Enhancing the Flavour Profile and Rheological Attributes of Processed Cheddar Cheese

By

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Affectionately Dedicated

to My Parents

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ABSTRACT

It has been realized that the lack of satisfactory flavour and lack of smooth velvet fullness in mouth, inability of keeping processed cheese blocks at ambient temperature, due to disappearance of sharp edges and rapid drying off followed by consequent rind formation lead to detrimental effects in terms of consumer acceptability of "Highland" processed cheese. Therefore prime objective of this research was to enhance the flavour profile and rheological attributes of "Highland" processed cheddar cheese.

First approach was to reformulate the existing processed cheese recipe, selecting the most appropriate type and amount of natural cheese blend, fat source and emulsifying salt. Changing the proportions of young-mild-matured cheese, fat source (i.e., butter, fresh cream, homogenized fresh cream, ghee) and emulsifying salt (i.e., trisodium citrate, Joha C/Joha T, Joha C/trisodium citrate), 12, 4 and 3 experimental cheese samples were prepared respectively and overall acceptability was assessed by 30 untrained sensory panelists. Highest ratings were observed in cheese blend that contains young, mild and matured cheese in the ratio of 1.5:2.5:3.3, fat source of homogenized fresh cream and emulsifying salt of Joha C/trisodium citrate. Thereupon, different quantities of selected ingredients were incorporated to reformulate the existing recipe. Cheese samples from reformulated recipes and existing recipe were sensed against a popular market sample as a control, showed a relatively lesser improvement. The most acceptable sample showed 41.26% of moisture, 30.5% of fat, 0.6% of acidity and 0.93-0.97 of a_w. One-way ANOVA was performed in statistical analysis.

As the study reveals, processed cheese ingredients have a minor effect in this aspect, in the detailed evaluation on extent of proteolysis and casein breakdown fractions was monitored by Dye-binding method and 12% SDS-PAGE respectively. Relative Casein Content (RCC) of young, mild and matured cheese was 84%, 54% and 37% respectively and for the same of the selected cheese blend was 52%, elaborating poor slicing ability. Percentage proteolysis showed an exactly linear pattern over the period (R^2 = 96.8) and reaches 56% at the end of proteolysis. Thus, It could be speculated that enough intensity of flavour precursors have developed during the 4 month ripening. At the onset of maturation two distinct bands appeared in gel-elctrophoretogram, are most probably α-S_1 and β caseins. Proceeding with ripening concomitant increase of α-$S_1$and β caseins derived bands were appeared along with consistent spreading faint stains. This reveals that concentration of small fragments are insufficient, which are responsible in giving rise to better flavour attributes. Since Salt/Moisture ratio undesirably influence the rate of bacterial and enzyme activity, 5.66% of it could govern to above defects.

CONTENTS

LIST OF ABREVIATIONS

AMF	Anhydrous Milk Fat
AOAC	Association of Official Analytical Chemists
AR	Analytical Reagent
CCP	Colloidal Calcium Phosphate
CEP	Cell wall Proteinase
GMP	Glyco-Macro-Peptide
CN	Casein
Conc.	Concentrated
ERH	Equilibrium Relative Humidity.
FDM	Fat in Dry Matter
GE	Genetically Engineered
ISP	Isoelectric Point
ES	Emulsifying Salts
hrs	hours
kD	kilo Dolton
kP	kilo Pascal
LAB	Lactic Acid Bacteria
LDPE	Low Density Poly Ethylene
MW	Molecular Weight
MVTR	Moisture Vapour Transmission Rate
NSLAB	Non Starter Lactic Acid Bacteria
RCC	Relative Casein Content
SLSI	Sri Lanka Standard Institute
SLS	Sri Lankan Standard
SNF	Solid Non Fat
w/w	weight to weight

LIST OF FIGURES

LIST OF TABLES

Tables **Pages**

CHAPTER 01

INTRODUCTION

1.1. Background:

Milk Industry of Lanka Company Ltd. (MILCO) is a leading company in Sri Lanka, engaged in the process of manufacturing range of milk based products including milk powder, butter, condensed milk, sterilized milk, pasteurized milk, cheese, yoghurt, ghee etc. and marketed under the brand name "Highland". It is proud to be a Sri Lankan company to manufacture their dairy products with a Sri Lankan identity.

Processed cheeses are extremely versatile foods, appealing to most consumer segments, because of their long-term stability and tailor-made functionality (Dairy Management Inc, 2004), is a complex system principally composed of protein, fat, minerals and water obtained by blending, mixing and heating natural cheeses of different maturity, emulsifying salts and water (Caric *et al.*, 1985). Although cheddar remains a commodity cheese, diversifying cheddar cheese flavor will help boost sales and will be of economic interest to cheese producers, as it opens ways to meet growing variations in demand and increasingly specific requirements created by the changing life styles of consumes (Linden and Lorient, 1999). Hence, food industry needs continuously updating knowledge on cheese technology in order to display a huge capacity for innovations and continuous improvements in their products.

It has been realized that lack of satisfactory flavour and lack of smooth velvet fullness in mouth and inability of keeping processed cheese blocks at ambient temperature due to, disappearance of sharp edges and rapid drying off followed by consequent rind formation lead to detrimental effects in terms of consumer acceptability, makes a relatively lower demand for "Highland" processed cheese in present market dynamics. To be ahead in the market it is indispensable to enhance overall sensory attributes up to the level in which consumer utmost satisfaction prevails.

Food texture is important as a quality indicator that consumers use to accept or reject a food product (Guinard and Mazzucchelli, 1996). Proper control of the parameters that describes the texture would therefore enable food processor to make products with the highest quality and sensory acceptability exhibiting wide range of textures. Texture of processed cheese is influenced by the chemical composition and the processing conditions used during cheese manufacture as well as by the type and amount of emulsifier incorporated (Blazquez *et al.*, 2006). Therefore raw material for processing, especially initial blend of natural cheese and

emulsifiers must be carefully selected and combined to produce the correct consistency in a spreadable product, a block cheese or a sliced cheese (Banks, 1998).

Although processed cheese ingredients enable us to modify sensory qualities, it is impossible to achieve excellence where cheese base is inferior in quality since processed cheese ingredients play a relatively minor role. Therefore multiple aspects of cheese making must be examined to fully understand where flavour and texture defects are originated and then efforts should be made to consistent control and manipulate rheological and textural characteristics as well as flavour properties. In natural cheese manufacturing process, ripening stage is the most crucial stage that is responsible for the appearance of the basic flavour and texture (Gripon, 1997). Among three major biochemical processes occur during ripening period, proteolysis is the principle and most complex event with regards to cheddar, thus assessment of this would reveal key reasons that responsible for flavour and texture defects in processed cheese. In addition, the cheese industry has also identified several specific processing and ingredient interactions potentially responsible for such defects (Dairy Management Inc, 2001) and need to investigate those, both for natural cheese and processed cheese.

1.2. Overall Objective:

- Enhancing the flavour profile and rheological attributes of processed cheddar cheese.

1.3. Specific Objectives:

- Selecting the most appropriate types and amounts of ingredients and developing a newer recipe for processed cheese in order to offer the highest flavour and texture appeal.

- Selecting the most affordable packaging material for processed cheese in order to customize the physical and sensory properties of the finished product.

- Evaluating the chemical and physical influences and ripening changes of natural cheese that govern the flavour and texture attributes of processed cheese.

CHAPTER 02

LITERATURE REVIEW

2.1. Cheese: An Overview

Cheese is a generic name for a group of fermented milk based products produced throughout the world in a great diversity of flavours, textures and forms (Fox *et al.,* 2000) giving appeal to a wide range of palates (Gordon, 1997). Cheese enjoys epicurean status, has desirable nutritional properties, is the classical convenience food and may be consumed as the main component of a meal, as a dessert or as a food ingredient (Fox and McSweeney, 1998).

2.1.1. Definition

The word "cheese" is commonly used as a collective term for widely variable products such as matured and non-matured cheese made with rennet, acid curd cheese, fresh cheese, and even processed cheese. Most of these fit the definition established by the Food and Agriculture Organization of United Nations (FAO), i.e., cheese is the fresh or matured solid or semi-solid product obtained by coagulating milk, skimmed milk, partly skimmed milk, cream, whey cream, buttermilk or any combination of these materials, through the action of rennet or other suitable coagulating agents, and by partially draining the whey resulting from such coagulation (Gordon, 1997).

2.1.2. History and Origin of Cheese Making

Cheese making is one of the oldest methods practiced by man and it provides a very elegant solution to the problem of preserving the protein and fat in milk, which is highly perishable and nutritional foodstuff. The exact origin of cheese making is difficult to establish, but according to the definite archaeological evidence cheese production dating perhaps from 6000-7000 BC in a rich agricultural area known as "Fertile Crescent" situated between the rivers Euphrates and Tigris in Iraq (Tamime, 1993). The acid coagulated cheeses originated from the preservation of milk by drying in the warm climates of the Eastern Mediterranean countries. Rennet type cheeses have been derived from the storage and transport of milk in the stomachs of animals in warm climates. However, the development of art of cheese making to a science has been comparatively recent and fundamental knowledge on biochemistry and microbiology has progressed in the last 50 years (Banks, 1998).

2.1.3. Production and Consumption

Cheese is mainly produced in Europe, North and South America, Australia, and New Zealand and to a lesser extent in North Africa and the Middle East, where it is originated during the

Agricultural Revolution (Fox and McSweeney, 1998). The growing importance of health and wellness has significantly altered consumption and buying behaviors. The per-capita consumption of all cheeses range from below 2Kg/year in Japan and South Africa through to 15-20Kg/year in countries such as France, Germany and Italy (Tamime, 1993). Cheese is accounting for 30% of total milk usage. World production of cheese is around 15×10^6 tones per annum at a rate of around 2.3% since 1985 (Guinee and Law, 2000).

It was recorded that average monthly milk production (cow and buffalo milk) in Sri Lanka during the year 2005 existed at about 16,061, 800 liters, from which cow milk attributed to 13, 484, 700 (Department of Census and Statistics, 2005). However Sri Lanka is not much interested in producing cheese comparable to other milk based foodstuffs even though consumers who demand cheese are dramatically increased. Therefore much of the consumption depends on imports.

2.1.4. Dietetic Importance

In the past, the nutritional aspects of cheese were rarely considered. Apart from water, man requires five major groups of nutrients in food, namely fat, protein, carbohydrate, vitamins and minerals. Cheese made from normal whole milk is known to contain most of the essential fatty acids, i.e., linolenic, linoleic and arachidonic acid and is also a rich source of proteins since it contains all the essential amino acids (Muir, 1998). The main protein in cheese is CN, which is somewhat inferior to whey protein in its biological value due to lower content of sulphur amino acids. However, digestibility of proteins in cheese is higher than that of whole milk (Renner, 1999). Although milk does contain milk sugar (lactose-a carbohydrate), cheeses that have been ripened do not contain appreciable amounts of lactose, since it is lost in whey or during maturation. Therefore People with lactose intolerance can complement their diet with cheese in order to gain nutrients in milk. Cheese contains appreciable levels of minerals, of which calcium, iron and phosphorus are the most important. Indeed, 100g of hard cheese can supply the daily calcium requirement of an average adult, plus nearly 50% of the phosphorus requirement, while the bioavailability is good compared to other food sources. In addition to nutritional benefits cheese is believed to have anti-carcinogenic effect and defensive action against dental caries (Robinson and Wilbey, 1998).

2.1.5. Classification of Cheese Varieties

The great range of cheese varieties, excluding minor local varieties, makes classification of cheese extremely complicated. Worldwide, there are more than 2000 types of cheese, sometimes made by very different manufacturing processes. The classification can be based on several aspects, and is done in different countries according to different criteria (Spreer

and Mixa, 1998). General and internationally recognized criteria is based on the method of coagulating CN in curd making (acid, rennet or both), moisture content (hard, semi hard and soft), principle microorganisms used for ripening (bacteria, mould) and texture (round-eyed, granular and close-textured) of cheese (Banks, 1998).

However, the popular types of natural cheeses could be categorized as soft-unripened (e.g., cottage cheese, cream cheese, Mozzarella), soft-surface ripened with moulds (e.g., Brie, Camembert), semi-soft-surface ripened with bacteria (e.g., Brick, Munster), semi-hard-internally ripened with moulds (e.g., Roquefort, Stilton), semi-hard (e.g., Edam, Gouda), hard-with eyes (e.g., Emmental, Gruyere), hard-without eyes (e.g., Cheddar, Provolone) and extra-hard (e.g., Parmesan, Romano). Examples of processed cheeses include American cheese and various cheese spreads, which are made by blending two or more varieties of cheese or blending portions of the same type of cheese that are in different stages of ripeness (Robinson and Wilbey, 1998).

2.2. Natural Cheese Manufacture

Cheese manufacturing is aimed at making an attractive and durable product in which important nutrients of the milk are concentrated. Cheese must be left for ripening to acquire desirable flavor and consistency. To achieve this, cheese is kept for a variable time under favorable conditions. Cheese making is a complicated process, involving many processing steps and several biochemical transformations. All of these variables affect to the yield, composition and quality of the cheese and its by-products (predominantly whey), and often in different directions (Walstra *et al.*, 1999).

Cheese manufacture is essentially a dehydration process in which the fat and casein in milk are concentrated between 6-12 fold depending on the variety, whereas the other milk components, especially water, are mainly removed along with whey. None of the milk components are fully retained, and other substances may be added, notably salt. This is illustrated in figure 2.1. The yield and composition of the cheese are determined especially by properties of milk, and by the manufacturing practice (ELSoda, 1997).

2.2.1. Basic Raw Materials

2.2.1.1. Milk:

Milk is a dispersion of milk fat globules and casein micelles in a continuous phase of water, lactose, whey proteins, and minerals. Although milk from many different species of mammal is used in cheese manufacture, cow's milk is used predominantly in industrial cheese making (Banks, 1998). Typically cow's milk contains 87.3% of water, 3.9 % of milk fat and 8.8% of

solids-not-fat. SNF constitutes 3.25% of protein (3/4 casein), 4.6% of lactose, 0.65% of minerals (Ca, P, citrate, Mg, K, Na, Zn, Cl, Fe, Cu, sulfate, bicarbonate etc.), 0.18% of acids (citrate, formate, acetate, lactate, oxalate) (Walstra *et al.,* 1999). Like in most dairy products, first cheese milk must be clarified, separated, standardized and then be subjected to pasteurization (Hill, 2006).

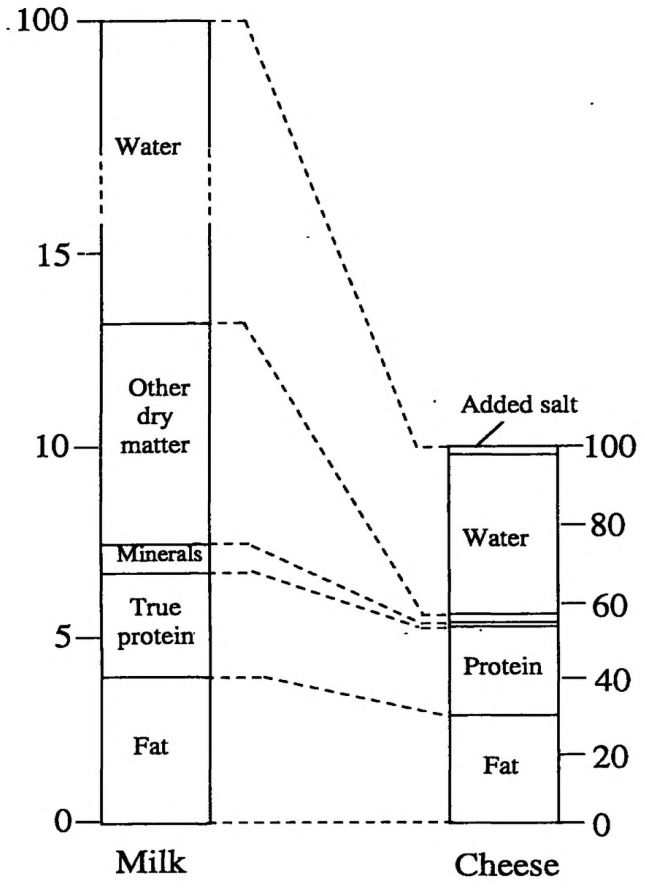

Fig 2.1. Components transfer from milk to cheese (Source: Walstra *et al.,* 1999)

2.2.1.2. Coagulants:

Rennet is the predominant coagulating agent, is an enzyme preparation isolated from the forth stomach (abomasum) of the calf. Commercial rennet preparations contain chymosin and pepsin in varying proportions and usually chymosin accounts for 70-80%. Since its molecular weight is 35600 and isoelectric point is 4.65, soluble in nature (Walstra *et al.,* 1999). The shortage of calf vials has compensated by means of alternative coagulants, such as mixture of bovine and porcine pepsin with calf rennet (50:50), fungal enzymes from *Rhizomucor miehei, Rhizomucor pussilus* and *Cryphoneptera paracitica,* enzymes derived from plants and bacteria (Gordon, 1997) and recombinant chymosins. The coagulants produced by gene manipulation of *Aspergillus niger, Kluveromyces lactis* and *Escherichia coli* are known respectively as Chymogen, Maxiren and Chy-Max are widely used for cheese manufacture in UK and USA (Banks, 1998).

2.2.1.3. Cheese Starters:

Production of cheese depends on fermentation of lactose by LAB to form mainly lactic acid. This imparts a fresh, acid flavour to curd cheeses, assists in the formation of rennet coagulum and causing shrinkage of the curd and moisture expulsion. Selected strains with predictable acid development and production of flavourful products are used as strains to obtain a steady rate of acidity through out the curd making process (Helen and Sharpe, 1981). Starters used in the cheese industry belong to the genera *Lactococcus, Streptococcus, Leuconostoc and Lactobacillus.* Of these organisms, the *Lactococci* are the most widely used. *Lactococcus lactis subsp. lactis* and *Lactococcus lactis subsp. cremoris* are mesophilic starter cultures, which are used in the production of variety of cheeses in which the processing techniques incorporate only moderate temperatures, up to about 40^0C (Banks, 1998).

2.2.1.4. Additives:

Calcium choride, nitrates, colours, hydrogen peroxide and lipases serve as optional ingredients in cheese making. Calcium choride is added to replace calcium redistributed during pasteurization. Milk coagulation by rennet during cheese making requires an optimum balance among ionic calcium and both soluble insoluble calcium phosphate salts. Because calcium phosphates have reverse solubility with respect to temperature, the heat treatment from pasteurization causes the equilibrium to shift towards insoluble forms and depletes both soluble calcium phosphates and ionic calcium. So $CaCl_2$ is added to restore ionic calcium and improve rennetability.

Sodium or potassium nitrate is added to the milk to control the undesirable effects of *Clostridium tyrobutyricum* in cheeses such as Edam, Gouda, and Swiss. Because milk colour varies from season to season, colour may be added to standardize the colour of the cheese throughout the year. Annato, Beta-carotene, and paprika are used. The addition of hydrogen peroxide is sometimes used as an alternative treatment for full pasteurization. Lipases, normally present in raw milk, are inactivated during pasteurization. The addition of kid goat lipases is common to ensure proper flavour development through fat hydrolysis (Hill, 2006).

2.2.2. Basic Operations:

As illustrated in figure 2.2, manufacture of cheese may involve many different processing steps, from which some are essential for all cheese varieties and are known as the basic operations include, (1) clotting of the milk by means of enzymes or acid, or both (a gel is formed, due to the casein particles aggregating into a network, enclosing fat globules), (2) removal of whey (comparable to milk serum) by means of syneresis of the gel (the resulting curd makes up 10% to 30% of the original volume of milk; the drier the curd, the firmer and

the more durable the cheese will be), (3) acid production in the cheese during its manufacture due to the conversion of lactose into lactic acid by lactic acid bacteria (the resulting pH of curd and cheese affects such parameters as syneresis, consistency, and ripening of the cheese), (4) salting, (5) fusion of curd grains into a coherent loaf that is easy to handle, and (6) ripening (microbial, biochemical, chemical, and physical processes during ripening are responsible for changes in composition and structure of the cheese; hence flavor and texture).

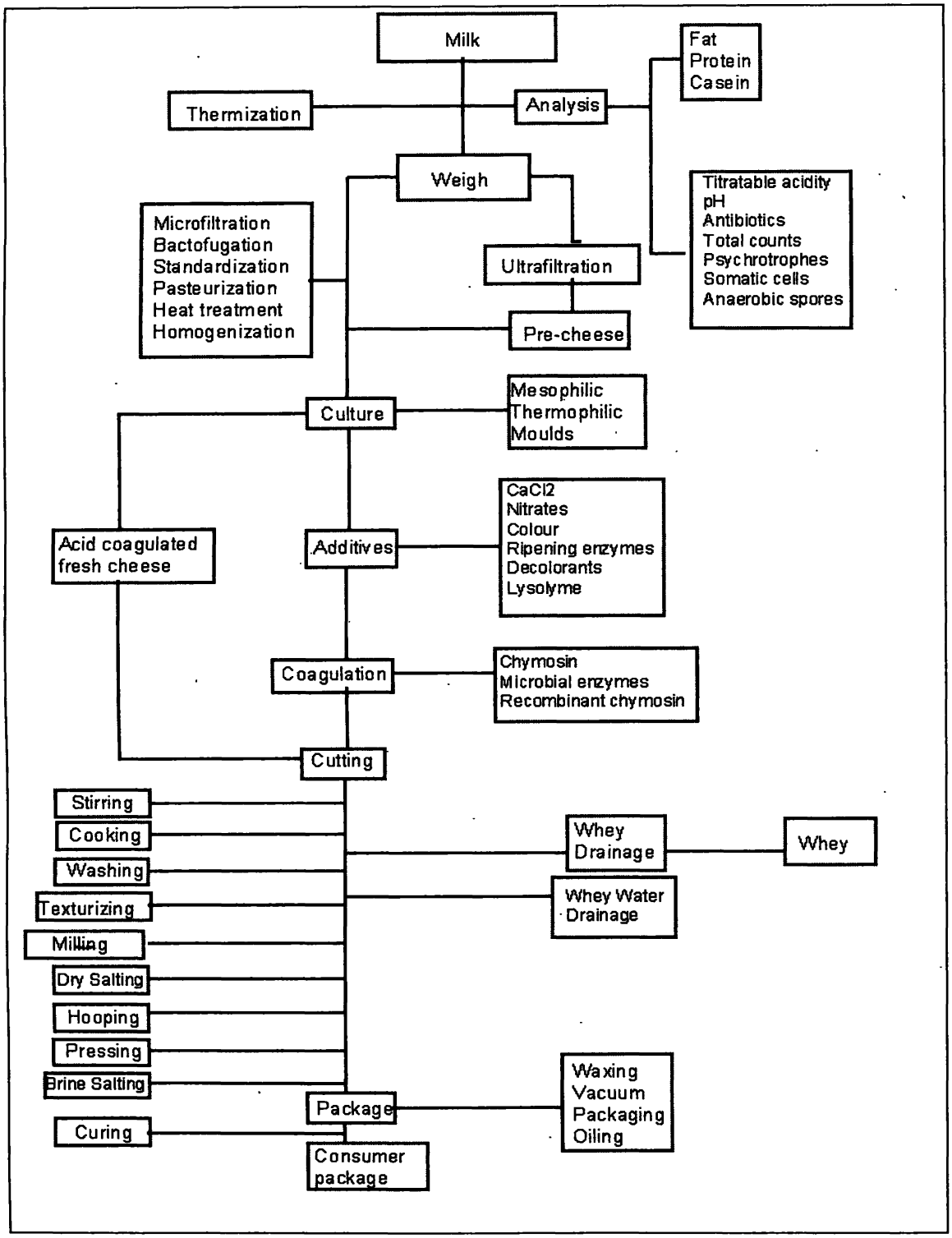

Fig 2.2. Flow Diagram of Cheese Making Process (Hill, 2006)

Fusion of curd grains and ripening are typical processing steps of ripened cheese; when these are not carried out, the product is referred to as fresh or young cheese (Walstra *et al.*, 1999). With the objective of diminishing variation in the course of the manufacture of the cheese and in its properties, some additional process steps have been applied: pasteurization of the milk and addition of cultures of microorganisms to the milk. By using different biochemical processes, the tasteless dairy protein is converted into tasty and easily digestible cheese with different flavors (Spreer and Mixa, 1998).

2.2.3. Functionality of Casein and Mechanism of Coagulation

2.2.3.1. Structure of Casein:

The CN content of milk represents about 80% of milk proteins. The principal CN fractions are alpha (s_1) and alpha (s_2)-CN, beta-CN (β-CN) and kappa-CN (k-CN). The distinguishing property of all CNs is their low solubility at pH 4.6. The common compositional factor is that CNs is conjugated proteins, most with phosphate group(s) esterified to serine residues. These phosphate groups are important to the structure of the CN micelle. Calcium binding by the individual CNs is proportional to the phosphate content. Table 2.1 summarizes the chemical characteristics.

Table 2.1. Distribution and Characteristics of milk proteins

Component	Approximate Concentration		Approximate MW	ISP	Groups per Mole		
	5% of Skim Milk Proteins	g/l^{-1}			P	-S-S-	-SH
Caseins	78-85	27.2		4.6	8	0	0
α_{s1}-CN	45-55	13.6	23,500	5.1	5	0	0
β-CN	25-35	8.2	24,000	5.3	1	0	2
k-CN	8-15	4.1	19,000	3.9	--	--	--
γ-CN	3-7	1.4	--	5.8	--	--	--
"γ_1"	--	--	20,500		1	0	0
"γ_1"	--	--	11,800		0	0	0
"γ_3"	--	--	11,500		0	0	0

(**Source:** Walstra *et al.*, 1999)

The conformation of CNs is much like that of denatured globular proteins. The high number of proline residues in CNs causes particular bending of the protein chain and inhibits the formation of close-packed, ordered secondary structures. CNs contain no disulfide bonds. As well, the lack of tertiary structure accounts for the stability of CNs against heat denaturation because there is very little structure to unfold. Without a tertiary structure there is

considerable exposure of hydrophobic residues. This results in strong association reactions of the CNs and renders them insoluble in water. Within the group of CNs, there are several distinguishing features based on their charge distribution and sensitivity to calcium precipitation.

alpha(s1)-CN: (molecular weight 23,000; 199 residues, 17 proline residues) Two hydrophobic regions, containing all the proline residues, separated by a polar region, which contains all but one of eight phosphate groups (figure 2.3).

Fig 2.3. Amino acid sequence of α S_1-casien (Source: Fox, 1998)

alpha(s2)-CN: (molecular weight 25,000; 207 residues, 10 proline residues) Concentrated negative charges near N-terminal and positive charges near C-terminal. It can also be precipitated at very low levels of calcium.

β -CN: (molecular weight 24,000; 209 residues, 35 proline residues) Highly charged N-terminal region and a hydrophobic C-terminal region. Very amphiphilic protein, acts like a detergent molecule. Self-association is temperature dependant; will form a large polymer at 20° C but not at 4° C. Less sensitive to calcium precipitation (figure 2.4).

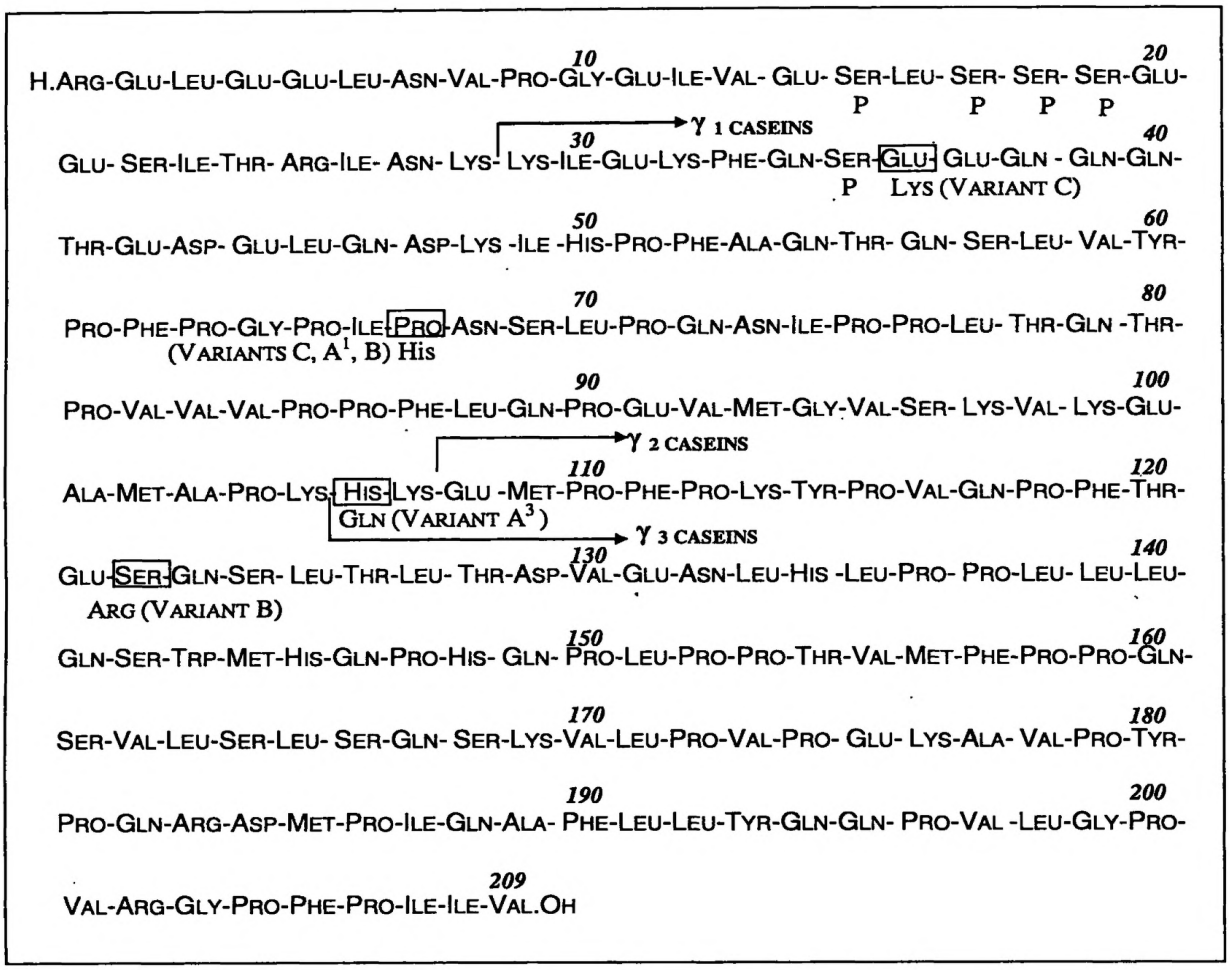

Fig 2.4. Amino acid sequence of β-casien (Source: Fox, 1998)

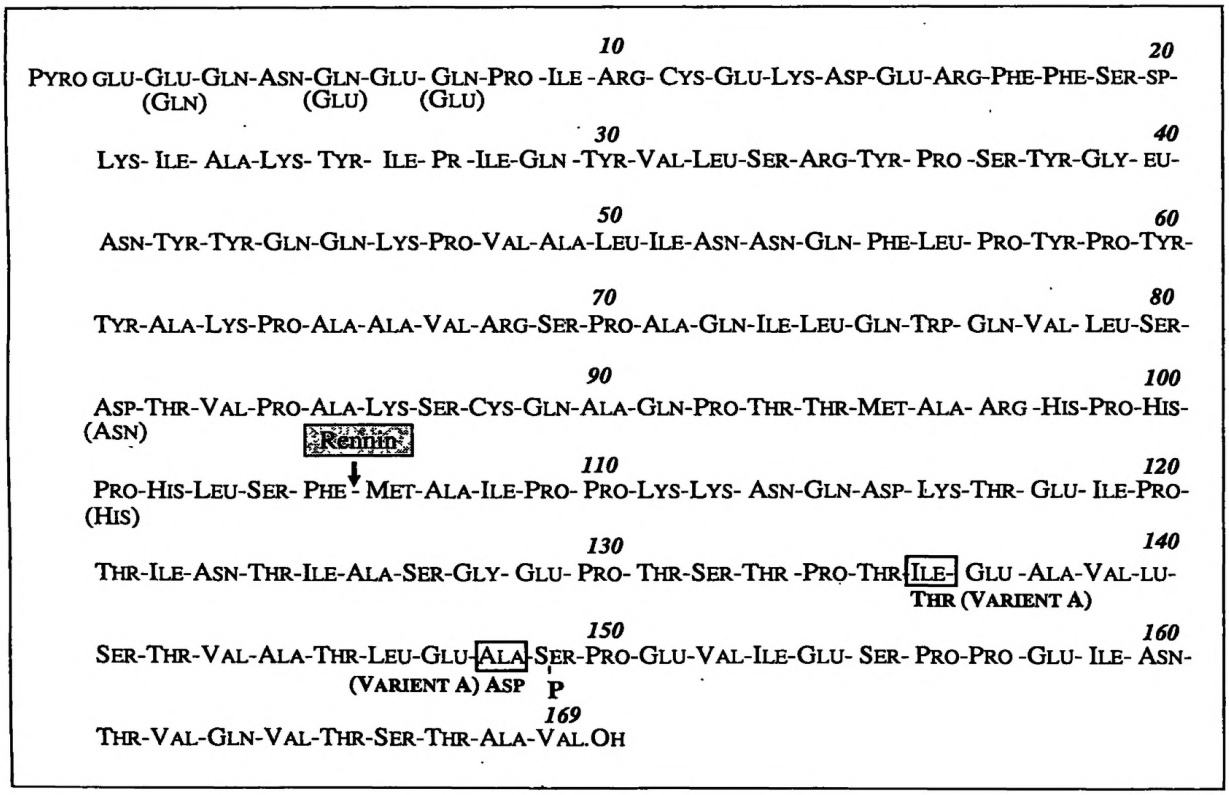

Fig 2.5. Amino acid sequence of k-casien (Source: Fox, 1998)

kappa-CN: (molecular weight 19,000; 169 residues, 20 proline residues) 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 a hydrophilic portion GMP, or more accurately, CMP (figure2.5).

2.2.3.2. Structure of the Casein Micelle:

Most (80%), but not all, of the casein proteins exist in a colloidal particle known as the casein micelle, with diameters of from 90 to 150 nm. Besides casein protein, calcium and phosphate, the micelle also contains citrate, minor ions, lipase and plasmin enzymes, and entrapped milk serum. These micelles are rather porous structures, occupying about 4 ml/g and 6-12% of the total volume of milk (Fox and McSweeney, 1998).

Two models have been developed for explaining the structure of CN, namely sub-micelle model (figure 2.5) and open structure. According to sub-micelle model, 10 to 100 CN molecules aggregate to form structures called sub-micelles. It is thought that there are two different kinds of sub micelle; with and without kappa-casein. These sub micelles contain a hydrophobic core and are covered by 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 open model also suggests there are more dense and less dense regions within the micelle, but there is less of a well-defined structure. In this model, calcium phosphate nanoclusters bind caseins and provide for the differences in density within the casein micelle (Hill, 2006). CCP acts as cement between the hundreds or even thousands of sub micelles that form the casein micelle (figure 2.6). Binding may be covalent or electrostatic. Sub micelles rich in kappa-casein occupy a surface position, whereas those with less are buried in the interior. The resulting hairy layer, at least 7 nm thick, acts to prohibit further aggregation of sub micelles by steric repulsion. The casein micelles are not static and maintain dynamic equilibria between the micelle and its surroundings (Walstra *et al.*, 1999).

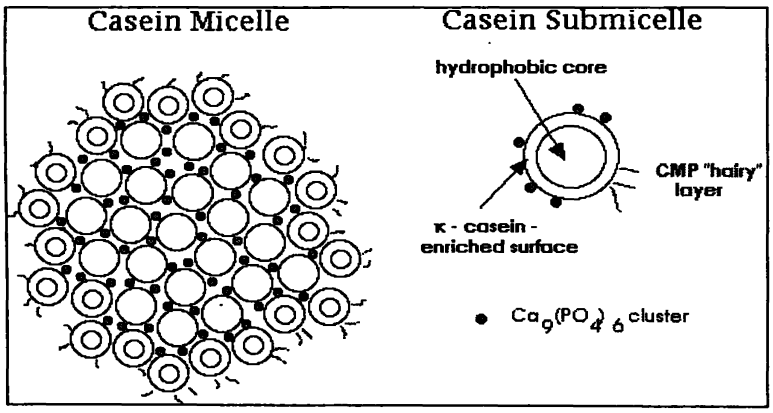

Fig 2.6. Casein micelle and sub-micelle structure (Source: Hill, 2006)

2.2.3.3. Mechanism of Coagulation:

Although the casein micelle is fairly stable, aggregation can be induced by enzyme or by acids. Chymosin, or rennet, is most often used for enzyme coagulation. During the 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. Reactive sites should be come into contact before aggregation of the para-casein micelles begins (Green and Grandison, 1999).

During the secondary stage, the micelles aggregate. This is due to the loss of steric repulsion of the kappa-casein as well as the loss of electrostatic repulsion due to the decrease in pH. As the pH approaches its ISP (pH 4.6), the caseins aggregate. The casein micelles also have a strong tendency to aggregate because of hydrophobic interactions (Foltmann, 1999). Calcium assists coagulation by creating isoelctric conditions and by acting as a bridge between micelles. The temperature at the time of coagulation is very important to both the primary and secondary stages. With an increase of 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 paracasein identity as the milk curd firms and syneresis begins (Fox and McSweeney, 1998). On the other hand acidification causes the casein micelles to destabilize or aggregate by decreasing their electric charge to that of the isoelectric 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 (Hill, 2006).

2.2.4. Cheddar Cheese

Cheddar cheese is coming under the textured cheese. Due to local influences and market preferences a number of slightly different cheddars have evolved, e.g., traditional English, New Zealand, American and Canadian cheddars. These differ from other groups in that after the whey is drained away the curds are manipulated to give a texture before being molded and pressed (Robinson and Wilbey, 1998).

2.2.4.1. Manufacture of Cheddar Cheese:

The manufacture of rennet-coagulated cheeses, such as Cheddar, can be divided into two more or less distinct phases i.e., conversion of milk to curd, which is essentially completed within 24 hrs and ripening of the curd as illustrated in figure 2.7 (Singh *et al.*, 2003)

Standardized clean fresh milk of good microbiological and chemical quality with an acidity of approximately 0.16% is heat treated at 72^{0}C for 15 seconds and left at starter temperature of not less than 21^{0}C. Starter is added to milk at the rate of 1% of the milk volume and kept for 45 minutes to 1hour. At this stage acidity should have been increased by 0.02%. In view of possibility of bacteriopage attack the starter addition may be increased to 2-5% and the fermentation time reduced to 5-10 minutes. If coloured cheese is required then annatto may be added to the milk at the rate of 0.015-0.03%. Rennet at the rate of 0.022-0.025% of milk diluted with cold water 6-10 times is added in to the fermenting milk. When using bulk starter acidity should be 0.19-0.22% before renneting.

The coagulum is cut when firm enough, in about 45 minutes with multi-bladed steel knives or nylon string frames to small size pieces. The curd-whey mixture is stirred slowly at first to allow the cut surface of the curd to heal. Then the temperature of the mixture is 0.5^{0}C per 5 minutes, rising to 1^{0}C per 5 minutes. After 45 minutes the temperature should be 39^{0}C. Stirring of the mixture should be continued for a further 45 minutes before the curd is 'pitched' (i.e., allowed to sink in whey). The acidity of this stage should be 0.15-0.175%. When the acidity of the whey reaches 0.20-0.25%, i.e., about 2.25-2.75 hours after renneting, the whey is removed from the vat and the curds collected along the sides of the vat to leave a drainage channel down the centre.

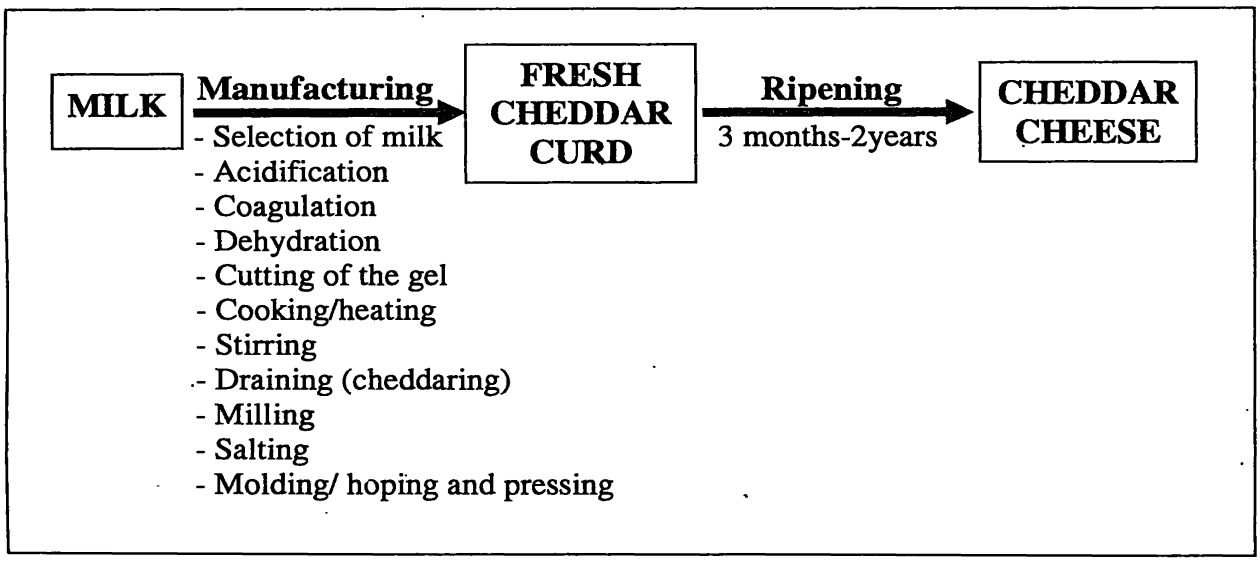

Fig 2.7. General description of cheddar cheese manufacture (Source: Sing *et al.*, 2003)

The next stage is one unique to cheddar cheese. The curds whilst still draining are cut in to large blocks, which are turned over and after 10-15 minutes, piled one on other. This process is repeated every 15 minutes. This piling produces 'chicken breast' structure of the curd (i.e., cheddared curd). When the acidity of draining whey reaches 0.68-0.85% and cheddaring is

completed, the curd is milled in to walnut-size pieces and salt is sprinkled over it at the rate of 2% w/w. Then well-mixed curd is molded and then pressed for 2hours at19 kPa (Robinson and Wilbey, 1998).

2.2.4.2. Cheddar Cheese Properties

Cheddar cheese is classified as a hard cheese, with a long shelf life and without a surface flora. It is about 45% to 50% fat in the dry matter and minimum dry matter of 62% (Spreer and Mixa, 1998). It has a buttery but firm body with close texture and a clean nutty flavor (Varnam and Sutherland, 2001). Cheddar cheese contains little active milk proteinase, active rennet, and a large pool of proteolytic enzymes from lactic acid bacteria; most of the fast acid-producing strains are also strongly proteolytic. At the low curing temperature (usually below 10°C) the proteolysis in the depth is relatively slow, whereas the degradation in the width is fast. It may be cured for varying lengths of time, from 3 to 15 months (Early, 1998). The curing room is around 85% percent relative humidity at 4°C (40°F) and the cheese is held for 60 days or longer. The peak flavor is usually attained in 9 to 12 months (Kosikowski and Mistry, 1997).

Defects that may occur in cheddar cheese include: open texture which may lead to formation of cracks due to gas production during maturation, "seaminess" which refers to the appearance of whitish "veins" seen in a cross-section of the cheese, incomplete acid production that often is responsible for insufficient flavor and abnormal consistency, contaminating bacteria that may cause defects, especially at high pH, low salt content, and high ripening temperature, difficulties in cooling down the interior of the cheese when made in very large blocks, and bitter flavor development if the salt content is low and the curing temperature is high (Walstra *et al.*,1999).

2.3. Ripening of Cheese

Rennet coagulated fresh or young cheese curd is rather flavourless, tough and rubbery, hence it is ripened, or matured, at various temperatures and times until the characteristic flavour, body and texture profile is achieved (Fox and McSweeney, 1998). The three primary biochemical events responsible for changes in chemical and physical properties during ripening of cheese are; (1) glycolysis, (2) Proteolysis, and (3) Lipolysis (Fox *et al.,* 1999) and the relative importance of which depends on the variety. These primary changes are followed and overlapped by a host of secondary catabolic changes, including deamination, decarboxilation and desulfurylation of amino acids, β-oxidation of fatty acids and even some synthetic changes that is esterification. The above mentioned primary reactions are mainly responsible for the basic textural changes that occur in cheese curd during ripening and are

also largely responsible for the formation of whole range precursors of basic flavor compounds cheese. However, the secondary transformations are mainly responsible for the finer aspects of cheese flavor and modify cheese texture (Singh *et al.,* 2003). Series of biological, biochemical and chemical changes are affected by the size and composition of young cheese, and are controlled by the conditions of temperature and humidity (Helen and Sharpe, 1981).

These changes are brought about by various types of enzyme systems, are classified in to several groups as; (a). Proteolytic enzymes (i.e., endopeptidases or proteinases, which hydrolyze proteins to peptides and exopeptidases, which split peptides into smaller ones and amino acids; this group includes amino peptidases, carboxy peptidases, di and tri peptidases), (b). Enzymes that decompose amino acids produced by exopeptidases (i.e., decarboxilases, deaminases, transaminases, demethiolases), (c). Lipases which breaks down triglycerides in to free fatty acids, di and mono glycerides, (d). Enzymes that break down fatty acids in to their derivatives, (i.e., dehydragenases and decarboxylases) (Walstra *et al.,* 1999).

The potential sources of these enzymes are (i) the lactic acid bacteria of the starter culture, (ii) the rennet, rennet paste and rennet substitute, insofar they are transferred to the cheese during manufacture and remain active (iii) miscellaneous, non-starter bacteria present in milk and surviving pasteurization (iv) Extracellular proteinases and lipases originating from psychrotrophic bacteria growing in the raw milk (v) indigenous milk enzymes, especially proteinases and lipoprotein lipase and (vi) other micro organisms growing within or on the rind of cheese (Singh *et al.,* 2003).

2.3.1. Glycolysis

In Glycolysis, remaining lactose (about 96% of lactose is removed the whey as lactose and lactic acid) is fermented by lactococcus starters to L-lactate by few weeks in cheddar cheese. Then NSLAB racemized L-lactate lactic within about 3 months and small amount is oxidized to acetic acid. However lactic acid may be metabolized to butyric acid, CO_2 and H_2, if the cheese is contaminated with *Clostridium tyrobutyricum* (Fox and McSweeney, 1998).

2.3.2. Citrate Metabolism

Bovine milk contains relatively low levels of citrate (~8 mM). Approximately 90% of the citrate in milk is soluble and most is lost in the whey; however colloidal citrate is concentrated in cheddar curd and is important in cheese made using mesophilic starters. Cheddar cheese contains 0.2 to 0.5% (w/w) citrate that is not metabolized by *Lc. lactis subsp. lactis or subsp. Cremoris* (Fox *et al.,* 2000), but is metabolized by *Lc. lactis biovar*

diacetylactis and *Leuconostoc spp,* with the production of diacetyl and CO_2. Early workers showed that the concentration of citrate in cheddar cheese decreases slowly to almost zero at 6 months, presumably as a result of metabolism by *Lactobacilli.* Among the principal flavor compounds derived from citrate (i.e., acetate, diacetyl, acetoin, and 2, 3-butandiol) only acetate and diacetyl contributes to cheddar flavour (Singh *et al.,* 2003).

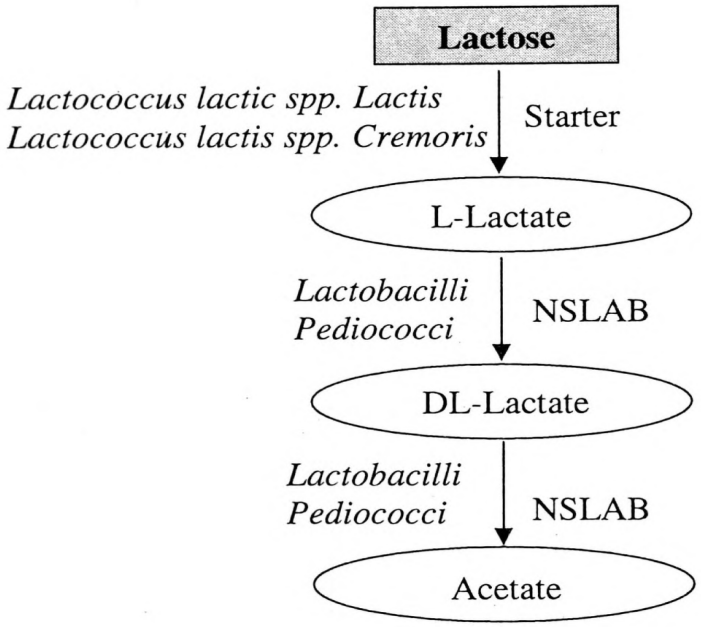

Fig 2.8. Pathway for the metabolism of lactose in Cheddar cheese (Source: Singh *et al.,* 1999)

2.3.3. Proteolysis

Proteolysis is the principle and most complex biochemical event (Fox *et al.,* 2000), which takes place during ripening and its degradation products, amino acids and peptides, have a considerable influence on the sensory characteristics of cheese. Primary proteolysis leads to the formation of large water-insoluble peptides and smaller water-soluble peptides (Poveda *et al.*, 2006). A gradual decomposition of caseins occurs due to the combined action of various enzymes as illustrated in figure 2.4 (Singh *et al.,* 2003). In Cheddar, enzymes from the coagulant (i.e., GE Chymosin, Chymosin/Pepsin mixture from calf stomach) serve as the principle proteolytic agent while Plasmin, Cathepsin responsible to a lesser extent. CEP and peptidases from starter and nonstarter lactic acid bacteria plays a relatively minor role in the hydrolysis of intact caseins and polypeptides (Fox *et al.,* 2000).

Proteolysis contributes to cheese ripening in at least four ways. (1) A direct contribution to flavour via amino acids and peptides, (2) Greater release of sapid compounds during mastication and perception of flavour, (3) Changes in pH via formation of ammonia, (4) Changes in texture arising from break down of protein network, increase in pH and greater water binding by newly formed amino and carboxyl groups. However, It is primarily

responsible for textural changes-in hardness, elasticity, cohesiveness, fracturability, stretchability, meltability and emulsifying properties. Unfortunately some small peptides give rise to bitter flavour once it is present in sufficient concentrations (Helen and Sharpe, 1981).

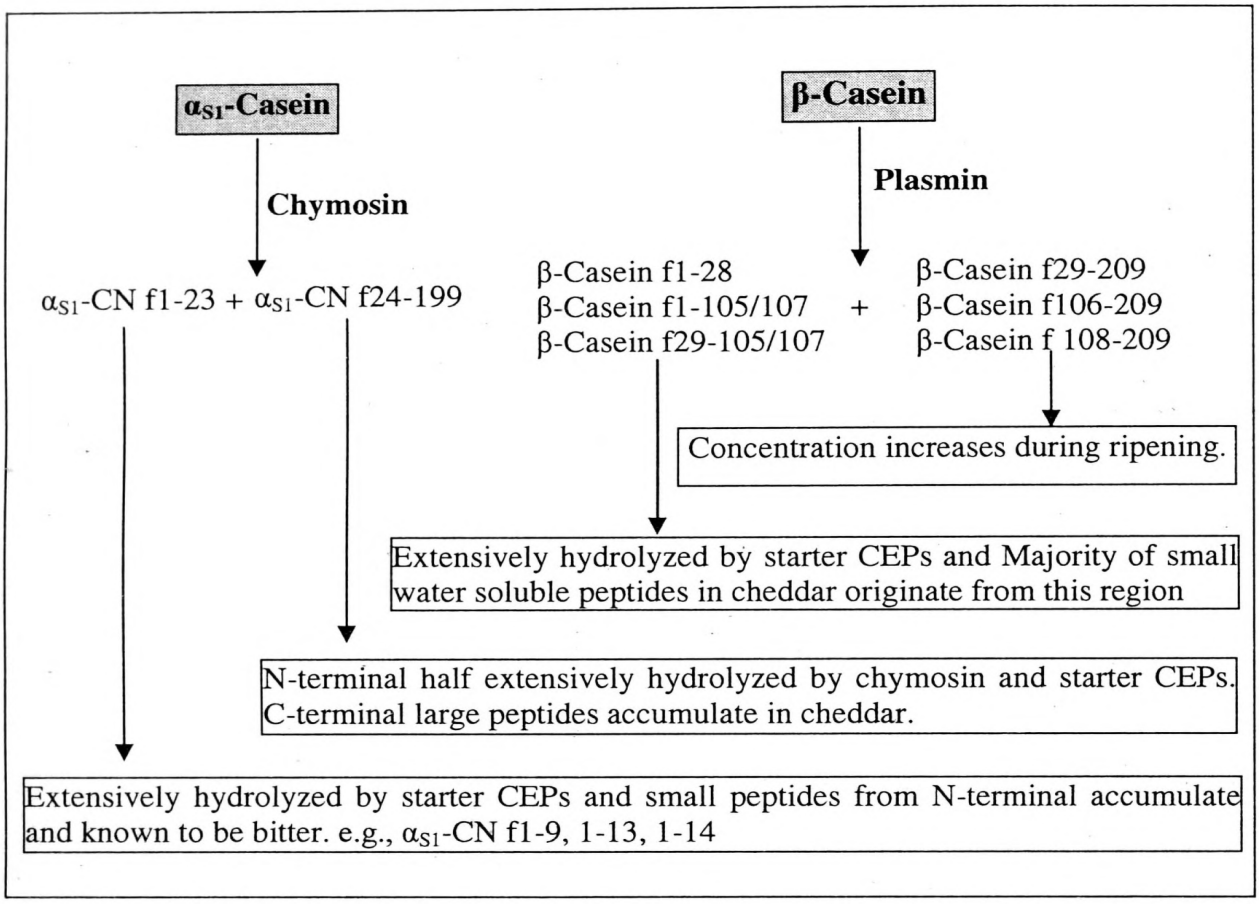

Fig 2.9. Pathway for the casein degradation cheddar cheese (Source: Singh *et al.,* 2003)

2.3.4. Lipolysis

Like all types of food with a high fat content, lipolytic (enzymatic hydrolysis by lipases and esterases) and oxidative (chemical) changes are likely to occur in cheese. The hydrolysis of triglycerides, which constitute more than 98% of cheese fat, is the principal biochemical transformation of fat during ripening, which leads to the production of free fatty acids (FFA), di and monoglycerides and possibly glycerol. FFA contributes to the aroma of cheese. Individual FFA, particularly acids between C4:0 and C12:0 have specific flavors (rancid, sharp, goaty, soapy, and coconut-like). The flavor intensity of FFA depends not only on the concentration, but also on the distribution between aqueous and fat phases, the pH of the medium, the presence of certain cations (that is, Na^{+}, Ca^{2+}) and protein degradation products. The pH of cheese has major influence on the flavor impact of FFA. At the pH of Cheddar (pH ~5.2), a considerable portion of FFA is present as salts, which are nonvolatile, thus reducing

their flavor impact. It is considered that too much of lypolysis is undesirable in Cheddar type cheeses, containing even moderate levels of free fatty acids to be rancid (Singh *et al.*, 2003).

2.4. Processed Cheese

The exact history of processed cheese is as opaque as the product itself, with various countries and companies claiming ownership of the concept, patents and processing methods. While history might be a bit sketchy, processed cheese performance shines brightly in the food industry. These products combine flavor and function with a long shelf life to help the food processing industry find the unique combination of melt, flow, shred and flavor to serve their needs (Turner, 2003). Processed cheese is an extremely versatile food that can be customized with a wide variety of flavor, texture and cooking attributes to appeal to most consumer segments. Because of their long-term stability, tailor-made functionality and availability in convenient sizes and packaging (e.g., as slices precut for sandwiches and hamburgers), processed cheeses in both their cold and heated forms are popular with fast food and other foodservice sectors as well as in the home (Dairy Management Inc., 2004).

Processed cheese represents an extremely delicate and complex system containing a wide variety of interacting components (Marchesseau *et al.*, 1997) including protein, fat, minerals, and water obtained by blending, mixing and heating one or more natural cheeses of different maturity, emulsifying salts and water (Caric *et al.*, 1985). It aids to produce a homogenous blend with desired flavor, color and functional characteristics. While this sounds like a relatively simple process, early attempts at making processed cheese were unsuccessful because heated cheeses tend to oil off, and moisture exudation commonly occurs during cooling and storage.

Two types are available:

(i) Block cheeses; this is a form, slicing cheese with a relatively low moisture (40%) and a high pH (5.7-6.3).

(ii) Cheese spread; this is a soft cheese with higher moisture content (50%) and a lower pH (5.4-5.8).

Numerous factors affect melting and textural characteristics of processed cheese. Many of the factors, which are not well understood, are interrelated and have a combined effect on meltability and texture. The factors that appear to have the most significant impact, however, include the characteristics such as chemical composition and nature of the natural cheese, levels of various types of dairy proteins, heat exposure during processing and cooling, manufacturing procedure and processing conditions used during manufacture, (Caric and

Kalab, 1999) the type and amount of emulsifier incorporated and pH (Marchesseau *et al.*, 1997). Some researchers (Olson *et al.*, 1958) showed that the morphology and firmness of the process cheese network are greatly affected by pH. Marchesseau and Cuq (1995) have previously shown a phenomenon of co-operativity in the various interactions created between protein polymers in the network of process cheese manufactured at a specific temperature. Thus, a basic understanding of the nature of the interactions among process cheese components, as affected by pH and ionic strength, is required to produce a product of good quality.

2.4.1. Critical Processed Cheese Ingredients

2.4.1.1. Initial Cheese Blend:

Cheese is the major ingredient in all processed cheese and significantly influences textural and melt properties. The degree of proteolysis in cheese has a major impact on the textural characteristics of the processed cheese. Aging of cheese decreases the level of intact casein. Model studies have shown that increasing the level of proteolysis (peptides with molecular mass <10kDa) by treatment of curd with an Aspergillus oryzae proteinase increases the softness of the processed cheese. It has long been recognized that block processed cheese with good sliceability and elasticity requires young cheese with 75%-90% intact casein, while medium aged cheese with 60%-75% intact casein works best for spreads. In general, using cheese with increasing amounts of proteolysis decreases firmness and elasticity but increases spreadability and heat-induced flowability in processed cheese. The base cheese provides body, texture and flavor. A proper blend of young and aged cheese affords optimal flavor and texture in the finished product. Excessive amounts of young cheese cause weak cheese flavor; excessive amounts of aged cheese result in poor body (Dairy Management Inc., 2003).

2.4.1.2. Emulsifying Salts:

ES or melting salts are of major importance in processed cheese production where they are used to provide a uniform structure during the melting-cooling processes. Phosphates and citrates are most commonly used at levels in the 1%-3% (w/w) range. These salts generally have a monovalent cation (i.e., sodium) and a polyvalent anion (i.e., phosphate, citrate). While these salts are not emulsifiers, they promote, with the aid of heat and shear, a series of concerted physicochemical changes in the cheese blend that result in rehydration of the aggregated paracasein matrix and its conversion into an active emulsifying agent (Caric and Kalab, 1999). These changes include calcium sequestration, upward adjustment and stabilization (buffering) of pH, paracasein hydration (solvation) and dispersal, emulsification of the free fat and structure formation (Fox *et al.*, 2000).

Five common ES are;

- Trisodium citrate (TSC; $2Na_2C_6H_5O_7$; pH of 1% solution: 8.55)
- Disodium phosphate (DSP; $Na_2 HPO_4.12H_2 O$; pH of 1% solution: 8.9-9.1)
- Trisodium pyrophosphate (TSPP; $Na_3HP_2O_7.9H_2O$; pH of 1% solution:6.7-7.5)
- Sodium acid pyrophosphate (SAPP; $Na_2H_2P_2O_7$; pH of 1% solution: 4.1)
- Sodium hexametaphosphate (SHMP; $(NaPO_3)_n$; pH of 1% solution: 6.0-7.5)

TSC readily binds calcium and, therefore, is commonly used for soft, easily melted cheeses. The use of SHMP (in an ES blend) produces firm, non-melting cheeses. Some research indicates that SHMP binds calcium better than TSC and that this is responsible for the reduced melt when using SHMP. SHMP may also cause increased cross-linking with caseins. Another issue that is not well understood is how much of the added ES dissolve during processing. Processed cheese of very low pH (<5.2) made with some acidic ES produces cheeses that do not melt, but it is unclear if this effect is due to specific ES-protein interactions or to the low pH caused by the addition of the acidic ES. The use of ES blends usually shifts the pH of the cheese from a typical value of 5.0-5.5 to 5.6-5.9 and increases the buffering capacity in the processed cheese. The increase in pH contributes to the formation of a stable product by increasing the calcium sequestering ability of the ES and the negative charge on the para-caseinate.

2.4.1.3. Fat:

Fat, one of the major constituents of milk, contributed to the physical properties of dairy products. The functional properties of milk fat are strongly related to its composition and to the amount and the type of crystals formed at the temperature of the application (Lopez *et al.*, 1995). Milk fat Gives desired composition, texture and meltability characteristics, hence butter, plastic (80% fat) cream, AMF are used as fat sources in processed cheese manufacture. Cream is a colloidal dispersion that displays non-Newtonian behavior (inverse relationship between apparent viscosity and shear rate, or hysteresis) at high fat contents and/or storage temperatures below 40°C (Fox and McSweeney, 1998). An increase in fat level has been found to increase apparent viscosity of cream while increasing temperatures result in a decrease in cream viscosity (Prentice *et al.*, 1999). AMF is produced with fresh cream or butter (Munro, 1998).

2.4.2. Packaging Materials for Processed Cheese:

Tin foil supported by a cardboard carton has been the preferred retail pack for processed cheese but after the Second World War it became prohibitively expensive. The obvious alternative was aluminium but it corroded quickly and suitable protective coatings were long

in coming. A few organizations are also packaging processed cheese in lacquered cans. So, metal foil has remained the preferred packaging material for processed cheese in cartons, while plastics (either in flexible films or rigid containers) have opened up other opportunities for the presentation of processed cheese products (Babu and Goyal, 1989).

2.5. Rheology and Texture of Cheese

Rheology is formally defined as the study of the deformation and flow of materials (Prentice *et al.,* 1999) when subjected to a stress and strain. The rheological properties of cheese are those that determine its response to a stress and strain (e.g., compression, shearing, or cutting) that is applied during processing (e.g., portioning, slicing, shredding or grating) and consumption (e.g., slicing, spreading or mastication and chewing). These properties include intrinsic characteristics such as elasticity, viscosity, and viscoelasticity that are related primarily to the composition, structure and strength of the attractions between the structural elements of the cheese.

The rheological properties of cheese are of considerable importance, since they affect; handling, portioning and packing characteristics, texture eating quality and ability to retain a given shape at a given temperature or when stacked. The rheology of cheese is a function of its composition, microstructure (i.e., the structural arrangement of its components), the physicochemical state of its components and macrostructure. The physicochemical properties include parameters such as the level of fat coalescence, solid fat: liquid fat ratio, degree of hydrolysis and hydration of para-casein matrix and level of intermolecular interaction between para-casein molecules (Fox *et al.,* 2000).

Texture is essentially a human experience arising from our interaction with food-its structure and behavior when it is handled. Texture is one of the main determinants in the quality of many dairy products. Texture is a psychological property, rather than a physical property (Lewis, 1993). Cheese texture may be defined as a composite sensory attribute resulting from a combination of physical properties and perceived by the sense of sight, touch (including kinesthesia and mouth feel) and hearing (Bemnan, 1998). Physical properties may include size, shape, number, nature, and conformation of constituent structural element.

Texture plays an important role in the overall acceptance of a product. Consumers expect certain products to have a particular texture. If the product does not live up to this expectation, then a loss of enjoyment can be experienced. Texture is therefore one of the major criteria used by consumers to assess the quality. It must be remembered that the texture perception of foods is a dynamic process, as the physical properties of the sample are continuously altered by chewing, salivation and body temperature.

According to the Szczesniak (1963) classification texture is sub divided in to three major aspects as mechanical characteristics, geometrical properties and other characteristics. The mechanical characteristics are related to the reaction of food to stress and it could further be separated in to primary parameters (hardness, cohesiveness, viscosity, elasticity and adhesiveness) and secondary parameters (brittleness, chewness and gumminess). Geometrical properties are related to the size, shape and orientation of the particles within the food. The other properties contribute to cheese texture include characteristics such as greasiness, oiliness, succulence and mouth coating associate with the presence of fat and moisture within the cheese.

Consumption of a piece of cheese involves a series of events; Visual assessment creates the first impression about the anticipated taste and texture of the cheese. Then the pressure exerted on the cheese by teeth, tongue and roof of the mouth during eating measures mechanical properties organoleptically. Eating occurs in four phases, i.e., placement in mouth, initial bite by teeth, chewing and mastication and finally swallowing. Table 2.2 explains the vocabulary of texture attributes, their definitions and mastication phases involved in sensory analysis of processed cheese samples. Characteristics such as hardness, brittleness, softness and springiness that are directly related to the intrinsic rheological properties (e.g., elasticity and viscosity) that determine the cheese's response to the stresses applied during biting, chewing, and salivation. Hence, cheese texture and cheese rheology are closely related, in that many of the textural properties are determined by its rheological properties.

Table 2.2. Vocabulary of texture attributes, definitions and mastication phases used in sensory analysis of processed cheese samples

Texture attribute	Definition	Mastication Phase
Firmness	The extent of the initial resistance offered by the cheese, Ranging from 'soft' to 'firm'	Phase 1: Judge on the first chew using front teeth
Rubbery	The extent to which the cheese returns/springs to its initial form after biting. Ranging from 'a little' to 'a lot'	Phase 2: Assessed during the first 2-3 chews
Creamy	The texture associated with cream that has been whipped. Ranging from 'a little' to 'a lot'	---
Chewy	The effort needed to break down the structure of cheese. Ranging from 'a little' to 'a lot'	Phase 3: Judged in the middle phase of mastication
Mouth coating	The extent to which the cheese clings to the inside of the mouth (roof, teeth, tongue, gums). Ranging from a 'a little' to 'a lot'	---
Fragmentable	Breaks down to smaller versions of itself. Ranging from a 'a little' to 'a lot'	Phase 4: probably judge towards the end of the chewing
Melting	The extent to which the cheese melts in mouth. Smooth velvet fullness in mouth. Ranging from 'a little' to 'a lot'	---
Mass formation	The extent to which the cheese forms a bolus or mass in the mouth after chewing. Ranging from 'a little' to 'a lot'	---
Greasy/Oily	The extent to which a greasy/oily residue is deposited in mouth after cheese is broken down. Ranging from 'a little' to 'a lot'	Phase 5: Judge at the end of the chewing sequence

Source: (Blazquez *et al.*, 2006)

2.6. Flavour of Cheese

In general, flavor is considered as a combination of aroma, taste and trigeminal perceptions from stimulation of the mouth and nasal area. Volatile molecules of foods lead to aroma perception. These components are sensed in the roof of the nose, at the nasal cavity during eating. Non-volatile molecules of foods may produce taste perceptions. The third component in flavor forms the activation of trigeminal nerve endings in the oral and nasal areas by volatile and non-volatile substances (Taylor and Linforth, 1996). In general, flavor is often judged as the most important food characteristic and thus, has very strong impact on food preferences and palatability (Schutz and Wahl, 1981).

The flavor profiles of cheeses are complex since it is variety and type specific. The volatile flavor compounds in cheese originate from degradation of the major milk constituents; namely lactose, citrate, milk lipids, and milk proteins (collectively called caseins) during ripening. The physicochemical parameters such as pH, water activity and salt concentration are necessary to direct biochemical reactions in the right direction and in case of deviation of any of these 3 parameters, cheeses could potentially develop texture and/or flavor inconsistencies.

A number of groups in the past have worked on identification of volatile flavor compounds from Cheddar cheese. The list of volatile flavor compounds identified in Cheddar is quite extensive and includes a wide variety of compounds; namely acids, alcohols, esters, aldehydes, ketones, sulfur-containing compounds, phenolics, and so on.

Only some of the compounds formed by glycolysis, lipolysis, and proteolysis directly contribute to cheese flavor; for example, short-chain fatty acids, acetaldehyde diacetyl, peptide, and amino acids. These changes are followed and/or overlapped by a concerted series of secondary catabolic reactions, which are responsible for the unique aroma profile of a particular variety or type of cheese. Products of proteolysis (that is, peptides and free amino acids) probably are significant in cheese taste, at least to "background" flavor and some off-flavors, for example, bitterness, but are unlikely to contribute much to aroma. Compounds arising from the catabolism of free amino acids contribute directly to cheese taste and aroma.

Texture sensation does not merely occur as a response to teeth, isolated from other stimuli, since taste, aroma and texture interact with each other during eating and they both affect food acceptability. It is often stated that flavor is more important than texture for overall acceptability of foods, but this is not the case in all food types or in all eating situations (Singh *et al.*, 2003).

2.7. Analytical Methods for Cheese

Cheese is subjected to chemical analysis for various reasons, such as to ascertain its composition for nutritional purposes, to ensure its compliance with standards of identity, to assess the efficiency of production or as index of quality (McSweeney and Fox 1999).

2.7.1. Methods for Compositional Analysis

Gross compositional analysis of cheese is conducted in accordance with standards methods published by the International dairy federation (IDF) and Association of Official Analytical Chemists (AOAC). Standard methods for moisture, ash, protein, fat, acidity and anion analysis are listed in table 2.3. (McSweeney and Fox, 1999). But there is no standard method for determination of pH (Fox *et al.*, 2000).

Table 2.3. Standard methods for compositional analysis of cheese

Constituent	Method
Total Solids	AOAC 926.08, AOAC 969.19 (1990)
Ash	AOAC 935.42 (1990)
Fat	AOAC 933.05 (1990)
Protein (Total)	AOAC 920.123 (1990)
Chloride	AOAC 935.43, AOAC 983.14 (1990)
Salt (NaCl)	AOAC 975.20 (1990)
Citrate	AOAC 976.15 (1990)
Nitrate/Nitrite	AOAC 976.14 (1990)
Acidity	AOAC 920.124 (1990)

Source: (McSweeney and Fox 1999)

2.7.2. Assessment of Extent of Proteolysis

Proteolysis is routinely monitored in studies on cheese ripening and is a useful index of maturity and quality. Variety of methods may be used, and falls into two general categories: Specific and non-specific. The latter include determination of nitrogen soluble or extractable by one of a number of solvents or precipitants. (e.g., water, pH 4.6 buffers, NaCl, ethanol, trichloroacetic acid) or permeable through ultra filtration membranes and quantified by any of several methods (e.g., Kjeldhal, Biuret, Lowry, Hull, absorbance at 280 nm) or by the formation of a reactive α-amino groups quantified by reaction with one of several reagents (e.g., trinitrobenzene sulphonic acid [TBNS], O-phthaldialdehyde [OPA], Cd-ninhydrin, Li-ninhydrin). Such methods are valuable for assessing the overall extent of proteolysis and the general contribution of each proteolytic agent. Non-specific techniques are normally relatively straightforward and some are suitable for assessment of ripening. However more

information about proteolysis is provided by techniques that resolve individual peptides (e.g., electrophoresis and chromatography) (Fox *et al.*, 2000).

2.7.3. Characterization of Proteolytic Pattern of Cheese

Electrophoresis is a technique used to separate and sometimes purify macromolecules in an electric field especially, proteins and nucleic acids that differ in size, charge and conformation. Proteins and nucleic acids are subjected to electrophoresis within a matrix or "gel'. Most commonly, the gel is cast in the shape of a thin slab, with wells for loading the sample. The gel is immersed in an electrophoresis buffer that provides ions to carry a current and some type of buffer to maintain the pH at a constant value (Sambrook *et al.*, 1989).

Electrophoresis has been applied widely to study primary proteolysis in cheese. Since only proteins and large peptides can be visualized by staining, the technique is limited to the assessment of casein loss and the formation and subsequent hydrolysis of primary products of casein proteolysis. In addition the difficulty of easy quantification of elctrophoretogram is also a serious limitation. However it is a powerful technique for studying proteolysis during the early stages of cheese ripening.

PAGE shows proteolytic pattern as well as its extent. Especially alkaline Urea-PAGE is used for cheese analysis and it has been concluded that SDS-PAGE and isoelectric focusing is inferior. In recent years, capillary electrophoresis is being applied increasingly to the analysis of peptides in cheese and has given very satisfactory quantifiable results.

2.7.3.1. SDS-PAGE: An Overview

Almost all analytical electrophoresis of proteins is carried out in discontinuous polyacrylamide gels as a support medium under conditions that ensure dissociation of the proteins into their individual polypeptide subunits and that minimize aggregation. In SDS-polyacrylamide gel electrophoresis, sodium dodecyl sulfate also called lauryl sulfate (SDS) is included in the matrix, buffer and samples.

Polyacrylamide gels are composed of chains of polymerized acrylamide that are cross-linked by a bifunctional agent such as N, N'-methylenebisacrylamide. The effective range of separation of SDS-polyacrylamide gels depends on the concentration of polyacrylamide used to cast the gel and on the amount of cross-linking. Polymerization of acrylamide in the absence of cross-linking agents generates viscous solutions since acrylamide alone forms linear polymers that are of no practical use. Cross-links formed from bisacrylamide add rigidity and tensile strength to the gel and form pores through which the SDS-polypeptide complexes must pass. The size of these pores decreases as the bisacrylamide: acrylamide ratio

increases, reaching a minimum when the ratio is ≈1:20. Most SDS-polyacrylamide gels are cast with a molar ratio of bisacrylamide: acrylamide of 1: 29, which has been shown empirically to be capable of resolving polypeptides that in size by as little as 3%.
The sieving properties of the gel are determined by the size of the pores, which is a function of the absolute concentrations of acrylamide and bisacrylamide used to cast the gel. Table 2.4 shows the linear range of separation obtained with gels cast with concentrations of acrylamide that range from 55 to 15%. Polymerization of acrylamide and bisacrylamide monomers is induced by ammonium persulphate (APS), which spontaneously decomposes to form free radicals. Tetramethylethylenediamine [TEMED $(CH_3)_2N\text{-}CH_2\text{-}CH_2\text{-}N(CH_3)_2$] a free radical stabilizer is generally included to promote polymerization (David, 1996).

SDS is an ionic detergent with strongly anionic head group and a lipophilic tail. It binds non-covalently to proteins with a stoichiometry of around one SDS molecule per two amino acids. SDS is used in combination with a reducing agent and heat to denature and dissociate the proteins before they are loaded on gel. The denatured SDS bound polypeptides become negatively charged. The negative charges on SDS mask the intrinsic charge of proteins, destroy most of the complex structure of proteins and are strongly attracted toward an anode (positively-charged electrode) in an electric field)

Table 2.4. Effective range of separation of SDS-polyacrylamide gels

Acrylamide[a] concentration (%)	Linear range of separation (kD)
15	12-43
10	16-68
7.5	36-94
5.0	57-212

[a] Molar ratio of bisacrylamide: acrylamide is 1: 29,

In most cases, SDS-polyacrylamide gel electrophoresis is carried out with a discontinuous buffer system in which the buffer in the reservoirs is of a different pH and ionic strength from the buffer used to cast the gel. The SDS-polypeptide complexes in the sample that is applied to the gel are swept along by a moving boundary creates when an electric current is passed between electrodes. After migrating through a stacking gel of high porosity, the complexes are deposited in a very thin zone (or stack) on the surface of the resolving gel. The ability of discontinuous buffer systems to concentrate all of the complexes in the sample into a very small volume greatly increases the resolution of SDS-polyacrylamide gels.

The sample and the stacking gel contain Tris.Cl (pH 6.8), the upper and lower buffer reservoirs contain Tris-glycine (pH8.3), and the resolving gel contains Tris.Cl (pH 8.8). All components of the system contain 0.1% SDS. The chloride ions in the sample and stacking gel form the leading edge of the moving boundary, and the trailing edge is composed of glycine molecule. Between the leading and trailing edges of the moving boundary is a zone of lower conductivity and steeper voltage gradient which sweeps the polypeptides from the sample and deposited them on the surface of the resolving gel. There, the higher pH of the resolving gel favors the ionization of glycine, and the resulting glycine ions migrate through the stacked polypeptides and travel through the resolving gel immediately behind the chloride ions. Freed from the moving boundary, the SDS-polypeptide complexes move through the resolving gel in a zone of uniform voltage and pH and are separated according to size.

SDS treated proteins have very similar charge-to-ratio, and similar shapes. Because the amount of SDS bound is almost always proportional to the relative molecular mass of the polypeptide and is independent of its sequence, SDS-polypeptide complexes migrate through the polyacrylamide gels in accordance with the size of the polypeptide. At saturation, approximately 1.4g of detergent are bound for 1g of polypeptide. By using the markers of known molecular mass, it is therefore possible to estimate the molecular mass of the polypeptide chain(s). In a gel of uniform density the relative migration distance of a protein (R_f) is negatively proportional to the log of its mass. If proteins of known mass are run simultaneously with the unknowns, the relationship between R_f and mass can be plotted, and the masses of unknown proteins estimated.

Further more SDS-PAGE can be used to determine the relative abundance of major proteins in a sample, and to determine the distribution of proteins among fractions. The purity of protein samples can be assessed and the progress of a fractionation or purification procedure can be followed. Different staining methods can be used to detect rare proteins and to learn something about their biochemical properties (Sambrook *et al.*, 1989).

Many systems for protein electrophoresis have been developed, and apparatus used for SDS-PAGE varies widely. Regardless of the system, preparation requires casting two different layers of acrylamide between glass plates. The lower layer (separating, or resolving, gel) is responsible for actually separating polypeptides by size. The upper layer (stacking gel) includes the sample wells. It is designed to sweep up proteins in a sample between two moving boundaries so that they are compressed (stacked) into micrometer thin layers when they reach the separating gel (David, 1996).

2.8. Sensory Evaluation of Cheese

2.8.1. Sensory Evaluation: A Brief Review

Sensory analysis is the identification, scientific measurements, analysis and interpretation of the properties (attributes) of a product as the y are perceived through the five senses of sight, smell, taste, touch and hearing. Sensory analysis answers questions of quality under three main headings-discrimination, description and preference. In discrimination questions aim to find out whether or not a difference exist between two or more products. In description, questions aim to describe and measure any difference that is found to exist between products. In preference, hedonic questions aim to identify liking or acceptability (Roland *et al.,* 2000).

The organoleptic qualities of processed cheese are traditionally assessed by sensory evaluation of flavour, body, texture, finish and overall acceptability by experienced judges or trained consumer panelists. However sensory analysis is subjective and although at present the best index of consumer acceptability, they provide data that are difficult to evaluate scientifically. Therefore instrumental analysis of cheese is carried out hence it enables objective, faster and less expensive assessment of quality, usually to complement sensory evaluation (Blazquez *et al.*, 2006).

2.8.2. Statistical Aspects of Sensory Evaluation

In planning sensory experiments, experimental design is of paramount importance, because it is need to control or minimize the potential sources of variability associated with the preparation of the test product, measurements and assessment process, including factors such as order effect, carry-over effect and assessor's fatigue. Choosing a statistical method and statistical package for analyzing data of a sensory evaluation is not an easy task, as there are so many available, but needs to critically determine before embarking on the sensory test.

The hedonic scales used for the collection of consumer liking data are usually ordinal scales with category descriptions of the form 'like extremely'. As a general rule of thumb, data collected from a trained sensory panel can be analyzed using parametric methods. Conversely, for the analysis of consumer data, it has normally recommended from a statistical point of view that non-parametric methods be used. In practice, where large numbers of consumers are used to provide the data, parametric analysis of variance is often used (Roland *et al.,* 2000).

CHAPTER 03

MATERIALS AND METHODOLOGY

3.1. Formulation of a Newer Processed Cheese Recipe

Materials:

Natural cheese reels of different maturity
Butter (80% fat)
Fresh Cream (72% fat)
Ghee (98% fat)
Joha C (Orthophosphate/ E 339)
Joha T (Polyphosphate/ E 452)
Trisodium citrate (E331)
Table salt
Citric acid (E 330)
Sodium benzoate (E 211)
Potassium sorbate (E 202)
Food colour [Annatto 160(b)]
Water
Sensory Evaluation ballot papers

Equipments:

Cheese shredder
Top loading balance (weighing up to 5.000 Kg, Accuracy = 0.001)
Cheese kettle
Vacuum packaging machine
Homogenizer
Refrigerator (maintained at 10 ± 1^0 C)
Trays with specific dimensions (molds)
Cheese knife (Stainless steel)

Methodology:

In order to determine the effect of changing critical ingredients on the processed cheese functionality, cheeses were manufactured in three different approaches at the MILCO pvt. Ltd, Digana. In first approach designed compositional variation was in cheese blend where as in approach 2, 3 fat source and emulsifying salt were varied.

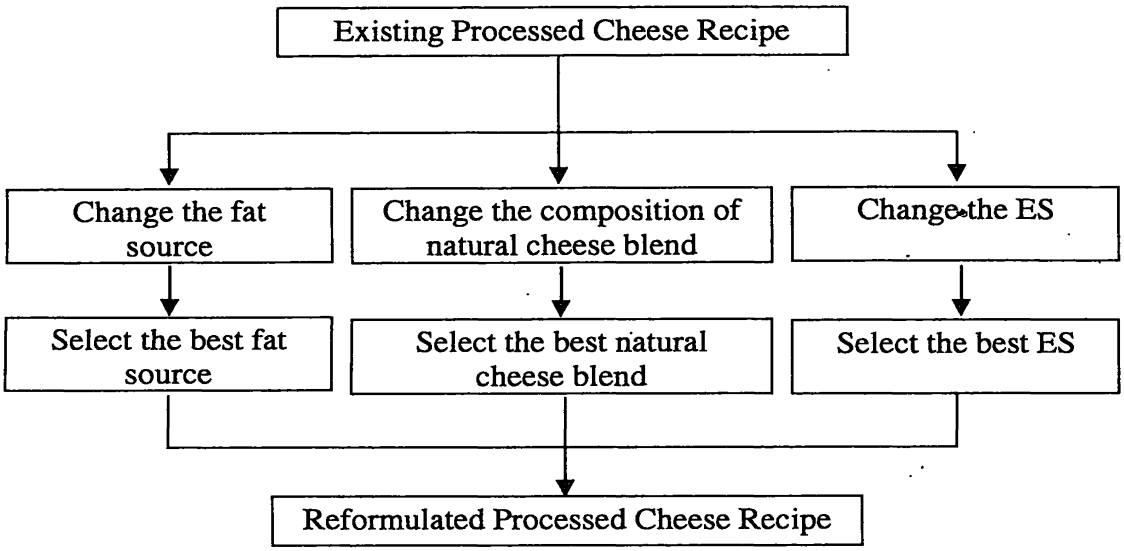

Fig 3.1. Outline of reformulation scheme of existing recipe

3.1.1. Selection of the Most Appropriate Blend of Natural Cheese:

Natural cheese (cheddar) reels of different maturity (young, mild, and matured) were taken out of the ripening rooms, scraped off the wax coating along with fungal mycelia and milled in the cheese shredder. Shredded cheese of different maturity were weighted and mixed in varying proportions as illustrated in table 3.1. Referring to the existing recipe (Table 3.2) all the other processed cheese ingredients were mixed equally with each of the above natural cheese blend in a cheese kettle and pasteurized at 90^0C for 4 minutes. Then mixture was poured in to cleaned trays of specific dimensions (1.5ft×1.0ft×0.3ft) and kept at 10^0C for 8 hours. After it gets harden, large processed cheese blocks were cut into 200g small blocks and vacuum packed.

Prior to sensory assessment each cheese was cut into 5g cubes and equilibrated to room temperature (28 ^{0}C). Then 12 cheese samples were presented in covered dishes, labeling with a randomly selected 3-digit code. Cheeses were scored for overall acceptability on 9-point hedonic scale using 30 untrained panel, where twelve samples were presented in 4 sessions (i.e., three at a time) under the same conditions in order to minimize the sensory fatigue. Data was statistically analyzed with one-way ANOVA using MINITAB statistical analysis package

(version 14.1). Magnitudes of main effects were compared using main effects plots and presence of interaction was judged using interaction plot.

Table 3.1. Different blends of natural cheeses

Sample Code	Young (10 days)	Mild (3month)	Matured (4/5 month)
252	3.0 Kg	1.5 Kg	2.8 Kg
798	3.0 Kg	1.0 Kg	3.3 Kg
366	3.0 Kg	0.5 Kg	3.8 Kg
904	2.5 Kg	2.0 Kg	2.8 Kg
813	2.5 Kg	1.5 Kg	3.3 Kg
707	2.5 Kg	1.0 Kg	3.8 Kg
272	2.0 Kg	2.5 Kg	2.8 Kg
258	2.0 Kg	2.0 Kg	3.3 Kg
952	2.0 Kg	1.5 Kg	3.8 Kg
907	1.5 Kg	3.0 Kg	2.8 Kg
861	1.5 Kg	2.5 Kg	3.3 Kg
824	1.5Kg	2.0 Kg	3.8 Kg

Table 3.2. Existing processed cheese recipe

Ingredients	Quantity (g)
Cheddar Cheese Blend**	7300.0
Butter	300.0
Joha C (Orthophosphate/ E 339)	160.0
Joha T (Polyphosphate/ E 452)	40.0
Table Salt	16.0
Citric Acid (E 330)	11.0
Food Colour [Annatto/ E 160 (b)]	0.8
Potassium Sorbate (E 202)	3.0
Sodium Benzoate (E 212)	0.8
Any other/water added	668.4
Total	8500.0

** Cheddar cheese blend comprised of young, mil and matured cheese in the ratio of 3: 1:1

3.1.2. Selection of the Most Appropriate Fat Source:

Four different processed cheese samples were prepared according to the existing recipe as described in previous section (3.1.1), but with different fat sources. The amount of each fat

source calculated according to the Pearson-square method and amount of each incorporated in the recipes are shown in table 3.3. Then 4 samples were prepared for sensory evaluation and sensed for overall acceptability with 30 untrained panel and data was statistically analyzed as described in previous section.

Table 3.3. Quantity of each fat source incorporated in different formulae

Sample Code	Fat source	Quantity (per 7300 g cheese blend)
305	Butter (80% fat)	300g
547	Fresh cream (72% fat)	350g
592	Homogenized fresh cream (72% fat)	350g
617	Ghee (98% fat)	250g

3.1.3. Selection of the Most Appropriate Emulsifying Salt:

Three processed cheese samples were prepared according to the existing recipe as descried in previous section (3.1.1), changing only the emulsifying salt. Then samples were prepared for sensory evaluation and sensed for overall acceptability with 30 untrained consumer panel and data was statistically analyzed as described in previous section.

Table 3.4. Quantity of each ES/ES blend incorporated in different formulae

Sample Code	ES/ Combination of ES	Quantity (per 7300 g cheese blend)
192	JohaC/JohaT	160: 40 g
622	Joha C/Trisodium citrate	100:100 g
429	Sodium citrate	200g

3.1.4. Reformulation of the Existing Recipe:

After determination of the most appropriate natural cheese blend, fat source and ES, four different processed cheese recipes were formulated, incorporating them in the existing recipe, as illustrated in table 3.5. Thereupon cheese samples were prepared according to the each recipe as described in section 3.1.1.

Samples that were prepared according to the Formula1, Formula2, Formula3 and Formula 4 were coded in three digit numbers as 456, 218, 552, 707 respectively and sensed for their acceptability in terms of flavour, texture, colour and overall acceptability against the company sample (sample code 537) and leading market sample (292). Preparation of the samples for sensory evaluation and statistical analysis were carried out in the same manner as explained in

section 3.1.1. To check whether there is an improvement, sensory data refers to newly developed formulae were analyzed along with sensory rating scores of existing cheese.

Table 3.5. Reformulated Recipes with selected ingredients

Ingredients	Formula 1	Formula 2	Formula 3	Formula 4
Cheddar Cheese (1.5:2.0:3.8)	7300.0 g	7300.0 g	7300.0 g	7300.0 g
Fresh Cream (homogenized)	350.0 g	400.0 g	450.0 g	500.0 g
Joha C/Trisodium Citrate (1:1)	200.0 g	250.0 g	300.0 g	350.0 g
Table Salt	16.0 g	14.0 g	12.0 g	10.0 g
Citric Acid	11.0 g	11.0 g	11.0 g	11.0 g
Food Colour (Annatto)	0.8 g	0.8 g	0.8 g	0.8 g
Potassium Sorbate	3.0 g	3.0 g	3.0 g	3.0 g
Sodium Benzoate	0.8 g	0.8 g	0.8 g	0.8 g

3.2. Gross Compositional Analysis

The best sample selected from the above activities was analyzed for moisture, fat, protein, salt and acidity and conformance to SLS standard was evaluated.

3.2.1. Determination of Moisture and Total Solids (Air-Oven Method)

Apparatus:

Moisture dishes made of Aluminum
Oven maintained at 105±1^{0}C
Analytical balance (Weighing up to 220.0000g, Accuracy .0001)
Desiccator

Methodology:

Pre-dried 3 aluminum dishes were weighted to the nearest 0.1 mg on an analytical balance. Then 3-5 g of fragmented cheese was weighted quickly into the each aluminum dishes and kept at 105±1^{0}C for 4 hours. Thereafter samples were cooled in a desiccator and total dry weight was determined. Again, samples were kept for an additional 20 minutes inside the oven and reweighed. This was repeated several times until constant weight was attained. Once the difference between the two consecutive readings after the additional drying period was less than 1 mg, it was recorded as the final reading. (Kirk and Sawyer, 1991) Then total solids and moisture contents were recorded on weight percent basis as follows:

$$\text{Total Solids} = \frac{(\text{Initial Weight} - \text{Final Weight})}{\text{Initial Weight}} \times 100\%$$

$$\text{Moisture} = (100 - \text{Total Solids})\%$$

3.2.2. Determination of Fat (Gerber Method)

Materials:

Gerber sulphuric acid (90-91% w/v)

Amyl alcohol

Apparatus:

Cheese butyrometer

Analytical balance (Weighing up to 220.0000g, Accuracy .0001)

Water bath (maintained at 65^0C)

Weighing funnel

Methodology:

About 10ml of Gerber sulphuric was added to the butyrometer, followed by 6mm layer of warm distilled water (30-40^0C). 3g from the well mixed sample was weighted into a weighing funnel, cut into small pieces and transferred carefully into the butyrometer. Then 1ml of amyl alcohol and sufficient warm distilled water were added so that the butyrometer was filled to the shoulder below the neck. After fitting the stopper tube was shaken, inverted and placed in a water bath at 65^0C for 3 minutes. Then tube was centrifuged at 1100 rpm for about 5 minutes. Finally tube was returned to the water bath and after 5 minutes % of fat was read directly on the scale. This was triplicated (Pomeranze and Meloan, 1996).

3.2.3. Determination of Crude Protein (Micro Kjeldhal Method)

Materials: –

0.02M HCl

Conc. H_2SO_4

30% NaOH

4% Boric acid

Catalyst tablets (Selenium)

Kjeldhal indicator (Methyl red - methylene blue mixture)

Diethyl ether

Apparatus:

Kjeldhal Digestion unit

Kjeldhal distillation unit

Analytical balance

Common labouratory glasswares

Methodology:

0.5g of sample, 1g of catalyst mixture ($K_2SO_4.SeSO_4$) and 25ml of Conc. H_2SO_4 were added in to a Kjeldhal flask and placed in the micro Kjeldhal unit for 6 hours. After the digestion was completed, the flask was allowed to cool and the contents were transferred to a 100 ml volumetric flask using distilled water. Distillation unit was set up. 10ml of 4% boric acid was taken into a titration flask and 3 drops of indicator was added to it. Steam generator was fixed to the mouth of the distillation unit while other end that emits the gas out, was dipped in the titration flask that contains boric acid. Then 5ml of the diluted sample was added in to the distillation unit. 10% NaOH was added when contents get started to boiling. Steaming was continued until dark blue colour turns greenish and finally trapped ammonia was determined by titrating it with 0.02N HCl using phenolphthalein as the indicator. The amount of acid consumed was recorded. Triplicates and one blank was run (Chang, 1998).

$$\text{Nitrogen \%} = \frac{(\text{Sample titre - Blank titre}) * N_{HCl} * 14 * V_D * 100}{\text{Aliquot of the digestion taken} * \text{Wt. of the sample} * 1000}$$

$$\text{Protein \%} = \text{Nitrogen \%} * 6.38$$

Where;

N_{HCl} = Normality of HCl

V_D = Volume made up of the digestion

Wt = Weight

3.2.4. Determination of Salt

Materials:

$AgNO_3$ (0.05 mol/ℓ)

Conc. HNO_3 (Rela.den 1.42)

Urea

Nitrobenzene

Ammonium ferric sulphate $[(NH_4)_2SO_4.Fe_2(SO_4)_3.2\ H_2O]$

Potassium thiocyanate (0.05 mol/ℓ)

Methodology:

Cheese sample was grated and cut into small pieces quickly. About 2g of the sample was weighed (nearest 0.01g) in to an Erlenmeyer flask. 10ml of water and 25ml of $AgNO_3$ solution were added. Contents of the flask were warmed up to 75^0C-80^0C, swirling vigorously to facilitate the dispersion of the sample. Then 10 ml of Conc. HNO_3 was added and gently boiled for about 10 minutes. About 0.3g of urea was added to the hot solution, mixed and cooled. Then 1ml of nitrobenzene was added. About 2ml of ammonium ferric sulphate indicator solution and 50ml of water were added and determined the excess silver nitrate by titrating with potassium thiocyanate solution, until the appearance of an orange tint, which persists for 30 seconds. Then 25ml of silver nitrate solution was tracked, 2ml of ammonium ferric sulphate indicator was added and titrated with potassium thiocyanate solution until the same end point was reached as in previous case. (1ml of 0.5mol/l potassium thiocyanate is equivalent to 0.00292 NaCl.) (SLS 735: 1998)

$$\text{Salt content (as NaCl \% by mass)} = \frac{0.292 - (V_1 - V_2)\,C}{0.5\ m} \times 100\%$$

Where;

V_1 = Volume in ml of potassium thiocyanate required for the first titration of excess $AgNO_3$

V_2 = Volume in ml of potassium thiocyanate required for the second titration of $AgNO_3$

C = Concentration in mol/l of potassium thiocyanate

m = mass in grams of the sample taken for the test

3.2.5. Determination of Acidity

Materials:

0.1M NaOH

1% phenolphthalein

Labouratory glasswares

Methodology:

About 20 g of cheese sample was macerated with warm distilled water (at 40^0C) to produce a total volume of 250ml and filtered. 25ml of filtrate was titrated with 0.1M NaoH, using 1ml phenolphthalein as an indicator and acidity was calculated as lactic acid (Pomeranze and Meloan, 1996).

[1ml of 0.1M = 0.09 g lactic acid]

3.3. Prevention of Rapid Drying Off and Disappearance of Sharp Edges

3.3.1. Determination of ERH/ Water Activity of Processed Cheese

Several portions of the cheese samples (2.5g) were placed inside series of small test tubes. A series of hygroscopic salts (table 3.6.) with desired range of RH were selected and single crystal of each (pin-head size) were placed inside the tubes out of contact with food stuff and maintained the temperature at 25^{0}C. The ERH of the food sample was obtained as a range by observing the last dry crystal and first wet crystal in the series of salts (Smith, 1971).

Table3.6. Relative humidity of saturated salt solutions at 25^{0}C

Hygroscopic Salt	RH % at 25^{0}C
NaCl	75
$(NH_4)_2SO_4$	79
KBr	83
KCl	86
$K_2Cr_2O_7$	87
$BaCl_2$	90
KNO_3	93
K_2SO_4	97

(**Source:** Smith, 1971)

3.3.2. Selection of the Most Suitable Packaging Material

Packaging materials with different physical properties that demanded by high moisture foods [i.e., Al foil, Metalized Al foils, Laminated Al (LDPE-Al-LDPE)] were selected and chemically sterilized by keeping them in 200ppm chlorine solution for 3 minutes. Thereafter, the packages were air dried. Processed cheese blocks (200g) were packed in to the selected packaging materials alone and in combination with paper cartons. Al foils were wrapped tightly and further tighten by pressing. Metalized Al-packs were vacuum packed and laminated Al foil-packs were heat sealed. All of them were kept at ambient temperature and at refrigerated conditions along with the samples packed in nylon packs. Finally they were comparatively assessed by their external appearance over 30 days (Babu and Goyal, 1989).

3.4. Evaluation of Natural Cheese in Respect of Texture and Flavour

3.4.1. Chemical and Physical Influences of Cheese Ripening

Moisture, fat, salt and acidity of natural cheddar cheese were determined in the same manner as described in previous sections 3.4.1, 3.4.2, 3.2.4 and 3.4.5 respectively and pH was determined in the following manner.

Apparatus:

Mortar and pestle
pH meter

Methodology:

About 10g of cheese sample was thoroughly blended with 10ml of distilled water using a mortar and pestle, and the pH of the resulted slurry was measured potentiometrically (SLS 773:1987).

3.4.2. Assessment of Extent of Proteolysis – Dye Binding Method (Chang, 1998).

Materials:

Orange G dye (dye content 80% w/w, MW 452.37, $C_{16}H_{10}N_2Na_2O_7S_2$)
Standard protein (90% protein w/w)
Cheese samples at different maturity
pH 2.2 buffer (0.2M KCl: 0.2MHCl= 50:7.8)

Apparatus:

Spectrophotometer
Analytical balance (Weighing up to 220.0000g)
Centrifuge and centrifuge tubes
Glass crucibles (G3)
Common labouratory glass wares

Methodology:

Preparation of Dye Solution:

Orange G (0.5mM) dye solution was prepared by dissolving 0.2827g of dye in 1litre of pH 2.2 buffer and absorbance was measured spectrophotometrically at the 478nm.

Sample Preparation and Absorbance Measurement:

Representative natural cheese (cheddar) samples at different maturity (i.e. 0, 15, 30,60,75,90,120 days) were obtained using cheese trier. Then each and every sample were finely ground in to 60 mesh or smaller sizes and mixed well. About 0.4g of each was weighted to the nearest 0.01g in to centrifuge tubes and excess dye (10ml) was added to each. The content was shaken vigorously to equilibrate the dye binding reactions centrifuged for 5 minutes and filtered through glass crucibles. Absorbance of the unbound dye solution in the filtrate was measured at 478nm. This experiment was performed three times (n=3).

Construction of the Standard Curve:

About 0.025, 0.050, 0.075, 0.10, 0.125, 0.150, 0.175g and 0.200g protein samples (casein) were weighted in to centrifuged tubes and excess dye (10ml) were added to each. The content was shaken vigorously to equilibrate the dye binding reactions centrifuged for 5 minutes and filtered through glass crucibles. Absorbance of the unbound dye solution in the filtrate was measured at 478nm of wave length. This experiment was performed three times. Referring to the purity (w/w) figures, protein content of each casein sample was calculated. Regression analysis was preformed using MINITAB statistical analysis package (version 14.1), for protein content and their corresponding absorbance figures and fitted line plot was obtained.

Finally (unhydrolysed/intact) casein content of the unknown sample was estimated using regression equation calculated by the least squares method. Based on the protein content of cheese samples, percentage proteolysis during ripening period was calculated and plotted against time. In order to calculate the relative casein index, total N of cheese was determined using Kjeldhal method, and total casein content (content of unhydrolyzed protein x 93/100) was divided by the factor 6.38 in order to obtain the total casein N.

3.4.3. Characterization of Proteolytic Pattern of Cheese (Denise, 1998)

Materials: –

Acetone AR (M.W. 58.08, Assay min 99.5%)
Diethyl Ether AR (M.W. 74.12, Assay 99.5%, Stabilized with 1-2% ethanol)
30.8% Acrylamide/Bisacrylamide stock solution (30% acrylamide, 0.8%BIS)
1.5M Tris/ HC l/pH 8.80 buffer (Resolving gel buffer)
1 M Tris/ HC l/pH 6.80 buffer (Stacking gel buffer)
10% (w/v) SDS (Sodium Dodecyl Sulphate) solution
Freshly prepared 10% (w/v) APS (Ammonium per Sulphate) solution

TEMED (N, N, N', N'- tetramethylethylenediamine)

Tris base (0.5 M-pH 6.8, 0.25M)

Glycine (2.5M-pH 8.3)

10% glycerol (10%)

2% mercaptoethanol

1% bromophenol blue

Water saturated n-butanol

Methanol AR

Glacial Acetic acid

Commossie brilliant blue R- 250

Protein standard (with molecular markers of 10,15,20,25,37,50,75,100 kD)

Apparatus:

Electrical balance (ADP 720/L, Max 500.00g)

Autovotex

Micro centrifuge (15 000 rpm max.)

Gel electrophoresis mini gel system (500V, 250mA max)

Gel electrophoresis power supply unit (400mA, 400V Max)

Micro centrifuge tubes

Micro pipits (0-1000µl) and pipeiter tips (0-200µl , 200-1000µl)

Blunt hypodermic needle

Humiltan microliter syringe

Surgical knife

Slowly rotaing plat form

Labouratory glasswares

Methodology:

Preparation of Samples:

Cheese samples at different maturity (1 day, 1, 2, 3 and 4 months) were chopped finely with a knife and 50 µg of each was weighted in to micro centrifuge tubes. All cheeses were then extracted three times with 1ml of acetone to remove fat and water. Wet cheese SNF was rinsed once with diethyl ether and air-dried (Hekken and Thompson, 1992).

Preparation of Gel Loading Buffer/ Sample Buffer:

A volume of 4ml of 5×gel loading buffer was prepared by mixing appropriate concentration of following components and then 2ml of 1×gel loading buffer was prepared (Table 3.7).

Preparation of the Resolving Gel:

Resolving gel was prepared by using appropriate volume of solution containing the desired concentration of acrylamide according to the table 3.8. Components were mixed in the order shown. Without delay, mixture was swirl rapidly and proceeded to the next step.

Preparation of the Staking Gel:

Stacking gel was prepared by using appropriate volume of solution containing the desired concentration of acrylamide, according to the table 3.9 and components were mixed in the order shown. Without delay, mixture was swirl rapidly and proceeded to the next step.

Preparation of the Electrolytic Buffer:

A volume of 400ml of 5 × stock solutions was prepared by mixing appropriate concentration of following components and then 2 liters of 1×working solution was prepared (Table 3.10).

Table 3.7. Solutions for preparing 5×gel loading buffer

Component	**Component Volumes for 4 ml**
0.5 Tris/ pH 6.8 buffer	0.5 ml
Water	1.9 ml
Glycerol	1.6 ml
10% SDS	0.8 ml
2-mercapto ethanol	0.2 ml
1% Bromophenol blue	0.2 ml

(**Source:** Sambrook *et al.*, 1989)

Table 3.8. Solutions for preparing resolving gel for Tris-glycine SDS-PAGE

Solution Component	**Component Volumes for 10 ml of 12%**
Water	3.3 ml
30% Acrylamide mix	4.0 ml
1.5M Tris (pH 8.8)	2.5 ml
10% SDS	0.1 ml
10% Ammonium persulphate	0.1 ml
TEMED	4 µl

(**Source:** Sambrook *et al.*, 1989)

Table 3.9. Solutions for preparing stacking gel for Tris-glycine SDS-PAGE

Solution Component	Component Volumes for 4 ml of 5% gel
Water	2.70 ml
30% Acrylamide mix	0.67 ml
1.5M Tris (pH 8.8)	0.50 ml
10% SDS	40 µl
10% Ammonium persulphate	90 µl
TEMED	6 µl

(**Source:** Sambrook *et al.*, 1989)

Table 3.10. Solutions for preparing electrolytic buffer

Solution Component	Component Volumes for 400 ml of 5% gel
25 mM Tris base	6.04 g
250 mM Glycine (pH 8.3)	37.6 g
0.1% SDS	2.0 g

(**Source:** Sambrook *et al.*, 1989)

Procedure of SDS-Polyacrylamide Gel:

(01). The gel casting unit was assembled according to the manufacturer's instructions.Then prepared acrylamide solution was poured in to the gap between the glass plates, leaving sufficient space for the stacking gel. The acrylamide solution was overlaid carefully with water saturated n-butanol using a Pasteur pipette. Then gel was placed in a vertical position at room temperature.

(02). After polymerization was completed (~30 minutes), the overlay were poured off and washed the top of the gel several times with deionized water to remove any unpolymerized acrylamide. Fluid from the top of the gel was drained as much as possible, and then any remaining water with the edge of a paper towel was removed.

(03). Prepared stacking gel solution was poured directly onto the surface of the polymerized resolving gel and immediately a clean Teflon comb was inserted, being careful to avoid trapping air bubbles. More stacking gel solution was added to fill the spaces of the comb completely. The gel was placed in a vertical position at room temperature.

(04). While the staking gel is polymerized, samples were prepared by heating them to 95^{0}C for 5 minutes in 1 x SDS gel-loading buffer to denature the proteins. Then Mixture was votexed, centrifuged and kept in ice.

(05). After polymerization was complete (30 minutes), Teflon comb was removed carefully. Using a squirt bottle, wells were washed immediately with deionaized water to remove any unpolymerized acrylamide and straightened the teeth of the stacking gel with a blunt hypodermic needle attached to a syringe.

(06). The gel was mounted in the electrophoresis apparatus and Tris-glycine electrophoresis buffer was added to the top and bottom reservoir.

(07). About 2.5 µl and 5 µl volume from each of the samples and molecular marker were loaded into the bottom of the wells in the order as illustrated in the figure 3.1. An equal volume of 1 x SDS gel-loading buffer was loaded into any wells that are unused.

(08). Electrophorsis apparatus were connected to an electric power supply and voltage of 60V/cm was applied to the gel.

(09). The gel was run until the bromophenol blue reaches the bottom of the resolving gel (about 4 hours). Then the power supply was turned off. Glass plates were removed from the electrophoresis apparatus and orientation of the gel was marked by cutting a bottom corner of the gel that is closest to the left most well.

(10). The gel was slightly washed with distilled water and placed immersed in a Coomassie Brilliant Blue solution over night on a slowly rotating flat form for staining. Then gel was removed from the staining solution and immersed in the destaining solution until excess stain was washed off from the gel and bands became visible.

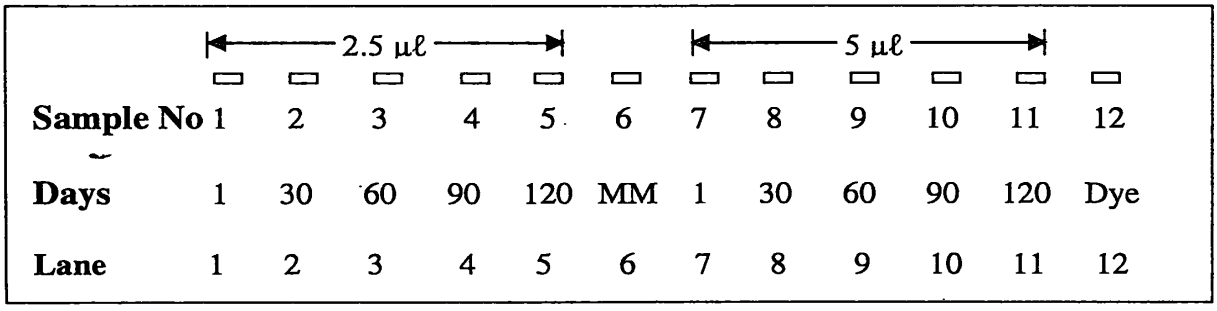

Fig. 3.2. Order of loading samples to the SDS-PAGE

(11). Referring to the distances, traveled by molecular markers calibration curve was constructed and molecular masses of casein fragments that representing distinct bands were calculated using fitted regression model.

CHAPTER 04

RESULTS AND DISCUSSION

4.1. Formulation of a Newer Processed Cheese Recipe

Due to the highly competitive nature in the market, processed cheese producers are often struggling to make the highest texture and flavour appeal in their products. Even though processed cheese-manufacturing sounds relatively simple in its definition, practically it is not so. Because obtaining the desired consistency, texture and flavour in the final product can only be achieved through careful selection and combination of the raw materials especially natural cheese blend, emulsifying salts in the correct ratio and by controlling the nature and extent of mechanical and thermal treatments. Therefore major part of this research was aimed at formulation of a newer processed cheese recipe manipulating these parameters at optimum conditions.

4.1.1. Selection of the Most Appropriate Blend of Natural Cheese

Table 4.1. Results of sensory evaluations - selection of correct blend of cheese (a)

Session	Sample Code	Mean	P-value
1	252	6.467	0.000
	366	7.800	
	798	7.133	
2	707	7.133	0.756
	813	7.033	
	904	7.300	
3	258	6.800	0.000
	272	5.533	
	952	5.233	
4	824	6.167	0.000
	861	8.200	
	907	4.333	

According to the experimental design, there are large numbers of possible combinations. Since this is a consumer targeted commercial activity, some of them seem to be not applicable due to economic aspects and due to organoleptic aspects. Hence 12 samples were selected neglecting others. In this experiment four levels of young cheese (3.0, 2.5, 2.0 and 1.5 Kg) was assigned to 12 different treatments so that three treatments getting the same level. Each of these three treatments with the constant level of young cheese was combined with three levels

of matured cheese (3.8, 3.3 and 2.8 Kg). In each case mild cheese was added to bring the total weight of each blend to 7.3 Kg.

When performing ANOVA for cheese samples (252, 366 and 798), the p-value (0.000) for overall acceptability indicates that there is sufficient evidence that not all the means are equal when alpha is set at 0.05. This implies that there is a statistically significant difference in overall acceptability. Since sample code 366 shows the highest mean it could be considered as the best one out of these three.

When performing ANOVA for cheese samples (707, 813, and 904), the p-value (0.756) for overall acceptability indicates that there is sufficient evidence that all the means are equal when alpha is set at 0.05. This implies that there is no statistically significant difference in overall acceptability. Therefore mean comparison is meaningless and sample code 904 could be selected as the best in an economic view point since it is comprised more mild cheese over matured cheese compared to other two.

When performing ANOVA for cheese samples (258, 272, and 952), the p-value (0.000) for overall acceptability indicates that there is sufficient evidence that not all the means are equal when alpha is set at 0.05. This implies that there is a statistically significant difference in overall acceptability. Since sample code 258 shows the highest mean it could be considered as the best one out of these three.

When performing ANOVA for cheese samples (824, 861, and 907), the p-value (0.000) for overall acceptability indicates that there is sufficient evidence that not all the means are equal when alpha is set at 0.05. This implies that there is a statistically significant difference in overall acceptability. Since sample code 861 shows the highest mean it could be considered as the best one out of these three.

In order to select the most appropriate natural cheese blend, sensory data referring to cheese samples that show the highest mean value in each session were again treated with ANOVA.

Table 4.2. Results of sensory evaluations - selection of correct blend of cheese (b)

Sample Code	Mean	P-value
258	6.800	0.000
366	7.800	
904	7.300	
861	8.200	

These four samples also show a significant difference ($p<0.000$). According to the multiple comparisons of mean, reveals sample (861) as the best one. But sample (366) also appears to have much more similarities with the above one because it is the immediately closer sample. Cheese blend (861) comprises young, mild and matured cheese in the ratio of 1.5:2.0:3.3 means comparatively higher level of matured cheese.

In order to infer how these individual factors are affected on responses main effect plots could be compared as follows.

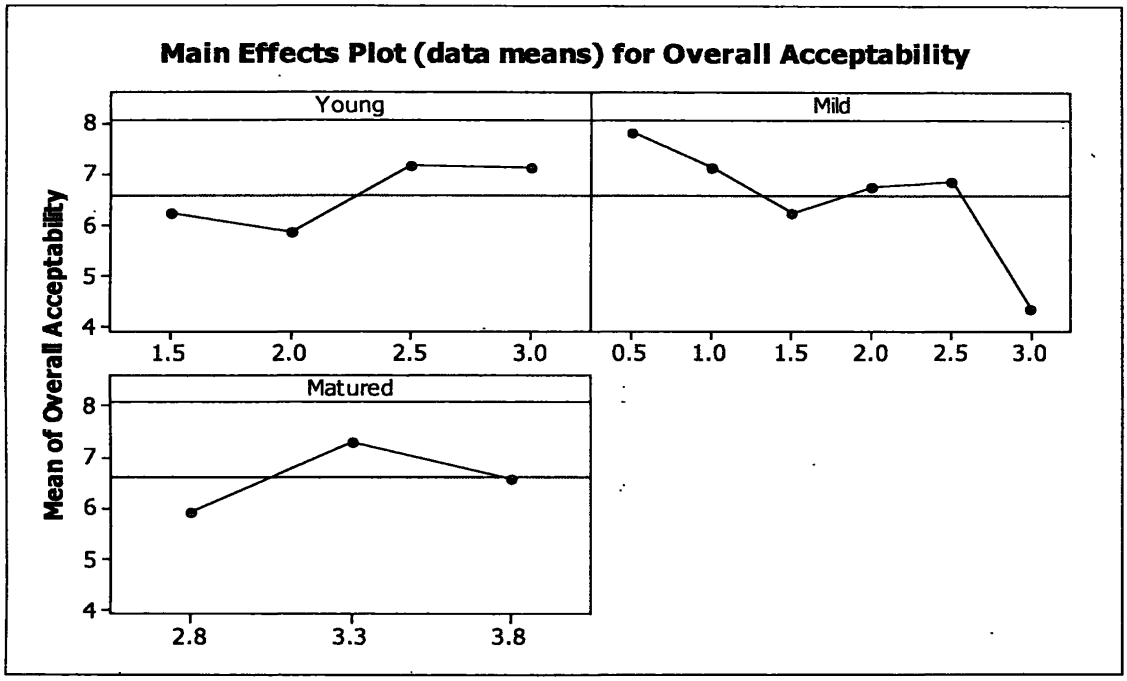

Fig 4.1. Main effect plots of young, mild and matured cheese

The points in the plot are the means of the response variable at the various levels of each factor, with a reference line drawn at the grand mean of the response data. The effects are the differences between the means and the reference line. The effect of mild cheese upon overall acceptability is larger compared to the effects of young cheese and matured cheese. According to the main effect plot of young cheese when quantity is increased, 2.5 Kg level offers the highest overall acceptability and beyond that level no more improvement is seen. According to the main effect plot of mild cheese, when quantity is increased, gradually decreasing the overall acceptability up to the level 1.5 Kg. Again it is increased at the levels 2.0 and 2.5 Kg and beyond that level no more improvement is seen. In the case of matured cheese, highest overall acceptability is seen at the 3.3Kg level. Although main effects behave in this manner interaction of these variables may have rather different effect. This could be observed using interaction plots, since interactions plots enable the visual assessment of interaction in the data.

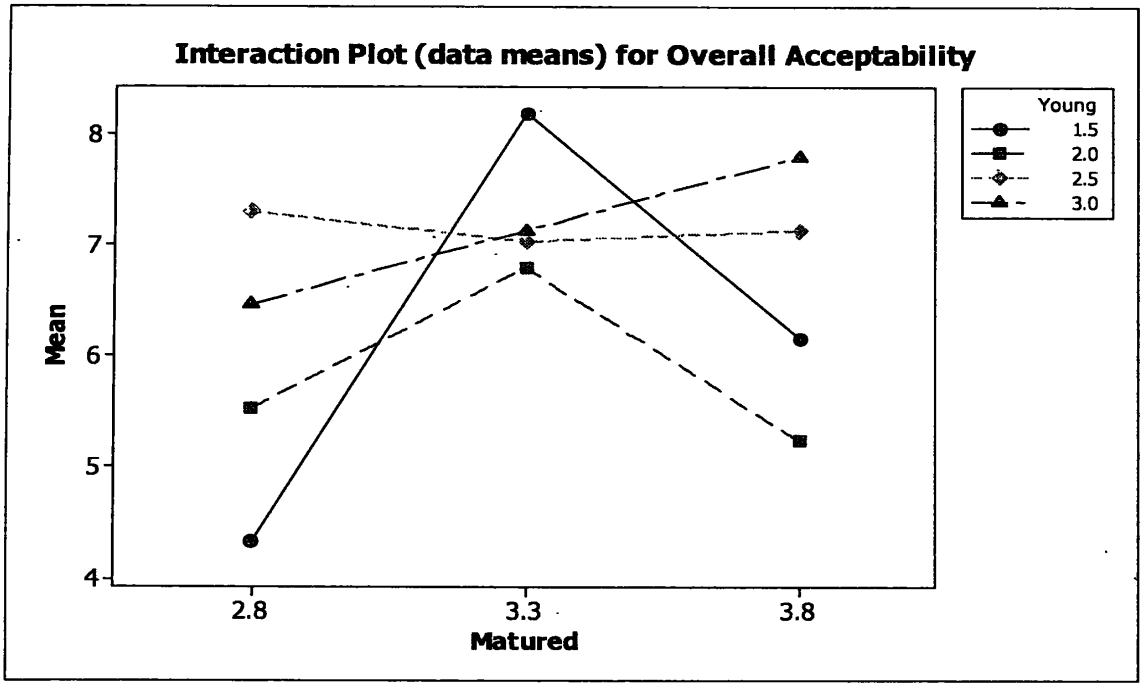

Fig 4.2. Interaction plot of young cheese and matured cheese

This interaction plot (Figure 4.2) shows the mean overall acceptability versus the quantity of matured cheese for each of the four levels young cheese. This plot shows apparent interaction because the lines are not parallel, implying that the effect of matured cheese upon overall acceptability depends upon the quantity of young cheese. 3.3 Kg of mild cheese in combination with 1.5Kg of young cheese seem to give the highest sensory rating.

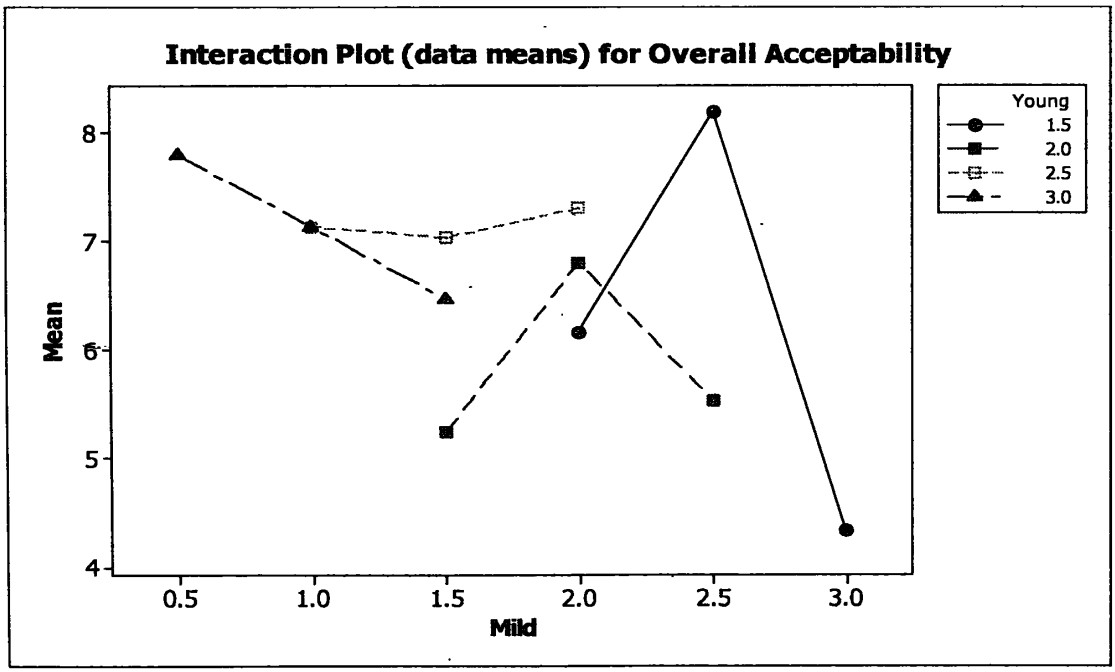

Fig 4.3. Interaction plot of young cheese and mild cheese

This interaction plot (Figure 4.3) shows the mean overall acceptability versus the quantity of mild cheese for each of the four levels young cheese. This plot shows apparent interaction because the lines are not parallel, implying that the effect of mild cheese upon overall acceptability depends upon the quantity of young cheese. 2.5 Kg of mild cheese in combination with 1.5Kg of young cheese seem to give the highest sensory rating.

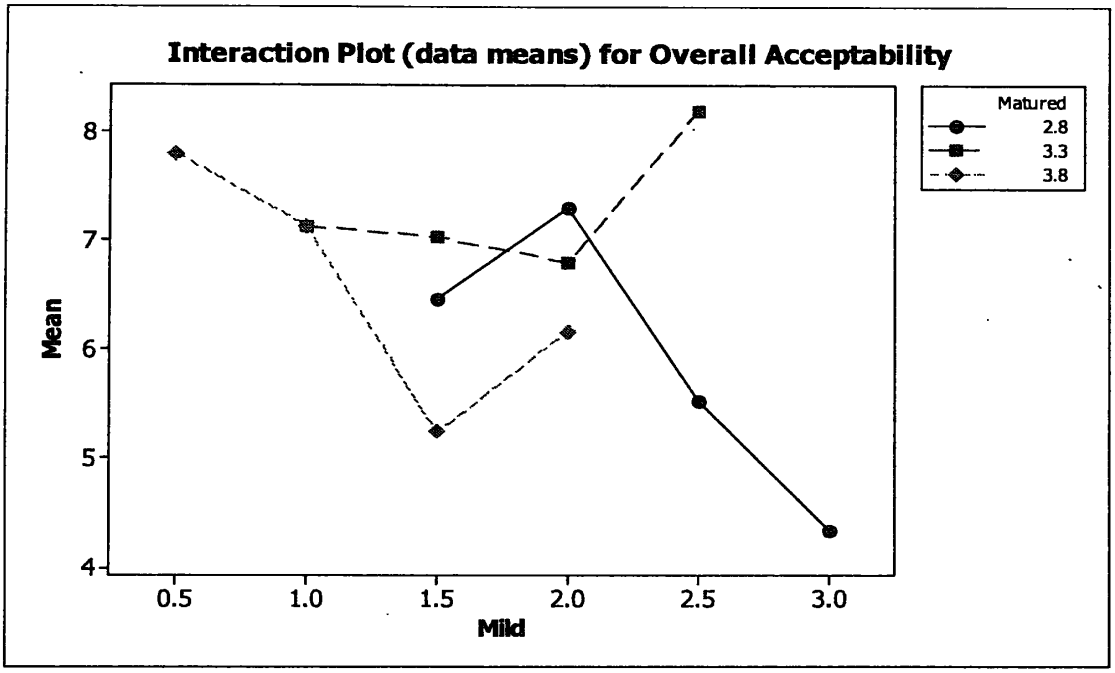

Fig 4.4. Interaction plot of mild cheese and matured cheese

This interaction plot (Figure 4.4) shows the mean overall acceptability versus the quantity of mild cheese for each of the three levels matured cheese. This plot shows apparent interaction because the lines are not parallel, implying that the effect of mild cheese upon overall acceptability depends upon the quantity of matured cheese. 2.5 Kg of mild cheese in combination with 3.3 Kg of young cheese seem to give the highest sensory rating.

4.1.2. Selection of the Most Appropriate Fat Source

Table 4.3. Results of sensory evaluations - selection of the best fat source

Sample Code	Mean	P-value
305	6.067	0.000
547	7.600	
592	8.003	
617	4.667	

Although different fat sources were used in above recipes, fat content was adjusted approximately to a same level. Therefore only the variable factor is fat source. P<0.000 implies that there is a statistically significant difference between 4 samples. Based on the results of mean comparisons, sample (592) could be selected as the best sample and thereby selected the homogenized fresh cream as the best fat source.

As demonstrated by Prentice (1972) an increase apparent viscosity is found to increase due to increase in fat level and due to homogenization (1992) that was attributed to protein adsorption to lipid globules and formation of homogenization clusters. Homogenization, with the resultant increase in fat globule surface area, typically stabilizes the cream emulsion. (Scott *et al,* 2003). Combination of these factors may be the key reasons to have the highest sensory rating for cheese sample that is prepared with homogenized fresh cream.

Interestingly, butter flavour differs from its parent cream, because additional flavour compounds are derived through lipolysis and oxidation reactions and during heating. But with excessive reactions lead to rancidity and off-flavours. To obtain the good plasticity there must be a suitable ratio of solid fat to liquid fat with many small fat crystals; otherwise faults such as brittleness or oiling-off are likely (Lane, 1998). Butter contains relatively higher content of diacetyl and use of butter as the fat source in processed cheese is not preferable, because amounts larger than 0.05mg/100g is frequently associated with flavour defects (Kosikowski, 1958). Since ghee is traditionally produced by open kettle boiling, it is able to contain oxidized flavour components, incorporating it in the processed cheese recipe will also cause inferior quality product.

4.1.3. Selection of the Most Appropriate Emulsifying Salt

Table 4.4. Results of sensory evaluations - selection of the best ES

Sample Code	Mean	P-value
192	6.966	0.000
622	7.367	
429	4.933	

P<0.000 implies that there is a statistically significant difference between 3 samples. Based on the results of mean comparisons, sample (622) could be selected as the best sample and thereby selected the Joha C/Trisodium citrate mixture (100:100) as the best ES.

Heating cheese during processing causes fat globules to be disintegrated, thus emulsifying salts must be added to prevent separation of fat. ES salts act upon the casein, open up the casein structure and help expose some monovalent cations and forms stable emulsion (Banks, 1998). Added citrate in cheese is thought to act as a calcium-chelating agent. Thus, by promoting CCP solubilization, citrate addition should decrease the content of bound calcium in cheese. This would decrease protein-protein interactions leading to increased emulsification of fat by caseins, and it would give the product the desired body and texture (Pastorino *et al.*, 2003). The proper ratio of emulsifying salts in formulation is important and manufacturers should stay within solubility limits and dissolve salts in an aqueous solution before introducing it to the cheese blend. Some factors to consider include the strength of the ion exchange required, the amount and direction needed to shift pH into proper range and how strongly the product will be creamed (Turner, 2003).

Since trisodium citrate is slightly alkaline (pH =8.55) and contributes to buffering, calcium binding and solubilization of protein, and assist to have long elastic structure, this was preferably used in manufacturing sliceable block cheeses. It was used in association with sodium dihydrogen orthophosphate (pH = 4.5) as well, because they maintained the pH of samples almost constant (6.0) and pH shift (6.5) lies very closer to the desired pH ranege as some experts recommend for processed cheese (between 5.2 and 6.0).

Polyphosphates are highly water soluble and effective in ion exchange and protein break down. Therefore polyphosphates are more preferable over citrates and monophosphates. Whatever the ES used quantity is largely dependent on the amount of unhydrolyzed casein content in natural cheese blend. The higher the relative casein content, the greater the emulsifier needs to be incorporated. But over dosage causes crystallization and undesirable changes in the sensory properties of cheese.

4.1.4. Reformulation of the Existing Recipe

Table 4.5. Sensory evaluation - reformulated recipes

Sample code	Flavour		Texture		Colour		Overall Acceptance	
	Mean	P value	Mean	P value	Mean	P value	Mean	P value
456	6.233	0.000	5.800	0.000	4.833	0.000	5.433	0.000
218	4.100		6.233		7.000		5.400	
552	7.433		5.900		6.300		7.700	
707	5.633		3.300		4.367		5.067	
537	5.633		4.333		6.300		5.53	
292	8.333		7.433		7.900		8.400	

Sensory evaluation reveals that there is a statistically significant difference in flavour, texture, colour and overall acceptability. Mean comparison results showed that market sample is the best in all disciplines, i.e. reformulated samples also did not reach to that level. When reformulated samples were compared with the cheese that was based on existing recipe, some what improvement was shown by the sample (552), while others showed no improvement. Average rating of sample (552) for overall acceptability was far closer to the market sample although statistically significant difference was existed. Therefore further modifications of the formula of the sample (552) would be assisted to have desired sensory qualities as appeared in market sample.

Sample (552) was prepared by adding 7300g of Cheddar cheese base(1.5:2.5:3.3), 450g of homogenized fresh cream, 300g of Joha C/Trisodium Citrate(1:1) blend and 12g of table salt. Once increasing the content of ES it was responsible to reduce the level of table salt. If both of them were increased simultaneously, final product would be too salty. However, salt content was also low in this recipe compared to the existing recipe. This implies for the correct balance of flavour salt content must be reduced while increasing the ES.

In all the above statistical analysis were to explore differences among the means, multiple comparison results were examined, where Hsu's MCB (Multiple Comparisons with the Best) was used since it compares each mean with the best (largest) of the other means. Data generated through sensory evaluation is ordinal and generally non parametric procedures are followed by such type of data. 9-point hedonic scale enables panelists to express their liking referring to a wider scale compared to 3-point and 5-point hedonics. This feature along with large number of observations makes non-normally distributed data normal, which in turn allows to use ANOVA. However it is quite suitable to check normality prior to analysis is carried out otherwise come up with erroneous results.

4.2. Gross Compositional Analysis

Table 4.6. Gross composition of processed cheese

Component	Percentage
Moisture	41.26 ± 1.53
Total Solids	58.74 ± 1.53
Fat	30.17 ± 0.29
FDM	51.36 ± 0.89
Protein	25.28 ± 0.73
Acidity	0.60 ± 0.05
Salt	2.32 ± 0.03

According to the SLS guidelines, processed cheese shall be of 35-80% of moisture, minimum 45% fat (on dry basis), 20-65% dry matter and maximum 3% of salt (Sri Lanka Standard Institution, 1987). As cheese samples tends to dry out rapidly on the out side, obtaining a representative sample is essential, thus all analysis were performed in triplicate and data were presented with their precision errors.

4.3. Prevention of Rapid Drying Off and Disappearance of Sharp Edges

It was seen that "Highland cheese" forms a hard rind readily once it is kept open at ambient temperature compared to a leading market sample of same type. That means there may be a difference between these two in the moisture retaining property. This may probably due to insufficient hydration of casein.

Marchesseau (1997) observed relationships between pH variation and the characteristics of process cheese that demonstrated the importance of pH control during the manufacturing process. Optimal pH conditions during manufacture ranged from 5.7 to 6.0. Small changes in ionic composition and strength modified the protein interactions substantially and had important repercussions on the final structure and quality of the protein gel that was established during processing of cheese. In addition to ionic interactions, hydrogen and hydrophobic interactions appeared to be important in the structural stabilization of process cheese (Pastorino *et al.,* 2003). At pH 5.2, there is increased solubilization of colloidal calcium phosphate and decreased interactions between proteins, which allows increased solvation of caseins. Thus, at pH 5.2, increased hydration of the protein matrix would be expected, leading to increased moisture content of cheese. However, lowering of pH, especially below 5.0, would promote protein-to-protein interactions as the caseins approach their isoelectric point and electrostatic repulsions are minimized (Marchesseau *et al.,* 1997) Thus, the ability of proteins to interact with water and the water-holding capacity of the protein matrix would decrease below pH 5.0, which then results in increased syneresis and decreased moisture content of cheese. Adding salt to cheese also seems to cause decreased hydration of caseins.

4.3.1. Determination of ERH/ Water Activity of Processed Cheese

According to the Sing (2003), Cheese is a medium-moisture food, containing about 30 to 50% moisture and water activity (a_w) varies from 0.98 to 0.87, and these values are highly correlated with the total nitrogen and ash content (mainly NaCl). Biochemical reactions that occur during the ripening of cheese contribute to the depression of a_w by increasing the number of dissolved low-molecular weight compounds and ions

ERH is an important practical property in food sample, since it is the dividing line between the ranges of atmospheric relative humidity in which the sample gains or loses moisture during storage (Smith, 1971). According to the method based on hygroscopisity of salts, the water activity of experimental cheese sample was in the range of 0.93-0.97. This method is based upon the fact that moisture will condense on to salt crystal only from an atmosphere which has a higher relative humidity. However this method does not yield an accurate result, but gives useful indication of the ERH of a sample between fairly narrow limits within about an hour. The degree of uncertainty in the results depends upon the interval between particular salts used.

It is difficult to keep this cheese outside, because moisture vapour will transfer from sample to air since it is moisture rich food. However, it is inevitable to match the ERH of cheese to ERH of air, because ERH dynamically change with the temperature. Modifying the product characteristics of cheese by the addition of food gums such as carrageen or other water binding agent and/or improve caseins to the extent at which higher moisture retention is possible provide means to avoid rapid drying off and retain the moisture within the food system. This is also a tedious task and adding gums may leads to defects in palatability issues. Therefore most affordable way is to enclose the cheese in a moisture proof material with minimum head space or after vacuuming.

4.3.2. Selection of the Most Suitable Packaging Material

At present "Highland" cheese blocks are packed in nylon packs. Even it is kept refrigerated, loose the sharp edges and undesirably change its shape, will render the consumers dissatisfaction. Obviously nylon is not ideal for packing foods that are rich in moisture, because it is not water impermeable although possess good barrier properties over air and aroma. Since MVTR of the packaging is critical in achieving the desired quality, safety, and shelf-life selection of the correct type of packaging is much more significant. Al foils and triple laminated Al foils are extremely resistant to water vapour transmission, thus seemed to give good sensory appeal in appearance. While metallized films have seen excellent cost-benefit compared to other barrier packages, nylon can be replaced by metallized films where the combination of light, moisture and oxygen barrier create a unique combinations of effective product protection. However further studies need to be carried out in order to verify the above facts.

4.4. Evaluation of Natural Cheese in Respect of Texture and Flavour

Reformulation of processed cheese recipe, changing only the processed cheese ingredients assisted lesser extent to texture and flavour development, Since nature and extent of ripening

strictly determines the same, attempts were made to identify and critically evaluate what sort of factors governs to texture and flavour defects in their natural cheese.

4.4.1. Chemical and Physical Influences of Cheese Ripening

Milk pretreatment procedures, ripening temperature and chemical parameters of fresh cheese curd, i.e., moisture, salt, and acid are vital to cheese ripening process. Prior to cheese making, the milk is subjected to a number of processing treatments, either to ensure the production of good quality cheese consistently or to impart specific textural and flavour characteristics to curd. In MILCO following pretreatment procedures are used.

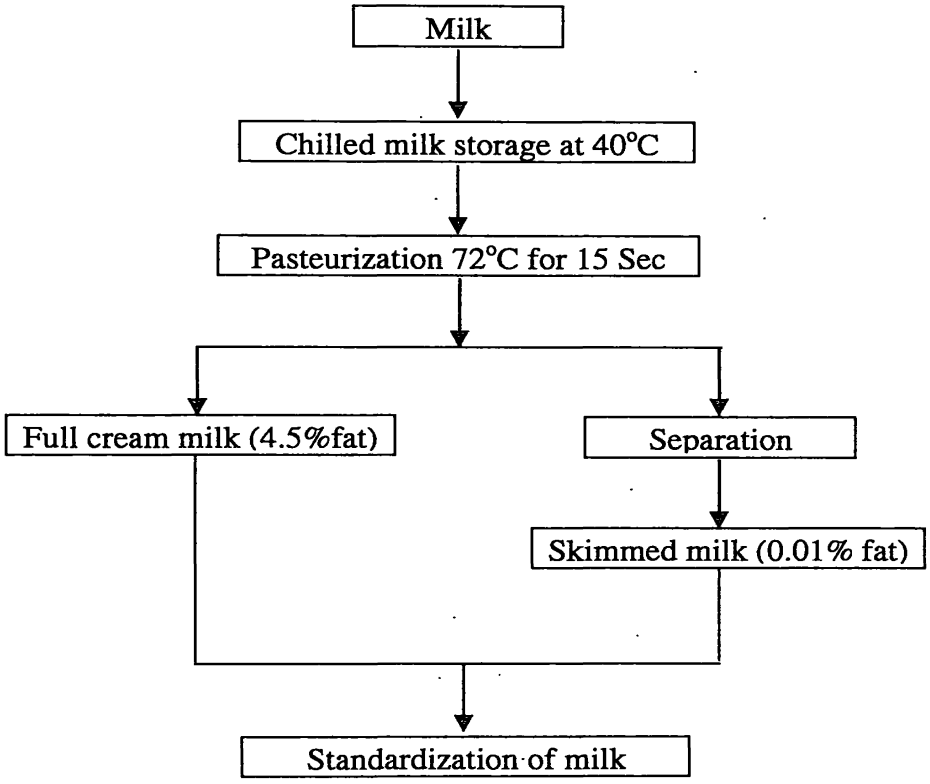

Figure 4.5. Milk pretreatment scheme

As most Cheddar cheese manufacturers do, they pasteurized chilled milk at 72^{0}C for 15 seconds (HTST) just before use. In general, cheese made from raw milk develops the characteristic cheddar flavor more rapidly (reaching its best flavor at 3 to 6 months) than cheese made from pasteurized milk (takes twice as long as that made from raw milk to develop the same flavor intensity) since pasteurization of milk causes very limited heat-induced interaction of whey proteins with casein and denatured whey proteins to remain in cheese. Finally this may influence the accessibility of caseins to proteinases during ripening (Sing *et al.*, 2003). On the other hand, pasteurization leads to inactivation of xanthine oxidase, which in turn increasing the risk of bacterial spoilage, thereby increasing the risk of bitter

flavour development in cheese (Walstra *et al.,* 1999). Since HTST is the technique used in this case heat exposure is minimal and effect of pasteurization could be negligible.

Ripening temperature also greatly affect on texture and flavour. Cheddar cheese from excellent hygienic quality develops more flavour in 4 months at 10^0C, 2 months at 16^0C than cheese held at 5^0C. At temperatures higher than 15^0C, usually have the effect of destroying the desirable cheddar body (Kosikowski, 1958). Although they maintain temperature near 16^0C, hygienic quality of milk is somewhat questionable, because they use old and degraded milk for cheese manufacturing where as poor hygiene inside ripening rooms also disagreed. So it is preferable to ripen cheese at 5^0C with such type of milk.

The final pH, moisture, S/M, temperature, and duration of ripening to a large extent control the proteolysis in cheese (Sing *et al,* 2003). High moisture and low salt concentration increase the ratio of fat, lactose and protein hydrolysis (Kosikowski, 1958). First grade young cheeses shall be of pH (4.85 to 5.20); S/M (2.5 to 6%); moisture in nonfat solids (50 to 57%) and FDM (50 to 56%). Young cheeses with a composition outside these ranges are considered unlikely to yield good quality matured cheese (Gilles and Lawrence 1973).

As chemical analysis reveals, composition of their young cheese was 41% of moisture, 28% of fat, 2.2% of salt and pH of 5.3. That means 47.46% of FDM and 5.36% of S/M. According to Gilles and Lawrence (1973) it is doesn't matter with S/M level. But quality of matured cheese is questionable referring to FDM. As Kosikowski, (1958) demonstrated many bacteria including lactic starter organisms grow poorly in brines above 4% salt. In "Highland" cheese actual brine concentration (5.36%) is far greater than this level.

The activity and the retention of coagulant depend on the amount of acid produced during the initial stages of manufacture. The role of pH in cheese texture is particularly important because changes in pH are related directly to chemical changes in the protein network of the cheese curd. As the pH of the cheese curd decreases, there is a concomitant loss of colloidal calcium phosphate from the casein micelles and, below about pH 5.5, a progressive dissociation of the sub-micelles into smaller aggregates occurs (Lawrence and others 1987). The solubilization of colloidal calcium phosphate, among other factors, affects curd (cheese) texture, stretchability, and meltability. The activity of enzymes in cheese is generally depends on the enzyme concentration and more over the parameters such as pH, NaCl content of the moisture in cheese (Guinee, and Fox, 1999), ripening temperature of cheese, water content of cheese and so on. To better control the ripening process it is essential to have optimum pH and temperature conditions, because temperature affects more on lipolysis rather than proteolysis. The lower the water content, smaller the diffusion coefficients and reaction

velocities since water content affects the composition of cheese (e.g., the calcium ion activity) and conformation of proteolytic enzymes (Walstra *et al.*, 1999).

The pH at whey drainage largely determines the mineral content of a cheese. The loss of Ca and phosphate from casein micelles determines the extent to which they are disrupted, and this largely determines the basic structure and texture of a cheese (Sing *et al.*, 2003).

4.4.2. Assessment of Extent of Proteolysis in Cheddar Cheese

Proteolysis during ripening contributes to textural changes in cheese during maturation (Graiver *et al.*, 2004). In young cheese, the greater part of the nitrogenous material present in the water insoluble form. Usually cheddar cheese undergoes less proteolysis and only 25-35% of protein is made soluble in a well-matured cheese (Helen and Sharpe, 1981). A high proportion of breakdown products are in the form of peptides and amino acids. Actually proteolysis is an index of quality, thus the extent of proteolysis was measured.

Anionic sulphonic dyes such as Orange-G, Orange-12, and Amaido-Black can bind to the positively charged lysyl, imidazole and arginyl residues of proteins at about pH 2.2 and leading to precipitation. If this coagulum is removed by centrifugation or filtration, the amount of dye remaining in the supernatant liquid is inversely proportional to the amount of protein in the sample. This is the principle used in dye binding method. However there are some limitations arising from method it self, because low molecular weight proteins and peptides react slowly, leading to poor separation and centrifugal supernatants (McSweeney and Fox, 1999).The dye reaction with protein is complex and non-uniform; hence the method is highly empirical and requires standardization or calibration (Kirk and Sawyer, 1991). Therefore calibration curve was made using known quantities of standard caseins as illustrated in figure 4.6, where casein content equivalent to 90% of the total weight taken.

Construction of Calibration Curve:

Table 4.7. Absorbance of remaining dye at different protein concentrations

Sample No:	Weight of Sample (g)	Protein Content (g)	Absorbance at 478 nm
1	0.0255 ± 0.0004	0.0229	0.541 ± 0.002
2	0.0505 ± 0.0004	0.0455	0.473 ± 0.002
3	0.0756 ± 0.0003	0.0680	0.459 ± 0.008
4	0.1003 ± 0.0004	0.0903	0.365 ± 0.002
5	0.1248 ± 0.0004	0.1123	0.309 ± 0.002
6	0.1506 ± 0.0002	0.1355	0.289 ± 0.002
7	0.1753 ± 0.0004	0.1578	0.191 ± 0.003
8	0.2002 ± 0.0005	0.1802	0.146 ± 0.002

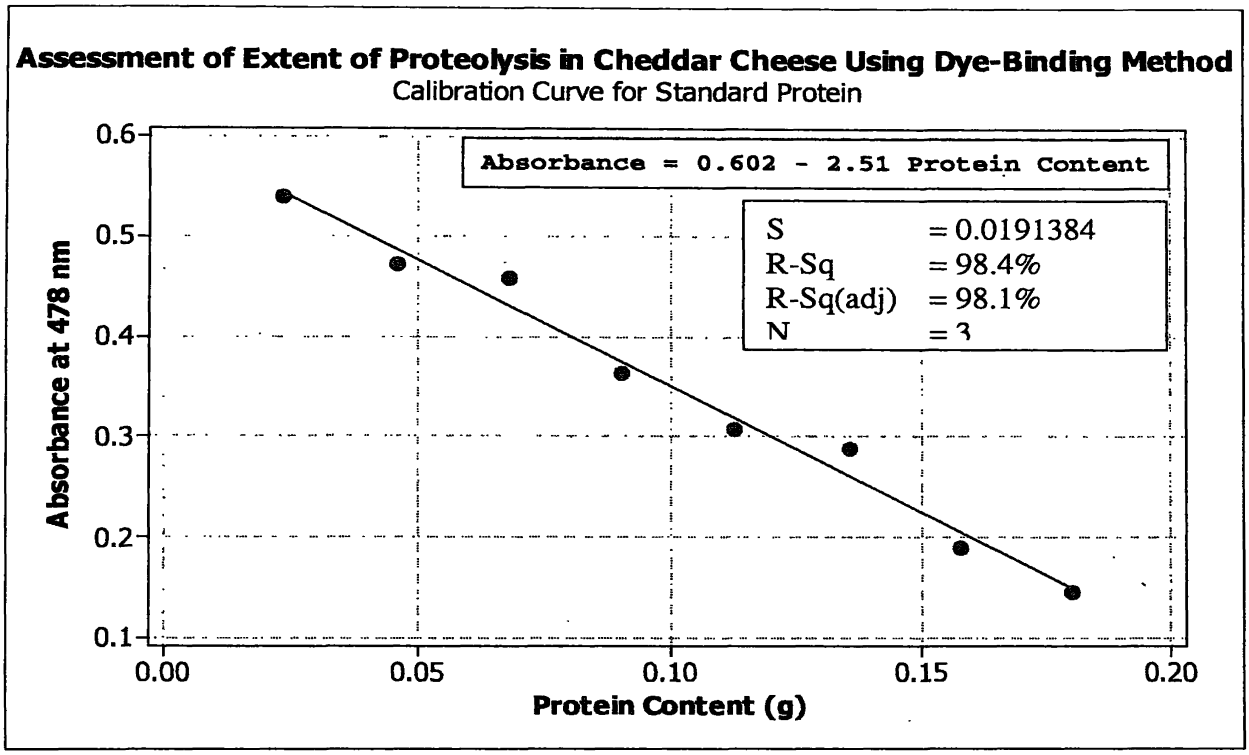

Fig 4.6. Calibration curve for casein at different levels of protein

In block cheese manufacturing, relative proportions of the young, mild and matured proportions in the cheese blend are critical in determining the correct consistency in the final product, which is primarily attributed by the level of unhydrolyzed casein remaining in the cheese blend. The level of unhydrolyzed casein in relation to the total nitrogen in the raw cheese is termed the RCC (Banks, 1998). Quantity of intact casein at each stage was calculated using above regression model. It could be expected that reliability of results are very high, because variation of absorbance is explained to a large extent by the protein content ($R^2 = 98.4$). Then total protein was determined by the Kjeldhal procedure and there by RCC values were calculated (table 4.8)

Table 4.8. Indices of proteolysis

Time (days)	UP %	UC %	TCN %	RCC %	P %
0	24.50	22.79	3.57	84.40	00.00
15	22.22	20.67	3.24	76.55	09.30
30	22.02	20.48	3.21	75.85	10.13
60	19.77	18.39	2.88	68.10	19.31
75	17.14	15.94	2.50	59.02	30.07
90	15.89	14.78	2.32	54.74	35.14
105	13.39	12.45	1.95	46.11	45.36
120	10.89	10.13	1.59	37.52	55.55

UP% : Percentage of Unhydrolyzed Proteins [Obtained from calibration curve]

UC% : Percentage of Unhydrolyzed Caseins [UP*(93/100)]

TCN% : Percentage of Total Casein Nitrogen [UC/6.38]

RCC% : Relative Casein Content [(TCN/TN)*100]

P% : Percentage Proteolysis [(UP1-UP2)/UP1*100]

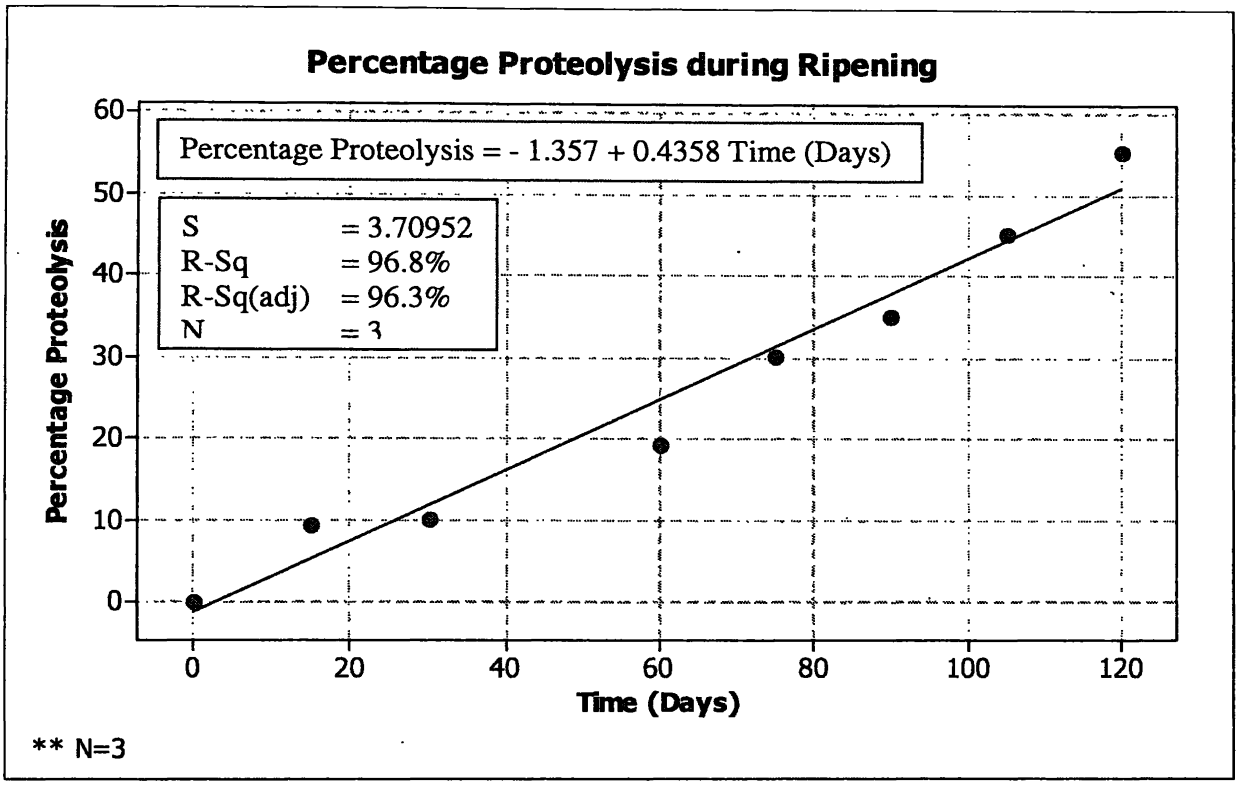

Fig 4.7. Percentage proteolysis during ripening

Block processed cheese with good slicing properties generally requires a raw material which has a long structure and relative casein content at or above 70%. That means blend of young and medium-ripe cheese i.e., predominantly young cheese. The selected blend of natural cheese in section 4.1 was with young: mild: matured proportion of 1.5:2.5:3.3 respectively. RCC values of these individual components prevail at 84.40%, 54.44% and 37.52% for young mild and matured respectively and thereby blend shows RCC of 52.95%. This implies that processed cheese prepared with this blend will offer unpleasant slicing properties. Since cheese blends that predominantly consisting young cheese offer weak flavour, at the same time it would have a long elastic structure, make a controversy in determining the most suitable blend. Therefore, one who attempt to make better texture, along with good flavour, based on this phenomenon only will mislead.

As literature reviews, flavour is of paramount importance against texture, because function and performance don't matter without flavour that would appeal to demanding consumer

palate and people used to reject any food that is lack in flavour although almost correct in texture. Merely primary proteolysis only sufficient to have texture, but extensive proteolysis is needed to have pleasant flavour profile. Percentage proteolysis figures show that young, mild and matured cheese had attained 9.30, 34.14 and 55.55% proteolysis at about 15, 90 and 120 days respectively. According to the graph 4.2, it is clear that proteolysis occurs at the rate of 2.51 and there is an exact linear relationship between time and percentage of proteolysis (R^2=96.8) thus, it is able to select cheese with a desired pre-determined level of proteolysis referring to the regression model that is calculated with least square method. At the end of 4 months period proteolysis reaches about 56%.

In order to select the natural cheese blend that affords optimal flavour and texture in finished product, it needs to concern both terms i.e., RCC and percentage proteolysis. Figure 4.8 shows, how those are interact.

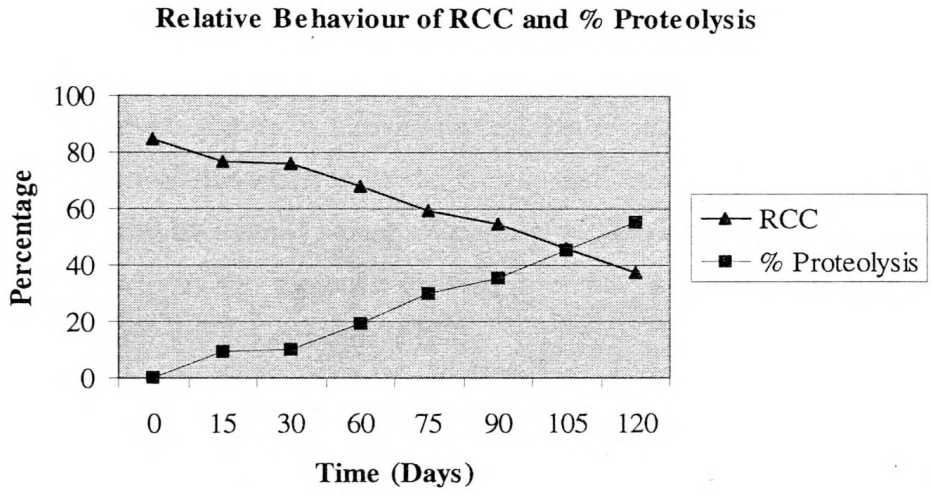

Fig. 4.8. Relative behaviour of RCC and proteolysis percentage

Above figure (4.8) show that about 100 days RCC and % proteolysis values become same. At about 60 days natural cheese reaches nearly 70% of RCC and 20% of proteolysis. Therefore incorporation of 60 days old cheese only, in manufacturing processed cheese may provide quite good texture but with mild flavour. However, flavour can be improved by the addition of enzyme modified cheese (EMC). This will also help eliminate the problem of protein instability of aged cheese and cost. Commercially many flavour producers look for the flavour "notes" desired, and offers in the liquid and dried mixture of proteases and lipases to achieve the right notes. Especially for cheddar butyric acid used as the flavour molecules (Turner, 2003).

In addition changing the starter culture and modification of fat level is also helpful for flavour development in cheddar cheese, since starter directly involves in proteolysis. The present culture used in the company is a mesophilic culture, and it comprises of *streptococcus cremoris, Streptococcus lactis, Streptococcus diacetylactic and Luconostoc citrovorum.* This combination is quite good for flavour development according to the view of Helan and Sharpe (1991), but other conditions need to be maintained at proper level, i.e. RH, Temperature, NaCl level etc. Fat importantly mediates as a solvent for hydrophobic flavour compounds. In addition lipolysis and formation of ketones from free fatty acids play a major part.

4.4.3. Characterization of Proteolytic Pattern of Cheddar Cheese

The most abundant proteins in milk are alpha s_1-casein, alpha s_2-casein, β-casein, k-casein and γ-casein, with molecular weights 23600, 25200, 23983, 19550, 20500 respectively (Waste et al., 1999). In the break up of casein into large peptides, chymosin is the main enzyme responsible for cheese proteolysis (Fox et al., 2000). Therefore, effectiveness of degradation depends on the chymosin concentration used, the amount of chymosin retained on the curd after the whey removal, and on its activity as function of the final curd pH, being that both, the percent retention of this enzyme in the cheese and its activity during ripening, are pH dependent and favored by low pH values (Walstra *et al.,* 1999). The chymosin action, besides pH, is also influenced by the presence of whey proteins, which tend to difficult chymosin access to caseins (Lelievre & Lawrence, 1988).

Alpha S_1-CN, rapidly degraded at the onset of maturation, with about 80% being decomposed within a one month. Later on rennet enzymes cause degradation larger peptides produced earlier, but at a slower rate (Vivex, 2006). In a latter stage of ripening, proteinase enzyme causes significant degradation of β-CN, although some 40% remains unaltered after 6 months (Walstra *et al.,* 1999).

Proteolytic pattern of cheddar cheese was verified by SDS-PAGE. This provides means to detect relative proportions of peptides present at each level of maturity and relative significance of enzyme sources on protein degradation. In order to minimize the uncertainties of the results same sample in two different concentrations were loaded and comparatively assessed. Using calibration curve molecular masses referring to each distinct band was calculated. Based on these results along with quoted literature bands and represented fragments there from were identified.

Table 4.9. Distances traveled by molecular markers when runs them in a SDS-PAGE

Distance (cm)	Log Distance(cm)	Molecular Mass (kD)
6.2	0.792392	10
5.2	0.716003	15
4.5	0.653213	20
3.0	0.477121	25
2.2	0.342423	37
1.3	0.113943	50
0.7	-0.154902	75
0.4	-0.397940	100

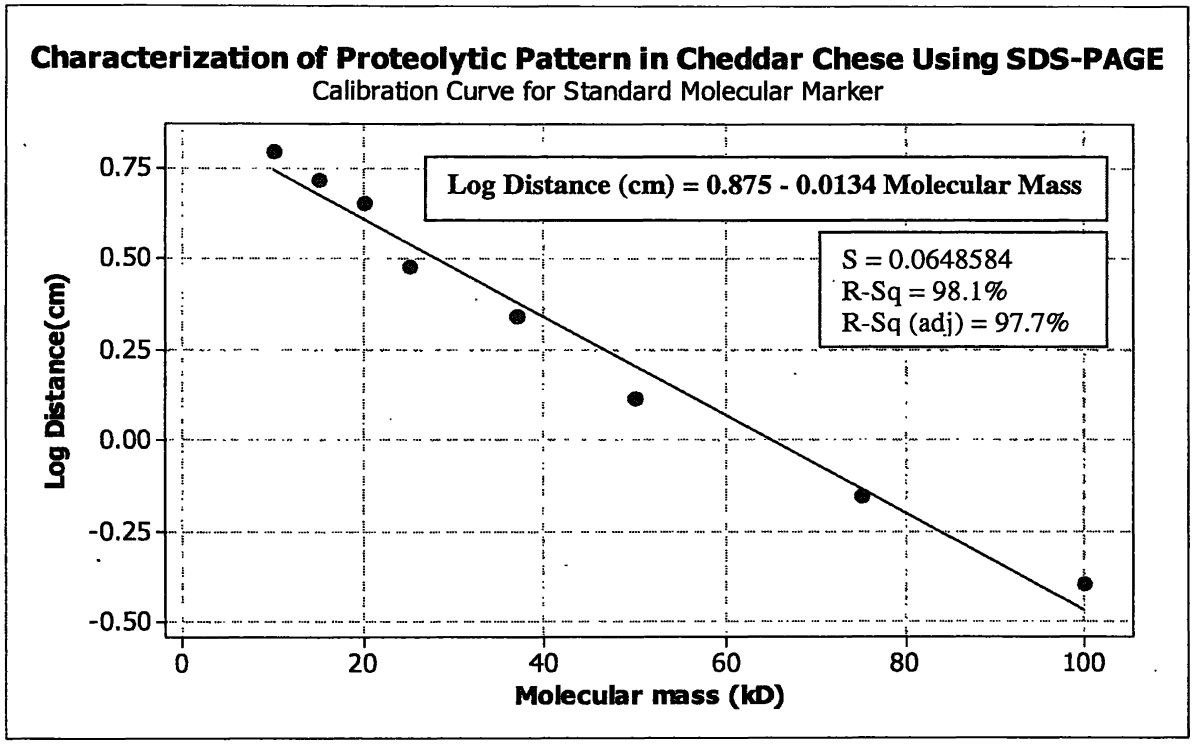

Fig 4.9. Calibration curve for standard molecular marker

According to the gel electrophoretogram (figure 4.10), at the onset of maturation more intense two distinct protein bands were appeared within the range, where 20kD and 25kD marker molecules exist. Their molecular masses are about 22 and 21 according to the calibration curve. Since corresponding molecular masses of β-CN and αS_1-CN are 24kD and 23kD respectively, it can be speculated that these bands are most probably indicating the unhydrolysed β-CN and αS_1-CN. This is further evident by their occurance in relatively higher quantities in starting material compared to other casein fractions. As literature reviews, αS_1-CN rapidly degraded at the onset of maturation (Walstra *et al.*, 1999) and basically

produces high molecular fragments such as αS_1-CN (1-169), αS_1-CN (f1-164), αS_1-CN (f1-159), αS_1-CN (1-156), αS_1-CN (1-153), αS_1-CN (1-149) and αS_1-CN (f24-199). These are also having the molecular weight close to 21 and 22 kD. Therefore observed fragments at onset of maturation also may be two of these.

It is apparent that bands get separated after 1 month and onwards along with a trend in decreasing intensity of the bands. Basically 5 bands were appeared in each lane within 1-4 months of period, reflecting extensive proteolysis. Approximately same observations were made in two phoretograms, although little dissimilarities. In 3 and 4 month matured cheese, band-width seemed to be gradually reduced, thinned and faded, while consistently dispersing faint stains were seen along these lanes especially within the inter-band regions reflects formation of low molecular weight fragments. Since 12% SDS-PAGE not facilitates the better resolution of proteins, especially low molecular fragments, appear them in the form of consistently dispersing faint stains. Not only the concentration of gel, but relative quantity of each newly created peptides also reason out the disappearance of distinct bands. Further work using 16% SDS-PAGE or urea-PAGE will most probably elaborate more on the smaller fragments. At the last two lanes (refers to 3 and 4 months respectively) fragments are appeared within the range of 15kD-10kD, implies initial fragments have broken down to 1 time lower weight.

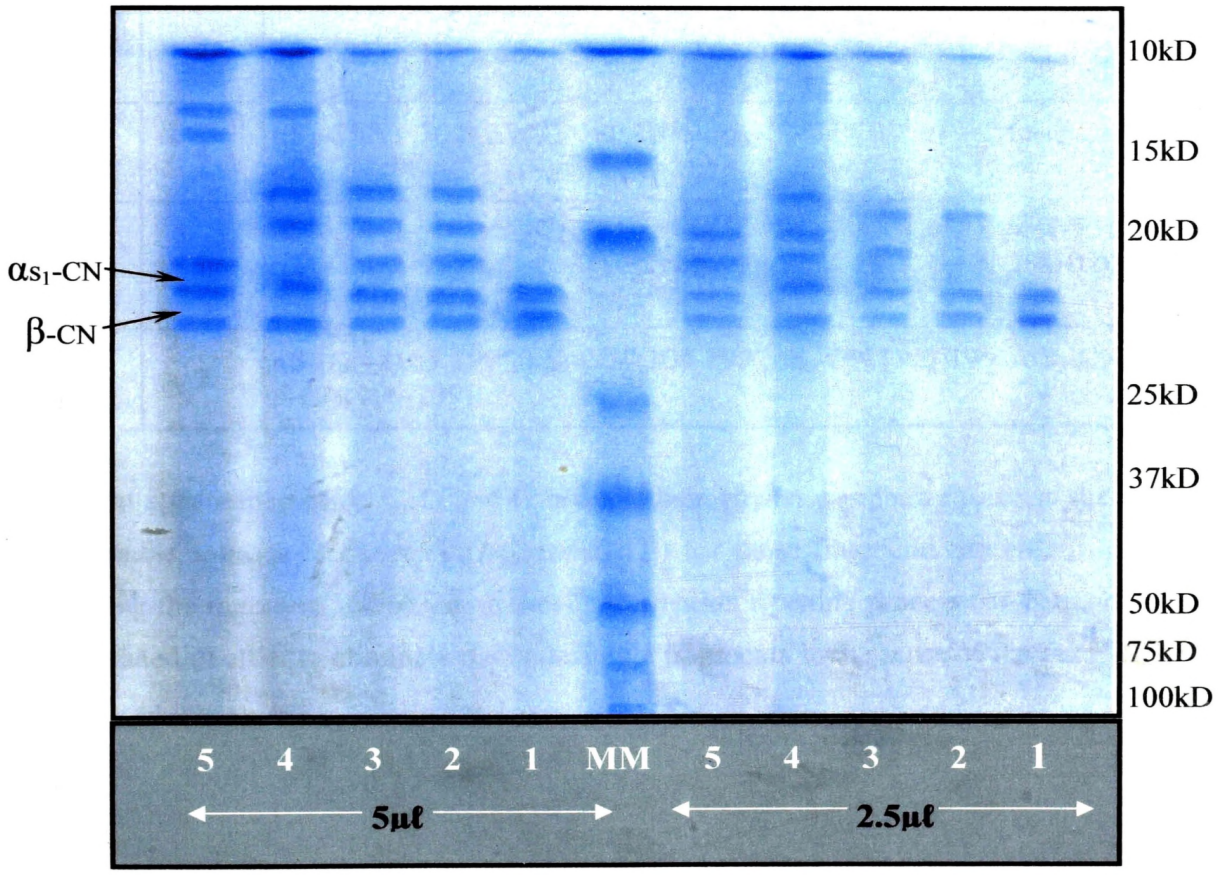

Fig. 4.10. SDS-PAGE gel electrophoretogram of water soluble fraction of cheddar cheese

Although 5 basic fragments are clear in phoretogram, molecular mass calculations reveals there are 11 different fragments (table 4.10)

Table 4.10. Different casein fragments identified in cheddar cheese

Coding Letter	Times Appear	Most Possible Fragment/ Fragments
A	7	αS_2-CN (1-188)
B	3	αS_1-CN (1-164), αS_1-CN (1-189),αS_2-CN(22-207), αS_2-CN (25-207), αS_2-CN (1-188), β-CN(1-189) β-CN(1-182)
C	5	αS_1-CN (1-159), αS_1-CN (1-156), αS_1-CN (1-153),αS_1-CN (23-199), αS_1-CN (22-164),αS_2-CN (1-179),αS_2-CN (1-174),β-CN(1-189)
D	6	αS_1-CN (1-149), αS_1-CN (1-151),αS_1-CN (29-189), αS_2-CN (1-163), αS_2-CN (1-174), β-CN(29-209) β-CN(1-139)
E	4	αS_1-CN (41-199), αS_2-CN (1-149), αS_2-CN (1-150), β-CN(1-163) β-CN(1-189), β-CN(1-164) β-CN(1-165)
F	1	αS_1-CN (1-124),
G	5	β-CN(1-107), β-CN(1-113), β-CN(1-183)
H	2	αS_1-CN (1-105), αS_2-CN (85-207),αS_2-CN (89-207), β-CN(1-105),
I	4	αS_1-CN (1-102),αS_1-CN (1-103), αS_1-CN (1-105),αS_2-CN (96-207), αS_2-CN (98-207), αS_2-CN (99-207),αS_2-CN (1-114),
J	1	αS_1-CN (1-90), αS_1-CN (91-199),αS_1-CN (1-102), αS_2-CN (1-95), αS_2-CN (1-97), αS_2-CN (1-98),αS_2-CN (115-207), β-CN(29-105), β-CN(29-107),k-CN (1-105)
K	2	αS_1-CN (103-199), αS_1-CN (104-199),αS_1-CN (105-199), αS_2-CN (1-88), β-CN(106-209),

Frequent appearance of A, C, D and G reveals their greater possibility to be in the ripening. Responsible sources of enzymes (Appendix XII) for these fragments are able to determine according the literature and based on those information ripening process can be modified and manipulated in order to eliminate the undesirable fragments in the sense of flavour.

CHAPTER 05

CONCLUSION AND FURTHER STUDIES

5.1. Conclusion

In processed cheese ingredients point of view, customize the texture and flavour attributes of processed cheddar cheese can be best achieved by blending 7300g of young, mild and matured cheese in the ratio of 1.5:2.5:3.3 along with the 450g of homogenized fresh cream, 300g of disodium orthophosphate / trisodium citrate (1:1) blend and 12g of table salt, under present conditions. Natural cheese blend intended for the above has a relative casein content of 52%, which in turn offer poor slicing properties and short texture in block processed cheese. The rate of proteolysis, 2.51 makes 56% of proteolysis within four months ripening, reveals natural cheese is with sufficient quality in the sense of flavour and consistent spreading of faint stains in gel electrophoretogram further emphasize on it. With these two contradictory aspects, the overall performance has become less satisfactory. Use of Aluminium-based packaging in processed cheese manufacture interestingly helps control the rapid drying off although water activity is in the range of 0.93-0.97.

5.2. Further Studies

A detailed investigation, both biological and biochemical aspects on natural cheese ripening needs to be carried out for further improvements of cheese base, since it is the major ingredient in processed cheese. Selection of an effective and affordable packaging material or devise a strategy to retain moisture of product itself to maintain sensory aspects of processed cheese is of utmost significance.

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APPENDIX I

Preliminary Sensory Analysis (Cheese blend, Fat source, ES)

Sensory Evaluation Form

Date: _____

Product Description: _____

You are kindly requested to assess food sample presented for the dictated sensory attributes, referring to the following scale.

9 - Like Extremely
8 - Like Very Much
7 - Like Moderately
6 - Like Slightly
5 - Neither Like nor Dislike
4 - Dislike Slightly
3 - Dislike Moderately
2 - Dislike Very Much
1 - Dislike Extremely

Sample code	Overall Acceptability

Comments:_____

_____

_____

_____

_____

Department of Food Science & Technology, FAPPSC, SUSL

APPENDIX II

Ultimate Sensory Analysis (Reformulated Recipes)

Sensory Evaluation Form

Date: _____

Product Description: _____

You are kindly requested to assess food sample presented for the dictated sensory attributes, referring to the following scale

9 - Like Extremely
8 - Like Very Much
7 - Like Moderately
6 - Like Slightly
5 - Neither Like nor Dislike
4 - Dislike Slightly
3 - Dislike Moderately
2 - Dislike Very Much
1 - Dislike Extremely

Sample code	Flavour	Texture	Colour	Overall Acceptability

Comments:_____
_____
_____
_____
_____

Department of Food Science & Technology, FAPPSC, SUSL

APPENDIX III

Selection of the Most Appropriate Blend of Natural Cheese - Part I

Session I

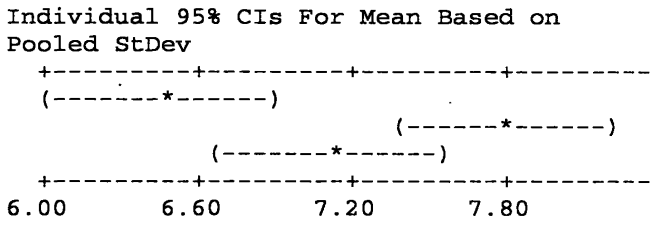
Normality test (Sample code 252-798-366)

H0: Data are normally distributed. Vs. H1: Data are not normally distributed.
The Kolmogorov-Smirnov test's p-value indicates that, at α levels smaller than 0.050, there is evidence that the data follow a normal distribution.

One-way ANOVA: Overall Acceptability versus Sample

```
Source        DF       SS     MS     F      P
Sample code    2    26.67  13.33  8.81  0.000
Error         87   131.73   1.51
Total         89   158.40

S = 1.231   R-Sq = 16.84%   R-Sq(adj) = 14.92%
```




```
Pooled StDev = 1.231

Hsu's MCB (Multiple Comparisons with the Best)

Family error rate = 0.05
Critical value = 1.94

Intervals for level mean minus largest of other level means

Level    Lower  Center  Upper
252     -1.950  -1.333  0.000
366      0.000   0.667  1.283
798     -1.283  -0.667  0.000
                      -2.0      -1.0       0.0       1.0
```

Interpreting the results

H0: $\alpha=0$ vs.
H1: $\alpha\neq0$ (α = 1.....i)

In the ANOVA table, the p-value (0.000) for overall acceptability indicates that there is sufficient evidence that not all the means are equal when alpha is set at 0.05. Hsu's MCB comparisons, compares the means of cheese samples (252 and 798) to the cheese sample (366) mean because it is the largest. Cheese sample (366) is best because the corresponding confidence interval contain positive values. No evidence exists that cheese samples (252 or 798) is the best because the upper interval endpoints are 0, the smallest possible value.

Session II

Normality Test (Sample Code 904-813-707)

H0: Data are normally distributed. Vs. H1: Data are not normally distributed.
The Kolmogorov-Smirnov test's p-value indicates that, at α levels smaller than 0.150, there is evidence that the data follow a normal distribution.

One-way ANOVA: Response versus Sample Code

```
Source        DF      SS    MS     F      P
Sample Code    2    1.09  0.54  0.28  0.756
Error         87  168.73  1.94
Total         89  169.82

S = 1.393   R-Sq = 0.64%   R-Sq(adj) = 0.00%

                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level   N   Mean  StDev   ---+---------+---------+---------+------
707    30  7.133  1.224     (-------------*-------------)
813    30  7.033  1.326   (-------------*-------------)
904    30  7.300  1.601           (-------------*-------------)
                          ---+---------+---------+---------+------
                           6.65      7.00      7.35      7.70

Pooled StDev = 1.393
```

Interpreting the results

In the ANOVA table, the p-value (0.756) for Overall acceptability indicates that there is sufficient evidence that all the means are equal when alpha is set at 0.05.

Session III

Normality Test(Sample Code272-258-952)

H0: Data are normally distributed. Vs. H1: Data are not normally distributed.
The Kolmogorov-Smirnov test's p-value indicates that, at α levels smaller than 0.150, there is evidence that the data follow a normal distribution.

One-way ANOVA: Response versus Sample Code

```
Source        DF      SS     MS      F      P
Sample Code    2   41.49  20.74  10.77  0.000
Error         87  167.63   1.93
Total         89  209.12

S = 1.388   R-Sq = 19.84%   R-Sq(adj) = 18.00%

                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level   N   Mean  StDev   --+---------+---------+---------+-------
258    30  6.800  1.690                        (------*------)
272    30  5.533  1.502      (------*------)
952    30  5.233  0.817   (------*------)
                          --+---------+---------+---------+-------
                          4.90      5.60      6.30      7.00

Pooled StDev = 1.388

Hsu's MCB (Multiple Comparisons with the Best)

Family error rate = 0.05
Critical value = 1.94

Intervals for level mean minus largest of other level means

Level    Lower  Center  Upper  ---------+---------+---------+---------+
258      0.000   1.267  1.962                      (----------*----)
272     -1.962  -1.267  0.000     (----*----------)
952     -2.262  -1.567  0.000  (-----*------------)
                               ---------+---------+---------+---------+
                                     -1.2       0.0       1.2       2.4
```

Interpreting the results

In the ANOVA table, the p-value (0.000) for overall acceptability indicates that there is sufficient evidence that not all the means are equal when alpha is set at 0.05. Hsu's MCB comparisons, compares the means of cheese samples (272 and 952) to the cheese sample (258) mean because it is the largest. Cheese sample 258 is best because the corresponding confidence interval contain positive values. No evidence exists that cheese samples (272 or 952) is the best because the upper interval endpoints are 0, the smallest possible value.

Session IV

Normality Test (Sample Code 907-861-824)

H0: Data are normally distributed. Vs. H1: Data are not normally distributed.
The Kolmogorov-Smirnov test's p-value indicates that, at α levels smaller than 0.150, there is evidence that the data follow a normal distribution.

One-way ANOVA: Response versus Sample Code

```
Source        DF      SS      MS      F      P
Sample Code    2  224.47  112.23  85.93  0.000
Error         87  113.63    1.31
Total         89  338.10

S = 1.143    R-Sq = 66.39%    R-Sq(adj) = 65.62%


                           Individual 95% CIs For Mean Based on
                           Pooled StDev
Level    N    Mean   StDev  -------+---------+---------+---------+--
824     30   6.167   1.020                (--*---)
861     30   8.200   0.761                                  (--*---)
907     30   4.333   1.516  (--*---)
                           -------+---------+---------+---------+--
                                4.8       6.0       7.2       8.4

Pooled StDev = 1.143

Hsu's MCB (Multiple Comparisons with the Best)

Family error rate = 0.05
Critical value = 1.94

Intervals for level mean minus largest of other level means

Level    Lower   Center   Upper   --+---------+---------+---------+-------
824     -2.606   -2.033   0.000          (--*---------)
861      0.000    2.033   2.606                        (---------*--)
907     -4.439   -3.867   0.000   (--*------------------)
                                  --+---------+---------+---------+-------
                                 -4.0       -2.0       0.0       2.0
```

Interpreting the results

In the ANOVA table, the p-value (0.000) for overall acceptability indicates that there is sufficient evidence that not all the means are equal when alpha is set at 0.05. Hsu's MCB comparisons, compares the means of cheese samples (907 and 824) to the cheese sample (861) mean because it is the largest. Cheese sample (861) may be best because the corresponding confidence interval contain positive values. No evidence exists that cheese samples (907 or 824) is the best because the upper interval endpoints are 0, the smallest possible value.

APPENDIX IV

Selection of the Most Appropriate Blend of Natural Cheese - Part II

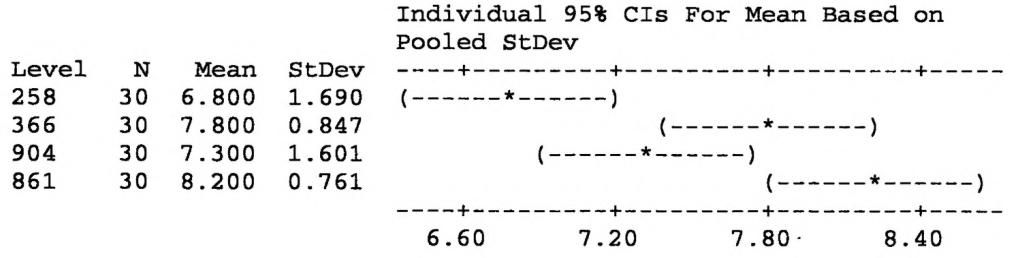
Normality Test (sample codes 366-904-258-861)

H0: Data are normally distributed. Vs. H1: Data are not normally distributed.
The Kolmogorov-Smirnov test's p-value indicates that, at α levels smaller than 0.150, there is evidence that the data follow a normal distribution.

One-way ANOVA: Overall Acceptability versus Sample

```
Source        DF      SS     MS     F      P
Sample code    3   36.10  12.03  8.52  0.000
Error        116  163.87   1.41
Total        119  199.97

S = 1.189   R-Sq = 18.05%   R-Sq(adj) = 15.93%
```




```
Pooled StDev = 1.189
```

```
Hsu's MCB (Multiple Comparisons with the Best)

Family error rate = 0.05
Critical value = 2.08

Intervals for level mean minus largest of other level means

Level    Lower  Center  Upper
258     -2.039  -1.400  0.000
366     -1.039  -0.400  0.239
904     -1.706  -1.067  0.000
861     -0.239   0.400  1.039
      -1.60    -0.80    -0.00     0.80
```

Interpreting the results

H0: $\alpha=0$ vs.
H1: $\alpha\neq0$ ($\alpha = 1.....i$)

In the ANOVA table, the p-value (0.000) for cheese indicates that there is sufficient evidence that not all the means are equal when alpha is set at 0.05. Hsu's MCB comparisons, compares the means of cheese samples (366,904 and 258) to the cheese sample (861) mean because it is the largest. Cheese sample 861 or 366 may be best because the corresponding confidence interval contain positive values. No evidence exists that cheese samples (904 or 258) is the best because the upper interval endpoints are 0, the smallest possible value. If cheese sample 366 is best, it is no more than 0.239 better than its closest competitor, and it may be as much as 1.039 worse than the best of the other level means.

APPENDIX V

Selection of the Most Appropriate Fat Source

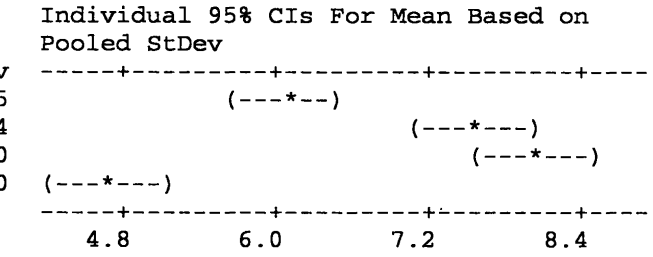
Normality Test (Sample Code 305-547-592-617)

H0: Data are normally distributed. Vs. H1: Data are not normally distributed.
The Kolmogorov-Smirnov test's p-value indicates that, at α levels smaller than 0.097, there is evidence that the data follow a normal distribution.

One-way ANOVA: Overall Acceptability versus Sample

```
Source         DF      SS     MS      F      P
Sample Code     3  212.29  70.76  41.73  0.000
Error         116  196.70   1.70
Total         119  408.99

S = 1.302   R-Sq = 51.91%   R-Sq(adj) = 50.66%
```




```
Pooled StDev = 1.302
```

```
Hsu's MCB (Multiple Comparisons with the Best)

Family error rate = 0.05
Critical value = 2.08

Intervals for level mean minus largest of other level means

Level    Lower  Center  Upper
305     -2.667  -1.967  0.000
547     -1.134  -0.433  0.267
592     -0.267   0.433  1.134
617     -4.067  -3.367  0.000
-3.0
-1.5
0.0
1.5
```

Interpreting the results

H0: $\alpha=0$ vs.
H1: $\alpha\neq0$ ($\alpha = 1.....i$)

In the ANOVA table, the p-value (0.000) for overall acceptability indicates that there is sufficient evidence that not all the means are equal when alpha is set at 0.05. Hsu's MCB comparisons, compares the means of cheese samples (305,617 and 547) to the cheese sample (592) mean because it is the largest. Cheese sample 547 or 592 may be best because the corresponding confidence interval contain positive values. No evidence exists that cheese sample 305 or617 is the best because the upper interval endpoints are 0, the smallest possible value. If cheese sample 547 is best, it is no more than 0.267 better than its closest competitor, and it may be as much as 1.143 worse than the best of the other level means.

APPENDIX VI

Selection of the Most Appropriate ES

Normality Test (Sample Code 192-429-622)

H0: Data are normally distributed. Vs. H1: Data are not normally distributed.
The Kolmogorov-Smirnov test's p-value indicates that, at α levels smaller than 0.150; there is evidence that the data follow a normal distribution.

One-way ANOVA: Re_OVERALL versus Sample Code

```
Source        DF      SS     MS      F      P
Sample Code    2  101.82  50.91  26.73  0.000
Error         86  163.80   1.90
Total         88  265.62

S = 1.380   R-Sq = 38.33%   R-Sq(adj) = 36.90%

                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level   N   Mean  StDev  ------+---------+---------+---------+---
192    29  6.966  1.210                      (----*----)
429    30  4.933  1.780  (----*----)
622    30  7.367  1.033                           (----*----)
                          ------+---------+---------+---------+---
                              5.0       6.0       7.0       8.0

Pooled StDev = 1.380

Hsu's MCB (Multiple Comparisons with the Best)

Family error rate = 0.05
Critical value = 1.94

Intervals for level mean minus largest of other level means

Level   Lower  Center  Upper  ------+---------+---------+---------+---
192    -1.099  -0.401  0.297                (-----*----)
429    -3.125  -2.433  0.000  (-----*------------------)
622    -0.297   0.401  1.099                          (----*-----)
                          ------+---------+---------+---------+---
                             -2.4      -1.2       0.0       1.2
```

Interpreting the results

H0: $\alpha=0$ vs.
H1: $\alpha\neq0$ ($\alpha = 1.....i$)

In the ANOVA table, the p-value (0.000) for Overall Acceptability indicates that there is sufficient evidence that not all the means are equal when alpha is set at 0.05. Hsu's MCB comparisons, compares the means of cheese samples (192 and 429) to the cheese sample (622) mean because it is the largest. Cheese sample 192 or 622 may be best because the corresponding confidence interval contain positive values. No evidence exists that cheese sample 429 is the best because the upper interval endpoints are 0, the smallest possible value. If cheese sample 192 is best, it is no more than 0.297 better than its closest competitor, and it may be as much as 1.099 worse than the best of the other level means.

APPENDIX VII

Sensory Evaluation of Reformulated Samples-Statistical Analysis

Flavour

Normality Test (Sample Code 456, 218, 552, 707, 537, 292)

H0: Data are normally distributed. Vs. H1: Data are not normally distributed.

The Kolmogorov-Smirnov test's p-value indicates that, at α levels smaller than 0.097; there is evidence that the data follow a normal distribution

Texture

Normality Test (Sample Code 456, 218, 552, 707, 537, 292)

H0: Data are normally distributed. Vs. H1: Data are not normally distributed.

The Kolmogorov-Smirnov test's p-value indicates that, at α levels smaller than 0.150; there is evidence that the data follow a normal distribution

Colour

Normality Test (Sample Code 456, 218, 552, 707, 537, 292)

H0: Data are normally distributed. Vs. H1: Data are not normally distributed.

The Kolmogorov-Smirnov test's p-value indicates that, at α levels smaller than 0.150; there is evidence that the data follow a normal distribution

Overall Acceptability

Normality Test (Sample Code 456, 218, 552, 707, 537, 292)

H0: Data are normally distributed. Vs. H1: Data are not normally distributed.

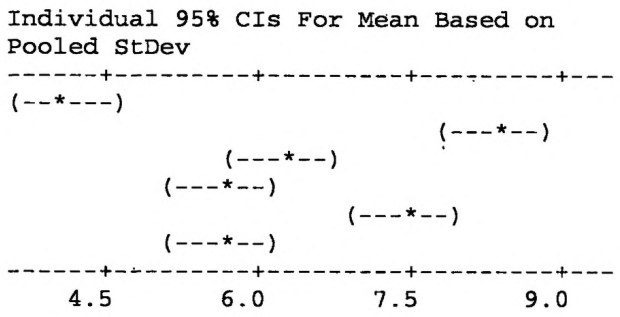
The Kolmogorov-Smirnov test's p-value indicates that, at α levels smaller than 0.150; there is evidence that the data follow a normal distribution

Comparison of Reformulated Samples, Market Sample and Company Sample

One-way ANOVA: Flavour versus Sample

```
Source   DF      SS     MS      F      P
Sample    5  333.63  66.73  28.04  0.000
Error   174  414.03   2.38
Total   179  747.66

S = 1.543   R-Sq = 44.62%   R-Sq(adj) = 43.03%
```




```
Pooled StDev = 1.543
```

```
Hsu's MCB (Multiple Comparisons with the Best)

Family error rate = 0.05
Critical value = 2.25

Intervals for level mean minus largest of other level means

Level    Lower   Center   Upper
218     -5.130   -4.233   0.000
292      0.000    0.900   1.796
456     -2.996   -2.100   0.000
537     -3.596   -2.700   0.000
552     -1.796   -0.900   0.000
707     -3.596   -2.700   0.000
-4.0
-2.0
0.0
2.0
```

Interpreting the results

H0: $\alpha=0$ vs.
H1: $\alpha\neq0$ ($\alpha = 1.....i$)

In the ANOVA table, the p-value (0.000) for flavour indicates that there is sufficient evidence that not all the means are equal when alpha is set at 0.05. Hsu's MCB comparisons, compares the means of cheese samples (456, 218, 552, 707 and 537) to the mean of cheese sample (292), because it is the largest. Cheese sample 292 is the best because the corresponding confidence interval contains positive value. No evidence exists that one of other cheese samples is the best because the upper interval endpoints are 0, the smallest possible value

One-way ANOVA: Texture versus Sample

```
Source   DF      SS     MS      F      P
Sample    5  321.80  64.36  21.99  0.000
Error   174  509.20   2.93
Total   179  831.00

S = 1.711   R-Sq = 38.72%   R-Sq(adj) = 36.96%

                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level   N   Mean  StDev   --+---------+---------+---------+-------
218    30  6.233  2.582                    (----*---)
292    30  7.433  1.040                              (----*---)
456    30  5.800  1.648                  (---*---)
537    30  4.333  1.422        (---*---)
552    30  5.900  1.605                  (---*---)
707    30  3.300  1.579  (---*---)
                          --+---------+---------+---------+-------
                          3.0       4.5       6.0       7.5

Pooled StDev = 1.711

Hsu's MCB (Multiple Comparisons with the Best)

Family error rate = 0.05
Critical value = 2.25

Intervals for level mean minus largest of other level means

Level   Lower  Center  Upper  ------+---------+---------+---------+---
218    -2.194  -1.200  0.000              (----*-----)
292     0.000   1.200  2.194                        (-----*----)
456    -2.628  -1.633  0.000            (----*-------)
537    -4.094  -3.100  0.000       (---*---------------)
552    -2.528  -1.533  0.000            (----*-------)
707    -5.128  -4.133  0.000  (----*-------------------)
                          ------+---------+---------+---------+---
                             -4.0      -2.0       0.0       2.0
```

Interpreting the results

H0: $\alpha=0$ vs.
H1: $\alpha\neq0$ ($\alpha = 1.....i$)

In the ANOVA table, the p-value (0.000) for flavour indicates that there is sufficient evidence that not all the means are equal when alpha is set at 0.05. Hsu's MCB comparisons, compares the means of cheese samples (456, 218, 552, 707 and 537) to the mean of cheese sample (292) because it is the largest. Cheese sample 292 is the best because the corresponding confidence interval contains positive value. No evidence exists that one of other cheese samples is the best because the upper interval endpoints are 0, the smallest possible value.

One-way ANOVA: Colour versus Sample

```
Source   DF      SS     MS      F      P
Sample    5  320.71  64.14  29.09  0.000
Error   174  383.60   2.20
Total   179  704.31

S = 1.485   R-Sq = 45.54%   R-Sq(adj) = 43.97%

                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level   N   Mean  StDev   --------+---------+---------+---------+-
218    30  7.000  0.830                        (---*----)
292    30  7.900  0.845                                (----*---)
456    30  4.833  1.315      (---*----)
537    30  4.533  1.776    (----*---)
552    30  6.300  2.231                  (---*----)
707    30  4.367  1.402   (---*----)
                          --------+---------+---------+---------+-
                                4.8       6.0       7.2       8.4

Pooled StDev = 1.485


Hsu's MCB (Multiple Comparisons with the Best)

Family error rate = 0.05
Critical value = 2.25

Intervals for level mean minus largest of other level means

Level    Lower  Center  Upper  -------+---------+---------+---------+--
218     -1.763  -0.900  0.000                  (----*-----)
292      0.000   0.900  1.763                            (-----*----)
456     -3.930  -3.067  0.000    (-----*-----------------)
537     -4.230  -3.367  0.000   (----*--------------------)
552     -2.463  -1.600  0.000              (-----*---------)
707     -4.396  -3.533  0.000  (----*----------------------)
                           -------+---------+---------+---------+--
                              -3.2      -1.6      -0.0       1.6
```

Interpreting the results

H0: $\alpha=0$ vs.
H1: $\alpha \neq 0$ ($\alpha = 1.....i$)

In the ANOVA table, the p-value (0.000) for colour indicates that there is sufficient evidence that not all the means are equal when alpha is set at 0.05. Hsu's MCB comparisons, compares the means of cheese samples (456, 218, 552, 707 and 537) to the mean of cheese sample (292) because it is the largest. Cheese sample 292 is the best because the corresponding confidence intervals contain positive value. No evidence exists that one of other cheese samples is the best because the upper interval end points are 0, the smallest possible value.

One-way ANOVA: Overall Acceptability versus Sample

```
Source   DF      SS     MS      F      P
Sample    5  300.84  60.17  42.32  0.000
Error   174  247.40   1.42
Total   179  548.24

S = 1.192   R-Sq = 54.87%   R-Sq(adj) = 53.58%

                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level   N   Mean  StDev   -+---------+---------+---------+--------
218     30  5.400  1.429     (---*---)
292     30  8.400  0.724                                (---*---)
456     30  5.433  1.006      (--*---)
537     30  5.533  1.525       (--*---)
552     30  7.700  0.877                          (--*---)
707     30  5.067  1.363   (--*---)
                          -+---------+---------+---------+--------
                         4.8       6.0       7.2       8.4

Pooled StDev = 1.192


Hsu's MCB (Multiple Comparisons with the Best)

Family error rate = 0.05
Critical value = 2.25

Intervals for level mean minus largest of other level means

Level   Lower  Center  Upper  -------+---------+---------+---------+--
218    -3.693  -3.000  0.000    (----*----------------)
292     0.000   0.700  1.393                          (----*---)
456    -3.660  -2.967  0.000     (---*----------------)
537    -3.560  -2.867  0.000     (----*----------------)
552    -1.393  -0.700  0.000                    (---*----)
707    -4.026  -3.333  0.000  (----*----------------)
                          -------+---------+---------+---------+--
                              -3.0      -1.5       0.0       1.5
```

Interpreting the results

H0: $\alpha=0$ vs.
H1: $\alpha \neq 0$ ($\alpha = 1.....i$)

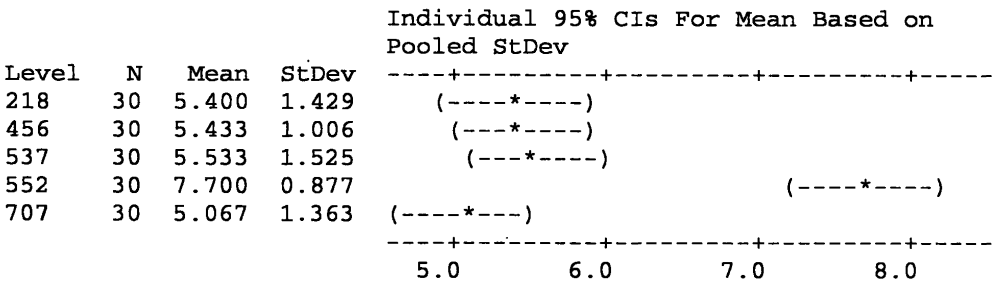
In the ANOVA table, the p-value (0.000) for overall acceptability indicates that there is sufficient evidence that not all the means are equal when alpha is set at 0.05. Hsu's MCB comparisons, compares the means of cheese samples (456, 218, 552, 707 and 537) to the mean of cheese sample (292) because it is the largest. Cheese sample 292 is the best because the corresponding confidence intervals contain positive value. No evidence exists that one of other cheese samples is the best because the upper interval end points are 0, the smallest possible value.

Comparison of Reformulated Samples with Company Sample

One-way ANOVA: Overall Acceptability versus Sample

```
Source      DF      SS     MS      F      P
Sample_1     4  135.29  33.82  21.12  0.000
Error      145  232.20   1.60
Total      149  367.49

S = 1.265   R-Sq = 36.82%   R-Sq(adj) = 35.07%
```




```
Pooled StDev = 1.265
```

```
Hsu's MCB (Multiple Comparisons with the Best)

Family error rate = 0.05
Critical value = 2.18

Intervals for level mean minus largest of other level means

Level    Lower  Center  Upper
218     -3.012  -2.300  0.000
456     -2.979  -2.267  0.000
537     -2.879  -2.167  0.000
552      0.000   2.167  2.879
707     -3.345  -2.633  0.000
-3.2
-1.6
-0.0
1.6
```

Interpreting the results

H0: $\alpha=0$ vs.
H1: $\alpha\neq0$ ($\alpha = 1.....i$)

In the ANOVA table, the p-value (0.000) for overall acceptability indicates that there is sufficient evidence that not all the means are equal when alpha is set at 0.05. Hsu's MCB comparisons, compares the means of cheese samples (456, 218, 707 and 537) to the mean of cheese sample (552) because it is the largest. Cheese sample 552 is the best because the corresponding confidence intervals contain positive value. No evidence exists that one of other cheese samples is the best because the upper interval end points are 0, the smallest possible value.

APPENDIX VIII

Assessment of Extent of Proteolysis

Construction of Standard Curve

Regression Analysis: Absorbance at 478 nm versus Protein Content (g)

The regression equation is
Absorbance at 478 nm = 0.602 - 2.51 Protein Content (g)

Predictor	Coef	SE Coef	T	P
Constant	0.60172	0.01497	40.20	0.000
Protein Content (g)	-2.5117	0.1315	-19.11	0.000

S = 0.0191384 R-Sq = 98.4% R-Sq(adj) = 98.1%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	1	0.13373	0.13373	365.10	0.000
Residual Error	6	0.00220	0.00037		
Total	7	0.13592			

Assessment of Extent of Proteolysis

Regression Analysis: Percentage Proteolysis versus Time (Days)

The regression equation is

Percentage Proteolysis = - 1.36 + 0.436 Time (Days)

Predictor	Coef	SE Coef	T	P
Constant	-1.357	2.387	-0.57	0.590
Time (Days)	0.43580	0.03223	13.52	0.000

S = 3.70952 R-Sq = 96.8% R-Sq(adj) = 96.3%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	1	2515.8	2515.8	182.83	0.000
Residual Error	6	82.6	13.8		
Total	7	2598.4			

APPENDIX IX

Electrophoretic Analysis of Proteolytic Pattern of Cheddar Cheese

Regression Analysis: Log Distance (cm) versus Molecular Mass (kD)

The regression equation is;

Log Distance(cm) = 0.875 - 0.0134 Molecular mass (kD)

Predictor	Coef	SE Coef	T	P
Constant	0.87510	0.03938	22.22	0.000
Molecular mass (kD)	-0.0134294	0.0007716	-17.41	0.000

S = 0.0648584 R-Sq = 98.1%. R-Sq (adj) = 97.7%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	1	1.2743	1.2743	302.94	0.000
Residual Error	6	0.0252	0.0042		
Total	7	1.2996			

Interpretation:

The p-value in the Analysis of Variance table (0.000), indicates that the relationship between Log Distance (cm) and Molecular Mass (kD) is statistically significant at an α-level of 0.05. The R^2 value shows that Molecular Mass (kD) explains 98.1% of the variance in, Log Distance (cm) indicating that the model fits the data extremely well. Regression equation can be rearranged as follows in order to determine the molecular masses of unknown fragments.

Molecular Mass (kD) = [0.875 - Log Distance (cm)] / 0.0134

APPENDIX X

Electrophoretic Analysis of Proteolytic Pattern of Cheddar Cheese

Table 2. Molecular masses of resolved fragments in SDS-PAGE

Sample	Volume (μℓ)	Distance (cm)	Log Distance (cm)	Molecular Mass (kD)	Coding Letter
1	2.5	3.8	0.579784	22.03104	B
		4.1	0.612784	19.56836	D
	5.0	3.7	0.568202	22.89537	A
		3.9	0.591065	21.18918	C
2	2.5	3.8	0.579784	22.03104	B
		4.1	0.612784	19.56836	D
		4.8	0.681241	14.45963	H
	5.0	3.7	0.568202	22.89537	A
		3.9	0.591065	21.18918	C
		4.3	0.633468	18.02478	E
		4.6	0.662758	15.83896	G
		4.9	0.690196	13.79134	I
3	2.5	3.8	0.579784	22.03104	B
		4.1	0.612784	19.56836	D
		4.5	0.653213	16.55127	F
		4.8	0.681241	14.45963	H
	5.0	3.7	0.568202	22.89537	A
		3.9	0.591065	21.18918	C
		4.3	0.633468	18.02478	E
		4.6	0.662758	15.83896	G
		4.9	0.690196	13.79134	I
4	2.5	3.7	0.568202	22.89537	A
		4.1	0.612784	19.56836	D
		4.3	0.633468	18.02478	E
		4.6	0.662758	15.83896	G
		4.9	0.690196	13.79134	I
	5.0	3.7	0.568202	22.89537	A
		4.1	0.612784	19.56836	D
		4.6	0.662758	15.83896	G
		4.9	0.690196	13.79134	I
		5.5	0.740363	10.04754	K
5	2.5	3.7	0.568202	22.89537	A
		3.9	0.591065	21.18918	C
		4.3	0.633468	18.02478	E
		4.6	0.662758	15.83896	G
	5.0	3.7	0.568202	22.89537	A
		3.9	0.591065	21.18918	C
		4.1	0.612784	19.56836	D
		5.3	0.724276	11.24806	J
		5.5	0.740363	10.04754	K

APPENDIX XI

Possible fragments resulting from proteolysis of α-S_1 casein

Clevage site	Fragments	Molecular mass (kD)	Agent
Phe $_{23}$-Phe $_{24}$	α-S_1 CN (f 1-23)	29.95500	Chymosin
	α-S_1 CN (f 24-199)	20.60450	
Phe $_{28}$-Phe $_{29}$	α-S_1 CN (f 1-28)	3.81250	
	α-S_1 CN (f 29-199)	19.78750	
Leu $_{40}$-Ser $_{41}$	α-S_1 CN (f 1-40)	5.39830	
	α-S_1 CN (f 41-199)	18.2010	
Leu $_{149}$-Phe $_{150}$	α-S_1 CN (f 1-149)	19.87741	
	α-S_1 CN (f 150-199)	3.72259	
Phe $_{153}$-Tyr $_{154}$	α-S_1 CN (f 1-153)	20.52811	
	α-S_1 CN (f 154-199)	3.07189	
Leu $_{156}$-Asp $_{157}$	α-S_1 CN (f 1- 156)	20.98866	
	α-S_1 CN (f 157-199)	2.61339	
Tyr $_{159}$-Pro $_{160}$	α-S_1 CN (f 1-159)	21.39005	
	α-S_1 CN (f 160-199)	2.20999	
Trp $_{164}$-Tyr$_{165}$	α-S_1 CN (f 1-164)	2.97861	
	α-S_1 CN (f 165-199)	1.62139	
Arg $_{90}$-Tyr $_{91}$	α-S_1 CN (f 1-90)	11.82733	Plasmin
	α-S_1 CN (f 91-199)	11.77267	
Lys $_{102}$-Lys$_{103}$	α-S_1 CN (f 1-102)	13.52443	
	α-S_1 CN (f 103-199)	10.06556	
Lys $_{103}$-Tyr$_{104}$	α-S_1 CN (f 1-103)	13.68063	
	α-S_1 CN (f 104-199)	9.91937	
Lys $_{105}$-Val $_{106}$	α-S_1 CN (f 1-105)	14.00803	
	α-S_1 CN (f 106-199)	9.59197	
Lys $_{124}$-Glu $_{125}$	α-S_1 CN (f 1-124)	16.54081	
	α-S_1 CN (f 125-199)	7.05919	
Arg $_{151}$-Gln $_{152}$	α-S_1 CN (f 1-151)	20.21681	
	α-S_1 CN (f 152-199)	3.38319	
Phe $_{23}$-Phe $_{24}$	α-S_1 CN (f 1-23)	29.95500	Cathepsin D
	α-S_1 CN (f 24-199)	20.60450	
Phe $_{28}$-Phe $_{29}$	α-S_1 CN (f 1-28)	3.81250	
	α-S_1 CN (f 29-199)	19.78750	

Possible fragments resulting from proteolysis of α-S_2 casein

Cleavage site	Fragments	Molecular mass (kD)	Agent
Phe $_{88}$-Tyr $_{89}$	α-S_2 CN (f 1-88)	10.62802	Chymosin
	α-S_2 CN (f 89-207)	14.25121	
Tyr $_{95}$-Leu $_{96}$	α-S_2 CN (f 1-95)	11.47343	
	α-S_2 CN (f 96-207)	13.40580	
Gln $_{97}$- Tyr $_{98}$	α-S_2 CN (f 1-97)	11.71498	
	α-S_2 CN (f 98-207)	13.16426	
Tyr $_{98}$-Leu $_{99}$	α-S_2 CN (f 1-98)	11.83575	
	α-S_2 CN (f 99-207)	13.04348	
Phe $_{163}$- Leu $_{164}$	α-S_2 CN (f 1-163)	19.68599	
	α-S_2 CN (f 164-207)	5.19324	
Phe $_{174}$-Ala $_{175}$	α-S_2 CN (f 1-174)	21.01450	
	α-S_2 CN (f 175-207)	3.86473	
Tyr $_{179}$-Leu $_{180}$	α-S_2 CN (f 1-179)	21.61836	
	α-S_2 CN (f 180-207)	3.38164	
Lys $_{21}$- Gln $_{22}$	α-S_2 CN (f 1-21)	2.53623	Plasmin
	α-S_2 CN (f 22-207)	22.34305	
Lys $_{24}$- Asn $_{25}$	α-S_2 CN (f 1-24)	2.89855	
	α-S_2 CN (f 25-207)	21.98068	
Arg $_{114}$-Asn $_{115}$	α-S_2 CN (f 1-114)	13.76812	
	α-S_2 CN (f 115-207)	11.11111	
Lys $_{149}$-lys $_{150}$	α-S_2 CN (f 1-149)	17.99517	
	α-S_2 CN (f 150-207)	6.88406	
Lys $_{150}$-Thr $_{151}$	α-S_2 CN (f 1-150)	18.11595	
	α-S_2 CN (f 151-207)	6.76328	
Lys $_{181}$-Thr $_{182}$	α-S_2 CN (f 1-181)	21.85991	
	α-S_2 CN (f 182-207)	3.14009	
Lys $_{188}$ –Ala $_{189}$	α-S_2 CN (f 1-188)	22.70532	
	α-S_2 CN (f 189-207)	2.17391	
Lys $_{197}$-Thr $_{198}$	α-S_2 CN (f 1-197)	23.79228	
	α-S_2 CN (f 198-207)	1.08695	

Possible fragments resulting from proteolysis of β-casein

Cleavage site	Fragments	Molecular mass (kD)	Agent
Leu $_{192}$-Tyr $_{193}$	β-CN (f 1-192 β-I^{I})	21.81440	Chymosin
	β-CN (f 193-209)	1.98740	
Ala $_{189}$- Phe $_{190}$	β-CN (f 1-189 β-I^{II})	18.0571	
	β-CN (f 190- 209)	2.18182	
Leu $_{163}$-Ser $_{164}$	β-CN (f 1-163 β-II)	18.16220	
	β-CN (f 164-209)	5.16748	
Gln $_{167}$-Ser $_{168}$	β-CN (f 1-167)	19.17711	
	β-CN (f 168-209)	4.70815	
Leu $_{139}$- Leu $_{140}$	β-CN (f 1-139 β-III)	19.30512	
	β-CN (f 140-209)	7.92347	
Leu $_{127}$- Thr $_{128}$	β-CN (f 1-127)	14.58379	
	β-CN (f 128-209)	9.30147	
Lys $_{28}$- Lys $_{29}$	β-CN (f 29-209 γ_1-CN)	20.39427	Plasmin
	8 slow β-CN (f 29-105)	11.17440	
	β-CN (f 29-107)	11.47580	
	8 fast β-CN (f 1-28)	3.44425	
Lys $_{105}$-His $_{106}$	β-CN (f 106-209 γ_2-CN)	9.06467	
	5 (β-CN f 1-105)	14.76912	
	β-CN (f 1-107)	15.06452	
Lys $_{107}$-Glu $_{108}$	β-CN (f 108-209 γ_3-CN)	8.77137	
Lys $_{113}$-Tyr $_{114}$	β-CN (f 1-113)	15.90242	
	β-CN (f 114-209)	8.08057	
Arg $_{183}$- Asp $_{184}$	β-CN (f 1-183)	20.62300	
	β-CN (f 184-209)	3.36050	

Possible fragments resulting from proteolysis of k-casein

Cleavage site	Fragments	Molecular mass (kD)	Agent
Phe $_{105}$-Met $_{106}$	k-CN (f 1-105)	12.14645	Chymosin
	k-CN (f 106- 169)	7.40352	

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