must be due to this enzyme.

5. CONCLUSIONS

The UHT treated milk coagulates mainly due to age gelation. This UHT treated and aseptically, packed whole milk had no growth of micro-flora or spores. However, some microbial enzymes could survive this short duration heat treatment. These types of enzymes are usually released by the psychrotropics.

The enzymes that could be present in milk are lipases, phosphotases, and ureases. Proteases etc. Enzyme activity of the UHT treated milk was neither due to Urease activity nor due to lipase activity. But protease activity was observed around pH 2-8 the activity of this enzyme was high at 3.5 -4.5. These enzymes are resistant to the heat treatment of UHT processing.

Although the fundamental mechanism and source of enzyme is still not clear, there was a heat resistant (more than UHT treatment) enzyme, which needed to be identified. Relevant to the studies that may be from a microbial source. However, if there were native plasmin enzymes (which are heat resistant and native to milk) then the enzyme activity should be present in UHT treated normal control milk sample. Therefore it confirms the source of enzyme may be from a micro-flora such as psychrotrophic bacteria, which could not identify without raw milk samples of the coagulated batch.

Structural changes of casein molecule may due to the polymerization of casein and whey proteins or formation of kappa-casein-beta-lactoglobulin complexes, which needed further studies to identify.

RECOMMENDATIONS

Establish a system to identify the individual micro floras, (species) especially for *Peudomonas fluoescens* and *Bacillus cereus*, if there is high count of these species, the bulk should not be processed.

Establish proper hygienic conditions; train the farmers to maintain a low count.

At raw milk storage, minimize the storage time as much as possible, (at long time storage in low temperature conditions psychotropic bacteria can be multified and they will increase the specificity count that will lead to age gelation after processing)

At process milk storage minimize the temperature as much as possible that will helpful to expand the shelf life of the products.

At heat treatment S-S bonds are dissociate in rapid rate, addition of food gums like caregeenm will helpful to increase S-S bonds, but this should be carried out after proper study of the gum and its' properties. Addition of caregeenm gums (iota- caregeenm) will modify S-S bonds of milk emulsion and it rearrange as elastic electrolytes that will minimize the aggregation of molecules.

Addition enzyme will be helpful to minimize the problem, but unfortunately enzyme inhibitors in food grade rarely be found and before that additional studies and enzyme purification should be taken place.

Addition of polyphosphates (average of 4.8 phosphorus atoms per chain) will minimize the gelation for some extend, but the quantity and the best polyphosphate should be identified.

6. REFERENCES

1. Adams, D. M. Brarach, J. T., Spek, M. I., 1975. Heat resistant proteases produced in milk by psychrotrophic bacteria of dairy origin. J. Dairy Sci., 58: P 828-854.
2. Andrews, A. T., 1975. J. Dairy Res., 42: P 89.
3. Anon, S., 1972. IDF Monograph on UHT milk, FIL-IDF Ann., Bull., part V, Gen. Secretariat, Belgium.
4. Anson, M. L., 1937. J. Gen. Physiol. 20: 565-574. Cited in Barret, A. J. 1977. Introduction to classification of tissue proteases (P 1-55). Proteases in mammalian cells and tissues. Bio Medical press, North Holland.
5. ASnoeren, J.H.M., Koops, J. and Westerbeek, D., 1976. Neth. Milk dairy J. 30: P 29.
6. Ausubel, F. M. R., Brent, R. E., Kingston, D. D., Seidman J. G., Smith, J. A., and Struhal, K., (eds) 1991. Current protocols in molecular biology. Vol. 1. John willey and sons. New York.
7. Bockelmann, B. V., 1994. Long life products from raw material to finished products. Tetra pak, Lund, Sweden.
8. Dalgleish, D. G., 1986. The enzymatic coagulation of milk. Development series, Fox, P. F. (ed). P 157-187. Applied science publishers. New York.
9. Dalgleish, D. G., 1999. Dairy chemistry and physics. P 1-20.
10. Dalgleish, D. G., 1999. UHT processing. P 1-8.
11. Early, R., 1997. Liquid milk and cream. The technology of dairy products. P 1-46 Chapman and hall. New York.
12. Fox, P. F., 1986. The enzymatic coagulation of milk. Development series. P 157-187. Applied science publishers. New York.
13. Furihata, C.J., Kawachi, and sugimura, T., 1972 Biochem. Biophys. Res. Commun 47 (4): 705-711. Cited in Athauda, S. B. P., 1992, studies on the structure and purification of aspartic proteases. Ph D. thesis. Life Sci. Inst. Japan
14. Graf, E. and Bauer, H. 1976. International food emulsions. Fnberg, S (ed). p 295 Marcel Dekker, inc., New York.
15. Green, M. L., and Morant, S V. J., 1981 J Dairy Sci., 48 P 57
16. Harwakar, V. R., 1986. The age gelation of sterilized milk Development series Fox P F. (ed). P 189-288 Applied science publishers New York

17. Horadagoda, A., 1990. Studies on the composition of indigenous buffalo milk in Sri Lanka. M.Phil. thesis. University of Peradeniya Peradeniya.
18. Hostettler, H., 1972. IDF Monograph on UHT milk, FIL-IDF Ann., Bull., part V, Gen. Secretariat, Belgium.
19. Hostettler, H., 1981. IDF Monograph on UHT milk, FIL-IDF Ann., Bull. Doc 133: P 65-68, Gen. Secretariat, Belgium.
20. Moor, C. V., 1975. J. Dairy Sci., 58: P 977.
21. Payens, T. A. J., 1976. Neth. Milk dairy J. 30: P 55.
22. Payens, T. A. J., 1978. Neth. Milk dairy J. 32: P 55.
23. Perera, H. K. I., 1999. Isolation, purification and characterization of acid proteases from porcine overy. M.Phil. thesis. University of Peradeniya Peradeniya.
24. Perkins, a. G., 1985. UHT processing methods. J. Society of dairy Tec., 38, 69-73.
25. Renner, E., and Schemidt, R., 1981. Chemical and physico-chemical aspects. P 49-64. IDF Monograph on UHT milk, FIL-IDF Ann., Bull., Doc 133.
26. Samel,R, Weaver, R. W. V. And Gammack, D. S., 1971. J. Dairy Res., 38: P 323.
27. Singh, H., McCarthy, O. J., and Lucey, J. A., 1997. Physiochemical properties of milk, Advanced dairy chemistry vol. 3, Fox, P. F., (ed) Chapman and Hall, New York.
28. Snoeren, J.H.M., Vanderspek, C. A., Dekker, R. and Both, P., 1979. Neth. Milk dairy J. 33: P 31. Cited in IDF Monograph on UHT milk. 1981. FIL-IDF Ann., Bull., doc 133 Gen. Secretariat, Belgium.
29. Tetra Pak. 1990. Tetra Pak aseptic packaging machine TBA/8. Tetra pak, Lund, Sweden.
30. Tetra Pak. 1994. Information on forming and checking of Tetra Brick aseptic cartons. Tetra pak, Lund, Sweden.
31. Varnum, A. H., and Sutherland, J. P., 1994. Milk and milk products, Chapman and Hall, New York.
32. Zadow, J.G., and Chituta, F., 1975. Aust J. Dairy Tech., 30 P 104.

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