THE EFFECT OF IRRADIATION AND ELEVATED TEMPERATURE ON THE RIPENING OF CHEDDAR CHEESE.

By

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This thesis is dedicated to my parents,
Mota and Matieho, my sisiter, Puleng
and my husband, Kabelo.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

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CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

Ripening of cheese is an expensive and time consuming process depending on the variety and the intensity of flavour desired e.g. Cheddar cheese is typically ripened for 6-9 months, Mozzarella for about 3 weeks and Parmesan for two years (Wilkinson, 1993). Acceleration of ripening of cheese will therefore lower the production cost. This will be beneficial to both the producers and consumers. Acceleration of ripening of cheese can be attained by elevating the ripening temperature and by irradiation (Fedrick, 1987 and Abd El Baky et al., 1986).

1.2 Cheese ripening

Three primary events occur during cheese ripening i.e. glycolysis, proteolysis and lipolysis. These primary reactions are mainly responsible for the basic textural changes that occur in the cheese curd during ripening and are also largely responsible for the basic flavour, odour and body of cheese (Fox *et al.*, 1993). The basic composition and structure of cheese is determined by the curd manufacturing operations but it is during ripening that the individuality and unique characteristics of each cheese variety develop, as influenced by the composition for the curd and other factors, e.g. the microflora established during manufacture. Some bacterial growth does occur in cheese during ripening, especially of the non-starter lactic acid bacteria, and of moulds in the case of the moulds ripened varieties. Although the actual growth of these micro-organisms does contribute to cheese ripening, perhaps very significantly in some varieties, cheese ripening is essentially an enzymatic process (Fox *et al.*, 1993).

Four, and possibly five, agents are involved in the ripening of cheese; 1) rennet or rennet substitutes (i.e. chymosin, pepsin and microbial proteinase);

2) indigenous milk enzymes, which are particularly important in raw milk cheeses; 3) starter bacteria and their enzymes, which are released after the cells have died and lysed; 4) enzymes from secondary starter (e.g. propionic acid bacteria, *Brevibacterium linens,* yeasts and moulds, such as *Penicillium roqueforti* and *Penicillium candidum* are of major importance in some varieties; 5) non-starter bacteria, i.e. organisms that either survive pasteurisation of the cheese milk or gain access to the pasteurised milk or curd during manufacture; after death, these cells lyse and release enzymes (Fox *et al.*, 1993).

Ripening involves the production, via various pathways, of a pool of sapid compounds which give the flavour typical of the intended variety (Wilkinson, 1993). Lactic acid producing bacteria are most important in Cheddar cheese ripening, they can also grow at low temperatures and contribute to the three primary events that occur during cheese ripening which are responsible for texture and flavour, i.e. glycolysis, lipolysis and proteolysis.

1.2.1 Enzymes in cheese ripening

1.2.1.1 Rennet

Proteinases in cheese include plasmin, rennet and proteinases of the starter and non-starter bacteria. Peptidases originate from cell wall, cell membrane and intracellular locations of the starter and non-starter bacteria (Wilkinson, 1993). Approximately 6% of the rennet added to cheese-milk remains in the curd after manufacture and contributes significantly to proteolysis during ripening. It was concluded that sufficient peptides were produced by the normal level of rennet used and increasing the rennet level did not further stimulate the production of amino acids and flavour development but did lead to bitterness (Wilkinson, 1993).

The proportion of chymosin retained in the curd is strongly influenced by the pH at whey drainage, increasing as the pH decreases (Creamer *et al.*, 1985 and Holmes *et al.*, 1977). According to Green and Foster (1974), little active pepsin remains in Cheddar but O`Keeffe *et al.* (1978), found significant

activity; presumably the discrepancy can be explained by differences in cheese pH since porcine pepsin is very unstable in the pH range 6.5-7.0. A small proportion of microbial rennet is retained in the curd and this is independent of pH. Very little, if any, coagulant survives the cooking condition used for Swiss cheeses although some appears to survive in Mozzarella, as indicated by the formation of α_{s1}-I casein (Wilkinson, 1993).

All the principal commercial rennets are acid proteinases that show specificity for peptide bonds to which hydrophobic residues supply the carboxyl group; all show generally similar specificity on the β-chain of insulin. The proteolytic specificity of calf chymosin and the principal rennet substitute on α_{s1} and β-chain is fairly well and these findings can, largely, be extended to cheese.
The β-casein in solution is sequentially hydrolysed at bonds 192-193, 189-190,163-164 and 139-140 to yield the peptides β-I^{1}, β-I^{II}, β-II and β-III, respectively; bonds 165-166, 167-168 may also be hydrolysed to yield peptides indistinguishable electrophoretically from β-II and at low pH (2-3), bond 127-128 is also hydrolysed to yield β-IV (Creamer *et al.*, 1971 and Carles *et al.*, 1984).

The C-terminal region of β-casein is very hydrophobic and undergoes temperature-dependent hydrophobic interactions. It is likely that such associations occur in cheese and render the chymosin-susceptible bonds, which are located in this region, inaccessible to chymosin. Presumably the effect is related to water activity (a_w), but the influence of varying a_w directly on the proteolysis of individual caseins has not been studied. When animal rennets are used, β-casein is quite resistant to proteolysis in bacterially ripened cheese throughout ripening (Visser; 1977, Thomas and Pearce 1981). The β-peptides normally produced by rennet, i.e. β-I, β-II do not appear, suggesting that plasmin and or bacterial proteinases are responsible for the hydrolysis of β-casein in these cheeses. NaCl in cheese is undoubtedly an inhibitory factor but even in the absence of NaCl, the extent of β-casein hydrolysis by animal rennet is slight (Phelan *et al.*, 1973). The hydrolysis of β-casein by chymosin is strongly inhibited by 5%, and completely by 10%

NaCl (Fox and Walley, 1971). The reasons for this inhibition are not clear but a similar effect is produced by sucrose or glycerol (Creamer *et al.*, 1971) or by high protein concentrations.

In Cheddar and Dutch-type cheeses, α_{s1}-casein is completely degraded to α_{s1}-I and some further products by the end of ripening (Visser, 1977 and Creamer *et al.*, 1988). In mould-ripened cheese, α_{s1}-casein is completely degraded to at least α_{s1}-I prior to the mould-ripening phase and very extensive degradation occurs thereafter due to the action of fungal proteinase and peptidases (Godinho and Fox, 1982). The specificity of *M. miehei* proteinase on α_{s1}-casein appears to be generally similar to that of chymosin but the relative rates of hydrolysis of the various susceptible bonds by the two enzymes differed consequently, α_{s1}-I casein does not accumulate in cheese made with the microbial rennet (Creamer *et al.*, 1985,1988 and Phelan, 1985). Apparently the activity of *M. miehei* proteinase on isolated α_{s1}-casein is very low in the absence of NaCl but is markedly stimulated by the presence of 2% NaCl (Phelan, 1985). Although the γ-casein contains the chymosin susceptible bonds of β-casein, γ-caseins accumulate in cheese during ripening; presumably these bonds are inaccessible in γ-caseins, as they are in β-casein. The action of chymosin on γ-casein in solution does not appear to have been reported (Fox *et al.*, 1993).

The secondary proteolytic action of the coagulant influences flavour in three ways:

1. Some rennet-produced peptides are small enough to influence flavour. Unfortunately some of these peptides are bitter and excessive proteolysis, e.g. due to too much or excessively proteolytic rennet or unsuitable environmental conditions, e.g. too much moisture or too little NaCl, leads to bitterness.
2. Rennet-produced peptides serve as substrate for microbial proteinases and peptidases which produce small peptides and amino acids. These contribute at least to background flavour, and perhaps, unfortunately, to bitterness if the activity of such enzymes is excessive. Catabolism of

amino acids by microbial enzymes, and perhaps alteration via chemical mechanism, lead to a range of sapid compounds (amines, acids, NH_3, thiols), which are major contributors to characteristic cheese flavours.

3. Alteratons in cheese texture appear to influence the release of flavourous and aromatic compounds, arising from proteolysis, lypolysis, glycolysis and secondary metabolic changes, from cheese during mastication which may be the most significant contribution to cheese flavour (Fox *et al.*, 1993).

1.2.1.2 Chymosin

Chymosin (EC 3.4.23.4), which is the principle proteinase in traditional rennets used for cheesemaking, is an aspartyl proteinase from gastric secretion by young mammals. The principle role of chymosin in cheesemaking is to specifically hydrolyse the Phe_{105}-Met_{106} bond of the κ-casein (the micelle-stabilizing protein) as a result of which the colloidal stability of micelle is destroyed, leading to gelation at temperatures ≥20 °C. Most of the rennet added to cheesemilk is removed in the whey, but some is retained in the curd and plays a major role in the initial proteolysis of caseins in many cheese varieties. More or less 6% of the chymosin drainage added to the cheesemilk is retained in the curd but the amount increases as the pH of whey drainage decreases (Creamer *et al.*, 1985). Pepsin, especially porcine pepsin is more pH sensitive than chymosin and hence the amount of these coagulants retained in the cheese curd is very strongly dependent on the pH of milk at setting and shortly thereafter; in fact, increasing the pH of the curd whey to ~7 after coagulation of milk by porcine pepsin is one of the methods used to produce rennet-free cheese curd (O' Keeffe *et al.*, 1977,1978). Only 2-3% of *Mucor* rennets are retained in the curd and appear to be independent of pH (Creamer *et al.*, 1985). In high-cooked cheeses, e.g. Emmental, chymosin is extensively denatured and makes little or no contribution to ripening (Singh and Creamer, 1990).

The primary site of chymosin action on α_{s1}-casein is Phe_{23}-Phe_{24} (McSweeney *et al.*, 1993a). Cleavage of this bond is believed to be responsible for the softening of cheese texture (De Jong, 1976) and the small peptide (α_{s1}-CNf 1-23) is rapidly hydrolyzed by starter proteinase. Chymosin cleaves α_{s1}-casein in solution at Phe_{23}-Phe_{24}, Phe_{28}-Pro_{29}, Leu_{40}-Ser_{41}, Leu_{149}-Phe_{150}, Phe_{153}-Tyr_{154}, Leu_{156}-Asp_{157}, Tyr_{159}-Pro_{160}, and Trp_{164}-Tyr_{165}. These bonds are also hydrolyzed at pH 5,2 in the presence of 5% NaCl (e.g. conditions in cheese) together with Leu_{11}-Pro_{12}, Phe_{32}-Gly_{33}, Leu_{101}-Lys_{102}, Leu_{142}-Ala_{143} and Phe_{179}-Ser_{180} are also cleaved by chymosin (McSweeney *et al.*, 1993).

The hydrolysis of α_{s1}-casein by chymosin is influenced by pH and ionic strength (Mulvihill and Fox, 1977,1980). Although the amount of α_{s1}-I peptide increases during ageing of cheese, with a concomitant decrease of the native of α_{s1}-casein, the relationship between the amounts of α_{s1}-casein and α_{s1}-I peptide in cheese is very weak, probably because α_{s1}-casein can be hydrolyzed to other products of low molecular weight (less than 11 000 Da) that are not detected by polyacrylamide gel electrophoresis (PAGE) and also because α_{s1}-I undergoes further degradation by either rennet or by other proteinases. The α_{s1}-I peptide can be almost entirely degraded during ripening (Grappin *et al.*, 1985)

1.2.1.3 Pepsin

Calf rennet contains about 10% bovine pepsin (EC 3.4.23.1) and many calf rennet preparations contain up to 50% bovine pepsin. The proteolytic products produced from Na-caseinate by bovine pepsin are similar to those produced by chymosin, although the specificity of bovine or porcine caseins has not been determined. The hydrolysis of bovine, ovine, caprine and porcin β-casein by chymosins and pepsins from these species, suggested generally similar specificities for chymosins and pepsin on the large peptides produced, although differences are apparent in the short peptides. Pepsins are more proteolytic than the corresponding chymosins (Fox and McSweeney, 1997)

1.2.2 Microflora in cheese ripening

Pasteurisation causes a number of changes, in addition to killing off the indigenous micro-organisms, e.g., indigenous enzymes are inactivated, whey proteins may be denatured and may interact with caseins, salts equilibria may be altered and vitamins and other growth factors may be destroyed. Until recently it was not possible to distinguish between and quantify the significance of these heat-induced changes in cheese ripening. However the development of microfiltration makes it possible to remove the indigenous microorganisms (>99.9% removal) without other concomitant heat-induced changes. The results of a study by (McSweeney *et al.*, 1991), in which the quality and biochemical parameters of Cheddar cheese made from raw, pasteurised or microfiltered (MF) milk were compared, indicated that the indigenous micro-organisms were the most significant factor.

The pasteurised and MF cheeses were indistinguishable with respect to quality, rate and pattern of proteolysis, and extent of lipolysis. In contrast, the raw milk cheeses underwent more rapid and extensive proteolysis and the products of proteolysis were markedly different, especially when analysed by RP-HPLC. The flavour of the raw milk cheeses was much more intense than that of the pasteurised and MF cheeses but commercial graders regarded the raw cheeses as atypical and unacceptable.
The pasteurised and MF milks were free of non-starter lactic acid bacteria (NSLAB) at the start of manufacture while the raw milk contained ~200 NSLAB/ml. NSLAB grew in all cheeses during ripening, reaching maximum numbers after about 11 weeks when the pasteurised and MF cheeses contained ~10^7NSLAB/g while the raw milk cheese contained ~10^8 NSLAB/g (McSweeney *et al.*, 1991).

Possibly the principal difference between the starter- and non-starter lactobacilli in cheese is the greater variability of the latter. The starter lactobacilli are exclusively thermophillic, homofermentative strains while the non-starter lactobacilli are a heterogenous population and include

thermophilic and mesophilic heterofermentative as well as homofermentative species. In pasteurised milk cheese the non-starter lactobacilli originate mainly as post-pasteurisation contaminants from the factory environment. It is very likely that a unique, characteristic population of *Lactobacillus* species and strain will evolve in each factory. The population may vary over time and will be strongly influenced by the standards of hygiene in the factory (McSweeney *et al.*, 1991).

There is little detailed information on such variability, which is probably responsible for variations in the quality of cheese. In the case of raw milk cheeses, the non-starter lactobacilli originate from the milk supply as well as from the factory environment. Hence, the species of lactobacilli in raw milk and pasteurised milk cheeses might be expected to vary markedly. It appears that NSLAB can have a significant impact on cheese quality, however, variably in the number and type of NSLAB population, whether due to the use of raw or thermized milk, variations in the factory environment or to variations in cooling is a problem (McSweeney *et al.*, 1991).

1.2.3 Proteolytic enzymes from starter cultures

Proteolysis occurs in all cheese varieties and is considered to be a prerequisite for good flavour development. It is affected by a number of agents including residual coagulant, indigenous milk proteinases, and the proteinases and peptides of starter and non-starter bacteria (Wilkinson, 1993).

Proteolysis is the most complex of the three primary events during cheese ripening and is possibly the most important for development of flavour and texture, especially in internal bacterial ripened cheeses. Proteolysis contributes to cheese ripening in at least four ways; 1) a direct contribution to flavour via amino acids and peptides, some of which may cause off-flavours, especially bitterness, or indirectly via catabolism of amino acids to amines, acids, thiols, thioesters, etc; 2) greater release of sapid compounds during mastication; 3) changes in pH via the formation of NH_3; 4) changes in texture arising from breakdown of the protein network, increase in pH and greater

water-binding by the newly formed amino and carboxyl groups. Although the ripening of some varieties (e.g Blue and Romano) is dominated by the consequence of lipolysis, proteolysis is more or less important in all varieties. In the case of Cheddar and Dutch-type cheeses, and probably other varieties, many authors regard proteolysis as the most important biochemical event during ripening. A high correlation exists between the intensity of Cheddar cheese flavour and the concentration of free amino acids (Aston *et al.*, 1983 and Amantea *et al.*, 1986).

Attempts have been made to develop proteolytic indices of cheese maturity; although such indices correlate well with age and maturity, they fail to detect off-flavours and should therefore be regarded as complementary to the organoleptic assessment of quality (Fox *et al.*, 1993). Proteolysis by rennet is believed to be responsible for the softening of cheese texture early during ripening via the hydrolysis of α_{s1}-casein to α_{s1}-I, which is sufficient to break the continuous protein matrix (De Jong; 1976, Creamer and Olson, 1982).

Undoubtedly, further proteolysis by coagulant, plasmin and bacterial proteinases modifies the texture further. Even in surface mould-ripened cheeses, and probably in smear cheeses, coagulant is considered to be essential for the development of proper texture, e.g. in Camembert, although the very marked increase in pH (to 7) caused by the catabolism of organic acids and the production of ammonia (by deamination of amino acids) is also essential (Lenoir, 1984 and Noomen, 1983). The proteinases excreted by the mould diffuse into the cheese to only a slight extent and contribute little to proteolysis within the cheese, although peptides produced by these enzymes in the surface layer may diffuse into the cheese.

1.2.3.1 Proteinases

The first step in the cascade of reactions leading to the production of amino acids from casein involves proteinases. Lactococcal proteinases have been well characterized biochemically and genetically. It is now generally accepted

that they are cell wall-associated except in the case of *L. lactis* subsp. *cremoris* ML1 which secretes the proteinase into the culture medium.

1.2.3.2 Peptidases in lactococci

The lactococcal proteinase initiate degradaton of the casein substrate to polypeptides which are then further hydrolysed by peptidase to yield peptides and amino acids which are necessary for cellular nutrition. The principal peptidases in lactococci are exopeptidases which catalyse cleavage of one or two amino acids from the free N-terminal of the peptide chain. Exopeptidase activity in the lactococci is exemplified by amino-di-tri-and tetrapeptidases. Endopeptidases can cleave large peptides at some bond within the peptide, distance from the carboxyl or amino terminus. In this respect, a proteinase can be regarded as an endopeptidase (Fox *et al.*, 1993).

1.2.3.3 Endopeptidases

The term 'endopeptidase' is applied to enzymes that hydrolyse interior bonds of peptides, but not of proteins Yan *et al.* (1987) identified two endopeptidases, LEPI and LEPII, in *L. cemoris* H61, which, since they were inhibited by EDTA and activated by Mn^{2+},were classified as metalloenzymes. LEPI had a molecular weight of 98kDa and a high affinity for Gly-Asn peptide bonds. Its pH and temperature optima were 70 and 40%, respectively. LEPII was an 80kDa endopeptidase which hydrolyse peptide bonds involving amino group of hydrophobic amino acids. It had pH and temperature optima at 6.0 and 37%, respectively. These endopeptidases participate in the degradation of α_{s1}-casein (f1-23), the first peptide produced from α_{s1}-casein by chymosin. These enzymes were not inhibited by serine Protinase inhibitors and therefore differed from lactococcal proteinases. A combination of endopeptidases due to secondary coagulant action, indigenous milk proteinases, starter bacteria and secondary microflora act on cheese proteins to break down structure and bring about changes to cheese body and texture. These enzymes also provide peptide structure for the release of amino acids. The amino acids are

thought to contribute to savoury background flavour in cheese, and also act as precursors for flavour volatiles (Law, 1987).

1.2.3.4 Aminopeptidases

Aminopeptidases hydrolyse l-lysyl-p-nitroanilides, have an optimum temperature of 40% and are irreversibly inhibited by EDTA. These aminopeptidases have a broad specificity and hydrolyse large peptides produced from β-casein by lactococcal proteinases (Geis *et al.*, 1985).

1.2.3.5 Starter proteinases

Work on several varieties of cheeses made with a controlled microflora indicates that starter proteinases/peptidases are primarily responsible for the formation of small peptides and amino acids i.e. trichloroacetic acid soluble nitrogen (TCA-soluble N). Likewise, these studies indicate that starter proteinase contribute little to the formation of the larger, pH 4.6 or water-soluble peptides. However, the lactococcal proteinases are capable of hydrolysing intact caseins in solution, especially β-casein, relatively few strains are capable of hydrolysing α_{s1}-casein, i.e. only those with P-111-type proteinase, but this is probably not significant since this protein is rapidly hydrolysed by chymosin and other rennets. However, the cell wall–associated proteinases can readily hydrolyse α_{s1} f1-23 (produced by chymosin) at several sites; some of the resultant peptides have been demonstrated in Gouda cheese (Kaminogawa *et al.*, 1986 and Exterkate *et al.*, 1991). In Dutch and Cheddar cheeses, the concentration of β-casein decreases slowly during ripening with the formation of little, if any, β-I (suggesting the lack of chymosin activity) but with the formation of γ-caseins (indicating plasmin activity). The cell wall proteinase of *L. lactis* NCDO 763 cleaves five bonds in β-casein, i.e. Ser-Gln (166-167), Gly-Lys (175-176), Gln-Arg (182-183), Tyr-Gln (193-194) and Ile-Ile (207-208) (Monnet *et al.*, 1986).

Since chymosin does not hydrolyse β-casein in cheese, possibly because of intermolecular hydrophobic interactions, it is probable that the starter proteinases are also unable to hydrolyse this region of β-casein in cheese. Starter bacteria reach maximum numbers in Cheddar and Dutch cheeses at or shortly after the end of manufacture, and viable numbers decline quickly thereafter (Visser, 1977; Martley and Lawrence, 1972). It is generally assumed that the cells lyse after death, releasing intracellular enzymes that diffuse into the surrounding environment (Umemoto *et al.*, 1978).

1.2.4 Lipolytic enzymes

1.2.4.1 Lipases

In most varieties, relatively little lipolysis occurs during ripening and is considered undesirable; most consumers would consider Cheddar, Dutch and Swiss- type cheese containing even a moderate level of free fatty acids to be rancid. Bills and Day (1964) failed to find any significant differences, qualitatively or quantitatively, in the free fatty acids in Cheddar cheese of widely different flavour. The ratios of individual free fatty acids from $C_{6:0}$ to $C_{18:2}$ in cheese to the corresponding esterified acids in milk fat were very similar, indicating that these acids were released non-selectively. However, free butyric acid was always about double that in glycerides suggesting that it is selectively liberated or synthesized by microorganisms. Reiter *et al.* (1966) suggested that volatile fatty acids may contribute to the background flavours of Cheddar but felt that longer chain fatty acids ($>C_{4:0}$) are not important.

In extra-mature cheeses, fatty acids probably make a positive contribution to flavour when properly balanced by the products of proteolysis and other reactions. Exceptions to the above general situation are the Blue cheeses and certain hard Italian varieties, e.g. Romano and Parmesan.

Milk contains a very potential lipoprotein lipase which normally never reaches its potential in milk. Indigenous milk lipase probably causes significant

lipolysis in raw milk cheese and probably makes some contribution in pasteurized milk cheese, especially if the milk was heated at sub-pasteurization temperatures, since heating at 78 ^{o}C for 10 seconds is required to completely inactivate milk lipase. Milk lipase is highly selective for fatty acids on the sn-3 position; since most of the butyric acid in milk fat is esterified at the sn-3 position, this specificity probably explains the disproportionate concentration of free butyric acid in cheese. Good quality rennet extract contains no lipase activity. In contrast, the rennet paste used in the manufacture of some Italian cheese contains a potent lipase, pregastric esterase (PGE), which in some countries is added to the cheese milk in partially purified form. Literature on PGE has been reviewed by Nelson *et al.* (1977).

PGEs show high specificity for short chain fatty acids esterified at the sn-3 position. Since the short-chain acids in milk fat are fat predominantly at the sn-3 position, the action of PGE results in the release of high concentrations of short and medium chain acids which are responsible for the characteristic piquant flavour of the hard Italian cheeses.

The lipases of psychrothrophs are probably more significant in cheese, and butter, than their proteinases, which are water-soluble and are therefore lost in the whey (Bhowmik and Marth, 1989). Mould-ripened cheeses, especially blue cheese, undergo the highest level of lipolysis of all varieties and up to 25% of the total fatty acids may be liberated in some blue cheese. However, the impact of fatty acids on the flavour of these cheeses is less than that for the hard Italian varieties, possibly due to neutralization on the elevation of the pH during ripening, and also due to domination of blue cheese flavour by methyl ketones.

1.4.2.2 Phosphatases

Phosphatases are enzymes which hydrolyse the C-O-P linkage of various phosphate and phosphonate esters, they are classified into "acid" or "alkaline" groups depending on the effect of pH on their activity (Stauffer, 1979). Although both acid and alkaline phosphatases are present in cheese, the formery are more active due to the relative low pH (about 5.2) of cheese. During ripening, the caseins are cleaved by rennet, plasmin and bacterial proteinases into phosphorus-rich peptides (Schormuller, 1968). The phosphate residues exert a protective effect against further proteolytic hydrolysis of the peptides. Complete casein degradation during cheese ripening can be achieved only by the combined action of proteinases and phophatases (Larsen and Parada, 1988).

Therefore, phophatases may play an important role in cheese maturation and flavour development (Martley and Lawrence, 1972 and Dulley and Kitchen, 1972). However, the activity of acid phosphatase(s) in cheese is probably the least studied of the primary hydrolytic events during ripening and their significance is mainly putative. The level of acid phosphatase activity in cheese remains constant during ripening.

(Andrews and Alichanidis, 1975) found no change in activity during storage of Feta cheese for 9-12 months at 6 ^{o}C. Similar results were obtained for Tele`me cheese and for softer cheese such as Kasseri when stored up to 18 months. Examination of cheddar cheese of differing ages showed that over a 12 months period no significant changes in acid phosphatase activity occurred at 13 ^{o}C. The levels of enzyme activity were relatively low at $8.1\pm1.11\times10^{-3}$ units/g throughout ripening. These comparative low values for a hard cheese such as cheddar suggested the involvement of factors other than the concentration effect of the milk enzyme (Andrews and Alichanidis, 1975).

Other possible sources of phosphatase in cheese are the starter bacteria. Both primary starter such as lactococci, and secondary organisms (fungi and

yeasts) contain acid phosphatase (Schormuller, 1968). The cellular location of the enzymes in lactococci has not been reported but it would appear to be cell wall or membrane bound (Larsen and Parada, 1988 and Dulley and Kitchen, 1972). The starter enzyme has a high molecular weight. Martley and Lawrence (1972) indicated that a starter culture producing a good flavoured cheese should possess high acid phosphatase activity. This view is not shared by all researchers and many consider the role of the bacterial acid phosphatase in cheese to be minimal (Andrews and Alichanidis, 1975).

The lactococcal enzyme binds strongly to miccelar casein but it does not dephosphorylate the caseins to a significant extent. Lactococcal acid phosphatase may, however, be more active on small phophopeptides produced from casein. Optimum activity of the starter enzyme is at pH 5.2 while the milk enzyme has a pH optimum of about 5.0. Although the source of the acid phosphatase in cheese is not certain, most authors believes that dephosphorylation of peptides by acid phosphatase is an important reaction in ripening cheese. The only report of dephosphorylation of peptides during cheese maturation is that of Dulley and Kitchen (1972).

1.3 Accelerated ripening

Ripening is an expensive and time-consuming process, depending on the variety; e.g. Cheddar cheese is typically ripened for 6-9 months while Parmesan is usually ripened for two years. Owing to the cost of ripening cheese, there are obvious economic advantages to be gained by accelerating the process. Greater control of ripening may also be gained by manipulating the process whereby end product quality may be predicted with greater certainty (Fox, 1988/89). Acceleration of cheese ripening is, therefore, of benefit to the producer from both the economic and technological point of view, provided, of course, that the final product has the same flavour profile and rheological attribute as conventional cheese (Fox, 1988/89; Law, 1986; Wilkinson, 1990; El-Soda and Pandian, 1991 and Walstra, 1987).

Three principal biochemical events involved in cheese ripening are; a) glycolysis of residual sugars, b) lypolysis, and c) proteolysis involving the degradation of the caseins to lower molecular weight peptides and free amino acids. Acceleration of glycolysis, which occurs rapidly, is considered to be of no benefit in most or all cheese varieties. Acceleration of lypolysis may be of benefit in Blue or some Italian types where lypolysis plays a major role in the generation of characteristic flavour. The contribution of lipolysis to the flavour of cheddar or Dutch cheese is unclear, and acceleration of lipolysis in these types is not usually undertaken as a means of enhancing flavour development. The aim of accelerating the various biochemical pathways is to reduce the ripening time without adversely affecting flavour or texture (Wilkinson, 1993).

Approaches to accelerate ripening fall into five categories; 1) exogenous enzymes, 2) modified starters, 3) cheese slurries/ high moisture cheeses, 4) adjunts of non-starter bacteria and, 5) elevated temperatures (Wilkinson, 1993)

Each method has associated advantages and disadvantages. Exogenous enzymes are relatively cheap, have specific action and give a choice of flavour options, but the choice of useful enzymes is rather limited, there is a risk of over-ripening, difficulty with uniform incorporation and possible legal barriers. Modified starters are easy to incorporate and the natural enzyme balance is retained, but modification of starters, either by physical or genetic approaches, is technically complex (Wilkinson, 1993).

Enzyme-modified, high-moisture cheeses have been used successfully as food ingredients, but in general, do not develop flavour or texture characteristics of the corresponding natural cheese. Adjunct cultures of non-starter lactic acid bacteria (NSLAB) may have potential to accelerate ripening, but to date, their use has been limited by the availability of suitable strains. Cheese ripening at elevated temperatures is technically the simplest method for accelerating ripening and the lower refrigeration costs may provide overall savings to the producer. The drawbacks of this approach are an increased risk of microbial spoilage and non-specific increases in ripening

reactions, possibly leading to unbalanced flavour or off-flavours (Wilkinson, 1993)

1.3.1 Temperature

Enzymatic, as well as chemical reactions generally occurs at faster rate as the reaction temperature is increased. Therefore, it can be reasonably assumed that the biochemical reactions that generate flavour compound or flavour precursors in cheese will be accelerated by increasing the temperature at which the cheese is matured. Many cheese varieties are now ripened at low temperatures e.g. 6-8 ^{o}C for Cheddar (Fox, 1988/89 and Fedrick *et al.*, 1983). Enzymatic as well as chemical reactions generally occur at faster rates as the reaction temperature is increased. Therefore, it can be reasonably assumed that the biochemical reactions that generate flavour compounds or flavour precursors in cheese, will be accelerated by increasing the temperature at which the cheese is matured.

In a study on the influence of various factors, such as starter type, level of non-starter lactic acid bacteria (NSLAB) and ripening temperature, i.e. 6 or 13 ^{o}C, on the flavour intensity of Cheddar cheese after six or nine months ripening, Law *et al.* (1979) found that ripening temperature was the single most important factor. After a maturation period of six months, cheese ripened at 13 ^{o}C scored 4.4 on a 0 to 8 scale for flavour intensity (corresponding to medium or matured cheese) while cheeses stored at 6 ^{o}C scored 3.2, corresponding to mild cheddar. At 6 ^{o}C, bitterness was more marked, either due to a lower flavour intensity or because degradation of bitter peptides by peptidase was not favoured at this temperature (Wilkinson, 1993).

Ripening at elevated temperatures ($\leq$15 ^{o}C) has been recommended to accelerate the ripening of cheese of good chemical and microbiological quality (Fedrick, 1987). El-Soda and Pandian (1991) concluded that the use of elevated temperatures to accelerate ripening was likely to be limited to large cheese factories where very hygienic procedures are adopted during

manufacture and ripening. Most cheddar cheese is now produced from pasteurised milk in highly automated plants with high hygienic standards and, thus, ripening of Cheddar cheese at an elevated temperature may be feasible (Folkertsma *et al.*, 1996).

Cooling rate and ripening temperature have a marked influence on the development of the viable microflora of commercially made cheddar cheese. Proteolysis and lipolysis can be accelerated by ripening at higher temperatures, could be retarded by placing cheeses into a lower temperature environment at some point during ripening. Ripening of cheddar cheese can be accelerated and its flavour intensified by ripening at 16 ^{o}C. However, ripening at 12 ^{o}C is undoubtedly more prudent since the texture of cheeses ripened at 16 ^{o}C deteriorated after about six months. The rate of ripening can be accelerated or retarded by raising or lowering the temperature during ripening. Increasing the ripening temperature markedly increased the rate and extent of lipolysis. The rate of lipolysis decreased on transfer of cheeses from 16 to 8 ^{o}C and visa versa (Folkertsma *et al.*, 1996)

1.3.2 Micro-organisms

Most evidence to date indicates the important role played by the enzymes of starter and non-starter bacteria in the generation of flavour in various cheese varieties. The objective of using modified or attenuated starters is to increase the number of starter cells without detrimental effect on the acidification schedule during manufacture so that the cells contribute only to proteolysis and other changes during ripening. A number of approaches have been adopted to augment the contribution of these cultures in an attempt to accelerate ripening, i.e. the use of a) lyzozyme treated starters, b) heat or freeze-shocked cells and c) mutant cultures. Modified starter culture, with attenuated acid-producing abilities, are added with the normal starter culture during cheese manufacture and contribute to proteolysis during ripening (Wilkinson, 1993).

Cheddar cheese manufactured with defined strains starter, producing a clean, consistent flavour, does not satisfy the market for extra-mature cheeses. Indeed, it now appears that the focus of accelerated ripening may switch from the use of added proteinases and lipases which are subject to strict legislation in some countries, to the selection of starter strains with enhanced autolytic properties and increased peptidase activity. This will provide a more balanced enzyme complement than that obtained through the addition of exogenous enzymes (Wilkinson, 1993).

The effect of the level of starter proteinase on the development of bitterness in Cheddar cheese was investigated by many researchers using cultures containing varying proportions of prt^+ and prt^-cells for cheese manufacture. Cheese made with 45-75% prt^- cells were significantly less bitter than cheese made using only prt^+ cells, this implies that the cell wall proteinase has a role in the production of bitter peptides which may be removed by the action of intracellular peptidases.

Non-starter lactic acid bacteria (NSLAB) are considered to make a significant contribution to proteolysis and flavour development in cheese (Peterson and Marshall, 1990 and Broome *et al.*, 1990). Therefore, the addition of selected NSLAB, along with the normal starter, to increase the rate of casein degradation and flavour development has begun to receive research attention. Inoculation of milk for cheddar cheese with heterofermentative lactobacilli, *L. brevis* or *L. fermentum*, consistently caused flavour and body defects, e. g. fruity flavour, openness and late gas formation. When combined with homofermenters e.g. *L. casei* subspecies *casei* or *L. casei* subspecies *pseudoplatarium,* heterofermenters caused no significant downgrading compared to the control cheeses, but neither did they improve the flavour over the control (Laleye *et al.*, 1990 and Lee *et al.*, 1990).

The addition of *Micrococcus* or *Pediococcus* strains to low-fat Cheddar cheese has been reported to enhance proteolysis and flavour development over the control cheese (starter only) after three months ripening. After six

months, the cheese inoculated with pediococci graded highest, while off-flavour development was noted for cheeses supplemented with micrococci (Bhowmik *et al.*, 1990).

1.3.3 Enzymes

Since cheese ripening is essentially an enzymatic process, it should be possible to accelerate ripening by augmenting the activity of key enzymes. Addition of enzymes has the advantage of more specific action for accelerating flavour development compared to elevated temperatures that can accelerate off-flavour development just as much as flavour-forming reactions. Enzymes may be added to generate specific flavours in cheese, e.g. lipase addition for Parmesan or blue-type cheese flavour (Fox, 1988/89 and Law, 1986). On the negative side, enzyme addition is not permitted in all countries and the range of useful enzymes available is quite limited. Uniform distribution of enzymes in the curd can be difficult to achieve and may give rise to small concentrations if the enzyme is added with the salt. If enzymes are added to the cheese milk, 90% of the enzyme may be lost in the whey, proteolysis can occur in the vat during manufacture, generating small peptides which are lost in the whey, leading to reduced yield, and the whey may be rendered unsuitable for further processing. Over-ripening, with flavour and body defects, may occur because of the inability to control enzyme activity during ripening (Wilkinson, 1993).

A more balanced approach to the acceleration of ripening using mixtures of proteinases and peptidases, attenuated starter cells or cell-free extracts is now favoured. Intracellular cell-free extracts (CFE) of cheese starter bacteria in combination with Neutrase resulted in significant acceleration of flavour development (Law *et al.*, 1983).

CFE caused no increase in the level of primary proteolysis or 12% TCA soluble N but caused the rapid release of small peptides and free amino acids. In combination with Neutrase, the levels of small peptides and free amino acids were greater than when Neutrase was used alone (Wilkinson,

1993). Proteolysis (12% TCA soluble N) was significantly increased in experimental cheeses over the control (Marschke and Dulley, 1978). The enzyme used in the latter trial was from *Kluyveromyces lactis,* available as Maxilact (Gis-Brocades). In a further study by (Marschke *et al.*, 1980), on the use of Maxilact to accelerate the ripening of cheddar cheese it was found that the preparation contained a proteinase, which was responsible for the increased level of peptides and free amino acids and the improved flavour of experimental cheeses. No increase in starter cell numbers could be attributed to the action of these enzymes (Marschke *et al.*, 1980). Law (1986) reported that high starter cell numbers do not necessarily lead to increased flavour development and suggested that available evidence supports the proposal that accelerated ripening reported for Maxilact-treated cheese is due to contaminating proteinase(s).

Lipolysis plays a major role in the generation of flavour in certain cheese types such as Romano, Blue cheeses and Feta, but its importance in varieties such as Cheddar or Gouda is unclear. The volatile fraction of Cheddar cheese, containing fatty acids, contributes significantly to cheese aroma but not to taste, the water-soluble fraction has no taste or aroma while the non-volatile water-soluble fraction contributes most to flavour intensity (Aston and Creamer, 1986 and McGugan *et al.*, 1979).

Acceleration of flavour development in Ras and Domiati cheeses by addition of commercial animal lipases has been reported (Abd El Salam *et al.*, 1978 and El-Neshawy *et al.*, 1982). Ras cheese flavour was improved by addition of Capalase K (from goat gastric tissues). Enzyme- treated cheeses acquired mature flavour after 45-60 days compared to 90 days for control cheeses. In the case of Domiati cheese with added lipase (i.e. kid-goat-lamb or lamb PGE), the flavour intensity of four-week-old experimental cheeses was more pronounced than that of an 8 weeks-old untreated cheese; however, at higher levels of enzyme, rancidity developed after eight weeks (El-Neshawy *et al.*, 1982). Improvement of the flavour of Cheddar and Provolone was noted when rennet pastes were used or when gastric lipase was added with rennet extracts, however increased proteolysis occurred, indicating significant

contamination of this lipase by proteinases. The addition of combinations of various fungal proteinases and lipases to Cheddar cheese has been reported to reduce ripening time by 50%, with good quality medium sharp Cheddar being produced in 3 months at 10 ^{o}C, when matured at 4,5 ^{o}C, little acceleration of ripening was observed (Wilkinson, 1993).

β-Galactosidase (lactase) hydrolyses lactose to glucose and galactose. Treatment of milk with β-galactosidase prior to the manufacture of yoghurt, buttermilk or cottage cheese shortened the manufacturing time by 20% (Thompson and Gyuriscsek, 1974). Yoghurts were 'sweeter" and "less acid" and were generally regarded as being more acceptable than controls. Addition of lactase to Cheddar cheese milk has been reported to reduce manufacturing time, improve flavour and accelerate ripening by about 50% (Marschke and Dulley, 1978).

1.4 Irradiation

The types of radiation used in food are called ionizing radiations because they are capable of converting atoms and molecules to ions by removing electrons. Ionizing radiations can be energetic charged particles, such as electrons, or high-energy photons, such as X-rays or gamma rays. Not all types of ionizing radiation are suitable for foods, either because they do not penetrate deep enough into the irradiated material or because they make the irradiation material radioactive. The gamma rays are from the radionuclides ^{60}Co or ^{137}Cs and the X-rays are generated from machine sources operated at or below an energy level of 5 megaelectronvolt (5MeV) (Diehl, 1990).

Gamma rays and X-rays are part of the electromagnetic spectrum which reaches from the low-energy, long wavelength radio waves to the high-energy, short wavelength cosmic rays. X-rays and gamma rays are identical in their physical properties and in their effect on matter; they differ only in their origin, X-rays being produced by machines, and gamma rays coming from radioactive isotopes (Diehl, 1990).

Many of the practical applications of food irradiation have to do with preservation. Radiation inactivates food spoilage organisms, including bacteria, moulds and yeasts. It is effective in lengthening the shelf life of fresh fruits and vegetables by controlling the normal biological changes associated with ripening, maturation, sprouting, and finally aging. For example, radiation delays the ripening of green bananas, inhibits the sprouting of potatoes and onions, and prevents the greening of endive and white potatoes. Radiation also destroys disease-causing organisms, including parasites, worms and insect pests that damage food in storage. As with other forms of food processing, radiation produces some useful chemical changes in food. For example, it softens legumes (beans), and thus shortens the cooking time. It also increases the yield of juice from grapes, and speeds the drying rate of plums.

During the irradiation process, food is exposed to the energy source in such a way that a precise and specific dose is absorbed. To do that it is necessary to know the energy output of the source per unit of time, to have a defined spatial relationship between the source and the target, and to expose the target material for a specific time. The radiation dose ordinarily used in food processing ranges from 50Gy to 10kGy and depends on the kind of food being processed and the desired effect. Food irradiation plants vary as regards to design and physical arrangement according to the intended use, but essentially there are two types:

Studies carried out since the 1940s demonstrating the benefits of food irradiation, have also identified its limitations and some problems. For example, because radiation tends to soften some foods, especially fruits, the amount (or dose) of radiation that can be used is limited. Also, some irradiated foods develop an undesired flavour. This problem can be avoided in meat if irradiated while frozen. However, no satisfactory method has yet been found to prevent the development of an off-flavour in irradiated dairy products. In some foods, the flavour problem can be prevented by using smaller amounts of radiation. The small amount of radiation required to

control *Trichinella spiralis* in pork, for example, does not change the flavour of meat (Food Irradiation, 1988).

1.4.1 General background

In the beginning of food irradiation research, treatments aimed at inactivating of micro-organism, were categorised into two groups: radiation sterilisation and radiation pasteurization. These terminologies were considered unsatisfactorily, and since 1964 three new terms were suggested:

1) Radappertization: The application to food of a dose of ionising radiation sufficient to reduce the number and/ or activity of viable micro-organism, to such an extend that very few, if, any, are detectable in the treated food by any recognized method (viruses being excepted). No microbial spoilage or toxicity should become detectable in a food so treated, no matter how long or under what conditions it is stored, provided the package remains undamaged. The required dose is usually in the range of 25-45kGy.
2) Radicidation: The application to food of a dose of ionising radiation sufficient to reduce the number of viable specific non-spore- forming pathogenic bacteria to such a level that none are detectable when the treated food is examined by any recognized method. The required dose is in the range of 2-8kGy.
3) Radurization: The application to foods of a dose of ionising radiation sufficient to enhance its keeping quality by causing a substantial decrease in numbers of viable specific spoilage microorganisms. The required dose is in the range of 0.4-10kGy (Diehl, 1990).

1.4.2 Effects of irradiation on nutritional quality

Food processing and preparation methods in general tend to result in some loss of nutrients. As in other chemical reactions produced by irradiation, nutritional changes are primarily related to dose. The composition of the food and other factors, such as temperature and the presence and absence of air, also influence nutrient loss. At low doses, up to 1kGy, the loss of nutrients from food is insignificant. In the medium-dose range, 1-10kGy, some vitamins loss may occur in food exposed to air during irradiation or storage. At high dosages, 10-50kGy, vitamin loss can be mitigated by protective measures, irradiation at low temperatures and exclusion of air during processing and storage (Food Irradiation, 1988).

Carbohydrates, proteins, and fats are the main compounds of foods. (In most foods water is also a main component). These three components provide energy and serve as building blocks for the growth and maintenance of the body. Animal feeding studies have shown that irradiation of foods at any dose level that is of practical interest, i.e., up to about 50kGy, will not impair these functions of the main compounds. Chemical analysis does show effects of radiation on carbohydrates, fats, and proteins which increase with increasing dose of radiation but even in the dose range of 10-50kGy, these are so small and so unspecific that attempts to develop analytical methods capable of detecting whether a food has been irradiated or not have been met with limited success (Diehl, 1990).

1.4.2.1 Carbohydrates

In the presence of water, carbohydrates are attacked mainly by $^{\bullet}OH$ radicals. Solvated electrons and hydrogen atoms play only a minor role. The $^{\bullet}OH$ radicals abstract predominantly the hydrogen of C-H bonds, forming water. Depending on the molecular position of C=O formed by disproportination or dehydration, the resulting product can be an acid, a ketone, or an aldehyde. Through loss of Carbon the 6-carbon sugar glucose can also be converted to

the 5-carbon sugar, arabinose. Many investigations have been carried out with monosaccharides other than glucose, and similar reaction mechanisms were postulated. When disaccharides or polysaccharides are irradiated, the reactions observed with monosaccharides can also occur.

Irradiation of starch produces dextrins, maltose, and glucose. This reduces the degree of polymerisation in polysaccharides and leads to reduced viscosity of polysaccharides in solution. The solubility of starch in water increases with increasing radiation dose. When carbohydrates are irradiated as components of a food, they are much less radiation-sensitive than in pure form. For example, when radiolysis products of pure starch and of wheat flour, were compared, products formation from starch irradiated with a dose of 5kGy was about as much as from flour irradiated at 50kGy (Diehl, 1990).

1.4.2.2 Proteins

Proteins consist of chains of amino acids connected by peptide bonds. When proteins are irradiated in the presence of water, all the reactions that are possible with amino acids are also possible with a protein containing these amino acids. Irradiation in the absence of water has also been studied but is of much less interest in the context of food irradiation. Metal ions of Fe, Cu, and Zn, particularly when bound to a porphyrin ring, modify mainly the reactions of secondary radicals formed on the protein. They can introduce new pathways for reactions, presumably involving intramolecular electron transfer. No significant destruction of essential amino acids has been observed in irradiated beef, fish or many other foodstuffs, even when sterilization doses of radiation were used. The good growth observed in various animal species fed different kind of irradiated feeds supports the conclusion that digestibility and biological value of proteins are essentially unchanged by treatment with radiation doses even in the range of 50kGy. Even extremely high radiation doses did not adversely affect protein quality: 210kGy in the study of beans, 180kGy in a feeding study with irradiated lentils (Diehl, 1990).

1.4.2.3 Lipids

The lipid or fat portion of foods consists predominantly of triglicerides. Milk fat, for instance, contains 94%, soybean oil contains 88% triglycerides. What has been said up until now about radiolysis of lipids in the absence of air, and most investigation of radiation effects on lipids have been carried out under anoxic conditions. It is generally assumed that irradiation in the presence of oxygen leads to accelerated autoxidation, and that the pathways are the same as in light-induced and metal-catalyzed autoxidation. Some irradiated fishery products such as haddock, shrimps, and king- and Dungeness crab can be stored in air. Fatty fish, such as petrale sole and flounder, become rancid when irradiated and stored in air. Ozone, formed from atmospheric O_2 during irradiation, may be a significant cause of such rancidity development. Exclusion of O_2 both during irradiation and also in the subsequent handling is indicated for fatty fishery products (Urbain, 1986).

Nuts may be irradiated in order to secure various kinds of results, including 1) inactivation of pathogenic bacteria such as salmonellae and moulds which can produce aflatoxin, 2) insect disinfestations, and 3) sprout inhibition. Since nuts may contain substantial amount of oils, undesirable flavours may develop from radiation-accelerated lipid oxidation. Almonds, chestnuts, chalghoza, and peanuts (ground-nuts) exhibit no change of sensory characteristics with doses of up to 1kGy. Irradiation accomplishes the needed reduction of microbial content of spices and vegetable seasonings without causing chemical changes which can significantly affect their normal sensory characteristics and uses (Urbain, 1986).

Using TBA (thiobarbituric acid) number as an indication of oxidation, (Green and Watts, 1966) found less oxidation in irradiated than in unirradiated ground beef samples. There is no difference in peroxide value of the different samples immediately after irradiation. Only during storage (and only in the presence of air) did the result of irradiation become apparent. Other such post-irradiation effects are occasionally observed, not only in lipid system. Irradiation of aqueous system may produce hydrogen peroxide, particularly in

the presence of oxygen. During post-irradiation storage, hydrogen peroxide will gradually disappear, while some other constituents of the system are being oxidised. Obviously, some oxidised compounds not present, or present in lower concentration immediately after irradiation, will be present in higher concentration after hours or days. Many substances or foodstuffs undergo different chemical changes during storage depending on whether they have been cooked, frozen, dried, or left untreated. On the contrary, heating, drying and some other traditional methods may cause higher nutritional losses than irradiation (Diehl, 1990).

1.4.3 Application of irradiation in dairy products: cheese

Dairy products may develop objectionable changes in flavour, odour and colour when irradiated, even with doses as small as 500Gy. A dose of 45kGy applied to fluid milk at 5 ^{o}C produces a brown colour and a strong caramelised flavour. Irradiation at temperatures in the range of –80 to –185 ^{o}C eliminates the brown discolouration and caramelised flavour but causes the occurance of an extremely bitter flavour. There is no significant difference between whole and skim milk in these effects. With doses of up to approximately 20kGy, concurrent irradiation and vacuum distillation yield milk of acceptable flavour. Such milk, however, develops unacceptable browning on storage. Gelation also occurs at ambient temperatures. Skim-milk powder, of moisture content of about 5%, irradiated with doses in the range of 2-16kGy and reconstituted in the normal manner, exhibit the typical 'irradiated" flavour. Irradiation increases free, masked, and total –SH groups. The addition of ascorbic acid, and especially ascorbyl-palmitate, which presumably act as free radical scavengers decreases the amount of –SH group in milk powder.

Khoa, a milk product prepared by concentrating whole milk in open pans to a moisture content of about 35%, has acceptable flavour with doses up to 5kGy with the use of a wrapper treated with sorbic acid prevents mould growth for extended time periods. Turkish kashar cheese (similar to cheddar) and plain yoghurt have acceptable flavour and colour when irradiated with doses up

to1.5kGy. Larger doses cause off-flavour and pronounced fading of colour. Inhibition of mould growth occur at ambient temperatures. Doses of about 400Gy extend the storage life of kashar cheese and plain yoghurt by four to five times the normal (Urbain, 1986).

It is believed that irradiation can be used to accelerate the cheese ripening time of Ras cheese because it increases the acid and peroxide values of milk fat, as well as the time required for complete coagulation of milk (Abd El Baky *et al.*, 1986). Irradiation is used in food as sterilization method (Abd El Baky *et al.*, 1986); it has even gained more use advantage than heat pasteurisation e.g. Gamma irradiation of cheese milk reduced the total bacterial count and spore formers by 98.98% and 95.77%, respectively and eliminated the coliform and pathogenic bacteria. Exposure of Cheddar cheese, Mozarella cheese, ice cream, frozen yoghurt and non-fat dry milk to 5kGy irradiation resulted in a 1-3 log decrease in aerobic mesophiles (Hashisaka *et al.*, 1990).

Treatment with 10kGy resulted in the further decrease in counts of aerobic mesophiles, psychrotrophs, and lactic acid producing bacteria in cheddar cheese. After a total irradiation dose of 20kGy, only lactic acid producing bacteria were enumerated in Cheddar cheese (Hashisaka *et al.*, 1990). The irradiation of raw-milk Camembert cheese at doses from 2.25-3.50kGy has recently been authorized in France but only the chemical method based on the analysis of volatile radio-induced hydrocarbons has a relevant application to raw-milk Camembert cheeses, which are rich in fat.

The formation of volatile hydrocarbons resulting from the rupture of fatty acid chains is not in itself specific to irradiation. These chemical components can also form after heating or simply as a result of oxidation, some hydrocarbons seem to appear essentially during irradiation. Results have shown that the increase in hydrocarbon concentrations, observed as the irradiation dose is increased, is only due to the irradiation treatment and it can be demonstrated that there is a 99.8%, (except for heptadecane which is less), correlation that exists between the hydrocarbons concentration and the irradiation dose (Bergaentzle *et al.*, 1994).

Several investigators have shown that enzymes are relatively resistant to the effect of ionising radiation Desrosier, 1963). This low sensitivity of enzymes to radiation effect implies little or no interference with the fermentations and chemical reactions that takes place during ripening of cheese (Abd El Baky *et al.*, 1986).

The concentration of soluble nitrogen compounds in cheese produced from irradiated milk (CIM) as compared with cheese produced from heated milk (CHM) is higher. The soluble nitrogen compound of both CIM and CHM increased gradually during ripening. However, concentrations of protein degradative products remained slightly higher in CIM, compared with CHM, a result that may be explained on the basis that heat treated and gamma irradiation have different effects on milk enzymes, particularly proteinases which contribute to proteolysis during cheese ripening (Abd El Baky *et al.*, 1986).

(Ismail *et al.*, 1975) showed that gamma irradiation resulted in an increase in the activity of milk proteinase by about 8%. Irradiation of milk increases the tendency to form free fatty acids during ripening which add to flavour. Gamma irradiation did not affect the appearance of cheese, but enhanced its body and flavour intensity, probably by increasing the concentration of soluble nitrogen compound and free fatty acids. However, an oxidised flavour was detected in fresh and one- month- old CIM, probably because of the oxidation of milk fat by gamma irradiation (Abd El Baky *et al.*, 1986). This defect disappeared during ripening, whereas taste and odour of CIM from the second month of ripening onwards, were organoleptically acceptable and even better than those of CHM of the same age (Abd El Baky *et al.*, 1986).

1.5 Aim

Manipulation of the microflora and proteolytic and lipolytic processes by elevated temperatures was reported by Fedrick (1987) and Folkertsma *et al.* (1996). With application in ripening of cheese, irradiation was only employed in the ripening of Ras cheese, where it was shown to have increased the acid

and peroxide values of milk fat as well as decrease the time required for complete coagulation of milk (Abd El Baky *et al.*, 1986).

Since irradiation can be employed to kill or control microorganisms, which upon being killed, lyse and expose their enzymes, as well as affect the chemical breakdown of proteins, amino acids, lipids and fatty acids, it was thought worth studying with an application in the ripening of Cheddar cheese.

It was hypothesised that early ripening at elevated temperature would allow a production of high level of enzymes by the microorganisms, while after irradiation these organisms would be killed or reduced, exposing the enzymes to effect the ripening process. In this study, the hypothesis was tested by analysis of release of free fatty acids and peptides, as a result of the mentioned enzyme action and the effect on sensory attributes.

The aim of this investigation therefore was to study the effect that both elevated ripening temperatures and irradiation have on the ripening of Cheddar cheese, with emphasis on the breakdown of lipids and proteins and its effect on sensory attributes.

CHAPTER 2
PROCESSING, IRRADIATION AND SENSORY ANALYSIS OF CHEDDAR CHEESE

2.1 Introduction

One of the factors affecting the manufacturing cost of Cheddar cheese, is the long ripening period. Accelerated ripening would therefore cut these costs, and is an aim of several researchers, by employing altered ripening times (Fox, 1988/89) and employing enzymes (Wilkinson, 1993) and microorganisms other than the normal starter cultures (Laleye *et al.*, 1990 and Lee *et al.*, 1990). In this study, another approach is investigated i.e. the employment of irradiation a fairly modern technology in food technology.

The traditional manufacture of Cheddar cheese consists of: (a) coagulating milk, containing a starter culture, with rennet; (b) cutting the resulting coagulum into small cubes; (c) heating and stirring the cubes with the concomitant production of a required amount of acid; (d) whey removal; (e) fusing the cubes of curd into slabs by cheddaring; (f) cutting (milling) the cheddared curd; (g) salting; (h) pressing; (i) packaging and ripening. Although it is impossible to separate the combined effects of some of these operations on the final quality of cheese, they will if possible be considered individually (Lawrence *et al.*, 1993).

As discussed in Chapter 1, three events occur during cheese ripening, i.e. glycolysis, proteolysis and lipolysis. These primary reactions are mainly responsible for the basic textural changes that occur in the cheese curd during ripening and are also largely responsible for the basic flavour of cheese. However, numerous secondary changes occur concomitantly and it is these secondary transformations that are mainly responsible for the finer aspects of cheese flavour

Proteolysis contributes to cheese ripening in at least four major ways:

1) a direct contribution to flavour via amino acids and peptides, some of which may cause off-flavours such as bitterness (due to hydrophobic peptides)or indirectly via catabolism of amino acids to amines, acids, thiols, thioesters, etc.
2) greater release of sapid compounds from the cheese matrix during mastication
3) liberation of substrates (amino acids) for other flavour-generating reactions (e.g. deamination, decarboxylation and desulfuration).
4) Textural changes via
    a) breakdown of the protein network
    b) increase in pH due to the production of NH_3 by deamination of free amino acids (particularly in surface mould varieties)
    c) decrease in a_w through greater water binding by the newly formed (liberated) amino and carboxyl groups (Fox, 1989)

Dramatic changes in the manufacture of Cheddar cheese have occurred during the past 25 years. The single most important factor has been the availability of reliable starter cultures because the successful development of continous mechanized systems for Cheddar manufacture has depended upon the ability of the cheesemaker to control precisely both the expulsion of moisture and the increase in acidity required in a given time. This in turn has lead to the recognition that the quality of cheese, now being made on such a large scale in modern cheese plants, can only be guaranteed if its chemical composition falls within predetermined ranges. Nevertheless, Cheddar cheese is still a relatively difficult variety to manufacture since the long ripening period necessary for the development of the required mature flavour can also be conducive to the formation of off-flavours. In addition, its texture can vary considerably (Lawrence *et al.*, 1993).

2.2 Materials and methods

2.2.1 Cheddar cheese manufacture

Cheddar cheese was manufactured at Thirstle Dairies situated at the University of the Free State, in the Free State Province of South Africa. The cheese was made in an open vat with HTST-pasteurized (72 ^{o}C; 15s) 4% fat-standarized milk. Milk was cooled to the inoculating temperature of 32-35 ^{o}C and the starter culture was added (RST-745 phage control mesophilic homofermentative culture, 60 units/ 4 000 l freeze-dried Lactic Culture for direct vat set (DVS). Calcium chloride (45%) (1 000 ml/4 000 l) and annatto dye (150 ml/ 4 000 l) were added just before the rennet (18 ml/ 4 000 l). The milk was set for ~35 min at a temperature of ~31.5 ^{o}C, and the curd was cut into 1 cm^{3} cubes and held for 15 min without stirring. The temperature of the curd-whey mixture was raised to 36 ^{o}C over 30 min with gentle stirring and the mixture was then held at 36 ^{o}C until the pH was 6.2 and enough whey expelled from the curd. The whey was drained and the curd cheddared until the pH declined to 5.4 (~60min). The blocks of curd were cut into cubes that were dry-salted (11 kg/ 4 000 l), mellowed for 15 min, placed in moulds and pressed overnight. After pressing, the cheeses were vacuum-packed and ripened at 8 ^{o}C and 16 ^{o}C. After allowing bonding of the curd cubes for two days, the 10kg cheeses were cut into smaller pieces of about 250 g, vacuum packed and ripened at the same temperatures. This was done for easier handling and sampling.

2.2.2 Cheddar cheese ripening and sampling

Cheeses were sampled on day 0 and ripening was continued at 8 ^{o}C and 16 ^{o}C. Cheeses were sampled again after 14 days (2 weeks), 42 days (6 weeks) and 84 days (12 weeks) of manufacture.

2.2.3 Irradiation of Cheddar cheese

After 4 days of manufacturing and ripening, cheese samples ripened at 8 ^{o}C and 16 ^{o}C were irradiated at 4kGy, after which ripening at the specified temperatures was continued. Irradiation was carried out at the Atomic Energy Corporation, Pelindaba, South Africa. The dose of approximately 4kGy was predetermined as an optimum dose that retarded growth of microorganisms, but had the lowest immediate effect on taste and flavour. Cheeses were sampled at the same intervals as in 2.2.2.

2.2.4 Sensory analysis

Sensory characteristics of cheese was evaluated using preference ranking (Basker, 1988) and was carried out in a sensory analysis laboratory of the Department of Food Science of the University of the Free State. A consumer panel comprising of forty-eight panellists (22-62 years of age) was recruited from students (20) and staff (28) of the University of the Free State (34 females; 14 males). Panellists assigned ranks by using a scale with 1 = most preferred sample and 4 = least preferred sample. Tap water at room temperature was provided for rinsing between samples during taste sessions. Thinly sliced apples were also available to clean the palate from excessive coating. Four cheese samples cut into cubes of 12.5 x 10 x 7.5mm presented on white polystyrene trays were evaluated by panellists (i.e. 8^{o}C unirradiated, 8^{o}C irradiated, 16^{o}C unirradiated and 16^{o}C irradiated). Samples were coded using three-digit numbers picked from a table of random numbers. Evaluations were performed at room temperature (20-22^{o}C), in individual testing booths under red light in the sensory evaluation laboratory. Cheese that was evaluated was ripened for 12 weeks.

2.3 Results and discussion

2.3.1 Irradiation of Cheddar cheese

It was noted that the colour of the irradiated cheese was a light yellow compared to the orange colour of the unirradiated cheese. This was probably due to the annatto cheese colouring being damaged or destroyed by the irradiation. This was not investigated further, and colour was also excluded as a parameter for sensory analysis. A pungent taste was observed for freshly irradiated cheese, which subsided during ripening. Again, this was only noted, but not investigated further, as only cheese ripened up to twelve weeks was evaluated sensorically.

2.3.2 Sensory analysis

Table 2.1 Ranking test of the unirradiated and irradiated Cheddar cheese ripened at 8 ^{o}C and 16^{o}C

	A	**B**	**C**	**D**
Sample	**8^{o}C unirradiated**	**8^{o}C irradiated**	**16^{o}C unirradiated**	**16^{o}C irradiated**
Rank sum	95	126	121	115
Difference vs A		31	26	20
B			5	11
C				6

Table 2.2 Significance level of differences obtained by the ranking test of the irradiated and unirradiated Cheddar cheese ripened at 8 ^{o}C and 16^{o}C

Significance level	P= 0.05	P=0.01
Critical difference	32.5	39.4
Sample A	A	A
B	A	A
C	A	A
D	A	A

The p=0.05 significance level is attained when the rank sum differences are greater than or equal to 14.9 and the p=0.01 significance level is attained when the rank sum differences are greater or equal to 19.6. Sample A (the standard cheese) had the lowest rank sum (table 2.1) and appeared to be preferred over the other three samples. Due to p=0.05 and p=0.01 (Table 2.2) product A was not significantly preferred over products B, C and D. There were overlapping ranges of preference, the panel evaluated them as not being different in taste.

2.4 Conclusion

Although the standard Cheddar, ripened at 8^{o}C appeared to be preferred over the cheese ripened at 16^{o}C and irradiated cheeses, no significant difference was found by the taste panel. To provide an explanation for this observation on a chemical basis, the changes observed for lipids and proteins was investigated, which are discussed in the subsequent chapters.

CHAPTER 3
THE EFFECT OF IRRADIATION AND ELEVATED TEMPERATURE OF CHEDDAR CHEESE ON FAT

3.1 Introduction

McSweeney and Fox (1993) reported that the degree of lipolysis in cheese depends on the variety of cheese and varies from slight to extensive. The free fatty acids in cheese are derived from two major sources: (1) the breakdown of the fat by lipolysis and (2) metabolism of carbohydrates and amino acids by bacteria. Extensive lipolysis in internal bacterially ripened cheese (e.g. Cheddar, Gouda and Swiss) is undesirable, but in mould-ripened cheese, lipolysis is essential for flavour development. Free fatty acids (FFA) play a major role in flavours of many cheese varieties. They have been considered the backbone of Cheddar cheese flavour by Patton (1963) and generally have been acknowledged to contribute cheesiness in Cheddar flavour by Forss, 1979 and Forss and Patton, 1966. In addition to the desirable flavours contributed by FFA at appropriate concentrations, high amounts of FFA caused by excessive lipolysis produce undesirable rancid off-flavours.

Because flavour quality and accompanying economic value of green and aged cheeses depend heavily on concentrations of FFA, it would be desirable to monitor accurately concentrations of FFA during manufacturing and ageing period (Woo and Lindsay, 1982).

As in every type of food with a high fat content, lipolysis and oxidation are likely to occur. Lipolysis is known to be an enzymatic reaction. Milk lipases have been shown to be more active than starter lipases in Cheddar (Reiter and Sharpe, 1971). They seem to hydrolyse the fat selectively and to be active mainly on mono- and diglyceridess. Free fatty acids will partition between water and lipid phase and be present as soaps. The liberated fatty acids are involved in several types of reactions which vary in importance according to the type of cheese considered. The other possible reaction in which polyunsaturated and, perhaps, monounsaturated, fatty acids can be

involved, is oxidation. The amount of oxidation in cheese is, however, rather limited, as fat would normally be very susceptible to oxidation in the conditions which prevail in cheese. The existence of a low redox potential, together with the presence of natural antioxidants, could prevent the initiation of oxidation mechanisms, or create conditions in which the primary oxidation products are further reduced.

It is believed that the hydrolytic reactions, including lipolysis, provide the free oleic, linoleic or linolenic acids which can undergo more rapid autoxidation. Methyl ketones contribute a piercing sweet fruitines, ranging from C_3, pungent, sweet, through C_7 blue cheesy, to C_{11} fatty, sweet. The aliphatic acids contribute to the flavour by being sour, fruity, cheesy or animal-like. Their contribution ranges from C_2 vinegary, C_3 sour, Swiss cheesy, C_4 sweaty cheesy, C_8 goat cheesy, C_9 paraffinic, to C_{14}-C_{18} with very little odour (Hamilton, 1982). Allen, 1982 found that lipolysis is the hydrolysis of triglycerides to produce FFA, which possess undesirable flavour characteristics. Most lipolytic rancidity in dairy products arises from the enzymic action of lipases. Lipolysis is usually determined by measurement of the FFA in the fat of a sample, which is achieved by extracting the fat, dissolving it in a neutralised solvent and carrying out titration.

Rossell (1982) postulated that oxidative rancidity is caused by oxidation of fat, and takes place through several intermediates including hydroperoxides and peroxides, eventually leading to aldehydes and ketones, as well as other breakdown products. These secondary oxidation products, especially the aldehydes, have the off-flavours associated with rancid oils. Oxidative rancidity is measured by Peroxide Value, Anisidine Value, thiobarbituricAcid (TBA) test etc.

Irradiation was shown to affect lipids in the same way as autoxidation and lipolysis, resulting in the formation of free fatty acids and other components that may affect organoleptic quality in foods (Hamilton, 1982). For this research, and also because the effect of irradiation on lipids in cheese has not

yet been fully described, the effect of irradiation on lipids are monitored, and reported in this chapter.

3.2 Materials and methods

3.2.1 Manufacture of Cheddar cheese

The manufacture of cheese is as discussed in Chapter 2 section 2.2.1

3.2.2 Sampling of cheese

The cheese was ripened as described in section 2.2.2 and sampling was done at day 0, 2 weeks, 6 weeks, and 12 weeks

3.2.3 Analysis of free fatty acids

3.2.3.1 Crude fat extraction

Cheese samples of 5 g each were grated and weighed into a 250 ml flat-bottomed flask and 30 ml of diethyl ether added. The mixture was then homogenised with an ULTRA TURRAX T25 (JANKEL & KUNKEL, IKA – Labortechnik). The mixture was left overnight in a refrigerator after which it was filtered through a Whatman no paper 1 into a wide-mouthed 250 ml glass beaker. The ether was evaporated under nitrogen and the mixture was poured into a polytop glass vial. After the ether has been evaporated (assessed by smelling the gas mixture), the mixture was put into a vacuum oven overnight. The mixture was then cooled and covered under a nitrogen blanket.

3.2.3.2 Determination of free fatty acids

The free fatty acids (% oleic) of cheeses were determined in a 0.5 g sample using the method of Pearson (1968).

A volume of 20 ml of ethanol (95 %) was mixed with 20 ml of diethyl ether, and 1 ml of the phenolphthalein indicator (1 % in ethanol) was added. The mixture was neutralised by the addition of 0.01 N NaOH from a burette. The 0.5 g of sample was weighed into a beaker-flask (200-400 ml capacity). The solvent was added to the oil, swirled and titrated with 0.1 N NaOH. The mixture was shaken constantly, until a pink colour persisted for 15 s.

The amount of free fatty acids are calculated as follows:

$$\text{FFA (as oleic)} = \frac{V \times 0.0282 \times 1000}{W}$$

V = volume (ml) of 0.01N NaOH

W = mass (g) of sample

3.2.4 Determination of TBA-value

The thiobarbituric acid reactive substances were determined in a 5 g sample using the aqueous acid extraction method of Raharjo *et al.* (1992).

3.2.4.1 Standard curve for TBA-determination

A volume of 4 ml of 1,1,3,3- tetraethoxypropane (TEP) was put in a stoppered tube (the TEP was replaced with distilled water as blank). TBA (4 ml) was added and the tubes were sealed and then mixed on a vortex shaker. The mixture was heated in a water bath at 94 ± 1 ^{o}C for 5 minutes. After that the mixture was cooled to room temperature. Absorbance was measured at 523 nm after the spectrophotometer was zeroed against the blank. A standard curve was drawn with TEP 1-7 concentrations against absorbance.

3.2.4.2 TBA-determination of cheese

A sample of 5 g was weighed into a centrifuge tube and 20 ml 5 % trichloroacetic acid (TCA) was added. The mixture was homogenized for 1 minute. The mixture was then centrifuged at 10000 g for 5 minutes at 4 ^{o}C. The supernatant was filtered through Whatman no 1 paper into a 25ml volumetric flask. The flask was filled up to the mark with 5 % TCA, and reacted with TBA as in 3.2.2.1. Absorbance was measured at 523 nm and the concentration read from the 3.2.2.1 standard curve. The mg malonaldehyde / 1000 g of sample was then calculated.

3.2.5 Statistical analysis

Differences between treatment groups were determined by using an analysis of variance (ANOVA) procedure (NCSS, 2001). The Turkey-Kramer multiple comparison test (α= 0.05) was used to identify differences between treatment means (NCSS, 2001).

3.3 Results and discussion

The changes observed in fatty acid composition during ripening of the unirradiated and irradiated Cheddar cheese at different ripening temperatures are shown in Table 3.1 and Figure 3.1. The changes in oxidation of lipids, as expressed by TBA value, of these cheeses are shown in Table 3.2 and Figure 3.2. The tables are included to report the exact values obtained, together with the standard deviations.

3.3.1 Free fatty acid

The free fatty acid content of the Cheddar cheeses are shown in Table 3.1, and the ANOVA of irradiation, ripening temperature and ripening time in Table 3.2. Changes of free fatty acid content during ripening are shown in Figure 3.1.

Table 3.1 Changes in free fatty acid composition of the unirradiated and irradiated Cheddar cheese ripened at 8 °C and 16 °C

Time	8 °C	8 °C i	16 °C	16 °C i
Day 0	0.873 ± 0.006^{ab}			
2 weeks	1.009 ± 0.016^{abcd}	0.986 ± 0.009^{abcd}	0.976 ± 0.007^{abcd}	0.986 ± 0.033^{abcd}
6 weeks	1.043 ± 0.013^{bcd}	0.916 ± 0.008^{ab}	1.161 ± 0.01^{d}	1.099 ± 0.002^{cd}
12 weeks	0.918 ± 0.02^{abc}	0.833 ± 0.0007^{a}	0.958 ± 0.015^{abcd}	1.00 ± 0.01^{abcd}

Means with different superscripts differ significantly.
Probability level: $p < 0.001$
8 °C: Unirradiated cheese ripened at 8 °C
8 °C i: Irradiated cheese ripened at 8 °C
16 °C: Unirradiated cheese ripened at 16 °C
16 °C i: Irradiated cheese ripened at 16 °C

Table 3.2 Analysis of variance of irradiation, ripening temperature and ripening time, and the free fatty acid composition of Cheddar cheeses

Effects	Probability value
Irradiation	0.1539
Ripening temperature	0.0005
Ripening time	0.001
Interaction	
Irradiation × Temperature	0.0459
Irradiation × Time	0.1114
Temperature × Time	0.0512
Irradiation ×Temperature × Time	0.9409

Probability level: p-values < 0.05 are significant

Changes in FFA content were significant for ripening temperature and ripening time, but not for irradiation (Table 3.2). Interactions were only significant for irradiation × ripening temperature.

Results in Table 3.1 and Figure 3.1 showed no significant increase in FFA (expressed as % oleic, the most abundant unsaturated fatty acid) of the unirradiated cheese samples ripened at 8 ^{o}C from 0.873 ± 0.006 at day 0 to 1.009 ± 0.016 at 2 weeks, there is no significant increase in FFA from cheese ripened for 2 weeks to 6 weeks and from cheese ripened for 6 weeks to 12 weeks. There is also no significant difference between cheese ripened for 2 weeks and 12 weeks.

The unirradiated cheese ripened at 16 ^{o}C showed no significant increase from day 0 to 2 weeks of ripening and no significant decrease from 2 weeks to 6 and also from 6 weeks to 12 weeks of ripening. There is a significant increase of FFA in irradiated cheese ripened at 8 ^{o}C from day 0 to 6 weeks, followed by an insignificant decrease from 6 weeks to 12 weeks. There is no significant difference between cheese ripened for 2 weeks and cheese ripened for 12 weeks. The irradiated cheese ripened at 8 ^{o}C showed no significant increase from day 0 to 2 weeks of ripening and no significant decrease from 2 weeks to 6 weeks and from 6 weeks to 12 weeks of ripening. There is no significant difference between cheeses ripened for 2 weeks and cheese ripened for 12 weeks. There is no significant increase in FFA from day 0 to cheese ripened for 2 weeks in the irradiated cheese ripened at 16 ^{o}C and no significant increase in FFA at 6 weeks and 12 weeks. There is a significant increase of FFA in irradiated cheese ripened at 16 ^{o}C from day 0 to 6 weeks of ripening, followed by a non significant decrease from 6 weeks to 12 weeks. There is basically no significant difference between cheese ripened for 2 weeks and for 12 weeks.

The unirradiated cheese ripened at 8 ^{o}C showed no significant difference with the unirradiated cheese ripened at 16 ^{o}C at 2 weeks, 6 weeks and 12 weeks of ripening, even though it seems as if there is a high increase in FFA of the cheese ripened at 16 ^{o}C at 6 weeks. The unirradiated cheese ripened at 8 ^{o}C showed no significant difference at 2 weeks with the irradiated cheese ripened at the same temperature for the same duration. There is also no significant difference in FFA at 6 weeks and 12 weeks of ripening between the

two cheeses. Both cheeses ripened at 16 ^{o}C showed no significant difference at 2 weeks, 6 weeks and 12 weeks. However, cheeses ripened at 16 ^{o}C (both the unirradiated and irradiated) have a high FFA content than the standard cheese at day 0. Except for small increase or decrease at different ripening times, cheeses have the same FFA throughout ripening.

The results of the current study differ from those of many researchers who reported that the concentration of free fatty acids increases during ripening and has a major impact on cheese flavour and from results of (Wong *et al.*, 1975) who reported that a homologous series of δ-lactones (C_{10}-C_{18}) occur in Cheddar (and probably other) cheeses and their concentrations increase during ripening up to about two months after which they remain constant or decline slightly. (Nunez *et al.*, 1986), reported an increase in the FFA of almost 90 % in Manchego cheese ripened at 20 ^{o}C when compared with the cheese ripened at 5 ^{o}C.

Fresno *et al.* (1997), reported that the lipid degradation of the Armada goat`s milk cheese becomes very intense from the second month of ripening and becomes comparable to that reported for cheeses ripened by moulds. He found that the free fatty acids content increased 20-fold during ripening, reaching final values of 44.5 g/ kg at 120 days. Such drastic changes were, however, not observed in the current study, not even when cheese was irradiated and ripened at elevated temperatures.

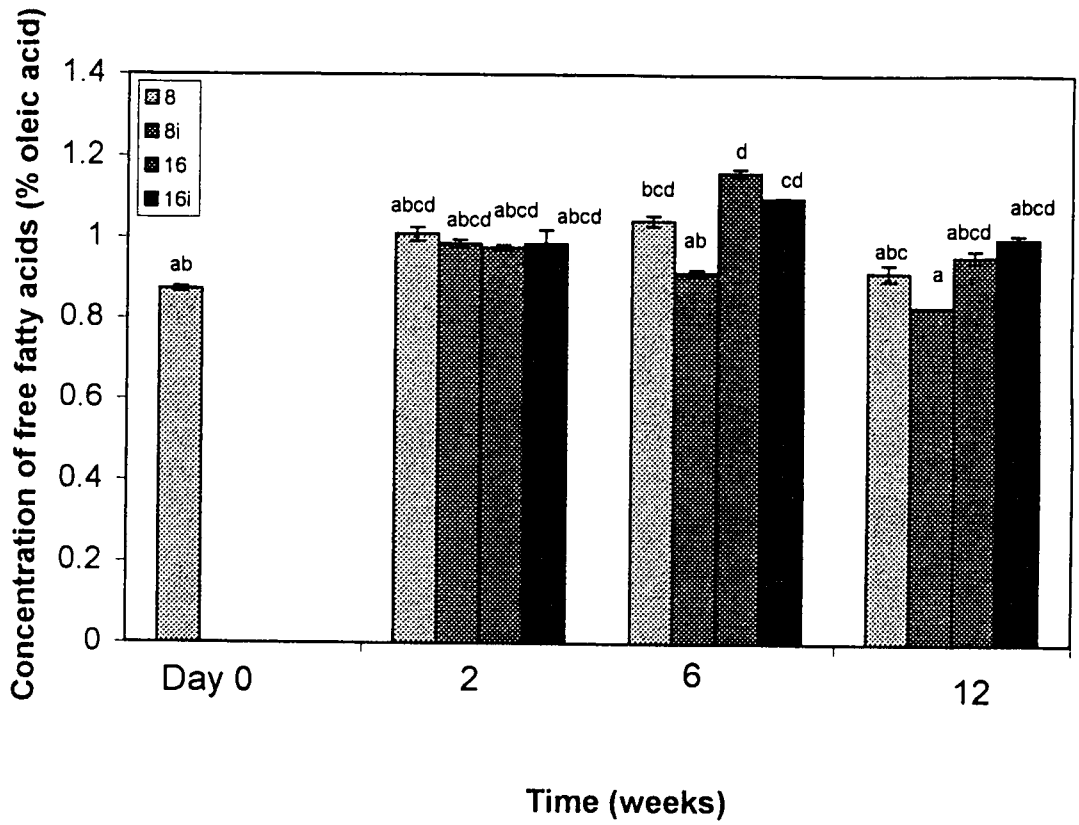

Figure 3.1 Changes in free fatty acid composition of the unirradiated and irradiated Cheddar cheese ripened at 8 ^{o}C and 16 ^{o}C

Means with different superscripts differ significantly
Probability level: $p < 0.001$
8: Unirradiated cheese ripened at 8 ^{o}C
8i: Irradiated cheese ripened at 8 ^{o}C
16: Unirradiated cheese ripened at 16 ^{o}C
16i: Irradiated cheese ripened at 16 ^{o}C

3.3.2 TBA value

The TBA-values of the Cheddar cheeses are shown in Table 3.3 and the ANOVA of irradiation, ripening temperature and ripening time in Table 3.4. Changes of TBA-values during ripening are shown in Figure 3.2.

Table 3.3 Changes in TBA value composition of the unirradiated and irradiated Cheddar cheese ripened at 8 ^{o}C and 16^{o}C

Time	8 ^{o}C	8 ^{o}C i	16 ^{o}C	16 ^{o}C i
Day 0	0.812 ± 0.099^{f}			
2 weeks	0.386 ± 0.136^{bc}	0.842 ± 0.099^{f}	0.697 ± 0.037^{def}	0.814 ± 0.073^{f}
6 weeks	0.234 ± 0.027^{a}	0.694 ± 0.079^{def}	0.521 ± 0.206^{ce}	0.644 ± 0.130^{cf}
12 weeks	0.244 ± 0.04^{ab}	0.717 ± 0.069^{ef}	0.457 ± 0.139^{c}	0.503 ± 0.086^{cd}

Means with different superscripts differ significantly
Probability level: $p < 0.001$
8 ^{o}C: Unirradiated cheese ripened at 8 ^{o}C
8 ^{o}C i: Irradiated cheese ripened at 8 ^{o}C
16 ^{o}C: Unirradiated cheese ripened at 16 ^{o}C
16 ^{o}C i: Irradiated cheese ripened at 16 ^{o}C

Table 3.4 Analysis of variance of irradiation, ripening temperature and ripening time, and the TBA-values of Cheddar cheeses

Effects	Probability value
Irradiation	0.0001
Ripening temperature	0.0036
Ripening time	0.0001
Interaction	
Irradiation × Temperature	0.0001
Irradiation × Time	0.8221
Temperature × Time	0.1056
Irradiation ×Temperature × Time	0.4687

Probability level: p-values < 0.05 are significant

Changes in TBA-values were significant for irradiation, ripening temperature and ripening time (Table3.4). Interactions were only significant for irradiation × ripening temperature.

Results in Figure 3.2 and Table 3.3 showed a significant decrease in TBA value of the unirradiated cheese samples ripened at 8 ^{o}C from 0.812 ± 0.099 at day 0 to 0.386 ± 0.136 in cheese ripened for 2 weeks, a further significant decrease from cheese ripened for 2 weeks to 0.234 ± 0.027 in cheese ripened for 6 weeks and no significant change afterwards. The TBA values of the unirradiated cheese ripened at 16 ^{o}C showed no significant increase from day 0 to cheese ripened for 2 weeks and no significant change from cheese ripened for 2 weeks to 6 weeks and from 6 weeks to 12 weeks of ripening, but cheese ripened for 6 weeks differ significantly from cheese ripened at day 0 and, cheese ripened for 12 weeks differ significantly from cheese ripened for 2 weeks and at day 0.

The TBA value of the irradiated cheese ripened at 8 ^{o}C showed an increase from day 0 to cheese ripened for 2 weeks, a decrease from cheese ripened for 2 weeks to cheese ripened for 6 weeks however, these changes were not significant. Ripening from 6 weeks to cheese ripened for 12 weeks showed no significant change. There is no significant increase from day 0 to cheese ripened for 2 weeks in the irradiated cheese ripened at 16 ^{o}C, and no significant decrease from cheese ripened for 2 weeks to cheese ripened for 6 weeks but a definite significant decrease from cheese ripened for 2 weeks to cheese ripened for 12 weeks. There is a constant decrease for irradiated cheese ripened at 16 ^{o}C from day 0 to 6 weeks of ripening, however, the changes are not significant. Ripening from 2 to 12 weeks indicated a significant decrease.

The unirradiated cheese ripened at 8 ^{o}C showed a significant lower TBA value than the unirradiated cheese ripened at 16 ^{o}C throughout ripening (from 2 weeks to 12 weeks). The same pattern is seen between the unirradiated and the irradiated cheese ripened at 8 ^{o}C and 16 ^{o}C, where the cheeses

ripened at 8 °C had lower TBA value than that ripened at 16 °C, however the changes are not significant. The unirradiated and the irradiated cheese ripened at 16 °C are not significantly different at 2 weeks, 6 weeks and 12 weeks. They are both not significantly different from the irradiated cheese ripened at 8 °C at 2 weeks and 6 weeks but significantly lower at 12 weeks. The TBA value of the standard cheese is significantly lower than the irradiated cheeses ripened at 8 °C and 16 °C and the unirradiated cheese ripened at 16 °C. In general, the TBA value of the irradiated cheeses is higher than for the unirradiated cheese though there is some overlapping results with the unirradiated cheese ripened at 16 °C. Therefore, it is correct to state that the TBA value of the standard cheese developed differently than in the other cheeses.

As the increase in TBA depicts oxidation, it can be postulated that ripening at elevated temperatures and irradiation cause an increase in oxidation as shown by high TBA values for these cheeses. The results seem to indicate that there is more oxidation at 16 °C than at 8 °C shown by lower values at the latter temperature. The decrease in TBA with ripening may represent an indication of a termination phase in oxidation, and the lower TBA values at 8 °C indicate that termination is faster at low temperature than at high temperature. It might also be that initiation of oxidation still continues at 16 °C.

Results of the current study differ from those of (Carballo *et al.*, 1994) who found a significant increase in of the Spanish hard goat cheese during cheese ripening. He found a TBA value of 0.082 ± 0.01 mg malonaldehyde/ 1000 g sample at day 0 compared to 0.812 ± 0.099 for our results. (Fresno *et al.*, 1997), reported a TBA that remains constant throughout ripening (from day 0 to 120 days) of the Armada cheese. He found a final value of 0.423 mg malonaldehyde/ kg of cheese at 120 days, when this value was compared to the values of other cheeses such as the Leon cow`s milk cheese (Prieto *et al.*, 1994), it was concluded that in Armanda cheese, the degree of autooxidative rancidity of fat was weak. The TBA-values during ripening of the irradiated

cheeses are also higher than in the unirradiated cheese ripened at 8 ^{o}C, almost comparable with the unirradiated cheeses ripened at 16 ^{o}C.

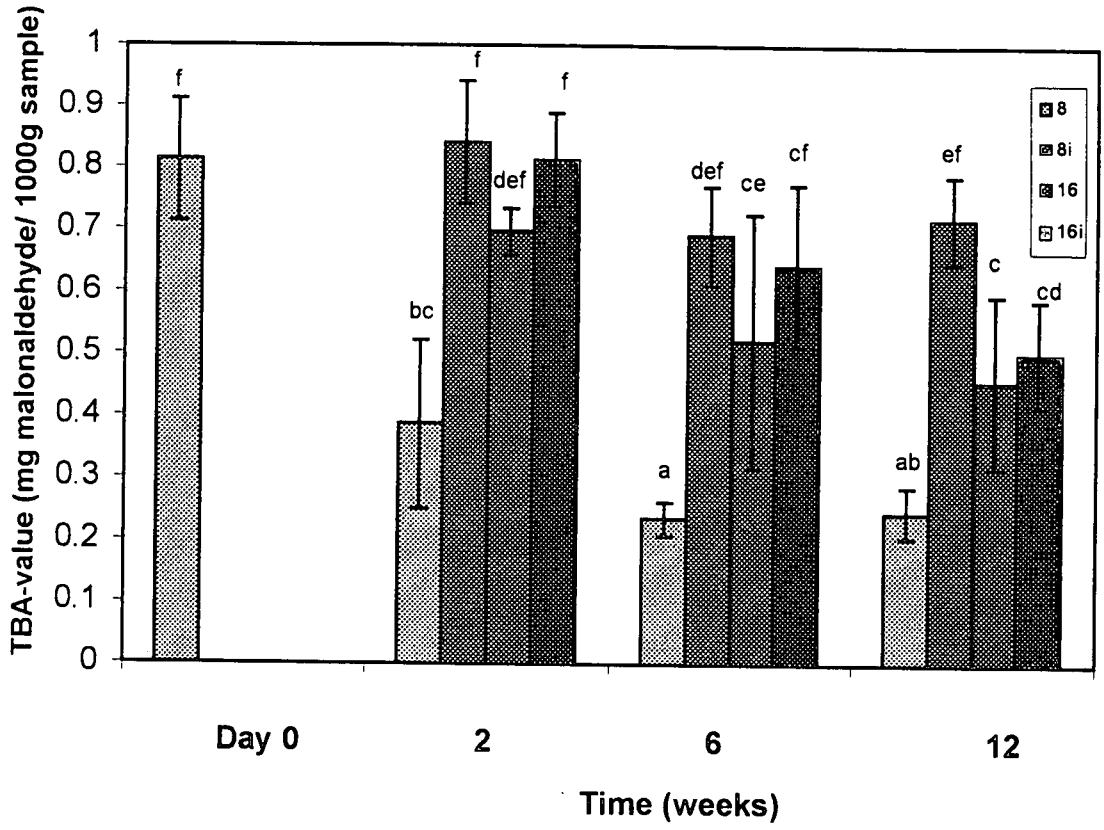

Figure 3.2 Changes in TBA value composition of the unirradiated and irradiated Cheddar cheese ripened at 8 ^{o}C and 16 ^{o}C

Means with different superscripts differ significantly
Probability level: $p < 0.001$
8: Unirradiated cheese ripened at 8 ^{o}C
8i: Irradiated cheese ripened at 8 ^{o}C
16: Unirradiated cheese ripened at 16 ^{o}C
16i: Irradiated cheese ripened at 16 ^{o}C

3.4 Conclusion

There is a high FFA (% oleic) in the cheeses as compared to the TBA value and this result implies that although there was evidence of hydrolysis of FFA from the lipid fraction, and the degradation of these fatty acids into peroxides,

there was no evidence of the development of rancidity in the cheeses by the end of ripening (though it was not tested for in the sensory evaluations, Chapter 2). These can be proven by the fact that the TBA values of all the cheeses were less than the critical value of rancidity for which TBA > 1. The high TBA values of irradiated cheeses (both 8 ^{o}C and 16 ^{o}C) can be due to the radiation breakdown products because even the cheese sampled at day 0 had a high TBA value, in fact the value is higher than in all the cheeses after ripening at 12 weeks. The slight decrease in FFA at week twelve can be attributed to the probable FFA degradation into volatile compounds. Kristoffersen et al. (1967) has discovered that when fatty acids and hydrogen sulfide are in proper ratio a desirable Cheddar flavour result, but if either fatty acids or hydrogen sulfide is too high or low a less desirable flavour is produced, it is therefore correct to postulate that the cheeses had just the right amount of fatty acids, because they did not have a soapy or a rancid taste that is linked to excessive lipolysis.

In Chapter 2 it was shown that the standard Cheddar, ripened at 8 ^{o}C appeared to be preferred (however not significantly) over the cheese ripened at 16 ^{o}C and irradiated cheeses. A pre-mature explanation can be that the TBA value of the standard Cheddar cheese is much lower and significantly different than that of the others after 12 weeks of ripening.

Bills and Day (1964) also observed that the balance between FFA and other flavour constituents is more important to Cheddar cheese flavour than the concentration of FFA alone. The following chapters will therefore be devoted to the role of proteins and their breakdown products during irradiation and ripening of Cheddar cheese.

CHAPTER 4

THE EFFECT OF IRRADIATION AND ELEVATED RIPENING TEMPERATURE OF CHEDDAR CHEESE ON PROTEINS ANALYSED BY FRACTIONATION AND ELECTROPHORESIS

4.1 Introduction

Aston *et al.* 1983, studied the expansion of research on cheese flavour and aroma that took place during the 1960`s and 1970`s which indicated the presence of hundreds of compounds that could contribute to cheese flavour and aroma. Most of these are present at very low concentrations, many below their flavour thresholds, but which may still affect cheese quality. Thus, cheese flavour is due to the correct balance of a mixture of compounds. In internally bacterially ripened cheeses such as Cheddar, there appears to be a fairly good agreement that the water- insoluble fraction (consisting mainly of proteins and large peptides) is devoid of flavour and aroma, that the water-soluble non-volatile fractions (small peptides, amino acids and organic acids) contain most of the compounds responsible for flavour while the aroma is principally in the volatile fraction. There also appears to be a strong support for the view that products of proteolysis are the principal contributors to cheese flavour (McGugan *et al.*, 1979; Aston *et al.*, 1983).

It has been reported by several researchers that non-volatile water-soluble fractions contribute to cheese flavour. McGugan *et al.*, 1979 reported that these components give an essential background flavour, while volatile components contribute to more characteristics "cheesy" qualities, Biede (1974) also reported that burned flavour correlated with proteolysis and (Barlow *et al.*, 1989) also found that cheese flavour correlate well with water-soluble nitrogen, lactic acid and H_2S. Biede and Hammond, 1979 reported that amino acids and dipeptides were responsible for brothy-nutty flavour, whereas burned and bitter flavours were due to trio-, penta- and

hexapeptides. Kowalesaska *et al.*, 1985 asserted that the water-soluble non-volatile fraction, including amino acids, peptides, salts, lactic acid, Ca^{2+} and Mg^{2+} ions, have a very intense flavour.

Aston and Creamer (1986) ascribe the taste intensity of a Cheddar cheese with bitter and brothy notes, to the water-soluble peptide-containing fraction. Hydrophobic peptides are bitter and responsible for the bitter defect in cheese. Cheese flavour components in the water-soluble fraction are of low molecular weight (Mr) (<1000); the flavour intensity is greatest in the fraction relatively rich in the free amino acids, Met and Leu. Because in this fraction the NaCl concentration is also highest, the latter is considered to have an additional effect on the total flavour intensity (Visser, 1993). Various proteinases and peptidases in cheese hydrolyse caseins to peptides and free amino acids. Small peptides and free amino acids contribute directly to the background flavour of cheese (McGugan *et al.*, 1979; Aston and Creamer, 1986; Cliffe *et al.*, 1993; Sigh *et al.*, 1994). Singh et al., 1994 discovered that most of the savory cheesy taste of the water-soluble extract of cheese is in the ultra-filtrate permeate (10 kDa. Nominal molecular weight cut-off), which contains small peptides, free amino acids, salts and other low molecular weight compounds, whereas the retentate, which contains intermediate size peptides, is bland.

Engels and Visser (1994) concluded that, with the exception of Edam, the ultra-filtrate permeate with a molecular weight (MW) <500, of Cheddar, Gouda, Gruye're, Maasdam, Parmesan and Proosdij cheeses contained the components responsible for flavour. The permeate of these cheeses contained low molecular weight peptides (probably not larger than tetrapeptides), amino acids, breakdown products of amino acids (e.g. γ -aminobutyric acid and ornithine), and short-chain fatty acids (<9). The majority of the peptides characterized in the water-soluble dia-filtrate permeate originate from α_{s1}-casein. Plasmin is mainly responsible for the primary hydrolysis of β-casein in cheese, resulting in the formation of

γ-caseins and proteose-peptones. Most of the peptides in the retentate of water-soluble extract of Cheddar cheese originate from the N-terminal half of β-casein. The concentration of γ-caseins increases during ripening of Cheddar cheese, but the proteose-peptones are further hydrolyzed by CEP (Singh *et al.*, 1995).

Although it was reported that irradiation, especially at the doses applied in this study, do not affect proteins, and therefore also enzymes, effects are probably not to be excluded (Diehl, 1990). Microorganisms are however affected, and therefore also their production of enzymes, providing a secondary effect on the breakdown of proteins. The effect of irradiation, whether direct or indirect, on the proteins are reported in the subsequent chapters.

4.2 Materials and methods

4.2.1 Manufacture of Cheddar cheese

The manufacture of cheese is as discussed in Chapter 2 section 2.2.1.

4.2.2 Sampling of cheese

The cheese was ripened as described in section 2.2.2 and sampling was at day 0, 2 weeks, 6 weeks and 12 weeks.

4.2.3 Extraction and fractionation of water-soluble nitrogen

In the current study extraction and fractionation of water-soluble nitrogen of Cheddar cheese was carried out according to the method of Kuchroo and Fox (1982 a, b) Figure 4.1.

A 20g sample of cheese was homogenized in 40 ml of water at 20 ^{o}C for 10 min and kept at 40 ^{o}C for 1hr. The mixture was centrifuged at 3 000 rpm for

30 min, and the water-soluble fraction collected; this contains about 70 % of the soluble nitrogen. To achieve a higher yield the procedure was repeated on the precipitate (Rank *et al.*, 1985). The water-soluble fraction was frozen at -20 ^{o}C until later analysis. Extraction was followed by fractionation of cheese water-soluble peptides. The (water-soluble nitrogen) WSN was ultra-filtrated through a regenerated cellulose membrane with a molecular cut-off of 10 000 Daltons (Millipore) at a trans-membrane pressure of 2kg/cm^2 The permeate obtained was further fractionated by precipitation with 5 % PTA, (Merck). The retentate was fractionated by 30 % EtOH (Illovo, SA) at pH 6.5, followed by adjustment of the filtrate to pH 5.5 with HCl (BDH).

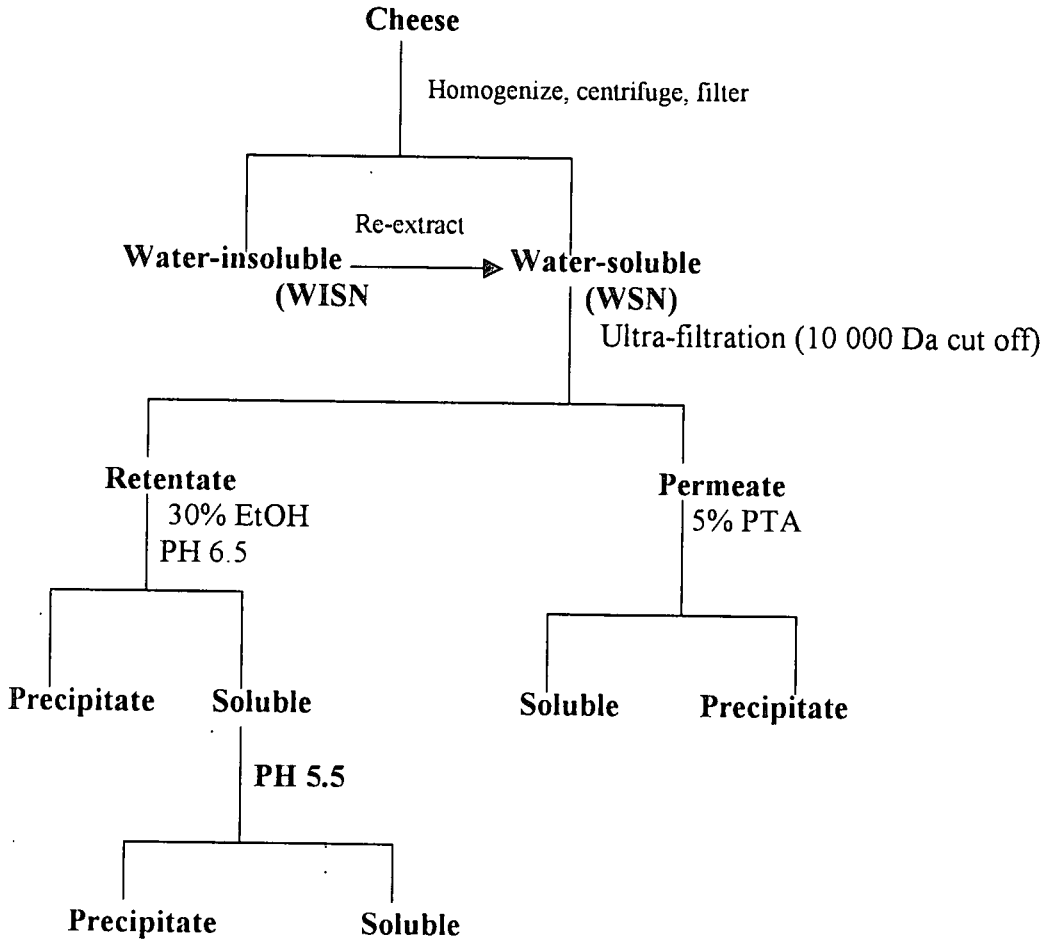

Figure 4.1 Flow diagram for the extraction and fractionation of the water-soluble nitrogen (WSN) of Cheddar cheese (taken from McSweeney and Fox 1993)

4.2.4 Nitrogen (N) determination

The nitrogen in cheese and WSN was determined by the macro Kjeldahl procedure according to the Buchi manual (slight variation on the AOAC method, 1970), and the WSN expressed as a percentage of total N.

4.2.5 Polyacrylamide gel electrophoresis

The method of Andrews (1983) was used to analyse the WISN and WSN-retentate, insoluble in 30 % ethanol samples by alkaline urea-polyacrylamide gel electrophoresis (Urea-PAGE). Samples were prepared as described by Shalabi and Fox (1987). Cheese sub-fractions were dispersed in a sample buffer of pH 7.6 (Shalabi and Fox, (1987) and aliquots of 1-20 µl were run on the gels consisting of a resolving gel (12,5 %T and 4 %C, pH 8.8) and a stacking gel (12,5 %T and 4 %C, pH 8.4) according to the method of Andrews (1983). Gels were run on a Mighty Small miniature slab gel electrophoreses unit, SE 260 (Hoefer Scientific Instruments), model PS 500X DC. Power supply was set at 210 volts (constant) for 2 gels running simultaneously.

The gels were stained by the direct-staining procedure (no destaining) of Blakesley and Boezi (1977) with coomassie brilliant blue G250 (Fluka) and were transferred to distilled water where the protein bands became more intense, the gels did not require de-staining and were stored in distilled water. The background was not stained and the protein bands were visualized directly. The electrophoretograms were photographed.

4.2.6 Statistical analysis

Statistical analysis were carried out as described in Chapter 3 section 3.4.3

4.3 Results and discussion

4.3.1 Nitrogen determination

The nitrogen analysis and calculations of N-contents for the treated and untreated cheeses ripened at different temperatures are given in Table 4.1, and the ANOVA on irradiation, ripening temperature and ripening time in Table 4.2. Changes in WSN content, expressed as a percentage of total N during ripening are shown in Figure. 4.2.

Table 4.1: N-analyses at different time intervals during the ripening of the unirradiated and irradiated Cheddar cheese

	% Moisture	% Dry matter	% WSN in wet cheese	% WSN in dry matter	% Total N in dry matter	% WSN/ Total N
Day 0	39.842	60.158	7.824	0.645	7.449	0.0705 ± 0.0025^{e}
2 Weeks A	42.149	57.851	0.519	0.044	6.810	0.007 ± 0.004^{a}
6 weeks A	43.279	56.721	2.043	0.177	7.934	0.022 ± 0.003^{ad}
12 weeks A	43.318	56.682	2.451	0.213	7.417	0.029 ± 0.009^{bcd}
2 Weeks B	41.935	58.065	2.305	0.194	7.093	0.0269 ± 0.005^{bcd}
6 weeks B	42.240	57.760	1.053	0.089	7.479	0.012 ± 0.006^{ab}
12 weeks B	40.374	59.626	1.893	0.156	7.461	0.021 ± 0.004^{ad}
2 Weeks C	40.691	59.309	2.561	0.209	6.756	0.031 ± 0.002^{d}
6 weeks C	40.982	59.018	2.295	0.191	6.917	0.028 ± 0.005^{bcd}
12 weeks C	41.240	58.760	2.824	0.237	7.302	0.032 ± 0.004^{d}
2 Weeks D	41.923	58.077	1.599	0.136	6.531	0.021 ± 0.007^{ad}
6 weeks D	41.307	58.693	1.127	0.094	7.431	0.013 ± 0.006^{ac}
12 weeks D	41.037	58.963	2.610	0.216	7.320	0.03 ± 0.002^{cd}

Means with different superscripts differ significantly.
Probability level: $p < 0.001$
A: Unirradiated cheese ripened at 8 ^{o}C
B: Irradiated cheese ripened at 8 ^{o}C
C: Unirradiated cheese ripened at 16 ^{o}C
D: Irradiated cheese ripened at 16 ^{o}C

Table 4.2 Analysis of variance of irradiation, ripening temperature and ripening time, and Water-soluble nitrogen (WSN) of Cheddar cheeses

Effects	**Probability level**
Irradiation	0.0008
Ripening temperature	0.0001
Ripening time	0.0001
Interaction	
Irradiation × Temperature	0.0001
Irradiation × Time	0.0001
Temperature × Time	0.1578
Irradiation × Temperature × Time	0.0001

Probability level: p-values < 0.005 are significant

Changes in WSN content were highly significant for irradiation, ripening temperature and ripening time (Table 4.2). Interactions were also highly significant for irradiation × ripening temperature, irradiation × ripening time and irradiation × ripening temperature × ripening time, but were not significant for ripening temperature × ripening time.

Quantitation and characterization of nitrogen in a water extract of cheese is a commonly used index of cheese ripening and WSN as a percentage of N was used to evaluate proteolysis in the ripening of cheese at normal and elevated temperature coupled with irradiation (Folkertsma *et al.*, 1996 and McSweeney *et al.*, 1994). The WSN ratio of the unirradiated cheese ripened at 8 ^{o}C showed a significant decrease from day 0 to ripening at 2 weeks. The ratio increased significantly from cheese ripened for 2 weeks to cheese ripened for 12 weeks, although there is no significant increase from cheese ripened for 2 weeks to 6 weeks and from cheese ripened for 6 weeks to 12 weeks.

The ratio for the unirradiated cheese ripened at 16 ^{o}C also decreased significantly from day 0 to ripening at 2 weeks, but there is no significant change from cheese ripened for 2 weeks to 6 weeks and from cheese ripened for 6 weeks to 12 weeks. There is not even a significant change in the WSN between cheese ripened for 2 weeks and cheese ripened for 12 weeks.

There was a significant decrease of WSN ratio of the irradiated cheese ripened at 8 ^{o}C from day 0 to ripening at 2 weeks, however there was no significant decrease from cheese ripened for 2 weeks to 6 weeks and no significant increase from cheese ripened for 6 weeks to 12 weeks. The cheese ripened to 2 weeks did not differ significantly from cheese ripened for 12 weeks.

The ratio for the irradiated cheese ripened at 16 ^{o}C decreased significantly from day 0 to cheese ripened for 2 weeks. There was no significant decrease from cheese ripened for 2 weeks to cheese ripened for 6 weeks, and no significant increase from cheese ripened for 6 weeks to 12 weeks. There was no significant difference between cheese ripened for 2 weeks and cheese ripened for 12 weeks.

Cheese sampled at day 0 differed significantly from all the other ripening stages (from 2 weeks to 12 weeks). There was no significant difference in the WSN between the irradiated cheese ripened at 8 ^{o}C, 16 ^{o}C and the unirradiated cheese ripened at 16 ^{o}C for 6 weeks or 12 weeks, but there is a definite significant difference between the unirradiated cheese ripened at 8 ^{o}C and the irradiated cheese ripened at 8 ^{o}C and 16 ^{o}C and the unirradiated cheese ripened at 16 ^{o}C only at 2 weeks of ripening. According to the WSN, irradiation and ripening at 16 ^{o}C of Cheddar cheese achieved the same levels of WSN (ca 0.029%) after two weeks of ripening than the 8 ^{o}C ripened cheese after 6 to 12 weeks. This result might suggest that irradiation be a useful process for accelerated ripening of chees

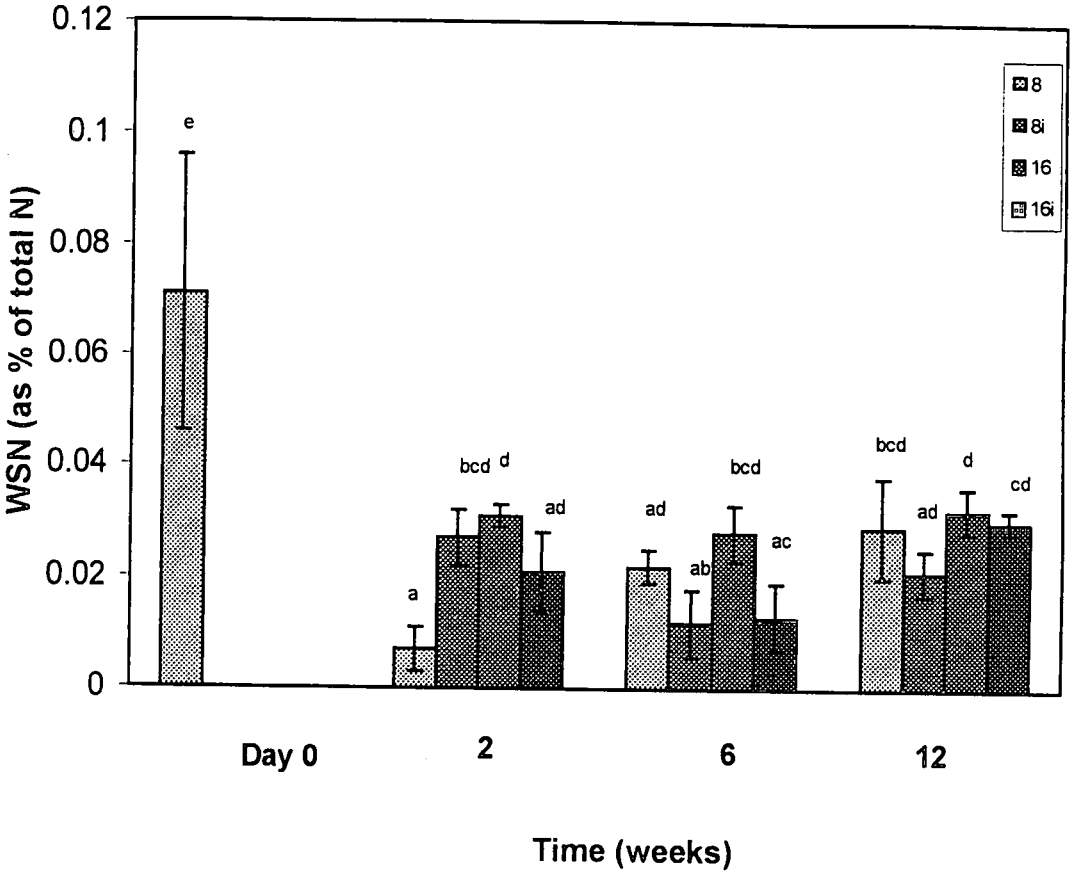

Figure 4.2 The Water-soluble nitrogen (WSN) as a percentage of total N during the ripening of Cheddar cheese.

Means with different superscript differ significantly
8: Unirradiated cheese ripened
Probability level: $p < 0.001$ at 8 ^{o}C
8i: Irradiated cheese ripened at 8 ^{o}C
16: Unirradiated cheese ripened at 16 ^{o}C
16i: Irradiated cheese ripened at 16 ^{o}C

4.3.4 Urea-PAGE analyses

As discussed in Chapter 1, proteolysis is regarded as the most important biochemical event in the ripening of Cheddar cheese. The principal water-insoluble peptides in Cheddar are produced either from α_{s1}-casein by chymosin (EC 3.44.23.4) or from β-casein by plasmin (EC 3.4.21.7) and this result into primary proteolysis, the specificity of which has been established by Fox et al., 1994. Electrophoresis has been applied widely to study primary proteolysis in cheese (Fox, 1989). Since only proteins and large peptides can be visualised by staining, the technique is limited to the assessment of casein loss and the formation and subsequent hydrolysis of the primary products of casein proteolysis. It was noted by (O`Sullivan and Fox, 1990) that the peptides in a 10 000 Da UF permeate do not stain on Urea-PAGE but the retentate of the WSN contains several detectable peptides, and these are what will be discussed in this chapter. Peptides in Figure 4.3 and Figure 4.4 were identified according to work by McSweeney et al. (1994).

In Figure 4.3 the electrophoretogram of WISF is shown, where the degradation of β and α_{s1} casein can be followed. There was a degradation of β-CN by plasmin which lead to the formation of the γ-peptides. There was a presence of γ_2(β-CNf106-209) in the standard cheese in Figure 4.3 (unirradiated cheese ripened at 8^{o}C) from the early ripening stages of cheese and the intensity of this band decreased with ripening up to week 12. The presence of γ_1(β-CN f29-209) and γ_3(β-CN f108-209) are unmistakable, and their intensity increased with ripening, up to 12 weeks. The intensity of the β-CN increased from day 0 to 2 weeks of ripening, and then gradually decreased up to 12 weeks. The intensity of the degradation of β-casein band, β-I(f1-189/192) also increased from day 0 to 2 weeks of ripening and then gradually decreased as ripening progressed up to 12 weeks. Marcos *et al.* (1979) reported that in the region of β-casein there is a major central band of β-CN between two minor bands, usually masked by the major one.

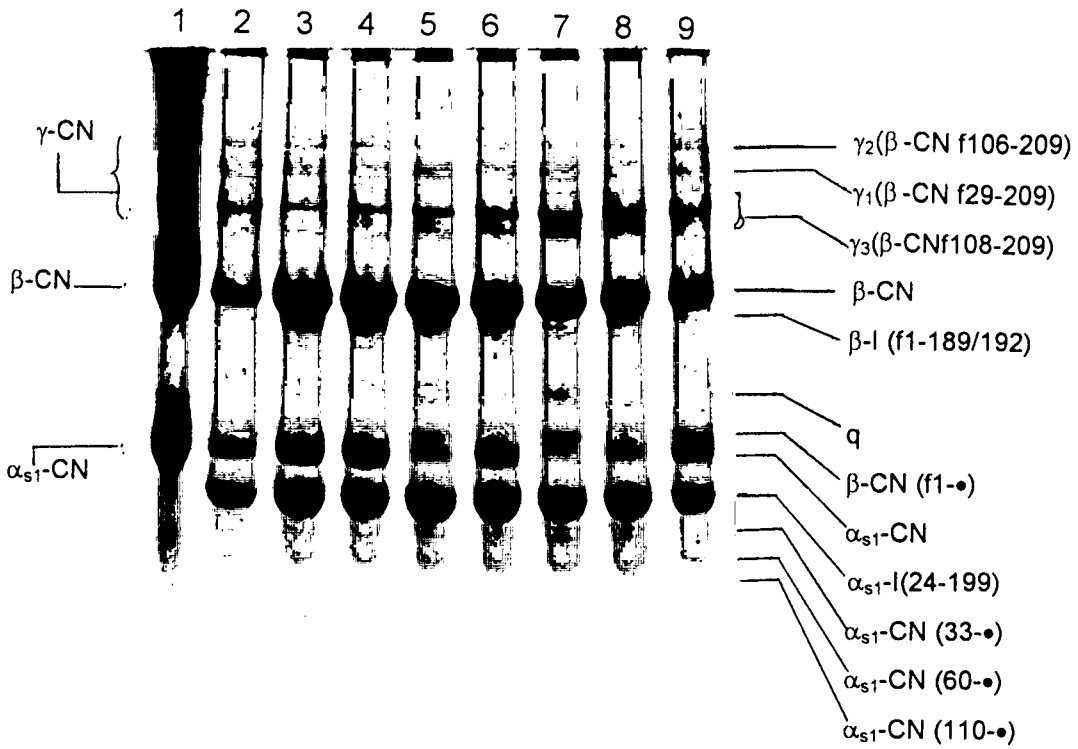

Figure 4.3 Urea-PAGE of the WISF of the Cheddar cheese ripened at 8 ^{o}C (irradiated and unirradiated) from day 0 to 12 weeks. Lane 1: marker, Na-caseinate, lanes 2: day 0, lane 3: 2 weeks unirradiated. Lane 4: 2 weeks irradiated, lane 5: 6 weeks unirradiated, lane 6: 6 weeks irradiated, lane 7: 12 weeks unirradiated, lane 8: 12 weeks irradiated.

The minor band that migrated slightly faster than the accompanying major protein probably represents a breakdown product named β-I (f1-189/192).

The peptide bands of peptide q are not clearly resolved in fresh cheese that was sampled at day 0 and in cheese ripened for 2 weeks but can be seen in cheese ripened for 6 weeks and 12 weeks. The presence of this band can be an indication that it appears in matured cheese or after 1 month of ripening. Richardson and Pearce, (1981) reported that this peptide is unique, because it is completely resistant to the action of chymosin, the extent of degradation of it in Cheddar cheese was related to the plasmin content in the cheese. The resolution of β-CN (f1-∘) was not well defined at day 0 and for cheese ripened for 2 weeks but started to show in cheese ripened for 6 weeks and intensified up to 12 weeks of ripening. The α_{s1}-CN increased from day 0 to 2 weeks of ripening, and then decreased up to 12 weeks. The peptide band of α_{s1}-I (24-199) stayed unchanged throughout ripening. The presence of this peptide is an indication of the major role played by rennet in the initial breakdown of (α_{s1}-casein), and this was present at least in the early stages of ripening as discussed by (Creamer and Richardson, 1974). Creamer (1975) found that this α_{s1}-I peptide can be almost entirely degraded during ripening. The formation of the α_{s1}-degradation peptides α_{s1}-CN (f33 -•) and α_{s1}-CN (f60 -∘) can be seen although they are not well resolved at day 0 and 2 weeks but the resolution improved at 6 weeks and 12 weeks. Peptide α_{s1}-CN (f110 -•) only developed after 12 weeks of ripening, and is visible as a very faint band.

Cheese ripened at 16 ^{o}C (fig. 4.4) showed a decrease in the intensity of peptide γ_2(β-CNf106-209), the band is darker at day 0 and for cheese ripened for 2 weeks than in cheeses ripened for 6 weeks and 12 weeks. The intensity of both the γ_2 (CN f29-209) and γ_3(β-CN f108-209), increased from day 0 to 12 weeks as ripening progressed. The β-CN seemed to have developed from day 0 to 2 weeks of ripening, and then was degraded after 6 weeks and 12 weeks of ripening.

The β-I(f1-189/192) which is the degradation band of the β-CN, developed from day 0 to 2 weeks and disappeared from 2 weeks to 12 weeks of ripening. Peptide q seemed to develop with ripening, where it is absent in cheese ripened for 0 days, it started to develop in cheese ripened for 2 weeks and is well developed in cheese ripened for 6 weeks and 12 weeks. The intensity of β-CN (f1-∘) also increased with ripening and this is clearly indicated in cheese ripened for 6 weeks and 12 weeks. The intensity of α_{s1}-CN increased from day 0 to 2 weeks and decreased thereafter in cheese ripened for 6 weeks and 12 weeks. The intensity of α_{s1}-I (24-199) increased from day 0 to 2 weeks, then stayed unchanged throughout ripening. The intensity of α_{s1}-CN (f33 -∘) and α_{s1}-CN (f60 -∘) also showed development with more intense bands after 6 weeks of ripening.

As discussed in Chapter 1, ripening of Cheddar cheese can be accelerated and its flavour intensified at 16 ^{o}C (Folkertsma *et al.*, 1996 and Fedrick, 1987) also reported that ripening at elevated temperatures (≤15 ^{o}C) has been recommended to accelerate the ripening of cheese of good chemical and microbiological quality. This is supported by the results of this current study. When comparing the two electrophoretograms, it can be seen that the γ_2(β-CNf106-209) is definitely better visible in cheese ripened at 16 ^{o}C than in cheese ripened at 8 ^{o}C. The same can be seen for peptides γ_3(β-CNf108-209), β-CN (f1-•) and peptide q. At 16 ^{o}C ripening, the development and subsequent breakdown of peptides β-CN and α_{s1}-CN is also faster. The higher ripening temperature seems to have had no effect on the development or degradation of peptides γ_1 (CN f29-209), β-I, (α_{s1}-CN (f33 -•), and (α_{s1}-CN (f60 -•) different from ripening at 8 ^{o}C.

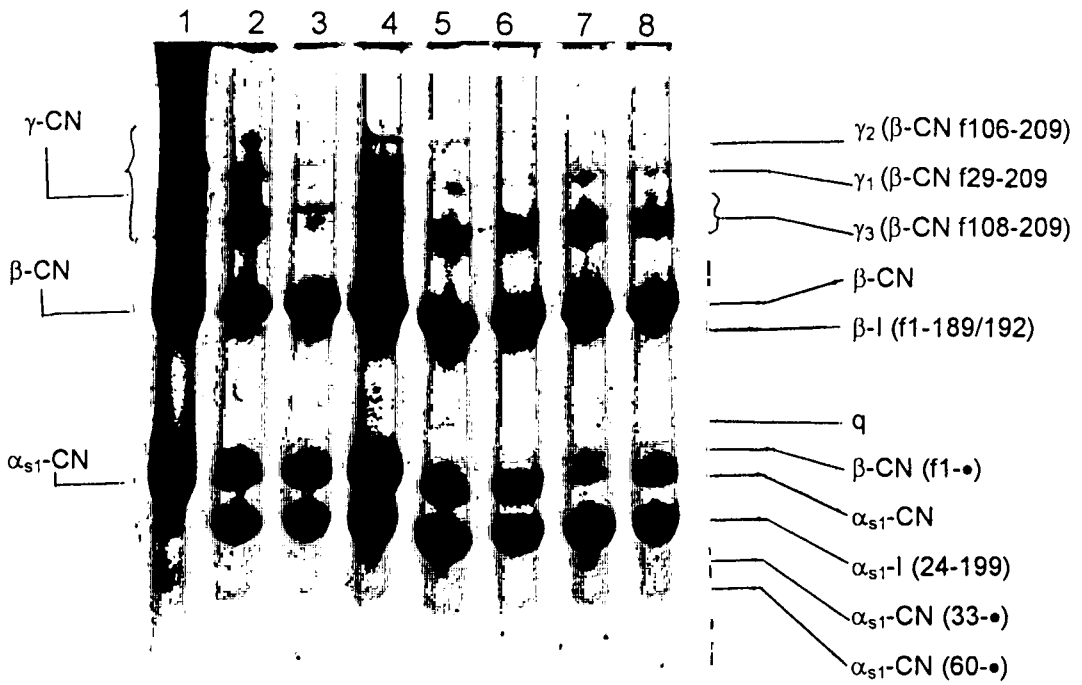

Figure 4.4 Urea-PAGE of the WISF of the Cheddar cheese ripened at 16 ^{O}C (irradiated and unirradiated) from day 0 to 12 weeks. Lane 1: marker, Na-caseinate, lanes 2,9: day 0, lane 3: 2 weeks unirradiated. Lane 4: 2 weeks irradiated, lane 5: 6 weeks unirradiated, lane 6: 6 weeks irradiated, lane 7: 12 weeks unirradiated, lane 8: 12 weeks irradiated.

The irradiated cheese ripened at the 8 °C (Figure 4.3) showed γ_2(β-CNf106-209) disappearing with ripening and the intensity of both the γ_1(β-CN f29-209) and γ_3(β-CN f108-209) increased with ripening. The intensity of (β-CN) increased with ripening but its degradation product (the β-I) showed a decrease in intensity as ripening progressed. Peptides q and β-CN (f1-∘) are present but not as well developed as in the unirradiated cheese. The intensity of α_{s1}-CN first increased up to 2 weeks of ripening, then decreased thereafter. The decrease is very much slower in the irradiated cheese than in the unirradiated cheese. The intensity of peptides α_{s1}-CN (f33 -∘) and α_{s1}-CN (f60 -∘) is the same in both the unirradiated and irradiated cheese.

Cheeses ripened at 8 °C show the same peptide bands for the irradiated and unirradiated cheeses, the only difference being the intensity. There is no difference in the band intensity of the γ_2(β-CNf106-209) peptide. The intensity of γ_1(β-CN f29-209) and γ_3(β-CN f108-209) bands is stronger in the unirradiated cheese ripened at 8 °C than in the irradiated cheese ripened at 8 °C. The β-CN developed the same in both the unirradiated and the irradiated cheeses. The same band is absent in day 0 and 2 weeks of the unirradiated cheese which is during the early stages of ripening. This band is the type that can only be found in mature cheeses because they develop later during the ripening of cheese i.e. after 1 month. The β-CN (f1 -•) is not well defined from the α_{s1}-CN in the irradiated cheese at 12 weeks but it is visible in the unirradiated cheese at the same ripening time. The α_{s1}-CN was degraded at 12 weeks in the unirradiated cheese but it is still strong in the irradiated cheese. The intensity of the α_{s1}-I peptide is the same in both the irradiated and the unirradiated cheeses. Peptide α_{s1}-CN (f33 -•) and (α_{s1}-CN (f60 -•) are present in both the irradiated and unirradiated cheese and their intensity increased with ripening in both, the only difference is that the bands are darker in the unirradiated cheeses than in the irradiated cheeses. The low intensity of the irradiated cheese can be due to the fact that irradiation may have had a negative effect on the starter cultures that help with the ripening of

cheese or even hindered the effectiveness of rennet, which is responsible for the breakdown of α_{s1}-casein.

The β-I(f1-189/192) peptide is very weak at day 0 and was unchanged during ripening at 16 °C. The γ_2(β-CNf106-209) peptide band of the irradiated cheese ripened at 16 °C showed a decrease in intensity with ripening, and the γ_1(β-CN f29-209) and γ_3(β-CN f108-209) showed the same pattern. The intensity of (β-CN) increased from day 0 to cheese ripened for 2 weeks and then decreased after ripening for 6 weeks and 12 weeks. Peptide q is very weak at day 0, and disappeared after irradiation. The β-CN (f1 -∘) is not present in cheese ripened for 2 weeks and 6 weeks but started to develop at 12 weeks. The α_{s1}-CN showed development after 2 weeks of ripening whereafter it was gradually degraded. The intensity of the α_{s1}-I (24-199) peptide band is the same throughout ripening. The intensity of peptides α_{s1}-CN (f33 -•) and α_{s1}-CN (f60 -•) increased with ripening for 2 weeks, 6 weeks and 12 weeks.

Comparison of unirradiated and irradiated Cheddar cheese ripened at 16°C showed that there is no difference between the γ_2(β-CNf106-209) peptide of the unirradiated and irradiated cheeses. The γ_1(β-CN f29-209) and γ_3(β-CN f108-209) showed a stronger development in the unirradiated cheese than in the irradiated cheese up to 6 weeks of ripening with no difference after 12 weeks. The β-CN developed at the same rate in both cheeses and β-I(f1-189/192) peptide was stronger in the unirradiated cheese ripened at 16°C, than in the irradiated cheese ripened at the same temperature.

Peptide q is also stronger in the unirradiated cheeses at 6 weeks and 12 weeks. The α_{s1}-CN is degraded at 6 weeks and 12 weeks in the unirradiated cheese but it is still present in the unirradiated cheese at 6 weeks and 12 weeks. The intensity of the α_{s1}-I (24-199) peptide is the same in both cheeses. Where peptides α_{s1}-CN (f33 -•) and α_{s1}-CN (f60 -•) continued their

intensity during ripening of the unirradiated cheese, they disappeared at 2 weeks, directly after irradiation, and only reappeared after 12 weeks of ripening.

Many researchers have researched the advantages and the disadvantages of accelerated ripening and came to the conclusion that elevated temperature is the simplest method to be used because it is easier to manipulate. Results in Figure. 4.3 and 4.4 have shown that cheese ripened at 16 ^{o}C showed the same peptide pattern at a shorter ripening time as the standard cheese (cheese ripened at 8 ^{o}C) for e.g. the unirradiated cheese ripened for 12 weeks in Figure. 4.3. has the same peptide pattern as cheese ripened for 6 weeks in Figure. 4.4. Furthermore, the irradiated cheese ripened at 8 ^{o}C for 12 weeks in Figure. 4.3 showed the same peptide bands as cheese ripened at 16 ^{o}C for 6 weeks in Figure. 4.4. However, no irradiated cheese ripened at either 8 ^{o}C nor 16 ^{o}C showed the peptide profile of the cheese ripened at 8 ^{o}C. The major difference between the unirradiated and the irradiated cheeses is visible in the dynamic changes of peptides β-CN (f1-e) and α_{s1}-CN.

According to Creamer and Richardson, (1974) rennet plays the major role in the initial breakdown of α_{s1}-I. Visser and De Groot-Mostert (1977) concluded that rennet is completely responsible for degradation of α_{s1}-CN. and β-CN during the first month of ripening, with enzymes from lactic acid bacteria contributing to proteolysis in the longer term, especially on β-CN. Fox *et al.*, (1994) concluded that primary proteolysis results from the action of chymosin and plasmin, with Marcos et al., (1979) stating that rennet and alkaline proteinases break casein into large peptides which are cleaved into small peptides by starter proteinases and peptidases. In the irradiated cheeses, β-CN was degraded at the same rate as in the unirradiated cheeses. This indicates that rennet could successfully hydrolyse this peptide, irrespective of irradiation. The α_{s1}-CN, however, was degraded at a slower rate in the irradiated cheeses. Since rennet is completely responsible for the breakdown

of the α_{s1}-CN during the early stages of ripening, it can be concluded that the α_{s1}-CN was affected by the irradiation in a way that hydrolysis by rennet was retarded, perhaps the irradiation caused a conformation change of the α_{s1}-CN. The quick formation of peptide q from α_{s1}-CN in the unirradiated and slow formation in the irradiated cheese can therefore also be explained.

According to McSweeney *et al.*, (1993) chymosin is responsible for the formation of peptides α_{s1}-I (24-199) and α_{s1}-CN (f33 -∞). Although not much can be concluded from the changes α_{s1}-I (24-199), the development of α_{s1}-CN (f33 -∞) was shown to be faster in the unirradiated cheese, also suggesting a conformation change of α_{s1}-CN due to irradiation which may lead to slower hydrolysis by chymosin. Since there are no differences in the changes of γ_2(β-CNf106-209) between the irradiated and unirradiated cheeses during ripening, but a more pronounced formation of γ_2(β-CNf29-209) and γ_2(β-CNf108-209), which are formed due to the action of rennet (Visser and de Groot-Mostert, 1977) a cause of conformation change of the β-CN after irradiation could also be suggested. Effects of irradiation on peptides formed from the action of starter organisms or their enzymes cannot be concluded at this stage.

Peptides of molecular size larger than 10 000 dalton were subjected to precipitation by 30% Ethanol. This WSN-EtOH ppt. was subjected to electrophoresis, and the peptide bands identified according to the work of McSweeney *et al.* (1994) and Singh *et al.* (1995). Peptides not identified by these researchers were numbered according to Bothma *et al.* (2001). The electrophoretograms of this fraction of cheeses ripened at 8 ^{o}C and 16 ^{o}C are shown in Figures 4.5 (8 ^{o}C) and 4.6 (16 ^{o}C).

Figure 4.5 (unirradiated cheese ripened at 8 ^{o}C) showed the presence of β-CN (1-?)(a) with intensity that disappeared with ripening, it seems as if it disappeared after 12 weeks. The intensity of β-CN (1-?)(b) seemed to

decrease from day 0 to cheese ripened for 2 weeks and increased in cheese ripened for 6 weeks and 12 weeks. The intensity peptide n band stayed the same throughout ripening. Compared to the results of other researchers, some peptides were not observed in the present works. These are β-CN (58-?) and β-CN (57-?) (Bothma *et al.*, 2001 and Singh et al., 1995). A possible explanation could be that the cheeses under discussion were not ripened for more than 12 weeks.

The intensity of peptide p decreased from day 0 to cheese ripened for 2 weeks and remained the same in cheese ripened for 6 weeks and 12 weeks. Peptide r showed an intensity that increase after 2 weeks then stayed unchanged. Peptide t disappeared after 2 weeks of ripening and peptide t_2 disappeared after 6 weeks of ripening. The intensity of peptide v increased after 6 weeks and continued to increase to 12 weeks. All other peptides seem to show no change.

The unirradiated cheeses ripened at 16 ^{o}C (Figure. 4.6) showed that β-CN (1-?)(a) disappeared with ripening, while the intensity of β-CN (1-?)(b) decreased. Peptide p decreased after 2 weeks of ripening and became very weak at 12 weeks of ripening. Peptide r had the intensity that increased from day 0 up to 6 weeks, then decreased after 12 weeks. Peptide t disappeared with ripening and peptide t_2 disappeared after 6 weeks of ripening. Peptide v showed an intensity that increased after 2 weeks up to 6 weeks, whereas all the other peptides seemed to show no changes.

When comparing the unirradiated cheeses ripened at 8 ^{o}C and 16 ^{o}C it can be seen that there is a faster disappearance of peptide p in cheese ripened at 16 ^{o}C than in cheese ripened at 8 ^{o}C. There is also a faster disappearance of peptide v and peptide t_2 in cheese ripened at 16 ^{o}C. All the other peptides do not show differences in the two cheeses.

The irradiated cheese ripened at 8 ^{o}C showed that the intensity of β-CN (1-?)(a) decreased at 2 weeks of ripening, reappeared at 6 weeks and decreased at 12 weeks of ripening. The β-CN (1-?)(b) disappeared after irradiation, and reappeared after 6 weeks of ripening. Peptide n decreased after irradiation, and reappeared at 12 weeks of ripening. Peptide o only appeared at 6 weeks of ripening where after it disappeared. Peptide p decreased after 2 weeks and almost disappeared at 12 weeks of ripening. Peptide t disappeared with ripening and the intensity of peptide t_2 increased at 2 weeks of ripening to 6 weeks and decreased towards 12 weeks of ripening. Peptide v stayed almost unchanged throughout ripening whereas all the other seemed to show no changes.

The irradiated and unirradiated cheeses ripened at 8 ^{o}C showed the same peptide bands with the difference is that the bands are more intense in the unirradiated than in the irradiated cheese. The major difference is the presence of peptide o in the irradiated cheese at 6 weeks of ripening and its absence in the unirradiated cheese. The β-CN (1-?)(a) and β-CN (1-?)(b) peptides are definitely more intense in the unirradiated cheese than in the irradiated cheese. The peptide n showed a darker band in the unirradiated than in the irradiated cheese. The other peptides of the two cheeses did not differ from each other.

The irradiated cheese ripened at 16 ^{o}C showed β-CN (1-?)(a) that disappeared after irradiation and the β-CN (1-?)(b) that disappeared after irradiation but reappeared after 6 weeks of ripening. Peptide n decreased after irradiation and reappeared at 6 weeks and 12 weeks of ripening. Peptide p decreased after 2 weeks of ripening and almost disappeared at 12 weeks of ripening. Peptide t disappeared with ripening and peptide t_2 developed after 2 weeks of ripening and almost disappeared at 12 weeks of ripening. Peptide v stayed throughout ripening while all other peptides did not show any changes

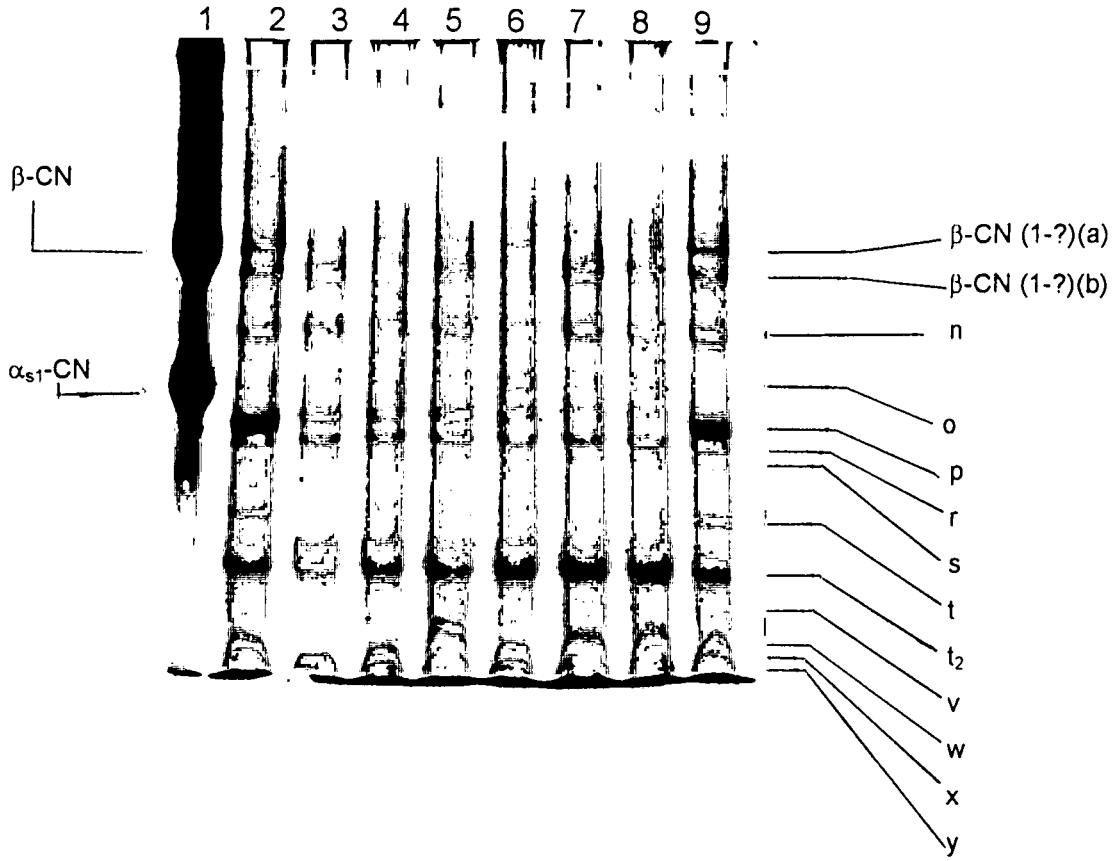

Figure 4.5 Water-soluble, ethanol-insoluble (retentate-ppt) of Cheddar cheese ripened at 8^{O} C (irradiated and unirradiated) from day 0 to 12 weeks. Lanes1: marker, Na-caseinate, lane 2: day 0, lane 3: 2 weeks unirradiated, lane 4: 2 weeks irradiated, lane 5: 6 weeks unirradiated, lane6: 6 weeks irradiated, lane 7: 12 weeks unirradiated, lane 8: 12 weeks irradiated.

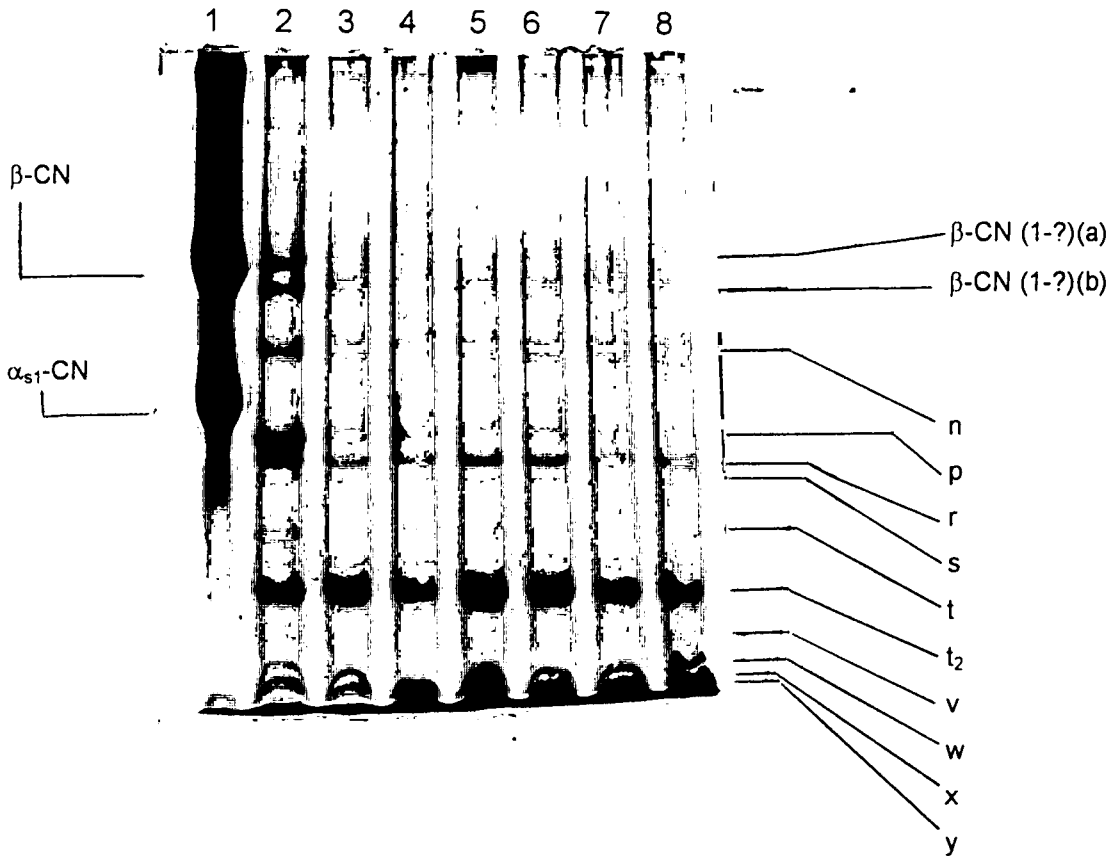

Figure 4.6 Water-soluble, ethanol-insoluble (retentate-ppt) of Cheddar cheese ripened at 16 ^{o}C (irradiated and unirradiated) from day 0 to 12 weeks. Lanes1: marker, Na-caseinate, lane 2: day 0, lane 3: 2 weeks unirradiated, lane 4: 2 weeks irradiated, lane 5: 6 weeks unirradiated, lane6: 6 weeks irradiated, lane 7: 12 weeks unirradiated, lane 8: 12 weeks irradiated.

The unirradiated and irradiated cheese ripened at 16 °C showed the presence of more or less the same bands with the major difference in the intensity of the individual bands. The β-CN (1-?)(b) disappeared in the unirradiated cheeses but reappeared at 6 weeks of ripening in the irradiated cheeses. Peptide t_2 disappeared after 6 weeks of ripening in the unirradiated cheese but developed after 2 weeks of ripening and almost disappeared at 12 weeks in the irradiated cheese. Peptide v stayed throughout in the irradiated cheese but increases after 2 weeks of ripening up to 6 weeks in the unirradiated cheese. All other peptides did not show any changes.

The irradiated cheeses showed peptide bands different from the unirradiated cheeses for the WSN-EtOH ppt, mainly of peptides o, β-CN (1-?)(b) and t_2. Since it is not clear from the literature whether peptides β-CN (1-?)(a) and β-CN (1-?)(b) are formed due to the action of rennet and chymosin, or also due to the starter organisms and their enzymes, it is not possible to conclude definitely on the differences in the development and degradation of these peptides in the irradiated and unirradiated cheeses.

4.4 Conclusion

Analysis of the WSN showed that only cheese ripened at 8 °C followed a different path of development of free N, with a low amount at 2 weeks of ripening, and then stabilizing at approximately 0.029% after 12 weeks of ripening. Ripening at 16 °C as well as irradiation, achieved the same level of WSN after 2 weeks of ripening, with, statistically, not much change afterwards. This would suggest that these treatments are useful for accelerated ripening of Cheddar cheese, but had to be proven by analysis of the breakdown of the caseins and changes in the peptide make-up.

Electrophoresis of WISF and WSF-EtOH ppt. Showed that ripening at 16 °C definitely accelerated ripening, as the peptide composition after 6 weeks of ripening was almost the same as that observed after 12 weeks when Cheddar

cheese was ripened at 8 ^{o}C. Irradiation and subsequent ripening revealed unique peptide profiles, which could not be matched with unirradiated cheese ripened at either 8 ^{o}C or 16 ^{o}C and at any time of ripening. The differences in peptide development or degradation of β-CN and α_{s1}-CN origin may possibly be ascribed to changes in conformation of the peptides, resulting in a slower hydrolysis by pepsin and rennin. An effect of irradiation on the starter organisms is not excluded, but could not be definitely concluded from the results obtained.

CHAPTER 5

THE EFFECT OF IRRADIATION AND ELEVATED RIPENING TEMPERATURE OF CHEDDAR CHEESE ON PROTEINS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

5.1 Introduction

It is not only the medium and highly volatile fractions (ketones, methyl and ethyl esters, alkanols, etc.) but also the water-soluble nitrogenous fractions that determine the flavour of the different types of cheese (Adda *et al.*, 1982). It was reported by McGugan *et al.*, 1979 that the peptides and amino acids freed through the action of proteolytic enzymes in the curd, the starter enzymes, moulds and so on are related to the intensity of the flavour, as well as to the age of the cheese (Aston and Dulley, 1982: Pham and Nakai, 1984). Proteolytic agents in cheese generally include indigenous milk proteinases, coagulant, starter and nonstarter proteinases and peptidases (Fox, 1989). In cheese ripening these enzymes hydrolyse casein to produce peptides and free amino acids, which are important in flavour development and the production of small peptides and free amino acids is due primarily to the activity of starter and non-starter proteinases and peptidases (Singh *et al.*, 1995).

The recognised importance of non-volatile water extractable fraction (McGugan *et al.*, 1979) has been interpreted as a direct effect of the proteolysis product. Bitterness in cheese results from the presence of low molecular weight peptides which are not further degraded to non-bitter peptides and amino acids by starters when the strains which are used are peptidase deficient (Adda *et al.*, 1982). Peptides also seem to contribute to other flavours. Although the volatile fraction of cheese contributes mainly to its aroma the water-soluble fraction (WSF) is mainly responsible for its taste (Aston *et al.*, 1983). Most of the nitrogenous compounds that contribute to

cheese flavour are soluble in aqueous solvent. PTA-soluble N was found by (Aston and Dulley, 1982) to correlate significantly with the age and flavour intensity and by Jarrett *et al.*, 1982 to contain small peptides and free amino acids.

Large peptides have been found to be important for the brothy flavour in Swiss cheese, whilst it was suggested that the typical sweet flavour results from an interaction of calcium and magnesium with the small peptides (Biede, 1977). McGugan *et al.*, (1979) have reported that the water-soluble fraction of ripened cheese, which consists mainly of peptides and amino acids, has a significant relationship to the intensity of cheese flavour and Fox *et al.*, (1994) has reported that the WISF of cheese is flavourless, but peptides in the water-soluble fractions are considered to be important to cheese flavour. A number of authors such as (Cliffe *et al.*, 1993; Engles and Visser, 1994) have shown that the very small peptides (< 500 Da) make a significant contribution to Cheddar flavour.

5.2 Materials and methods

5.2.1 Manufacture of Cheddar cheese

The manufacture of cheese is as discussed in Chapter 2 section 2.2.1.

5.2.2 Sampling of cheese

The cheese was ripened as described in section 2.2.2 and sampling was done at day 0, 2 weeks, 6 weeks and 12 weeks.

5.2.3 Extraction and fractionation of water-soluble nitrogen

The extraction and fractionation of protein peptides is as shown on the flow diagram on figure 4.1 and section 4.2.

5.2.4 Peptide analysis by high performance liquid chromatography

Lyophilized samples of the water-soluble, 5%PTA-soluble and 5%PTA-insoluble fractions (UF-permeate) were dissolved in deionized water (Milli-Q water) (1mg/500µl), and filtered through a 0,45 µm Cameo (Separation) pre-filter and stored frozen. Samples of the water-soluble, EtOH-soluble fraction (UF retentate) were directly injected into the HPLC chromatograph.

HPLC was carried out on a HPLC system, which comprised of a Hewlett Packard 1100 sample injector and quartenary gradient pump solvent delivery system, and a Shimadzu Variable Wavelength detector operated at 230 nm. Samples were chromatographed on a 5 µm Jupiter RP18 250 mm × 4,6 mm reversed-phase column (Separations) preceded by a Jour-Guard RP/C18- 5µm guard column (Separations) at ambient temperature. A linear gradient of 0,15% TFA (Trifluoroacetic acid), (ion-pairing agent) in water (eluant A) to 0,1 % TFA (Merck) in 80:20 (v/v) acetonitrile (Burdick & Jackson) in water (eluant B) was increased to 70 % B over 70 min at 1ml/min flow rate. A volume of 40 µl of filtered sample was injected for chromatography.

5.3 Results and discussion

Elution profiles of peptides are shown from, which discussions are derived. The numbering of peptide material eluting at different times was not assigned according to individual peaks or specific groups of peaks observed in each chromatogram, but according to groups of peaks that were clearly formed from a loose array of peaks after ripening of cheeses to 12 weeks of at least one of the treatments to ensure a better comparison between the treatments. Where necessary, clearly resolved individual peaks were identified by letters.

5.3.1 Peptide analysis by high-performance liquid chromatography

Peptide profiles (WS, EtOH-soluble retentate) of the unirradiated cheese ripened at 8°C are shown in Figure. 5.1. The figure shows a reduction of the peptides eluted at 15-20 min. with ripening and the same reduction is seen at peptides eluted at 22-32 min from day 0 to cheese ripened for 12 weeks. There is a reduction in peptides eluted at 33-43 from day 0 to cheese ripened at 2 weeks and a total change in these peptides from cheese ripened for 6 weeks to cheese ripened for 12 weeks. A complete change in the region 48-62 min. is observed from day 0 to 12 weeks of ripening. A clear development of peptides eluted at 63-72 min is seen from day 0 throughout ripening especially in cheese ripened for 6 weeks and 12 weeks.

The WS, EtOH-soluble retentate fractions of unirradiated cheese ripened at 16°C are shown in fig. 5.2. Peptides eluted at 15-20 min. showed some reduction from day 0 to cheese ripened for 6 weeks and a development in cheese ripened for 12 weeks. There is a development in peptides eluted at 22-32 min from day 0 to cheese ripened for 12 weeks. Peptides eluted at 33-43 min showed some reduction from day 0 to cheese ripened for 2 weeks and a total change into a new group from 2 weeks of ripening to 12 weeks. Peptides eluted at 45-62 also changes into a new group with ripening. Peptides that eluted at 63-72 min. developed from 2 weeks to 12 weeks of ripening.

The chromatogram of irradiated cheese ripened at 8 °C (Figure. 5.3) showed that peptides eluted at 12-20 min. did not disappear from day 0 to 12 weeks of ripening. There is a development in peptides eluted at 22-32 min from day 0 to cheese ripened for 12 weeks. Peptides eluted at 33-43 first showed a reduction from day 0 to 2 weeks of ripening and then a change into a new group of peptides in cheese ripened for 6 weeks and 12 weeks. Peptides eluted at 46-66 min change into a new and different group of peaks as ripening progressed.

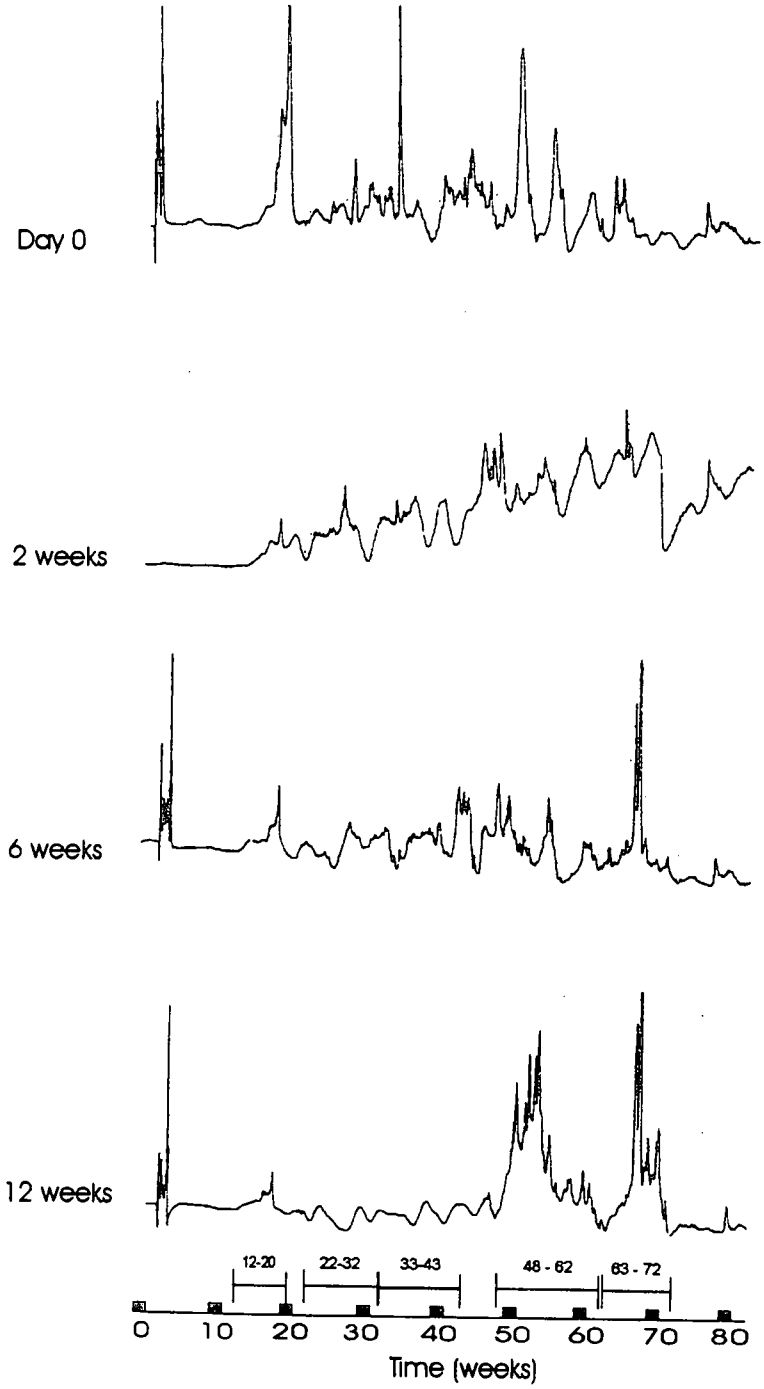

Fig. 5.1. The WS, EtOH-soluble retentate fraction of the unirradiated cheese ripened at 8 °C.

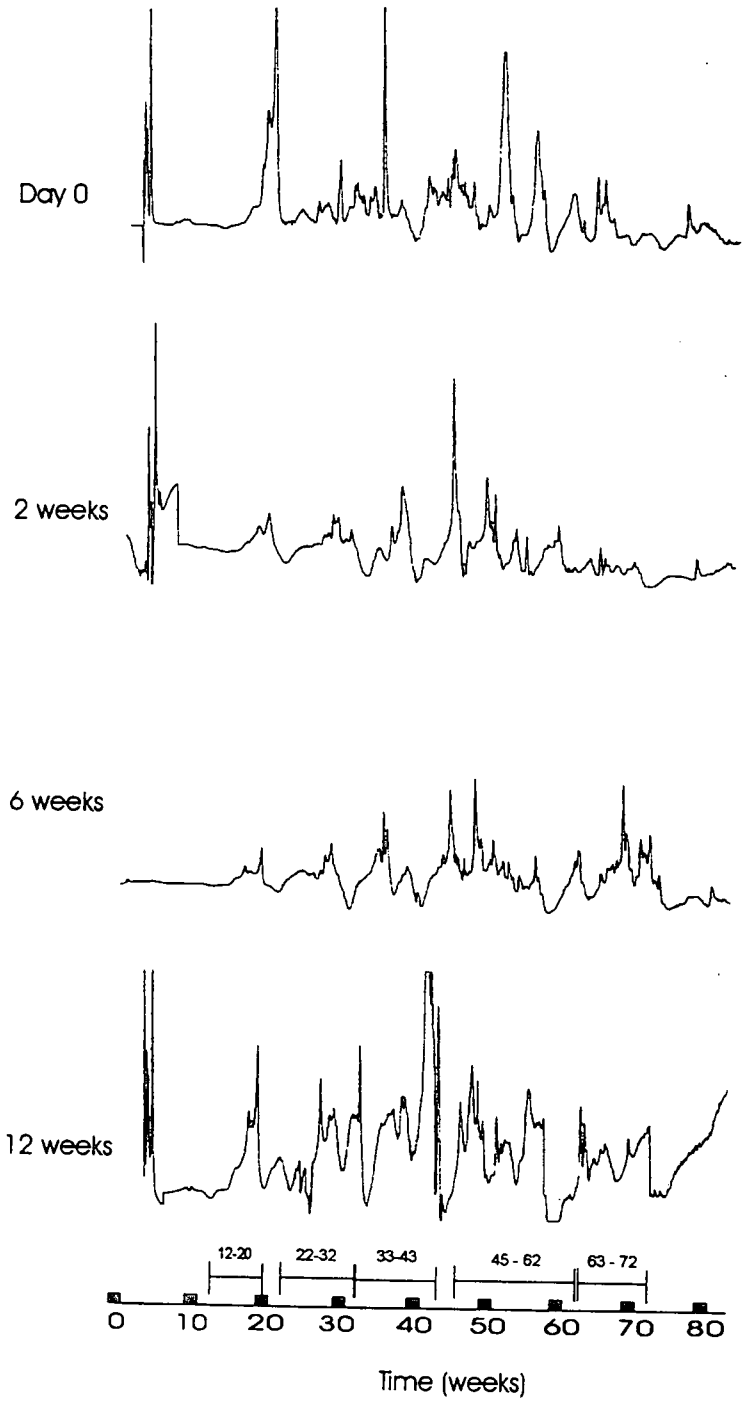

Fig. 5.2. The WS, EtOH-soluble retentate fraction of the unirradiated cheese ripened at 16 °C.

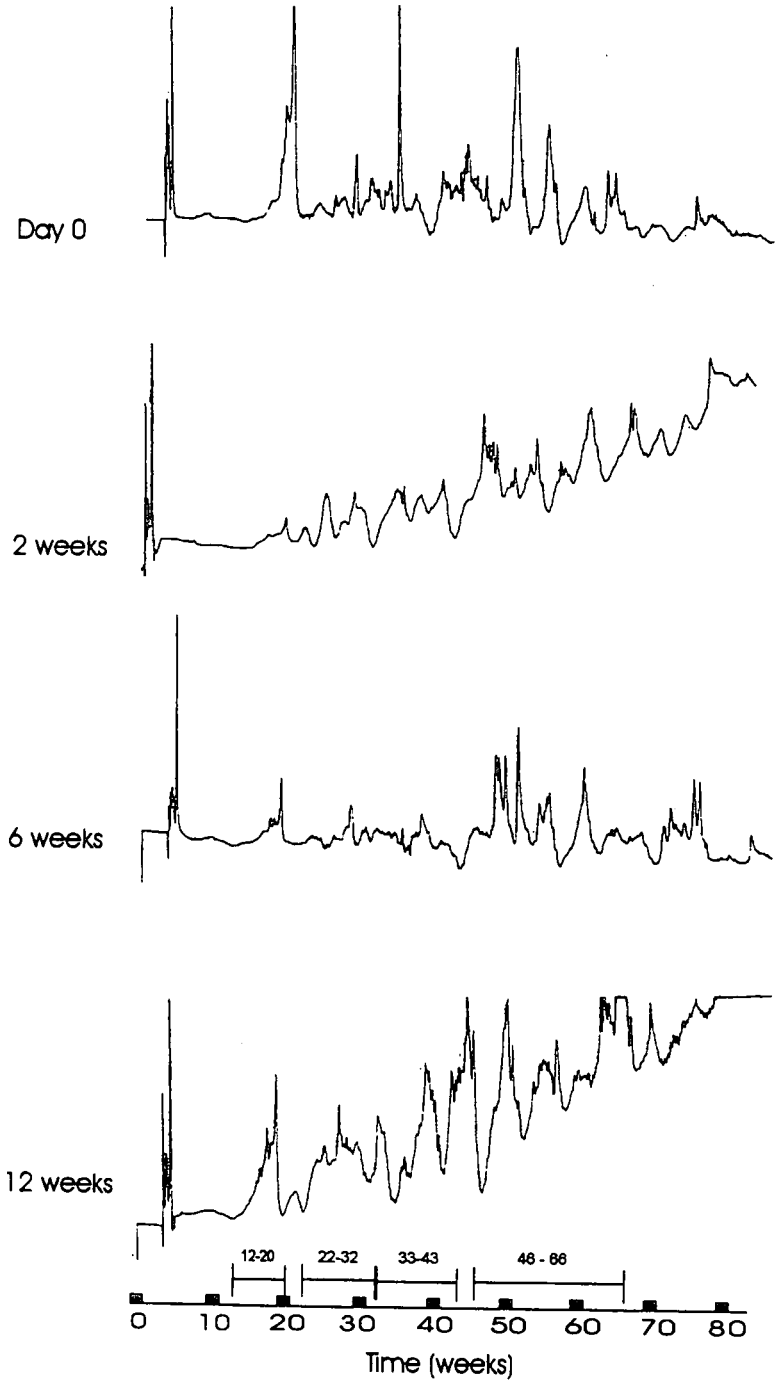

Fig. 5.3. The WS, EtOH-soluble retentate fraction of the irradiated cheese ripened at 8 °C.

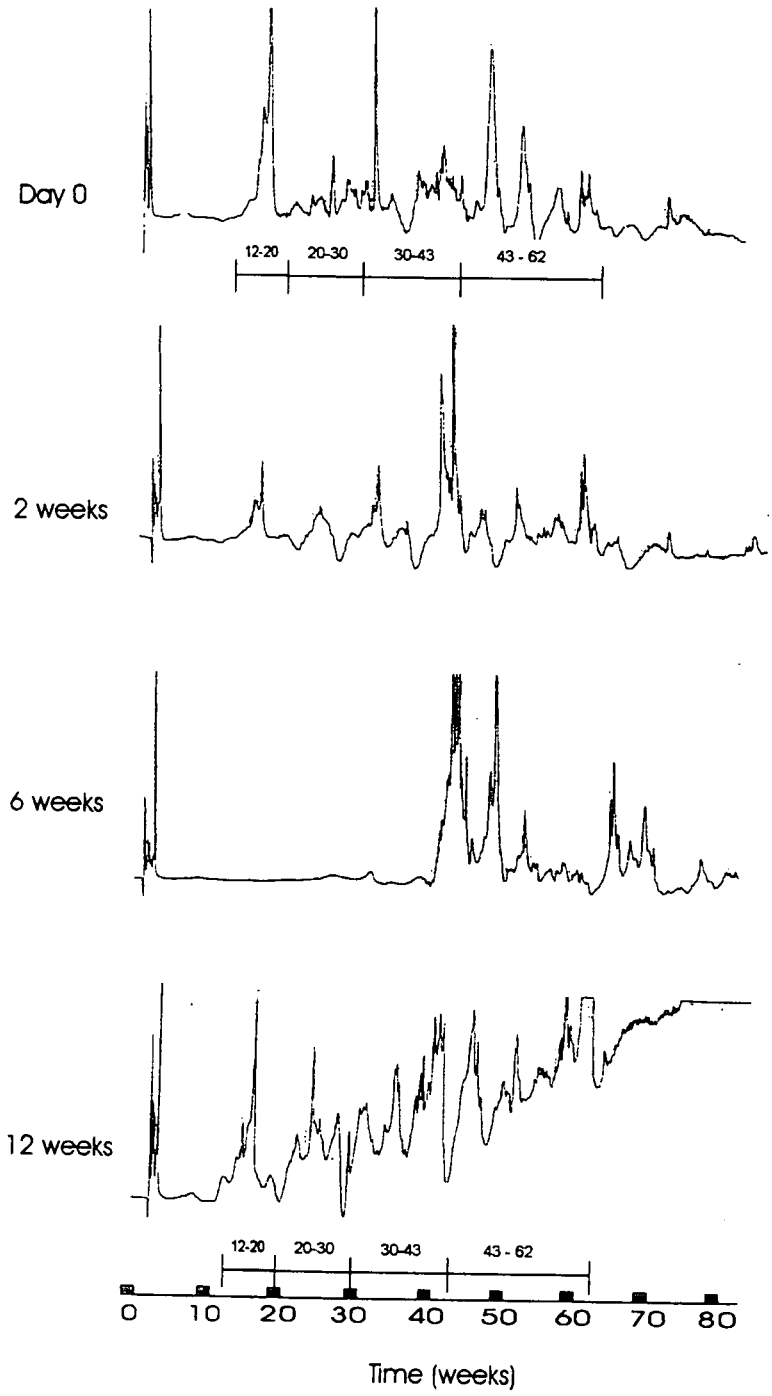

Fig. 5.4. The WS, EtOH-soluble retentate fraction of the irradiated cheese ripened at 16 °C.

Peptides (EtOH-soluble retentate) of the irradiated cheese ripened at 16 ^{o}C are shown in Figure. 5.4. Peptides eluted at 12-20 min were not changed much in cheese ripened for 12 weeks. There is a development of a peptide group eluted at 20-30 min from day 0 to cheese ripened for 12 weeks. There is also a development in peptides eluted at 30-43 min. and a change into new peptides in the group eluted at 43-62 min as cheese matured from day 0 to 12 weeks of ripening.

When comparing the EtOH-soluble retentate of the unirradited cheeses ripened at 8 ^{o}C and 16 ^{o}C, one finds that there is a reduction in peptides eluted at 12-20 min in both cheeses but the reduction at 12 weeks of ripening is more in the cheese ripened at 8 ^{o}C than in cheese ripened at 16 ^{o}C. Peptide peaks eluted at 22-32 min are higher in cheese ripened at 16 ^{o}C than in cheese ripened at 8 ^{o}C. The major difference is between groups eluted at 50-62 min in the cheese ripened at 8 ^{o}C and the peptides eluted at 45-62 min in the cheese ripened at 16 ^{o}C with a higher group of peaks at 52-60 min. The group of peptides eluted at 63-72 are higher in the cheese ripened at 8 ^{o}C than in the cheese ripened at 16 ^{o}C. The peptide profile of the cheese ripened at 16 ^{o}C is more complex at 12 weeks than the profile of cheese ripened at 8 ^{o}C. It seem as if the less complex peptide profile observed for the cheese at 6 weeks of ripening at 16 ^{o}C has some similarity with the profile of 12 weeks of ripening at 8 ^{o}C. This would indicate an accelerated ripening at 16 ^{o}C.

The unirradiated and irradiated cheeses ripened at 8 ^{o}C showed a reduction in peptides eluted at 12-20 min with more reduction in the unirradiated cheese than in the irradiated cheese. The reduction in peptides eluted at 22-32 min and 33-43 min is again higher in the unirradiated than in the irradiated cheese. The well developed peptide group that eluted at 45-62 and 63-72 minutes in the unirradiated cheese are not as clearly resolved as for the irradiated cheese. Again, the peptide profile of the irradiated cheese is more complex than that of the unirradiated cheese at 12 weeks of ripening.

Irradiated and unirradiated cheese ripened at 16 ^{o}C shows the development of the same peptides, especially those that eluted early (12-20 and 28-32 min) and their development and reduction is the same in the two cheeses. Peptides that eluted after 35 min are different in the two cheeses. Peptides that eluted at 30-43 and 43-62 minutes in the irradiated cheese ripened at 16 ^{o}C are almost the same as peptides that eluted at 33-46 and 46-66 minutes in the irradiated cheese ripened at 8 ^{o}C. The development and reduction of peptides that eluted at 15-20 minutes in the irradiated cheeses ripened at different temperatures (at 8 ^{o}C and at 16 ^{o}C) are almost the same. At 12 weeks of ripening, the peptide profile of the unirradiated cheese ripened at 8 ^{o}C is different from those of the unirradiated cheese ripened at 16 ^{o}C and the irradiated cheeses ripened at 8 ^{o}C and 16 ^{o}C, the latter three having more complex profiles. The elution of the EtOH-soluble retentate fractions of the cheese indicates that this cheese has hydrophillic and hydrophobic peptides eluting early and late respectively.

The peptide patterns of the WS, 5% PTA-insoluble fraction of the unirradiated cheese ripened at 8 ^{o}C are shown in Figure. 5.5. There is a large peak of a single peptide x and a low peak of a single peptide w at day 0. During ripening the amounts of peptide x are reduced whereas the amounts of peptide w are increased up to 12 weeks of ripening. Peptides eluted at 39-45, 46-50 and 52-65 min showed a strong development from day 0 to 12 weeks of ripening.

The chromatogram of the WS, 5% PTA-insoluble permeates fraction of the unirradiated cheese ripened at 16 ^{o}C is shown in Figure. 5.6. During ripening the amounts of peptide x are also reduced and those of peptide w increased with ripening. Peptides eluted at 32-38 min showed a strong development with time and the same pattern is seen with peptide eluted at 39-45. Peptides eluted at 48-56 min showed a quick development at 2 weeks of ripening and what looked like reduction at 6 weeks and 12 weeks of ripening. Two distinct peptides can be identified in this region, i.e. eluting at 48-52 and 53-56 min.

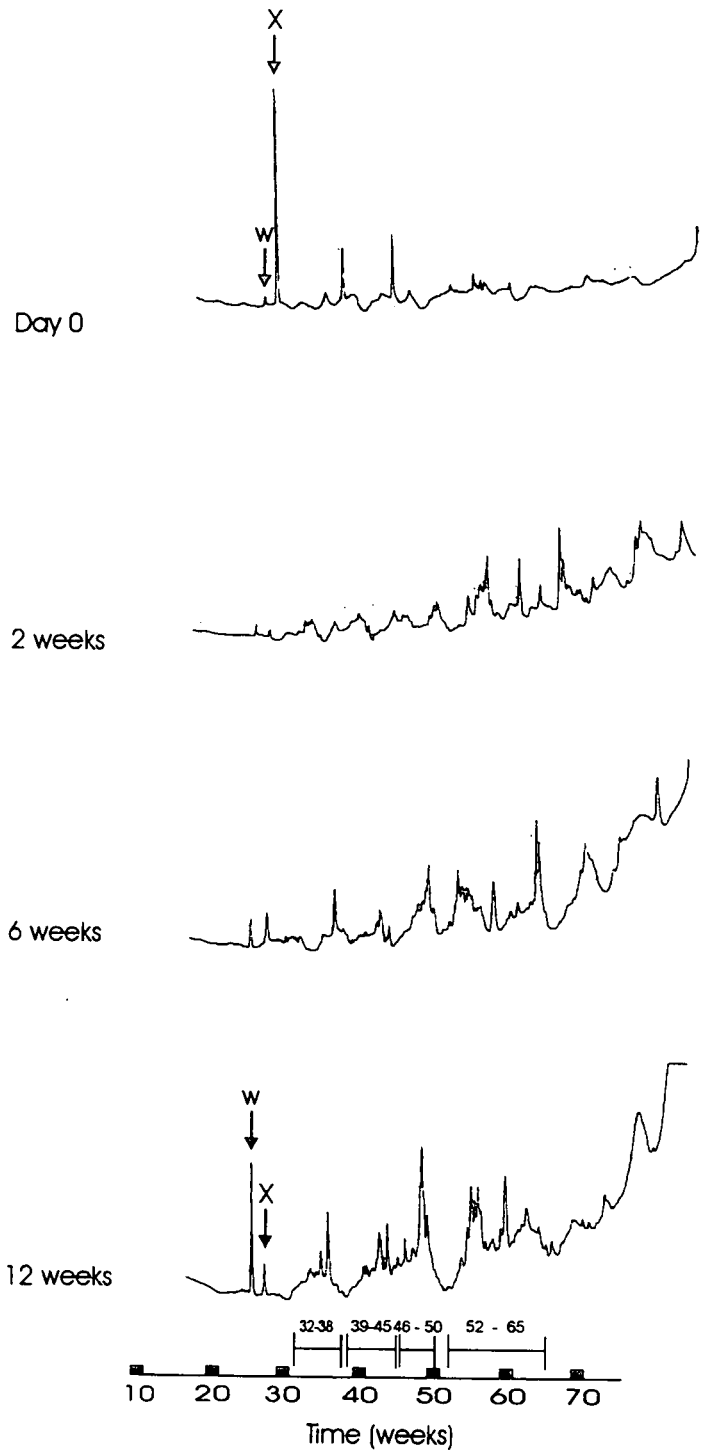

Fig. 5.5 RP-HPLC of the WS, 5% PTA-insoluble permeate fraction of the unirradiated cheese ripened at 8 °C.

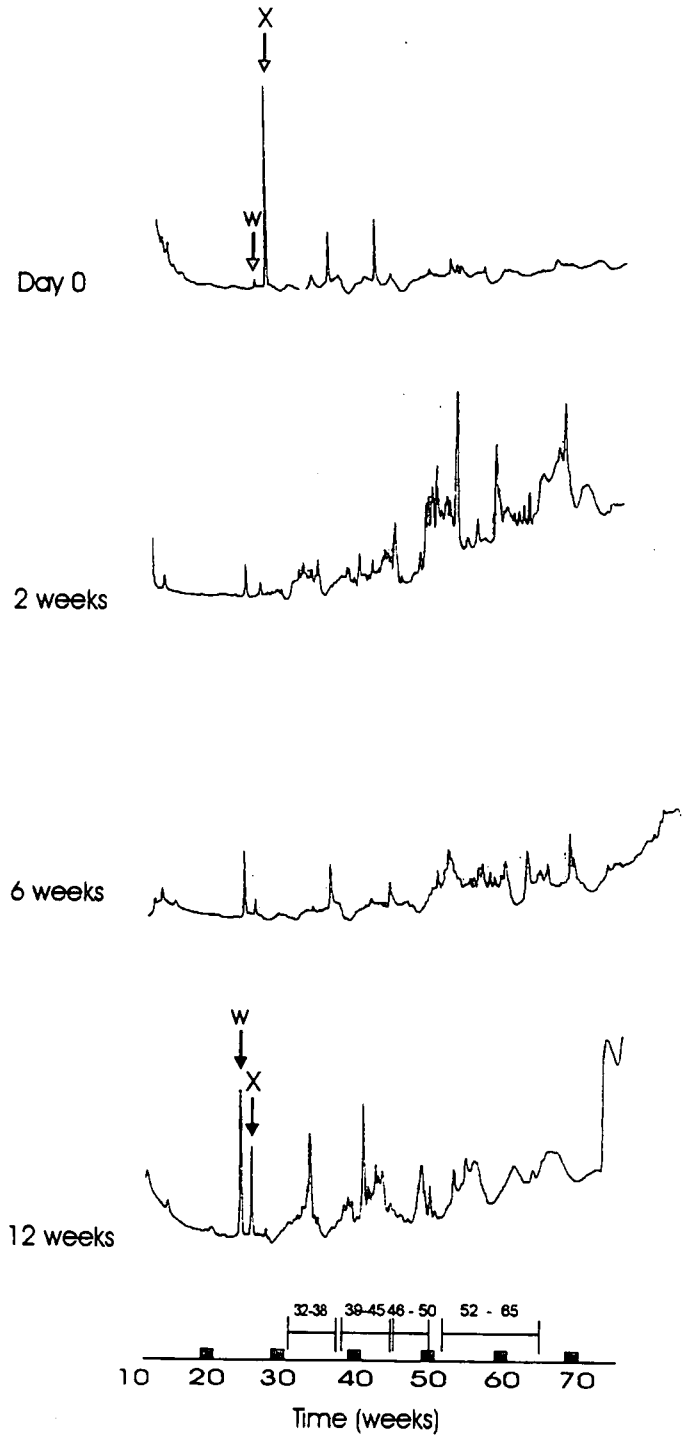

Fig. 5.6 RP-HPLC of the WS, 5% PTA-insoluble permeate fraction of the unirradiated cheese ripened at 16°C.

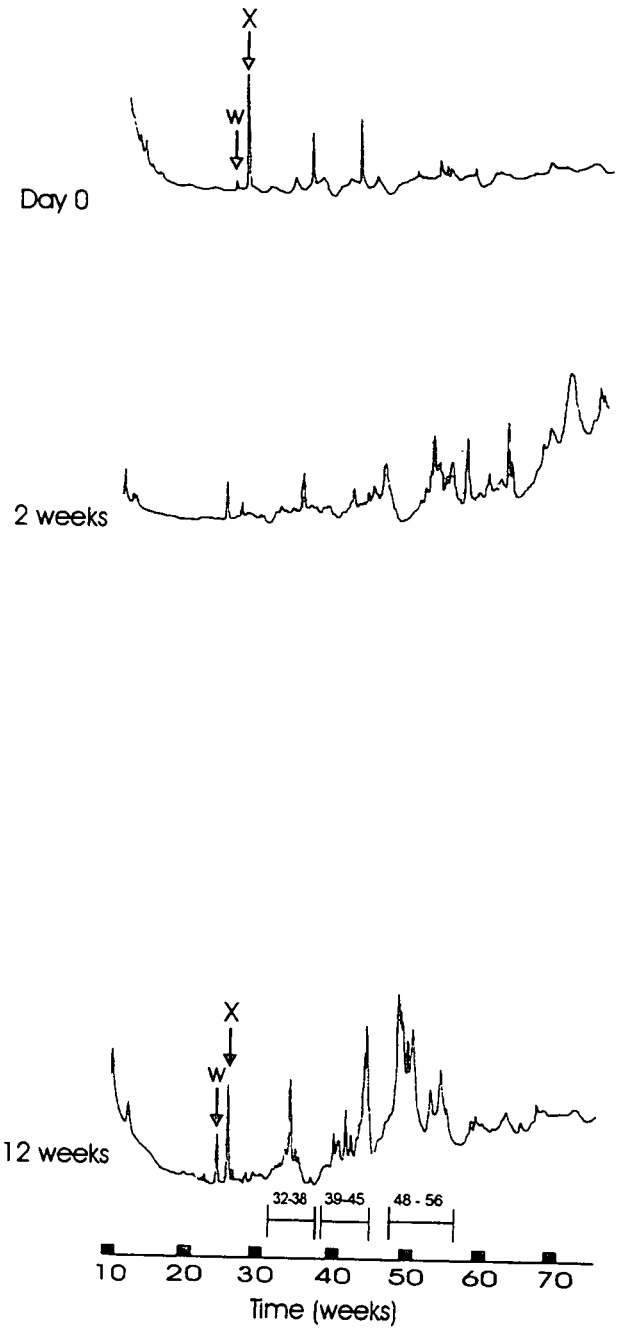

Fig. 5.7 RP-HPLC of the WS, 5% PTA-insoluble permeate fraction of the irradiated cheese ripened at 8°C.

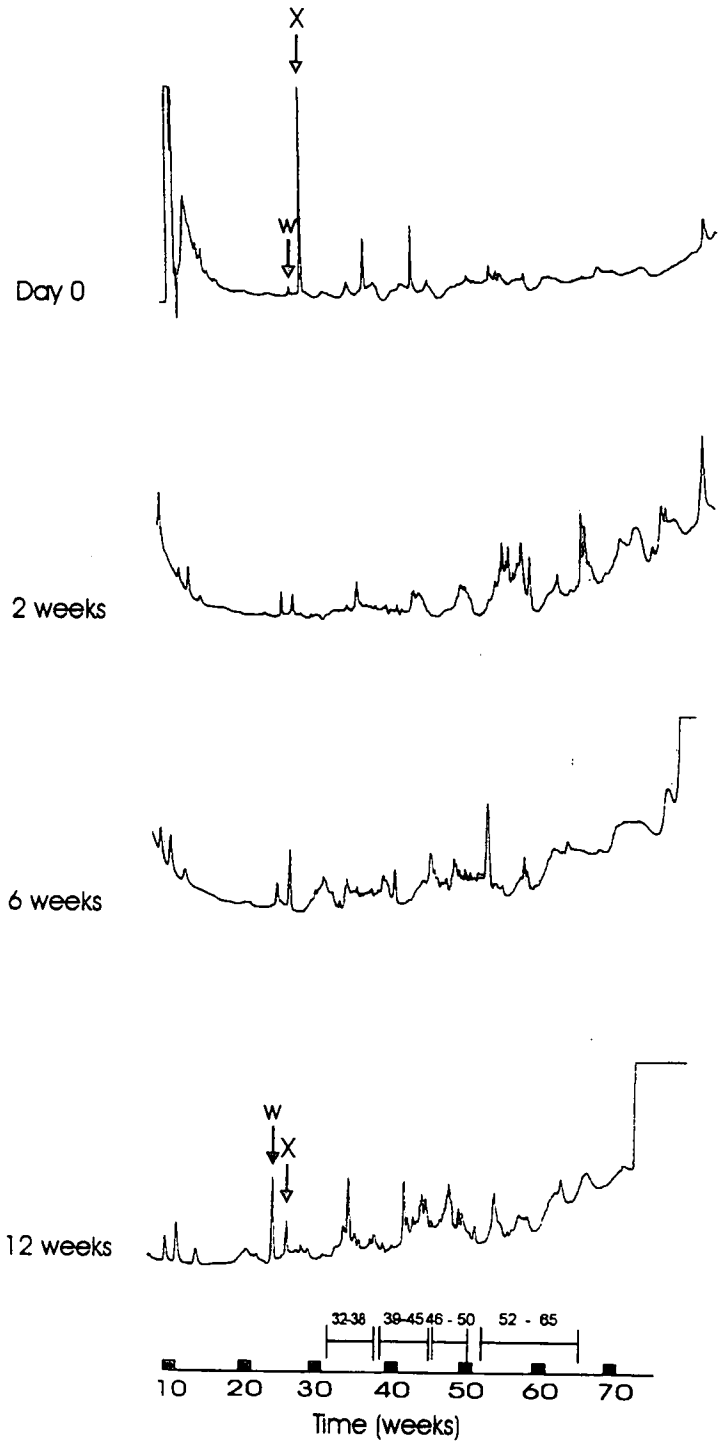

Fig. 5.8 RP-HPLC of the WS, 5% PTA-insoluble permeate fraction of the irradiated cheese ripened at 16 °C.

The patterns for irradiated cheese ripened at 8 ^{o}C are shown in Figure. 5.7. Although the chromatogram for 6 weeks is absent, one can see the development of peptide w from day 0 to 12 weeks of ripening whereas peptide x seems not to change, resulting in higher amounts than peptide w after 12 weeks of ripening. There is a development of peptides that eluted at 32-38 and at 39-45 min. Peptides eluted at 46-50 min also develop with ripening and peptides eluted at 52-56 min also showed some development with ripening.

The irradiated cheese ripened at 16 ^{o}C (Figure. 5.8) showed that the amounts of peptide x declined, while those of peptide w increased to reach higher amounts than peptide x after 12 weeks. Peptides eluted at 32-38 min showed some development with time from day 0 to 12 weeks and so are peptides eluted at 39-45 min. Peptides eluted at 52-56 min also showed some development with time.

The unirradiated cheeses ripened at 8 ^{o}C and 16 ^{o}C showed the same development of peptide w and reduction of peptide x, though peptide x occurs in higher amounts in cheese ripened at 16 ^{o}C for 12 weeks than in cheese ripened at 8 ^{o}C. They show the same development of peptides eluted at 32-38 min. The main difference is at the peptides eluted from 46-65 min, which are higher and better developed in cheese ripened at 8 ^{o}C at 12 weeks than in cheese ripened at 16 ^{o}C. In the cheese ripened at 16 ^{o}C, there is a quick development of peptides eluted at 48 –52 min which is not observed for the cheese ripened at 8 ^{o}C.

The unirradiated and irradiated cheeses ripened at 8 ^{o}C showed the same development of peptides eluted at 32-38 min. The difference is that peptide w occurs in larger amounts than peptide x in the unirradiated cheese whereas in the irradiated cheese the amounts of peptide x is larger than that of peptide w. The main difference is with the peptides eluted 46-65 min, which developed stronger in the irradiated cheese than in the unirradiated cheese. The

irradiated cheese has higher peptide peaks than the unirradiated cheese at 12 weeks of ripening. The difference between the unirradiated and the irradiated cheeses ripened at 16 ^{o}C is the high amounts of peptides w and x in the unirradiated cheese compared to the irradiated cheese. Peptides eluted at 32-38 min are more or less the same in the two cheeses. The main difference is the quick development of peptides eluted at 48-56 min in the unirradiated cheese and the slow development in the irradiated cheese.

The difference in RP-HPLC pattern of the WS, 5 %PTA-soluble permeate fractions are shown in Figure 5.9-5.12. Singh *et al.*, 1994 reported that most of the savoury, cheesy taste of the water-soluble extract of cheese is in the ultra-filtration permeate (10 kDa nominal molecular weight cut-off), which contains small peptides, free amino acids, salts and other low molecular weight compounds, whereas the retentate, which contains intermediate size peptides, is bland. Jarrett *et al.*, 1982 reported that the PTA-soluble N is a good index of cheese maturity. The PTA-soluble permeate fraction of the unirradiated cheese ripened at 8 ^{o}C (Figure 5.9) shows high amounts of peptide g and a low amount of peptide f. The difference between the two peptides is almost 10-fold. At the end of ripening (12 weeks) peptide g is still higher than peptide f but the difference is only, about 3-fold. The rest of the peptides do not show much differences or similarities to be noted.

The fraction of the unirradiated cheese ripened at 16 ^{o}C (Figure 5.10) started with high amounts of peptide g and low amounts of peptide f but ended up with the two peptides developed to the same amounts in cheese ripened for 12 weeks. The rest of the chromatogram also does not warrant discussion. The irradiated cheese ripened at 8 ^{o}C (Figure 5.11) has a high amount of peptide g and low amount of peptide f at day 0, and a higher peptide f peak than peptide g at 6 weeks and 12 weeks of ripening. The fraction of the irradiated cheese ripened at 16 ^{o}C showed a higher amount of peptide g than peptide f at day 0 and the two peptides developed to the same amounts at 12 weeks of ripening.

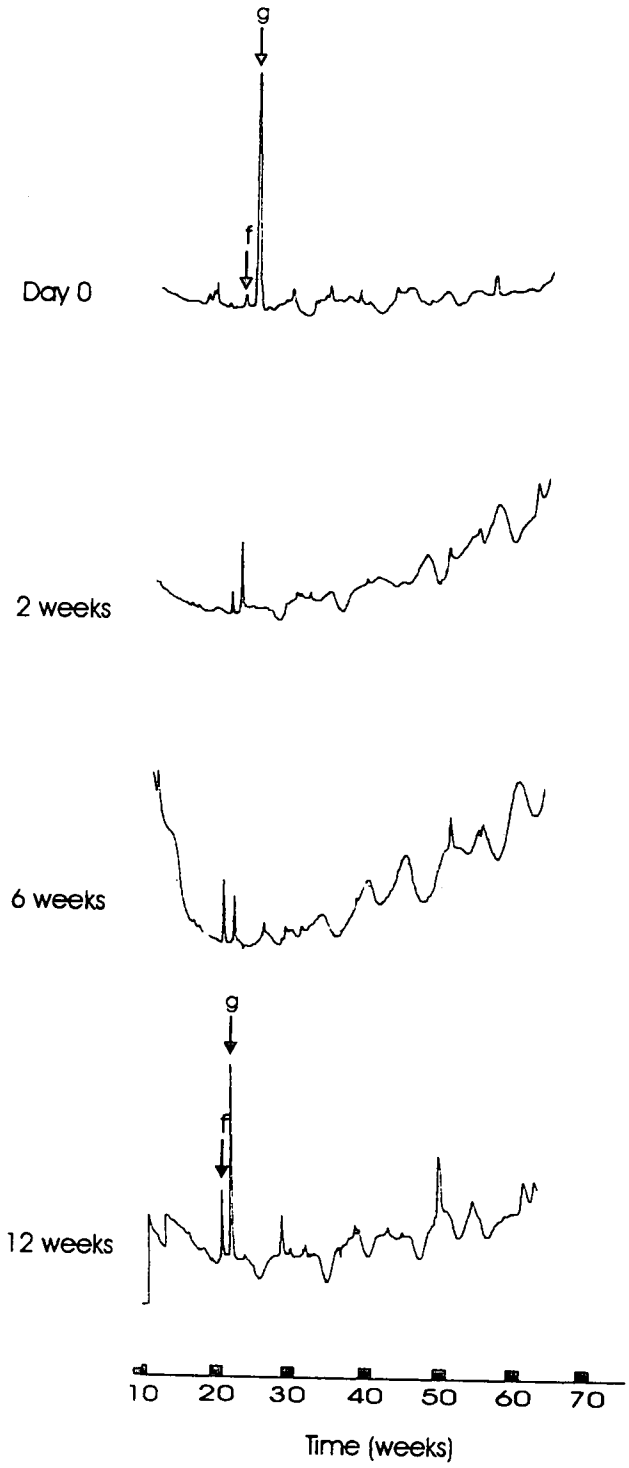

Fig. 5.9 RP-HPLC of the WS, 5% PTA-soluble permeate fraction of the unirradiated cheese ripened at 8 °C.

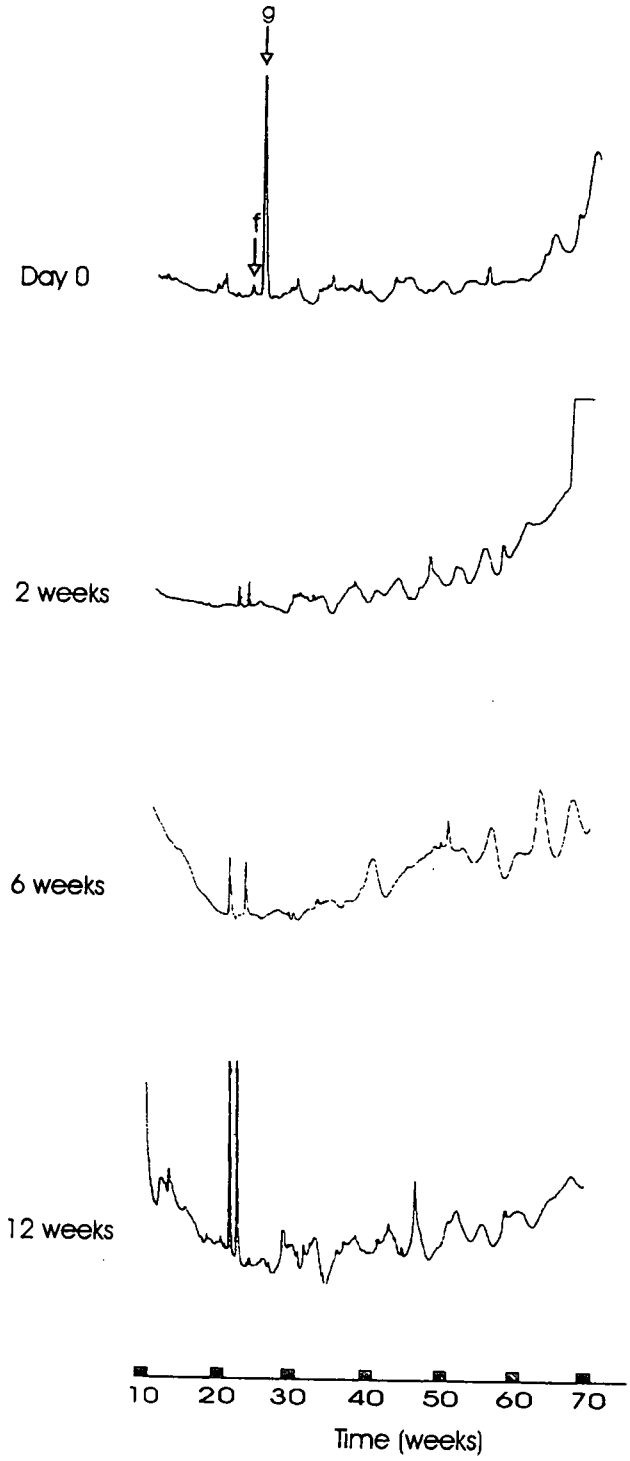

Fig. 5.10 RP-HPLC of the WS, 5% PTA-soluble permeate fraction of the unirradiated cheese ripened at 16 °C.

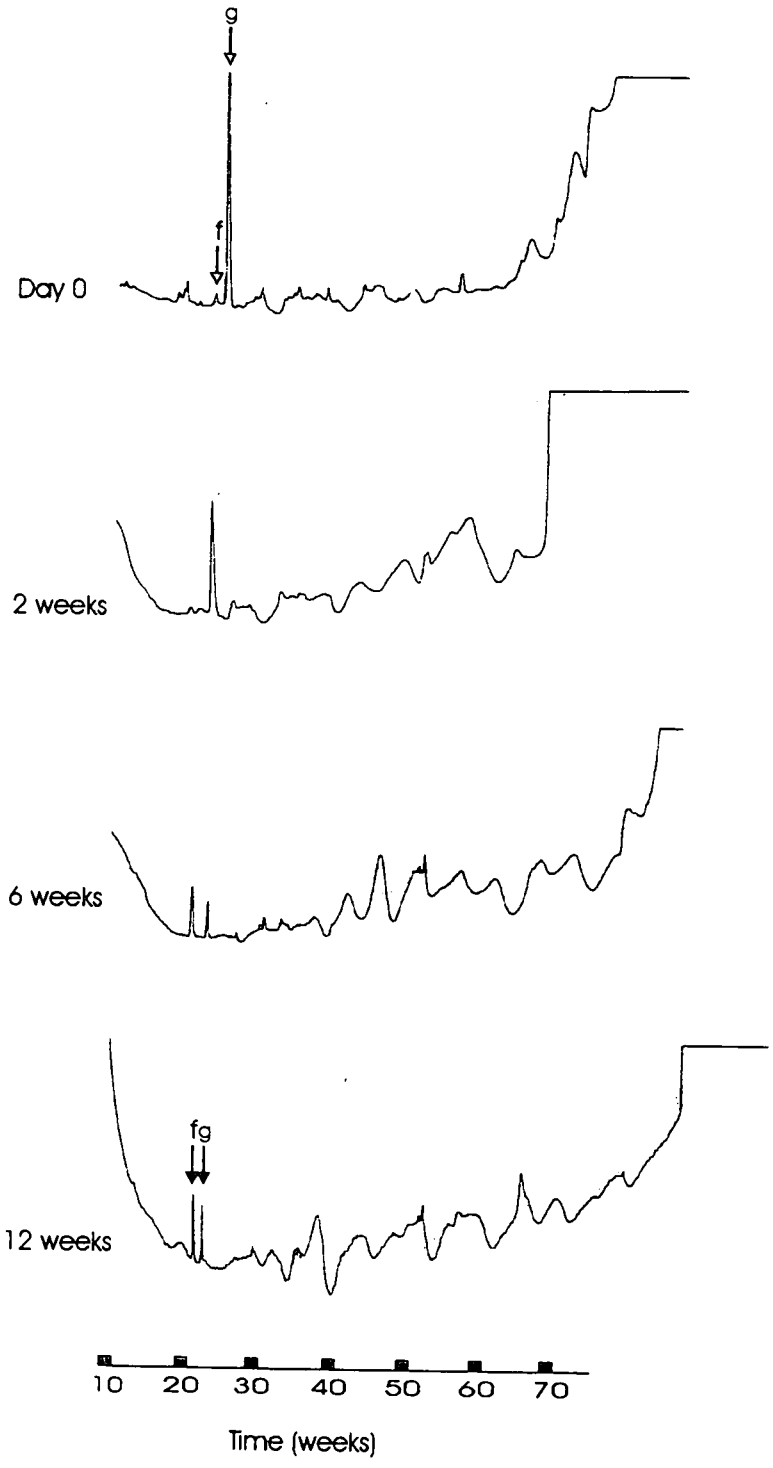

Fig. 5.11 RP-HPLC of the WS, 5% PTA-soluble permeate fraction of the irradiated cheese ripened at 8°C.

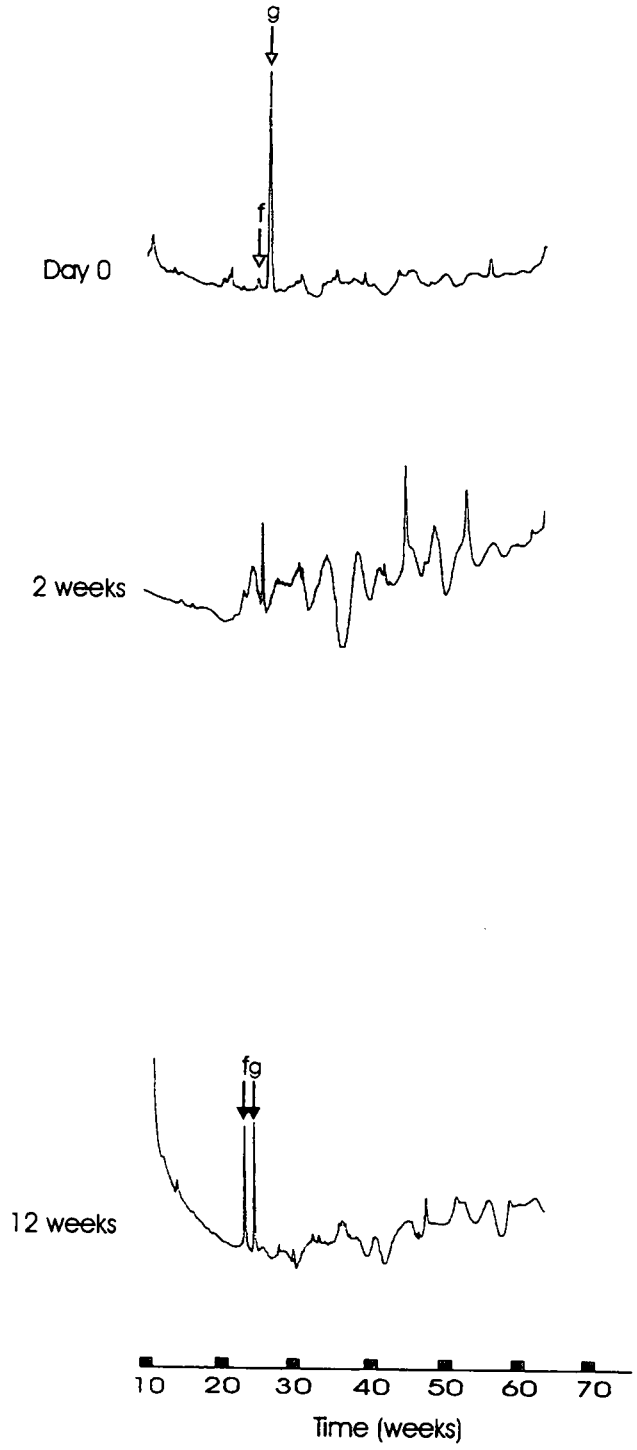

Fig. 5.12 RP-HPLC of the WS, 5% PTA-soluble permeate fraction of the irradiated cheese ripened at 16 °C.

Both unirradiated cheeses ripened at 8 ^{o}C and 16 ^{o}C have high amounts of peptide g and low amounts of peptide f at day 0. The major difference is at 12 weeks of ripening where cheese ripened at 8 ^{o}C showed higher amounts of peptide g and a low amounts of peptide f but the two peptides developing to amounts that do not differ in cheese ripened at 16 ^{o}C. The unirradiated and the irradiated cheese ripened at 8 ^{o}C also differ at 12 weeks of ripening. The two peptides have reached almost the same amounts at the end of ripening in the irradiated cheese but in the unirradiated cheese peptide f is in lower amount than peptide g. Peptide g and f reached the same amounts in the unirradiated and the irradiated cheeses ripened at 16 ^{o}C. The only difference is in the unirradiated cheese, where the peak height is almost twice that in the irradiated cheese.

Unirradiated and irradiated cheeses ripened at 16 ^{o}C have almost the same peptide profile of g and f as the irradiated cheese ripened at 8 ^{o}C. The peptide peaks changed from being of different amounts at day 0 to being of the same amount or almost the same amounts in the 3 cheeses ripened for 12 weeks. The unirradiated cheese ripened at 8 ^{o}C has a different peptide profile from the rest, peptide f developed with ripening but not to the same amounts as peptide g. At the end of ripening peptide f is still lower than peptide g.

5.4 Conclusion

Comparison of the RP-HPLC chromatograms of the WS-EtOH soluble retentate and 5% PTA-insoluble fractions of Cheddar cheeses of different treatments indicate that completely different peptide profiles were observed after 12 weeks of ripening. A more complex profile was observed for irradiated cheeses and the cheese ripened at 16 ^{o}C. This observation may also explain why the cheese ripened at 8 ^{o}C seemed to have been preferred (although not statistically significant) in the sensory analysis reported in Chapter 2.

Although not exactly similar, the HPLC peptide profile of the cheese ripened at 16 ^{o}C up to 6 weeks showed peptide peaks that could be matched with those observed at 12 weeks of ripening at 8 ^{o}C. This would confirm that ripening was accelerated at 16 ^{o}C. The peptide profile of the irradiated No conclusive explanation can be derived from the HPLC profiles of the 5 % PTA-soluble fractions.cheeses at any time were completely different from the unirradiated ones, so that no conclusion on accelerated ripening can be drawn. No conclusive explanation can be derived from the HPLC profiles of the 5 % PTA-soluble fractions.

Where the electrophoretograms of large proteins and peptides, reported in Chapter 4, could not explain much about peptide development or degradation by starter cultures and their enzymes or by the irradiation itself, the HPLC profiles do shed some light. After two weeks of ripening, the irradiated cheeses did not show excessive peptide formation. This would support the work of Diehl, (1990) that a dose of 4 kGy irradiation does not affect proteins much. Irradiation may affect the starter organisms in such a way that the caseins cannot be further degraded when they are killed. This is not observed, as a complex array of peptides was still developed after irradiation of cheeses during ripening to 12 weeks.

Thus, either enough enzymes were produced by the starter organisms, before being killed by irradiation to continue peptide degradation, or irradiation affected the peptides in such a way that peptides different from the unirradiated cheeses could be formed upon ripening to 12 weeks. These peptides could have been formed either as a result of breakage by the energy of irradiation, or a conformation change, caused by the irradiation, which may lead to a different degree of hydrolysis by enzymes.

CHAPTER 6
GENERAL CONCLUSION

In the dynamic world that changes regularly, people's lifestyle also changes readily. Cheese has become most people's delicacy, and, as the world population grows, so would the consumer demand for cheese. It is therefore advisable to develop new methods of cheese manufacturing that will be quicker and become beneficial to both the consumer and the producer because accelerating ripening will lead to lower production costs and lower selling price, this could also lead to the production of a different kind of cheese altogether to allow for consumers to have various choices.

Some cheese varieties, especially the acid-coagulated cheeses, are consumed fresh, but the majority of rennet-coagulated cheeses are ripened for periods ranging from 4 weeks to more than 2 years (Fox, 1989). During ripening, a multitude of chemical and biochemical changes occur in which the principle constitutes of the cheese, namely proteins, lipids and lactose are degraded to primary products. Among the principle compounds that have been isolated from several cheese varieties are peptides and amino acids, (from proteins), fatty acids and methyl ketones (from lipids) and organic acids, especially lactic acid and propionic acids (from lactose). In the right combinations these compounds are responsible for the characteristic flavours of various cheeses (Fox, 1989).

A high dose of irradiation has been used to kill microorganisms and prevent sprouting in vegetables, fruits, nuts and meats and it has been postulated by other researchers that irradiation can accelerate the ripening of cheese because it hinders the growth of microorganisms but does not have a negative effect on the enzymes (Food Irradiation, 1988).

The aim of the study was to determine the effect that irradiation and elevated temperature have on the ripening of Cheddar cheese, especially on fat,

proteins and flavour. This was done by means of sensorical, TBA-number, FFA, WSN, electrophoretical and chromatographical analyses. The analysed cheeses were the standard cheese (ripened at 8 ^{o}C), the irradiated cheese ripened at 8 ^{o}C, the unirradiated cheese ripened at 16 ^{o}C and the irradiated cheese ripened at 16 ^{o}C. A consumer taste panel appeared to prefer the standard cheese over the other 3 cheeses, but because of the overlapping preference and the standard being not significantly different from the others, it was concluded that the 4 cheeses were not different in taste. It was noted that the irradiated cheeses have a light yellow colour as compared to the intense yellow colour of the unirradiated cheeses and also that the freshly irradiated cheeses have a pungent taste. These were only noted but not tested for because the sensorical analyses were done to cheese ripened for 12 weeks and the light yellow colour might have been due to the annatto being damaged by irradiation. Being not different in taste did not, however, indicate that lipolysis and proteolysis took place in the same way in the cheeses, so a comparative research into the chemical and biochemical aspects of cheeses was done.

Free fatty acids (FFA) play a major role in flavour of many cheese varieties. They have been considered the backbone of Cheddar cheese flavour by Patton (1963) and generally have been acknowledged to contribute cheesiness in Cheddar flavour by Forss and Patton (1966) and Forss (1979). In addition to the desired flavours contributed by FFA at appropriate concentrations, high amounts of FFA, caused by excessive lipolysis produce undesirable rancid off-flavours. While non-rancid Cheddar cheeses have low, but still substantial concentrations of FFA, samples perceived as rancid should have distinctly larger amounts of FFA.

The FFA of the standard cheese was not significantly different from that of the other 3 cheeses at 12 weeks and other ripening times, although irradiated cheeses showed higher FFA-levels after 6 weeks of ripening. The TBA-value of all cheeses were less than 1 which is the critical value of rancidity. The

TBA-value of the standard cheese was lower and significantly different from that of the other cheeses throughout ripening, which might be an explanation for its apparent preference. A severe increase in TBA-value after irradiation was noted, and the TBA-value also decreased after ripening was continued.

In the internally ripened cheese such as Cheddar, there appears to be fairly good agreement that the water-insoluble fraction (consisting mainly of proteins and large peptides) is devoid of flavour and aroma, that the water-soluble non-volatile fraction (small peptides, amino acids and organic acids) contains most of the compounds responsible for flavour while the aroma is principally in the volatile fraction. There appears to be to be strong support for the view that products of proteolysis are the principal contributors to cheese flavour (McGugan *et al.*, 1979; Aston *et al.*, 1983).

Only cheese ripened at 8 ^{o}C followed a different path of the development of free N. The WISN and WSN-EtOH precipitate, analysed by Urea-PAGE, showed that ripening at 16 ^{o}C accelerate ripening of Cheddar cheese, as the peptide composition after 6 weeks of ripening was almost the same as that observed after 12 weeks of ripening when Cheddar cheese was ripened at 8 ^{o}C. Irradiation resulted in a different Urea-PAGE profile of proteins, showing a slightly slower peptide breakdown. This may suggest that irradiation caused a different conformation of peptides resulting in the slower hydrolysis of the β-CN and αs1-CN by pepsin and rennin.

Irradiated cheeses and cheese ripened at 16 ^{o}C showed more complex HPLC-profiles of WS-EtOH and 5% PTA-insoluble precipitate. This may also explain the apparent preference of cheese ripened at 8 ^{o}C in the sensory analysis (although not statistically significant). Cheese ripened at 16 ^{o}C for 6 weeks showed similar WS-EtOH soluble peptide profile as that of the cheese ripened for 12 weeks at 8 ^{o}C. The peptide development, as shown by HPLC, show that break down of proteins by irradiation at the low dose of 4kGy is not excluded, and that enzymes, rennet as well as enzymes from starter

organisms, still function after irradiation. However, a change in conformation of the substrate proteins and peptides by irradiation is suggested, because different peptides, and different amounts of specific peptides formed were noted, perhaps due to an altered enzyme-substrate interaction.

SUMMARY

Proteolysis and lipolysis are important biochemical events in the ripening of cheese to contribute to the development of flavour and texture. The characteristic proteolysis of each type of cheese is brought about by the enzymes used in the manufacturing process, e. g. rennet, as well as enzymes from the specific microbial cultures used in each cheese type. The microbial and chemical development can be manipulated by external conditions such as ripening temperature and irradiation. In the current study the effect of temperature together with irradiation on the ripening of Cheddar cheese was investigated. Cheddar cheeses were irradiated after 4 days of ripening at 8 ^{o}C and 16 ^{o}C at a dose of 4kGy and ripening continued up to 12 weeks with unirradiated cheeses ripened at 8 ^{o}C and 16 ^{o}C as controls. In a sensorical comparative study, the cheeses were evaluated as not being different in taste. There were no significant differences in the free fatty acid content of the cheeses but the irradiated cheeses and cheese ripened at 16 ^{o}C had higher TBA-values. The WSN/Total N (water-soluble nitrogen /total nitrogen) of the cheese ripened at 8 ^{o}C was significantly different than that of the other cheeses after 2 weeks, but then reached the same levels as the treated cheeses after 6-12 weeks. The Urea-polyacrylamide gel electrophoresis of the WISF indicated that the cheese ripened at high temperature matures faster than the cheese ripened at low temperature for both the unirradiated and the irradiated cheeses, whereas the irradiated cheeses showed different peptide development over the same period of ripening. The RP-HPLC of the cheeses indicated a difference in peptide profiles of the unirradiated and irradiated cheese ripened at 16 ^{o}C, and the irradiated cheese ripened at 8 ^{o}C from the unirradiated cheese ripened at 8 ^{o}C.

Keywords: Lypolysis, proteolysis, enzymes, sensorical, irradiation, RP-HPLC, TBA-value, free fatty acids, taste, Urea-PAGE

OPSOMMING

Proteolise en lipolise is belangrike biochemiese prosesse in die rypwording van kaas om by te dra tot die ontwikkeling van geur en tekstuur. Die karakteristieke proteolise van elke tipe kaas word te weeg gebring deur die ensieme wat in die verwaardigingsproses gebruik word, bv. rennien, sowel as die ensieme van die spesifieke mikrobiese kulture wat in elke kaassoort gebruik word. Die mikrobiologiese en chemiese ontwikkeling kan beheer word deur eksterne kondisies soos rypingstemperatuur en bestraling. In die onderhawige studie is die effek van temperatuur tesame met bestraling op die ryping van Cheddar kaas ondersoek. Cheddar kase is bestraal 4 dae na ryping by 8 ^{o}C en 16 ^{o}C met `n dosis van 4kGy en ryping is voortgesit met onbestraalde kase, wat by 8 $^{o}$C en 16 $^{o}$C rypgemaak is, as kontroles. In `n sensoriese vergelykende studie is kase as nie verskillend in smaak evalueer. Daar was geen betekenisvolle verskille in die vrye vetsuur inhoud van die kase nie, maar die bestraalde kase en kaas wat by 16 ^{o}C rypgemaak is, het hoër TBA-waardes vertoon. Die water-oplosbare stikstof /totale stikstof van die kaas wat by 8 ^{o}C rypgemaak is, het betekenisvol verskil van diè van die ander kase na 2 weke, maar het dieselfde vlakke bereik as die behandelde kase na 6 tot 12 weke. Die Ureum-poliakrielamied gel elektroforese van die water-onoplosbare fraksie het getoon dat die kaas wat by hoë temperatuur rypgemaak is, vinniger ryp geword het as diè kaas by lae temperatuur in beide die bestraalde en onbestraalde kase, terwyl bestraalde kase ander peptiedontwikkeling oor dieselde rypmakingstyd vertoon het. Die Tru-fase hoe drukvloeistof chromatografie van die kase het `n verskil in peptied profiele aangedui vir die onbestraalde en bestraalde kase wat by 16 ^{o}C rypgemaak is, en die bestraalde kaas wat by 8 ^{o}C rypgemaak het verskil van die onbestraalde kaas wat by 8 ^{o}C rypgemaak is.

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