Characterisation of Protein Degradation in Cheddar Cheese

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Thesis submitted to the University of Glasgow in accordance with the requirements for the Degree of Doctor of Philosophy in the Faculty of Science

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March 1998

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This thesis is dedicated to Ysobel Gourlay, whose patience and love helped me through difficult times more than she will ever know.

ACKNOWLEDGEMENTS

I would firstly like to thank the Scottish Office Agriculture Environment and Fisheries Department for funding this research project at the Hannah Research Institute, Ayr. I would also like to express my gratitude and sincere thanks to Professor Malcolm Peaker, Director of the Institute, for providing the excellent facilities with which to pursue my studies.

I am greatly indebted to my project supervisors, Dr. Jeffrey Leaver and Dr. Jean M. Banks, for all their expert guidance, encouragement and interest throughout the course of my research and during the writing of the thesis. Also, many thanks to Jeff for the car lifts, when the inclement Scottish weather prevented me from bicycling to and from work!

Many thanks to Dr. D. J. C. Pappin and Dr. M. Bartlet-Jones of the Protein Sequencing Laboratory, Imperial Cancer Research Fund, London, for generously providing the TFEITC protein sequencing reagent and coupling buffer, and for technical advice.

Many thanks also to Dr. P. L. H. McSweeney (Department of Food Chemistry) and Á. Healy (National Food Biotechnology Centre), University College, Cork, Ireland, for kindly agreeing to perform Edman sequencing analysis, and for interesting discussions on cheese proteolysis and peptide sequencing.

I would like to express my gratitude to Micromass UK Ltd. for kindly allowing me the use of computer facilities to complete my write-up. Without their generous assistance this thesis would certainly not have been completed as quickly.

I will always possess the fondest memories of my time at the Hannah, and a very big thank you must go all my friends and colleagues for making my three years there a thoroughly enjoyable, rewarding and memorable experience.

ABSTRACT

Considerable research attention over the last few years has highlighted the important role of protein degradation in cheese, and its pivotal role in flavour and texture development. While possible degradation pathways of the respective protein molecules involved have been determined, many such studies have been performed using model systems, which attempt to mimic the conditions present within a maturing cheese curd. The main aims of this study were however directed at identifying how such protein degradation pathways varied in Cheddar cheese manufactured under different processing variables.

The concentration of free amino acids, released through the combined action of rennet and microbial proteinase and peptidase enzymes, was found to show a marked heat treatment and pH dependent effect.

Qualitative analysis of the reversed phase chromatographic profiles of water-soluble peptides from Cheddar cheese manufactured from standard pH and reduced pH milk revealed peptide differences. Further, statistical analysis of the data suggested that the observed variations in the individual amino acid concentrations from the Cheddar cheese were closely associated with differences in the peptide precursor supply. Therefore, controlling the extent and type of peptides released may help eliminate flavour defects, such as bitterness, and lead to the identification of key peptides necessary to produce Cheddar cheese of good quality.

Characterisation of Cheddar cheese peptides was performed on a matrix-assisted laser desorption ionisation time-of-flight mass spectrometer, using a volatile degradation reagent, trifluoroethylisothiocyanate. Of the peptides identified, the majority came from α_{s1}- and β-caseins, and mainly from their N and C-termini respectively. This result, whilst already well documented, reinforces the important requirement of identifying key peptides, if the final acceptability of a young cheese is to be predicted with certainty.

Quantitative analysis of the caseins and their degradation products was achieved by the application of capillary electrophoresis. The approach highlighted significant differences in the hydrolysis of the α_{s1}-casein and α_{s1}-I-polypeptide f(24–199) between Cheddar cheese manufactured from standard and reduced pH milk.

CONTENTS

Chapter 2: Effect of manufacturing conditions on the concentrations of free amino acids in Cheddar cheese

Chapter 3: Reversed phase-high performance liquid chromatography analysis of the effect of manufacturing conditions on the levels of water-soluble peptides in Cheddar cheese

Chapter 4: Characterisation of water-soluble peptides from Cheddar cheese by mass spectrometry

ABBREVIATIONS

ACH	Alpha-cyano-4-hydroxycinnamic acid
CU	Clotting units
CZE	Capillary zone electrophoresis
DEAE	Diethylaminoethyl
DVI	Direct vat inoculation
EDTA	Diaminoethanetetraacetic acid
FPLC	Fast protein liquid chromatography
HFBA	Heptafluorobutyric acid
HPLC	High performance liquid chromatography
HTST	High temperature short time
MALDI-MS	Matrix-assisted laser desorption/ionisation-mass spectrometry
$[M+H]^+$	Protonated molecule
PITC	Phenylisothiocyanate
PTC	Phenylthiocarbamyl
RP-HPLC	Reversed phase-high performance liquid chromatography
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TFA	Trifluoroacetic acid
TFEITC	Trifluoroethylisothiocyanate
Tris	Tris (hydroxymethyl) aminomethane
UV	Ultraviolet

PUBLICATIONS

Gouldsworthy, A. M., Leaver, J. and Banks, J. M. 1996. Application of a mass spectrometry sequencing technique for identifying peptides present in Cheddar cheese. *Int. Dairy Journal.* **6**, 781–790.

Diaz, O., Gouldsworthy, A. M. and Leaver, J. 1996. Identification of peptides released from casein micelles by limited trypsinolysis. *Journal of Agricultural and Food Chemistry.* **44**, 2517–2522.

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IN PREPARATION

Gouldsworthy, A. M., Banks, J. M. and Leaver, J. Casein degradation in Cheddar cheese: A capillary electrophoresis study.

Chapter 1

General Introduction

1.1 Composition of milk

Milk is a complex fluid, the primary purpose of which is to sustain growth and development in the young by supplying their nutritional requirements. Differences occur in the quantitative composition of milk between species, with additional compositional variations existing within a given species, depending upon the age of the animal, its stage of lactation and the genetic variation.

Milk proteins from all species examined to date can be classified into two types, the caseins and the serum or whey proteins. The caseins exist as protein aggregates, known as micelles, the structure of which has been the subject of recent reviews (Holt, 1992; Rollema, 1992). Separation of casein from whey protein can be achieved by centrifugation, producing a micellar pellet, or the acidification to pH4.6 of raw skimmed milk held at 20^0C, a process which causes the caseins to precipitate.

1.1.1 Caseins

The caseins are a group of phosphate-containing proteins, which account for between 76–86% of the total milk protein. In bovine milk, they are expressed as four gene products, known as α_{s1}-casein, α_{s2}-casein, β-casein and κ-casein. The primary structures of most of the genetic variants of the four caseins have been confirmed by DNA sequence analysis; however, further heterogeneity occurs due to the extent of their post-translational modifications.

Phosphorylation of all the caseins occurs at varying levels at serine, and occasionally threonine, residues. The genetic variants of α_{s1}-casein, α_{s2}-casein, β-casein and κ-casein usually possess several phosphoseryl residues (Table 1.1), while the additional glycosylation of the κ-casein variants results in extensive heterogeneity within this family of proteins. Swaisgood (1992) reviewed the chemistry of the caseins, and detailed the primary structures of the α_{s1}-, α_{s2}-, β- and κ-casein known genetic variants (Figures 1.1–1.4).

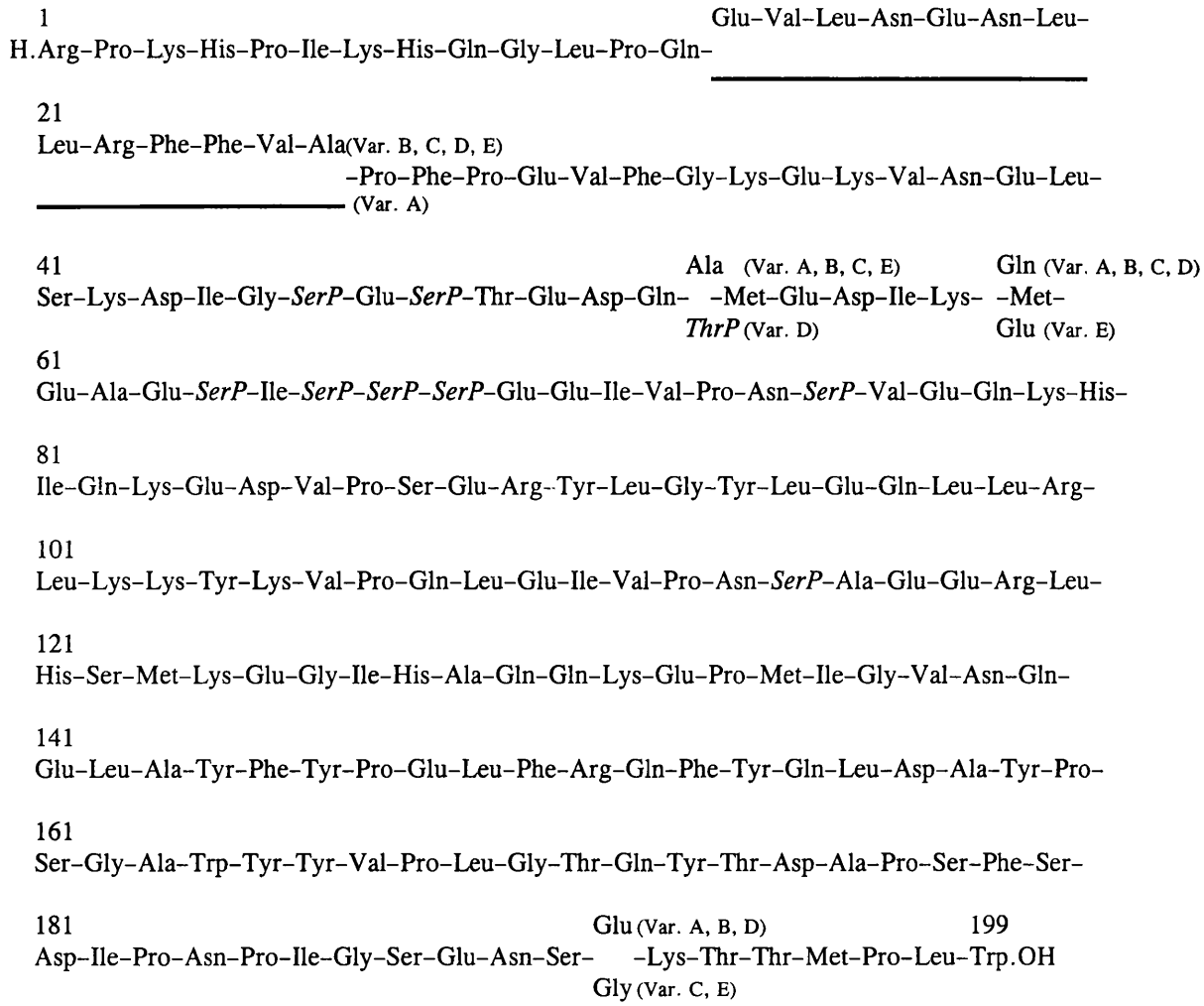
TABLE 1.1: Casein nomenclature[a] and phosphorylation.

α_{s1}-Casein	α_{s2}-Casein	β-Casein	κ-Casein
α_{s1}-CN A-8P	α_{s2}-CN A-10P	β-CN A^1-5P	κ-CN A-1P
α_{s1}-CN B-8P	α_{s2}-CN A-11P	β-CN A^2-5P	κ-CN B-1P
α_{s1}-CN B-9P	α_{s2}-CN A-12P	β-CN A^3-5P	
α_{s1}-CN C-8P	α_{s2}-CN A-13P	β-CN B-5P	
α_{s1}-CN D-9P		β-CN C-4P	
α_{s1}-CN E-8P		β-CN D-4P	
		β-CN E-5P	

[a] As recommended by the American Dairy Science Association (Eigel *et al.*, 1984).

Table adapted from Swaisgood (1992).

FIGURE 1.1: Primary structures of the known genetic variants of α_{s1}-casein.



Sites of post-translational phosphorylation are in italics.

Data from Swaisgood (1992).

FIGURE 1.2: Primary structure of α_{s2}-casein genetic variant A-11P.

1
H.Lys-Asn-Thr-Met-Glu-His-Val-*SerP*-*SerP*-*SerP*-Glu-Glu-Ser-Ile-Ile-*SerP*-Gln-Glu-Thr-Tyr-

21
Lys-Gln-Glu-Lys-Asn-Met-Ala-Ile-Asn-Pro-Ser-Lys-Glu-Asn-Leu-Cys-Ser-Thr-Phe-Cys-

41
Lys-Glu-Val-Val-Arg-Asn-Ala-Asn-Glu-Glu-Glu-Tyr-Ser-Ile-Gly-*SerP*-*SerP*-*SerP*-Glu-Glu-

61
SerP-Ala-Glu-Val-Ala-Thr-Glu-Glu-Val-Lys-Ile-Thr-Val-Asp-Asp-Lys-His-Tyr-Gln-Lys-

81
Ala-Leu-Asn-Glu-Ile-Asn-Gln-Phe-Tyr-Gln-Lys-Phe-Pro-Gln-Tyr-Leu-Gln-Tyr-Leu-Tyr-

101
Gln-Gly-Pro-Ile-Val-Leu-Asn-Pro-Trp-Asp-Gln-Val-Lys-Arg-Asn-Ala-Val-Pro-Ile-Thr-

121
Pro-Thr-Leu-Asn-Arg-Glu-Gln-Leu-*SerP*-Thr-*SerP*-Glu-Glu-Asn-Ser-Lys-Lys-Thr-Val-Asp-

141
Met-Glu-*SerP*-Thr-Glu-Val-Phe-Thr-Lys-Lys-Thr-Lys-Leu-Thr-Glu-Glu-Glu-Lys-Asn-Arg-

161
Leu-Asn-Phe-Leu-Lys-Lys-Ile-Ser-Gln-Arg-Tyr-Gln-Lys-Phe-Ala-Leu-Pro-Gln-Tyr-Leu-

181
Lys-Thr-Val-Tyr-Gln-His-Gln-Lys-Ala-Met-Lys-Pro-Trp-Ile-Gln-Pro-Lys-Thr-Lys-Val-

201 207
Ile-Pro-Tyr-Val-Arg-Tyr-Leu.OH

Sites of post-translational phosphorylation are in italics.

Data from Swaisgood (1992).

FIGURE 1.3: Primary structures of β-casein genetic variants A^1, A^2, A^3, B and C.

1
H.Arg–Glu–Leu–Glu–Glu–Leu–Asn–Val–Pro–Gly–Glu–Ile–Val–Glu–*SerP*–Leu–*SerP*–*SerP*–*SerP*–Glu–

(Var. C)
21 Ser Lys
Glu–Ser–Ile–Thr–Arg–Ile–Asn–Lys–Lys–Ile–Glu–Lys–Phe–Gln– –Glu– –Gln–Gln–Gln–
SerP Glu
(Var. A, B)

41
Thr–Glu–Asp–Glu–Leu–Gln–Asp–Lys–Ile–His–Pro–Phe–Ala–Gln–Thr–Gln–Ser–Leu–Val–Tyr–

61 Pro (Var. A^2, A^3)
Pro–Phe–Pro–Gly–Pro–Ile– –Asn–Ser–Leu–Pro–Gln–Asn–Ile–Pro–Pro–Leu–Thr–Gln–Thr–
His (Var. C, A^1, B)

81
Pro–Val–Val–Val–Pro–Pro–Phe–Leu–Gln–Pro–Glu–Val–Met–Gly–Val–Ser–Lys–Val–Lys–Glu–

101 His (Var. A^1, A^2, B, C)
Ala–Met–Ala–Pro–Lys– –Lys–Glu–Met–Pro–Phe–Pro–Lys–Tyr–Pro–Val–Glu–Pro–Phe–Thr–
Gln (Var. A^3)

121 Ser (Var. A, C)
Glu––Gln–Ser–Leu–Thr–Leu–Thr–Asp–Val–Glu–Asn–Leu–His–Leu–Pro–Leu–Pro–Leu–Leu–
Arg (Var. B)

141
Gln–Ser–Trp–Met–His–Gln–Pro–His–Gln–Pro–Leu–Pro–Pro–Thr–Val–Met–Phe–Pro–Pro–Gln–

161
Ser–Val–Leu–Ser–Leu–Ser–Gln–Ser–Lys–Val–Leu–Pro–Val–Pro–Gln–Lys–Ala–Val–Pro–Tyr–

181
Pro–Gln–Arg–Asp–Met–Pro–Ile–Gln–Ala–Phe–Leu–Leu–Tyr–Gln–Glu–Pro–Val–Leu–Gly–Pro–

201 209
Val–Arg–Gly–Pro–Phe–Pro–Ile–Ile–Val.OH

Sites of post-translational phosphorylation are in italics.

Data from Swaisgood (1992).

FIGURE 1.4: Primary structures of the known genetic variants of κ-casein.

1
PyroGlu-Glu-Gln-Asn-Gln-Glu-Gln-Pro-Ile-Arg-Cys-Glu-Lys-Asp-Glu-Arg-Phe-Phe-Ser-Asp-

21
Lys-Ile-Ala-Lys-Tyr-Ile-Pro-Ile-Gln-Tyr-Val-Leu-Ser-Arg-Tyr-Pro-Ser-Tyr-Gly-Leu-

41
Asn-Tyr-Tyr-Gln-Gln-Lys-Pro-Val-Ala-Leu-Ile-Asn-Asn-Gln-Phe-Leu-Pro-Tyr-Pro-Tyr-

61
Tyr-Ala-Lys-Pro-Ala-Ala-Val-Arg-Ser-Pro-Ala-Gln-Ile-Leu-Gln-Trp-Gln-Val-Leu-Ser-

81
Asn-Thr-Val-Pro-Ala-Lys-Ser-Cys-Gln-Ala-Gln-Pro-Thr-Thr-Met-Ala-Arg-His-Pro-His-

101
Pro-His-Leu-Ser-Phe-Met-Ala-Ile-Pro-Pro-Lys-Lys-Asn-Gln-Asp-Lys-Thr-Glu-Ile-Pro-

121 Ile (Var. B)
Thr-Ile-Asn-Thr-Ile-Ala-Ser-Gly-Glu-Pro-*Thr*-Ser-*Thr*-Pro-*Thr*- -Glu-Ala-Val-Glu-
Thr (Var. A)

141 Ala (Var. B)
Ser-*Thr*-Val-Ala-Thr-Leu-Glu- -*SerP*-Pro-Glu-Val-Ile-Glu-Ser-Pro-Pro-Glu-Ile-Asn-
Asp (Var. A)

161 169
Thr-Val-Gln-Val-Thr-Ser-Thr-Ala-Val.OH

Sites of post-translational phosphorylation and glycosylation in italics.

Data from Swaisgood (1992).

1.2 Cheese

Cheese is the generic name for a group of fermented milk products, which are commonly believed to have evolved in Iraq some eight thousand years ago. Production of these early cheeses was the result of lactic acid production by bacterial fermentation of the milk sugar, lactose, the resulting acidification of the milk causing coagulation of the caseins. Today, this process is termed isoelectric or acid precipitation (Fox, 1993).

In contrast, the properties of rennet curds are very different from those produced by acid precipitation. Rennet curds exhibit better syneresis properties, making it possible to produce low moisture cheese curd without hardening, and consequently rennet curds have come to predominate in the manufacture of cheese. Animal rennets were probably the first milk coagulants used, although today several genetically engineered rennets exist.

1.2.1 Cheese manufacture

Production of the vast majority of cheese varieties can be subdivided into two well-characterised phases, manufacture and ripening.

$$\text{Milk} \xrightarrow{\text{Manufacture}} \text{Fresh cheese curd} \xrightarrow{\text{Ripening}} \text{Mature cheese}$$

The manufacturing phase is defined as those operations performed during the first 24hours, and include the processes of coagulation, acidification, dehydration, shaping and salting. However, it is during the subsequent ripening phase that the characteristic flavour and texture of the individual cheese varieties develop, as through a series of consecutive and concomitant biochemical events involving the breakdown of protein and fat, products with highly desirable aromas and flavours are created.

1.2.2 Coagulation

Rennet-catalysed coagulation of milk is typically achieved using acid (aspartate) proteinases of animal or fungal origin, and proceeds in two clearly defined phases. A primary enzymatic phase causes the specific hydrolysis of the κ-casein at the peptide bond Phe_{105}-Met_{106}, resulting in the release of the protein's hydrophilic C-terminal segment - the caseinomacropeptide, κ-casein f(106–169) - thereby allowing the initiation of the clotting process.

The truncation of κ-casein to para-κ-casein - κ-casein f(1–105) - results in a destabilisation of the casein micelles, allowing their resultant coagulation through hydrophobic interactions in a secondary non-enzymatic phase. This aggregation occurs in the presence of a critical concentration of Ca^{2+}, and temperatures greater than about 20^0C (Green and Grandison, 1992).

1.2.3 Ripening

While some cheeses are consumed fresh, many varieties are not ready for consumption at the end of the manufacturing process, when the young cheese curd is generally tough, rubbery and lacking in flavour. To allow a cheese to develop its particular aroma and flavour, there is generally a period of ripening or maturation, the time of which can be as short as a few weeks, or as long as two years or more in length depending on the cheese variety. During this ripening period, a complex series of biochemical changes occurs through the combined catalytic action of the following agents:

1. Rennet and rennet substitutes, i.e. chymosin.
2. Indigenous milk enzymes, i.e. plasmin.
3. Starter bacteria and their enzymes.
4. Secondary starter microflora and their enzymes.
5. Non-starter bacteria, i.e. organisms surviving, or gaining access, after pasteurisation.

Primary biochemical changes occurring during ripening involve proteolysis, glycolysis and lipolysis, which are responsible for the fundamental structural changes and flavour development occurring in the cheese curd. These are followed, and overlapped, by a host of secondary catabolic changes including the deamination, decarboxylation and desulphurylation of amino acids and the β-oxidation of fatty acids.

Proteolysis represents the most complex of these primary events, the products of which, being non-volatile, contribute greatly to cheese flavour and texture. This area has been the subject of much research and investigation in recent years, with many extensive and comprehensive reviews appearing in the literature (e.g. Fox, 1989; Fox *et al.*, 1994). Contributions made by proteolysis include:

1. A contribution to flavour through the release of peptides and amino acids. Some of these products may directly cause off-flavours, especially bitterness, while indirectly the catabolism of released amino acids to amines, acids, thiols, thioesters, etc. will also affect cheese flavour.
2. Greater release of savoury compounds during mastication.
3. Changes in pH via the formation of NH_3.
4. Changes in texture arising from the breakdown of the protein network, an increase in pH, and greater water binding by the newly formed amino and carboxyl groups.

1.2.4. Ripening agents in cheese

The initial degradation of casein is effected mainly by the coagulating enzymes present in the rennet, and more specifically by the proteolytic enzyme chymosin. Further hydrolysis of the polypeptides formed is catalysed by enzymes derived from the deliberately added starter and adventurous non-starter bacteria, resulting in the release of peptides and ultimately free amino acids.

Proteolytic degradation of the casein during cheese maturation results in the release of increasing amounts of water-soluble protein material. This water-soluble fraction is generally composed of hydrophilic polypeptides, small peptides and free amino acids, and is believed to make the largest contribution to the intensity of the cheese flavour (McGugan *et al.*, 1979; Aston and Creamer, 1986).

In recent years, a considerable amount of work has been published concerned with the controlled degradation of isolated casein proteins by the individual proteolytic agents found in cheese. Such an approach attempts to define the hydrolytic action and specificity of the various proteolytic agents, allowing the possible identification of the enzymes responsible for the production of individual peptides isolated and characterised from the cheese matrix.

1.3 Proteinases from the coagulant

Chymosin (E.C. 3.4.23.4) is an aspartyl proteinase of gastric origin, which is secreted by the young of many mammalian species. Additionally, it is the principal proteinase found in traditional rennets used for making cheese. Only some 3–6% of the

rennet added to cheese milk is retained in the curd, the amount retained being greatly influenced by the type of rennet, the cooking temperature and the pH at whey drainage.

The specificity of chymosin is primarily for peptide bonds possessing an aromatic or hydrophobic residue on the N-terminal side of the scissile bond. The primary chymosin cleavage site in the milk protein system is the κ-casein peptide bond Phe_{105}-Met_{106}, hydrolysis of which causes the coagulation of milk. For β-casein and α_{s1}-casein the most susceptible peptide bonds to chymosin action are Leu_{192}-Tyr_{193} and Phe_{23}-Phe_{24} respectively. A measure of the affinity of an enzyme for a substrate is described by the parameter k_{cat}/K_m, which has been estimated as 1405, 20.6 and 1.8s^{-1} mM^{-1} for these peptide bonds in the κ-, β-, and α_{s1}-casein respectively (Carles and Ribadeau-Dumas, 1984; Carles and Ribadeau-Dumas, 1985).

Studies by Visser (1977) on the contribution of enzymes from rennet, starter bacteria and milk to proteolysis and flavour development in Gouda cheese showed the rennet to be responsible for the initial hydrolysis of casein and the greater part of soluble nitrogen production.

1.3.1 Chymosin action on α_{s1}-casein

Mulvihill and Fox (1977) studied the influence of pH and urea on the hydrolysis of α_{s1}-casein by chymosin, and found that pH affected the pattern of proteolysis. Ionic conditions were also found to affect α_{s1}-casein proteolysis (Mulvihill and Fox, 1979).

Pahkala *et al.* (1989) isolated and identified peptides liberated by the chymosin hydrolysis of a 1.5% solution of α_s-casein in 50mM phosphate buffer (pH6.0), at 40^{0}C, following incubation for four hours. Peptides released from α_{s1}-casein came from the N-terminal 23 residues, the middle region of the molecule comprising residues 99–149 and the C-terminal residues 150–199. Cleavage was identified at the following sites: Gln_{13}-Glu_{14}, Leu_{20}-Leu_{21}, Phe_{23}-Phe_{24}, Leu_{98}-Leu_{99}, Leu_{101}-Lys_{102}, Ile_{127}-His_{128}, Ala_{129}-Gln_{130}, Leu_{149}-Phe_{150}, Phe_{153}-Tyr_{154}, Leu_{156}-Asp_{157} and Trp_{164}-Tyr_{165}.

McSweeney *et al.* (1993b) also investigated the proteolytic specificity of chymosin on bovine α_{s1}-casein. Following a 24hour incubation in 100mM phosphate buffer (pH6.5) at 30^{0}C, the resulting pH4.6-soluble peptides were separated by RP-HPLC and identified from their amino acid sequence, with confirmation by mass spectrometry and/or amino acid composition analysis. Cleavage was identified at the following sites: 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}.

Under simulated cheese conditions of pH5.2 and 5% NaCl, all the above sites except Phe_{153}-Tyr_{154} and Trp_{164}-Tyr_{165} were cleaved, with the additional hydrolysis of peptide bonds Leu_{11}-Pro_{12}, Phe_{32}-Gly_{33}, Leu_{101}-Lys_{102}, Leu_{142}-Ala_{143} and Phe_{179}-Ser_{180} also occurring. The rate at which specific peptide bonds were cleaved was dependent on the ionic conditions. Differences existed between pH6.5 and pH5.2 in the presence of 5% NaCl, particularly at peptide bond Leu_{101}-Lys_{102} which was hydrolysed far faster at the lower pH.

Chymosin action on the α_{s1}-casein-(24–199)-fragment, under simulated cheese conditions of pH5.2 and 4% w/v NaCl, resulted in cleavage at 19 sites (Exterkate *et al.*, 1995). Pahkala *et al.* (1989) and McSweeney *et al.* (1993b), using α_{s1}-casein as a substrate, had previously described the hydrolysis of many of these peptide bonds.

1.3.2 Chymosin action on β-casein

Visser and Slangen (1977) investigated the chymosin hydrolysis of β-casein in 50mM sodium acetate buffer (pH5.4), identifying seven cleavage sites, Leu_{192}-Tyr_{193} > Ala_{189}-Phe_{190} > Leu_{165}-Ser_{166} ≥ Gln_{167}-Ser_{168} ≥ Leu_{163}-Ser_{164} > Leu_{139}-Leu_{140} ≥ Leu_{127}-Thr_{128}. At a high enzyme/substrate ratio, some cleavage in the region 140–145 of the protein molecule also occurred. It was observed that in five of the seven main peptide bonds cleaved the C-terminus of a leucine residue was involved.

The β-casein f(193–209), isolated from rennet-treated casein and from Gouda cheese, has been characterised as a bitter peptide. For the β-casein f(166–189) no cleavage of the Gln_{167}-Ser_{168} peptide bond was observed, as happened when whole β-casein had been incubated with the enzyme. This suggested that cleavage of one peptide bond in the region 163–167 suppresses the cleavage of the two other labile bonds.

Carles and Ribadeau-Dumas (1984) also established the order of production of large polypeptides from β-casein by chymosin. Mulvihill and Fox (1978) found NaCl inhibited the chymosin hydrolysis of β-casein to an extent dependent on pH.

1.3.3 Chymosin action on α_{s2}-casein

In contrast to α_{s1}-casein and β-casein, α_{s2}-casein is relatively resistant to degradation by chymosin. Chymosin was observed to have a weak proteolytic activity on α_{s2}-casein (Pihlanto-Leppälä *et al.*, 1993), and although the TFA precipitated peptides were not identified in the study it was presumed that they were long and hydrophobic.

McSweeney *et al.* (1994) studied the proteolytic specificity of chymosin on bovine α_{s2}-casein in 100mM sodium phosphate buffer (pH6.5) at 30^{0}C for 24hours. The resultant pH4.6-soluble peptides were separated by RP-HPLC, and identified from their N-terminal amino acid sequence. Cleavage was identified at the following sites: Phe_{88}-Tyr_{89}, Tyr_{95}-Leu_{96}, Gln_{97}-Tyr_{98}, Tyr_{98}-Leu_{99}, Phe_{163}-Leu_{164}, Phe_{174}-Ala_{175} and Tyr_{179}-Leu_{180}. Hydrolysis was generally restricted to those residues between 90–120 and 160–207, which represent the more hydrophobic regions of the α_{s2}-casein molecule, with the primary cleavage site appearing to be peptide bond Phe_{88}-Tyr_{89}.

1.4 Proteinases from the milk

Two main indigenous proteinases in milk are now recognised, plasmin (fibrinolysin - E.C. 3.4.21.7) and cathepsin D (E.C. 3.4.23.5). Plasmin is a trypsin-like proteinase, with a pH optimum of about 7.5 and a high specificity for peptide bonds containing a lysine residue. Its preferred substrates are β-casein and α_{s2}-casein. Cathepsin D is a heat-labile enzyme with a pH optimum of 4.0, and is sequestered within lysosomes. Incubation of the caseins with cathepsin D produces electrophoretograms that suggest a specificity very similar to that of chymosin, although the two enzymes differ with respect to the rates of cleavage of certain peptide bonds (McSweeney *et al.*, 1995).

The contribution of plasmin to proteolysis in Cheddar cheese was investigated by Farkye and Fox (1991), who noted differences in the electrophoretic patterns of control cheeses and cheeses containing the plasmin inhibitor, 6-aminohexanoic acid. Farkye and Landkammer (1992) found that proteolysis in cheese increased with plasmin activity, with a resultant improvement in the flavour and overall quality of the cheese after 3 and 6 months of ripening. A three-fold increase in the plasmin activity in milk consistently resulted in cheese of superior sensory quality.

The combined action of chymosin and plasmin could theoretically produce quite small peptides, as their specificities are complementary, especially on β-casein which

chymosin cleaves primarily towards the C-terminus, while plasmin cleaves mainly in the N-terminal region.

1.4.1 Plasmin action on α_{s1}-casein

Le Bars and Gripon (1993) studied the proteolytic activity of plasmin towards α_{s1}-casein in a 50mM ammonium hydrogen carbonate solution (pH8.0) at 37^{0}C. Following their isolation and purification by reversed-phase or ion-exchange chromatography, peptide fragments were identified by N-terminal sequencing and amino acid composition analysis, and found to correspond to bond cleavages at Arg_{22}-Phe_{23}, Lys_{34}-Glu_{35}, Lys_{79}-His_{80}, Arg_{90}-Tyr_{91}, Arg_{100}-Leu_{101}, Lys_{102}-Lys_{103}, Lys_{103}-Tyr_{104}, Lys_{105}-Val_{106}, Lys_{124}-Glu_{125}, Arg_{151}-Gln_{152} and Lys_{193}-Thr_{194}.

McSweeney *et al.* (1993c) also investigated the proteolytic specificity of plasmin on bovine α_{s1}-casein, incubating the protein in 50mM ammonium bicarbonate buffer solution (pH8.4) at 37^{0}C. Primary cleavage sites were at Arg_{22}-Phe_{23}, Arg_{90}-Tyr_{91}, Lys_{102}-Lys_{103}, Lys_{103}-Tyr_{104}, Lys_{105}-Val_{106}, Lys_{124}-Glu_{125} and Arg_{151}-Gln_{152}, with the protein also being cleaved at a further 11 other bonds. These results are consistent with the findings of Le Bars and Gripon (1993) with respect to the initial cleavage sites of α_{s1}-casein by plasmin.

Identification of the primary plasmin cleavage sites, and the order of production of the small pH4.6-soluble peptides, suggested that α_{s1}-casein is cleaved initially towards its centre and at peptide bond Arg_{22}-Phe_{23}, with further hydrolysis of the polypeptides subsequently produced.

1.4.2 Plasmin action on β-casein

β-Casein in solution is hydrolysed at five primary sites, Lys_{28}-Lys_{29}, Lys_{105}-His_{106}, Lys_{107}-Glu_{108}, Lys_{113}-Tyr_{114} and Arg_{183}-Asp_{184}. Such proteolytic action results in the formation of polypeptides known as the γ-caseins; β-casein f(29–209) (γ_1-casein), β-casein f(106–209) (γ_2-casein), β-casein f(108–209) (γ_3-casein), β-casein f(114–209) (γ_4-casein) and β-casein f(184–209) (γ_5-casein). Complementary to the formation of the γ-caseins are the proteose peptones; β-casein f(1–105) and β-casein f(1–107) (proteose peptone 5), β-casein f(29–105) and β-casein f(29–107) (proteose peptone 8-slow), β-casein f(1–28)

(proteose peptone 8-fast) and β-casein f(29–113?) (proteose peptone T), and probably the fragments β-casein f(113–183), f(106–113) and f(108–113) (Fox *et al.*, 1994).

1.4.3 Plasmin action on α_{s2}-casein

Hydrolysis of α_{s2}-casein by plasmin in solution occurs at eight sites, Lys_{21}-Gln_{22}, Lys_{24}-Asn_{25}, Arg_{114}-Asn_{115}, Lys_{149}-Lys_{150}, Lys_{150}-Thr_{151}, Lys_{181}-Thr_{182}, Lys_{188}-Ala_{189} and Lys_{197}-Thr_{198}, creating about 14 peptides (Fox *et al.*, 1994).

1.5 Proteinases and peptidases of lactococci

The most comprehensively understood proteolytic system of the lactic acid bacteria is that of lactococci, with attention over the last few years focused primarily on the mesophilic cheese starter strains of *Lactococcus lactis*. The extensive proteolytic system possessed by lactococci is necessary because of their inability to synthesise some essential amino acids for growth, and a knowledge of the specificity of the enzymes involved can aid in assessing their significance and possible contribution to the generation of cheese flavour.

There are three major components to the lactococci proteolytic system. Primary enzymatic degradation of the milk casein is through the action of a cell envelope proteinase, which generates a range of polypeptides. A series of peptidases then act to catalyse the hydrolysis of these polypeptides into smaller peptides and free amino acids. Finally, a series of transport systems enable the uptake of these proteolytic products into the bacterial cell (Figure 1.5). For reviews of the proteolytic system in lactic acid bacteria see Pritchard and Coolbear (1993) and Tan *et al.* (1993a).

1.5.1 The cell envelope proteinase

The cell envelope proteinase of *Lactococcus lactis* catalyses the partial hydrolysis of one or more of the casein components of bovine milk to a number of polypeptide products. In ripening cheese, the proteinase activity additionally degrades casein fragments generated by the action of renneting enzymes.

The enzyme, following post-translational processing of the primary gene product, has an approximate molecular weight of 180kDa, and has been found to exhibit close

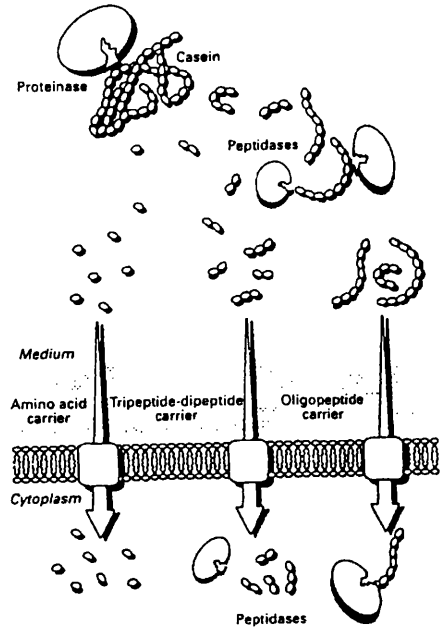


FIGURE 1.5: Proposed scheme for the breakdown and utilisation of casein by lactococci. From Tan *et al.*, 1993a.

homology with a subfamily of serine proteases, called the 'subtilases' (Siezen *et al.*, 1991).

Knowledge of the enzymatic specificity of the cell envelope proteinase has been achieved mostly through studies of the action of the purified, cell-free enzyme on various milk casein substrates. From such studies, two different types of lactococcal cell envelope proteinase are recognised. One proteinase type, designated the P_I-type, preferentially cleaves the β-casein component of milk, while a second enzyme, the P_{III}-type, can cleave both β-casein, although with an electrophoretic pattern different to that obtained from the P_I-type proteinase, and α_{s1}-casein (Visser *et al.*, 1986a). Cleavage sites in α_{s1}-casein are located throughout its sequence, but the major low molecular weight products resulting from proteinase action arise, as with β-casein, from the C-terminal region of the protein.

Additionally, some bacteria strains possess proteinases exhibiting a pattern of casein degradation intermediate between that of the described P_I- and P_{III}-types.

1.5.1.1 Cell envelope proteinase action on α_{s1}-casein

Reid *et al.* (1991a) partially purified the P_{III}-type cell wall-associated proteinase from *Lactococcus lactis* subsp. *cremoris* strain SK11, and incubated it with α_{s1}-casein substrate for various times up to a total of 48hours. Following separation by RP-HPLC, 16 peptide degradation products were characterised by the complete determination of their amino acid sequence and mass spectrometry. Eleven of the peptides were derived from the C-terminal 78 residues, three from the N-terminal region and two from the central region of the protein. A total of 19 cleavage sites were identified; however, no obvious consensus sequence for residues flanking the hydrolysed bonds could be determined.

Three polypeptides, generated by the chymosin hydrolysis of the α_{s1}-casein f(24–199) (α_{s1}-I), were used to investigate the *in vitro* enzymatic activity of the P_I-type proteinase from *Lactococcus lactis* subsp. *cremoris* strain HP (Exterkate *et al.*, 1995). Under conditions prevailing in cheese (viz. pH5.2 and 4% w/v NaCl), α_{s1}-casein f(24–98) and f(102–149) were cleaved in a manner largely corresponding to the degradation of α_{s1}-casein by the P_{III}-type proteinase (Reid *et al.*, 1991a). In the case of the third polypeptide, α_{s1}-casein f(165–199), cleavage occurred initially at five sites, Leu_{169}-Gly_{170}, Gln_{172}-Tyr_{173}, Asp_{175}-Ala_{176}, Glu_{189}-Asn_{190} and Asn_{190}-Ser_{191}, releasing relatively small

peptides, with secondary cleavages observed on prolonged incubation. However, only the cleavage at Leu_{169}-Gly_{170} had been previously observed by Reid *et al.* (1991a) for this peptide with the P_{III}-type proteinase.

Exterkate *et al.* (1991) determined the specificity of the P_I- and P_{III}-type proteinases from *Lactococcus lactis* subsp. *cremoris* strains HP and AM_1 respectively, on the α_{s1}-casein f(1–23), the initial peptide product formed on chymosin cleavage of peptide bond Phe_{23}-Phe_{24}. At pH6.5, and under relatively low ionic strength conditions, a characteristic and mutually different cleavage pattern was observed for the two proteinases. Peptide bonds cleaved by the P_I-type proteinase were identified as His_8-Gln_9, Gln_9-Gly_{10} and Gln_{13}-Glu_{14}, with secondary conversion of the initial degradation product, α_{s1}-casein f(1–13), to f(1–8) and f(1–9) occurring when the initial α_{s1}-casein f(1–23) substrate had almost completely disappeared. In contrast, the P_{III}-type proteinase cleaved peptide bonds Leu_{16}-Asn_{17}, Asn_{17}-Glu_{18} and Leu_{21}-Arg_{22}, with secondary conversion of the initial products being less evident. Properties of the residues N-terminal to the cleaved bonds were used to explain the hydrolysis patterns, in terms of the structural and various interactive features of the proteinases respective binding regions, predicted on the basis of their close sequence similarity with the subtilisin family.

However, under conditions prevailing in cheese (viz. pH5.2 and 4% NaCl in the moisture), conversion of the α_{s1}-casein f(1–23) peptide by the same cell-free lactococcal proteinases appeared to change markedly (Exterkate and Alting, 1993). In the case of the P_I-type proteinase, cleavage at peptide bonds His_8-Gln_9 and Gln_9-Gly_{10} was not observed, with hydrolysis of bond Gln_{13}-Glu_{14} being enhanced, and the splitting of the Leu_{16}-Asn_{17} and Asn_{17}-Glu_{18} peptide bonds, specific for the P_{III}-type proteinase, also increasing. For the P_{III}-type proteinase, cleavage of peptide bond Leu_{21}-Arg_{22} was almost completely eliminated, and hydrolysis of bond Asn_{17}-Glu_{18} slightly reduced with cleavage of Leu_{16}-Asn_{17} and the P_I-type specific bond Gln_{13}-Glu_{14} being enhanced.

Incubation of the α_{s1}-casein f(1–23) peptide with whole cells of *Lactococcus lactis* subsp. *cremoris* at pH5.2 in the presence of NaCl, resulted in an initially similar degradation pattern (Exterkate and Alting, 1993). In contrast to the cell-free proteinases, however, cleavage of peptide bond Gln_9-Gly_{10} by the HP cells (P_I-type proteinase) was still observed, although mainly as a secondary action, with the AM_1 cells (P_{III}-type proteinase) showing no detectable cleavage of bond Leu_{21}-Arg_{22}.

1.5.1.2 Cell envelope proteinase action on β-casein

Monnet *et al.* (1986) investigated the hydrolysis of bovine β-casein by the P_I-type cell envelope proteinase from *Streptococcus lactis* NCDO763. Following a 48hour incubation in 100mM sodium phosphate buffer (pH6.0) at 35^{0}C, five TFA-soluble peptides were characterised by amino acid composition analysis, N-terminal Edman degradation sequencing and C-terminal digestion with carboxypeptidases A and Y. The peptides identified corresponded to bond cleavages at Ser_{166}-Gln_{167}, Gln_{175}-Lys_{176}, Gln_{182}-Arg_{183}, Tyr_{193}-Gln_{194} and Ile_{207}-Ile_{208}. From the limited data available, an assessment of the specificity of the proteinase was difficult, with the only notable feature being the occurrence of a hydrophobic residue at position P3 in four of the five peptide bonds hydrolysed.

Visser *et al.* (1988) investigated the specificity of the P_I-type proteinase from *Streptococcus cremoris* strain HP on β-casein. Following a 16hour digestion in 20mM ammonium acetate buffer (pH6.2) at 15^{0}C, the pH4.6-soluble peptides were separated by semi-preparative RP-HPLC. Ten chromatographic peptides were identified, accounting for at least seven cleavage sites, all of which were in the C-terminal fifty residues of the protein. Additionally, five of the peptides had a Gln residue on the N- or C-terminal side of the scissile bond. These results corroborate those of Monnet *et al.* (1986); who could have detected further cleavage points had they identified the minor peaks in the HPLC chromatogram.

Peptide bonds in β-casein cleaved by the P_{III}-type proteinase from *Lactococcus lactis* subsp. *cremoris* strains SK11 (Reid *et al.*, 1991b) and AM1 (Visser *et al.*, 1991a) have also been identified.

Comparison of the β-casein peptide bonds cleaved by the P_I- and P_{III}-type proteinases reveal more similarities than differences. However, the relative rates of hydrolysis of the various bonds by the lactococci P_I-type proteinase from strain H2 and the P_{III}-type proteinase from strain SK11 indicate some very significant differences (Reid *et al.*, 1991b). The Tyr_{193}-Gln_{194} peptide bond was rapidly hydrolysed by both proteinase types, releasing the β-casein C-terminal f(194-209), while the adjacent bond, Leu_{192}-Tyr_{193}, was much more susceptible to cleavage by the P_{III}-type than the P_I-type proteinase. Conversely, the bonds Gln_{182}-Arg_{183}, Gln_{175}-Lys_{176} and Ser_{166}-Gln_{167} were very rapidly cleaved by the P_I-type proteinase, but were cleaved either much more slowly or

not at all by the P_{III}-type proteinase. Cleavage in the N-terminal 1–163 region of β-casein by the P_I-type proteinase occurred only very slowly, although several bonds within this sequence were rapidly hydrolysed by the P_{III}-type proteinase, viz. Asp_{43}-Glu_{44}, Gln_{46}-Asp_{47}, Phe_{52}-Ala_{53}, Gln_{123}-Ser_{124} and Asn_{132}-Leu_{133}.

Recently, Juillard *et al.* (1995) identified more than 100 different oligopeptides resulting from the hydrolysis of β-casein by the P_I-type proteinase of *Lactococcus lactis*. Following a 24hour incubation of the β-casein in 50mM Tris-HCl (pH6.5), containing 20mM $CaCl_2$, the TFA-soluble peptides were fractionated by HPLC and sequenced by collision induced dissociation using a coupled ion spray mass spectrometer. A total of 91 cleavage sites were determined, representing 44% of all peptide bonds in β-casein, illustrating the very broad specificity of the P_I-type proteinase. Eleven peptide bonds were preferentially cleaved, including all but the Gln_{167}-Ser_{168} bond observed by Visser *et al.* (1988) as being cleaved by the *Streptococcus cremoris* strain HP P_I-type proteinase.

1.5.2 Peptidases

Further degradation of the polypeptides is due primarily to the action of a range of peptidases. Given the multitude of peptidases known to be present in lactococci, this provides several possible routes for their complete degradation. Only a brief discussion of the types and specificities of peptidases characterised from lactococci will necessarily follow, as this area has been substantially reviewed in recent years (Pritchard and Coolbear, 1993; Tan *et al.*, 1993a).

To date, a wide range of peptidases have been isolated, purified and characterised with respect to their subunit structure, isoelectric points, pH and temperature optima, reaction to various chemical reagents, and substrate specificities. Table 1.2 summarises details of some of the peptidases purified and characterised from lactococci.

1.5.2.1 Endopeptidases

Endopeptidases degrade large peptides, usually up to approximately 25 residues in size, and often with a broad specificity. Such enzymes are likely to play a major role in the degradation of peptides released from casein by the cell envelope proteinase.

1.5.2.2 Aminopeptidases

Aminopeptidase N (PepN) is generally regarded as possessing a broad specificity, capable of hydrolysing a range of different peptides. The PepN isolated from *Lactococcus lactis* subsp. *cremoris* strain Wg2 by Tan and Konings (1990), has an approximate M_w of 95kDa, and was found to hydrolyse a broad range of peptides, although it was not active on dipeptides possessing an N-terminal alanine. While Tan *et al.* (1993a) classified the PepN as a general aminopeptidase, Niven *et al.* (1995) found it to have a marked preference for substrates containing arginine at the N-terminus, with cleavage only to a lesser extent at other residues such as lysine and leucine.

Tan *et al.* (1993b) investigated the degradation and debittering of a tryptic digest of β-casein, using the purified PepN from *Lactococcus lactis* subsp. *cremoris* strain Wg2. Incubation with PepN caused the degradation of the tryptic peptides, resulting in the hydrolysis of hydrophobic peptides and a subsequent decrease in the bitterness of the initial reaction mixture.

Aminopeptidase A (PepA) is specific for N-terminal aspartate and glutamate residues, and consequently would compliment the activity of PepN. Exterkate and de Veer (1987) purified to homogenity the PepA from *Lactococcus lactis* subsp. *cremoris* strain HP, finding it to have a M_w of approximately 130kDa and composed of three catalytically inactive subunits, each of approximately 43kDa. Niven (1991) determined the specificity of the PepA from *Lactococcus lactis* subsp. *lactis* NCDO 712, finding of the eighteen aminoacyl-alanine dipeptides tested the enzyme hydrolysed Asp-Ala, Glu-Ala and Ser-Ala. This PepA was found to have a M_w of 245kDa by gel-filtration chromatography, with a single band corresponding to 41kDa detected following SDS-PAGE of the purified enzyme, indicating it to be hexameric.

An intracellular aminopeptidase (PepC) was isolated by Neviani *et al.* (1989) from cell extracts of *Lactococcus lactis* subsp. *cremoris* AM2. The enzyme was characterised as having a M_w of 300kDa, and found to be composed of six identical subunits not linked by disulphide bridges. The purified enzyme possessed a broad specificity of action, hydrolysing β-naphthylamide derivatives, dipeptides and tripeptides, with the highest activity observed when histidine was the N-terminal amino acid residue.

However, the most extensively characterised aminopeptidase in lactococci is the X-prolyl-dipeptidyl enzyme, which specifically hydrolyses X–Pro–Y–.... containing

TABLE 1.2: Peptidases purified and characterised from lactococci (Adapted from Pritchard and Coolbear, 1993).

Peptidase	Abbreviation[a]	Specificity	M_w (native) (kDa)	M_w (subunit) (kDa)
X-propyl dipeptidyl aminopeptidase	PepX	X-Pro ↓ Y-....	160–190 117	82–90 ND[b]
Aminopeptidase N	PepN	X ↓ Y-Z....	93–95	95
Aminopeptidase C	PepC	X ↓ Y-Z....	300	50
Aminopeptidase A	GAP	Asp(Glu) ↓ Y-Z....	130 245 440–520	43 41 approx.40–43
Pyrrolidone carboxylyl peptidase	PCP	pGlu ↓ Y-Z	80	40
Prolyl iminopeptidase	PIP	Pro ↓ Y-Z	110	50
Dipeptidase	DIP	X ↓ Y	49 100	49 ND
Prolidase	PRD	X ↓ Pro	42–43	ND
Tripeptidase	TRP	X ↓ Y-Z	103–105	52–55
Endopeptidases	PepO NOP LEP I LEP II MEP	....W-X ↓ Y-Z....	70 70 98 80 180	70 70 98 40 70 (or 35)

[a] Provisional nomenclature for lactococcal peptidases proposed by Tan *et al.* (1993a).
[b] ND - Not Determined.

peptides by cleaving off the N-terminal X-Pro dipeptide. Such X–Pro–Y–.... motifs are frequently found in the caseins due to their relatively high proline content, with β-casein possessing the highest number of proline residues; 35 in a total of 209. In particular, the C-terminal end of this protein has a very high proline content, and thus, it is probable that X-prolyl-dipeptidyl aminopeptidase activity can contribute significantly to cheese flavour development, as this region is a source of many potentially bitter tasting peptides.

1.5.2.3 Dipeptidases and tripeptidases

The products released by the action of the endopeptidases and aminopeptidases are amino acids and various small peptides, i.e., dipeptides and tripeptides. For an efficient proteolytic process, several dipeptidases and tripeptidases are needed to release the maximum amount of amino acids.

A consequence of the hydrolytic action of the X-prolyl-dipeptidyl enzyme is the release of several X-Pro peptides. Their subsequent degradation is achieved through the action of a prolidase. Kaminogawa *et al.* (1984) purified and characterised the prolidase from *Lactococcus lactis* subsp. *cremoris* H61, finding that it hydrolysed dipeptides containing C-terminal proline such as Leu-Pro, Phe-Pro, Ala-Pro and Val-Pro, but not Gly-Pro.

1.6 Bitterness in cheese

Bitterness is a flavour defect common amongst dairy products, and is caused by the accumulation of bitter tasting peptides following the degradation of protein. During cheese manufacture and the subsequent maturation period, the milk proteins, α_{s1}-, α_{s2}-, β- and κ-casein, are hydrolysed by proteolytic enzymes derived from a variety of sources, releasing a range of polypeptides, peptides and ultimately free amino acids.

Because cheese is biochemically and biologically a very active material, off-favours, including excessive bitterness, are not the result of real off-tasting substances, but rather the consequence of an imbalance allowing the normal constituent bitter peptides to be pushed to too high a concentration. An extensive review of the formation, isolation and identification of bitter peptides from caseins has been made by Lemieux and Simard (1992).

1.6.1 The perception of bitter taste

There are four fundamental taste qualities that can be recognised by the human tongue; sweet, sour, salty and bitter, represented by sucrose, hydrochloric acid, sodium chloride and quinine, respectively. A fifth taste quality, termed "umami", is attributed to monosodium-L-glutamate.

It is now accepted that the bitter flavour produced during the enzymatic degradation of casein is due to certain types of peptides. However, other substances, including amino acids, amines, amides, substituted amides, long chain ketones and some monoglycerides present in cheese may also contribute to the perception of bitterness (Ney, 1979).

1.6.2 Bitterness is related to peptide hydrophobicity

Ney (1971) established a hypothesis, which described the degree of hydrophobicity exhibited by a peptide as being the critical predictor of its bitterness. Ney derived the Q-rule value as a measure of a peptide's hydrophobicity, calculated using the average free energy values for the transfer of the amino acid side chains from ethanol to water;

$$Q = \frac{\Sigma \Delta f}{n}$$

where Δf = the free energy of transfer of the side chains of the amino acid residues (hydrophobicity; cal.mol^{-1}) according to Tanford (1962); n = number of amino acid residues; Q = average hydrophobicity of a peptide.

Ney decided that for peptides to exhibit a bitter taste, they must possess a Q-value $>$ 1,400 cal.res^{-1}, and additionally have a molecular weight of between 100 and 6,000Da. Peptides with a Q-value $>$ 1,400 cal.res^{-1} but having a molecular weight over 6,000Da, or peptides having a Q-value $<$ 1,300 cal.res^{-1} with a molecular weight ranging from 100 to 10,000Da, have a non-bitter taste. If the calculated Q-value lies between 1,300 and 1,400 cal.res^{-1} no prediction can be made about the peptide's bitterness.

Ney's Q-rule is empirically based, reflecting the assumption that hydrophobic interaction is essential for the sensation of bitter taste. The rule is qualitative and applicable only to peptides tasted in pure solution. Despite the occasional occurrence of bitter peptides with Q-values below 1,300 cal.res^{-1} (Guigoz and Solms, 1976; Wieser and Belitz, 1976), it is now accepted that Ney's rule is broadly applicable for assessing the hydrophobicity of small peptides.

Bigelow and Channon (1976) produced average hydrophobicity values - $H\phi_{ave}$ - for pure proteins using revised Δf values. Based on amino acid side chain solubility in both ethanol and dioxane (Nozaki and Tanford, 1971), Bigelow and Channon's Δf values are recommended for calculating the average hydrophobicity of protein hydrolysates, as extrapolation of the Q-rule to mixtures of peptides has been refuted (Alder-Nissen, 1986).

1.6.3 Peptide bitterness and primary structure

Importantly, the positional effect of an amino acid in a peptide sequence with regard to perceived bitterness cannot be determined by the Q-rule. Leucine-containing peptides are hydrophobic and associated with bitterness, with stronger bitterness always found when a leucine residue was located at the C-terminus of a chemically synthesised peptide (Ishibashi *et al.*, 1987a).

Investigations into the role of phenylalanine and tyrosine residues in the bitter taste of peptides (Ishibashi *et al.*, 1987b) indicated that bitterness was more intense when phenylalanine was located at the C-terminus, and when the content of phenylalanine or tyrosine was increased in peptides. Additionally, when evaluating the taste of peptides containing phenylalanine at the C-terminus, it was found that when the hydroxyl-containing amino acids, serine or threonine, or a basic amino acid was present in the peptides, diverse tastes were observed in addition to the bitterness. This indicates that a C-terminal phenylalanine does not always ensure bitter taste.

Studies on the taste of proline-containing peptides showed that, contrary to the case of peptides containing typical hydrophobic amino acids such as phenylalanine or leucine, there was no specific enhancement of bitterness when proline was located at the C-terminus (Ishibashi *et al.*, 1988). Rather, the most significant role of proline on peptide bitterness was dependent on a conformational alteration of the peptide molecule,

caused by a folding of the peptide skeleton due to the imino ring side chain of the proline residue.

Such studies reaffirm the reported observations of Kanehisa *et al.* (1984), who on synthesising the C-terminal octapeptide of β-casein; H-Arg^{202}-Gly-Pro-Phe-Pro-Ile-Ile-Val^{209}-OH, found it to be extremely bitter. Subsequent synthesis of the closely related heptapeptide; H-Arg-Gly-Pro-Phe-Pro-Ile-Val-OH, in which one of the C-terminal hydrophobic isoleucine residues in the original octapeptide was removed, resulted in an approximate 2,700% decrease in bitter taste perception. It was observed that both an N-terminal basic amino acid residue and a C-terminal hydrophobic residue can produce a strong bitter taste. Bitter taste is highly dependent on a peptide's spatial structure, and therefore ultimately on its primary structure. These observations were confirmed by Kato *et al.* (1984), who also noted that although the quantitative examination of bitterness is important for studies on the use of casein as food, or in foods, it is seldom done.

1.7 Matrix-assisted laser desorption/ionisation-mass spectrometry

Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) is a technique allowing the analysis of a variety of biomolecules with masses up to at least 300,000Da. Historically, attempts to use laser light as a mass spectrometric ionisation process began in the 1970s. However, these early experimental investigations on laser desorption were restricted to the analysis of highly absorbing molecules, generally with molecular weights below 2000Da. The inability of high molecular weight samples to be sufficiently excited at the laser wavelengths used fuelled searches for a matrix material which would allow ionisation and desorption, irrespective of a compound's individual absorption characteristics (Karas *et al.*, 1987; Karas and Hillenkamp, 1988).

1.7.1 Matrices

An important property of the matrix for achieving sample desorption is its ability to undergo some specific photochemistry, leading ultimately to proton transfer either to or from the analyte molecules under investigation. From many hundreds of potentially useful compounds that have been tested for their suitability as matrices, a small collection has been identified as being of real practical worth. Table 1.3 lists some of the more

popular matrices, together with their useable wavelengths and areas of predominant application.

A complete understanding of the MALDI process has not yet been achieved, although it is suggested that the matrix serves three main functions (Bahr *et al.*, 1994):

1. Absorption of energy from the incident laser light.
2. Isolation of the biomolecules from each other.
3. Ionisation of the biomolecule.

Consequently, different perceptions exist in the literature as to the appropriate matrix compound to use for any given application. Without access to sample observation, matrices which tend to form relatively homogeneous arrays of very small crystals, such as sinnapinic acid or α-cyano-4-hydroxycinnamic acid (ACH), usually perform best. In contrast, single-crystal irradiation usually requires that observation takes place, in order to locate the optimum position on the sample support slide, as in the case of the matrix 2,5-dihydroxybenzoic acid.

1.7.2 Sample preparation

Sample preparation is a very important procedural step in performing MALDI-MS. However, this fact is often lost when simply describing the mixing of a few microlitres of an analyte solution with a similar volume of a saturated matrix solution, and spotting microlitre volumes of the resulting mixture on to an inert metal sample support slide.

The matrix should be present in a large, typically 10,000 fold, molar excess over the analyte, which, following evaporation of the solvent, forms a random array of microcrystals, in which the analyte molecules are embedded. The potential solubility and hydrophobicity of the biomolecule to be analysed should be considered carefully during sample preparation. Aqueous solutions are used in many instances, although depending on the analyte or matrix solubility, organic solvents or solvent mixtures may be necessary. For protein and peptide analysis, small additions of trifluoroacetic acid or heptafluorobutyric acid are often made to ease solubilisation and subsequent transfer.

TABLE 1.3: Selected matrices for matrix-assisted laser desorption/ionisation mass spectrometry (MALDI-MS).

Matrix	Form	Wavelengths	Main Application
2,5-Dihydroxybenzoic acid (DHB)	Solid	337nm, 355nm	Proteins
DHB + 10% 5-methoxy salicylic acid	Solid	337nm, 355nm	Proteins > 20,000Da
Sinnapinic acid	Solid	266nm, 337nm, 355nm, 2.94μm, 10.6μm	Proteins
α-Cyano-4-hydroxycinnamic acid	Solid	337nm, 355nm	Proteins
2-Nitrobenzyl alcohol	Liquid	266nm	Proteins
Nicotinic acid	Solid	266nm, 2.94μm, 10.6μm	Proteins
3-Hydroxypicolinic acid	Solid	337nm, 355nm	Nucleic acids/glycoconjugates
2-(4-Hydroxyphenylazo)benzoic acid	Solid	266nm, 337nm	Proteins/glycoconjugates
Succinic acid	Solid	2.94μm, 10.6μm	Proteins/Nucleic acids
Glycerol	Liquid	2.94μm, 10.6μm	Proteins
2-Nitrophenyl octyl ether	Liquid	266nm, 377nm, 355nm	Synthetic polymers

Adapted from Hillenkamp, 1994; Bahr *et al.*, 1994.

1.7.3 Mass spectrometry analysis of proteins and peptides

Of all the biomolecules, proteins and peptides have turned out to be especially amenable to MALDI analysis. In sharp contrast to electrospray ionisation mass spectrometry, where there usually exists one charge per 1000Da molecular weight, in MALDI-MS the singly charged parent molecule usually forms the dominant spectral peak. Depending on the protein being analysed and the matrix used, ions of higher charge states and multimers can also appear in the MALDI spectrum, although generally with lower intensities. Existence of such multimers and multiply charged ion signals can be useful in improving the accuracy of the mass determination.

Glycoproteins with a carbohydrate content of over 50% have also been analysed by MALDI-MS (e.g. Hillenkamp *et al.*, 1991), usually being identified in spectra by their pronounced peak width resulting from the carbohydrate heterogeneity. Hydrophobic and membrane proteins usually require addition of detergent to enable them to dissolve in a suitable solvent, ensuring the necessary homogeneous protein-matrix crystallization can be achieved (Rosinke *et al.*, 1995).

Sample preparation and short measurement time, coupled with the high sensitivity, typically a few hundred femtomoles to a few picomoles of protein, make MALDI-MS an excellent choice for monitoring the preparation of small quantities of biological material. With the amount of material actually consumed in generating a spectrum lying within the attomole range, precious samples can be retrieved for further characterisation, for example by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (Cerpa-Poljak *et al.*, 1995).

Analysis of mixtures by MALDI-MS is feasible, although individual component sensitivity is often highly dependent on the matrix used, and therefore a test of the different matrices is generally advisable for any particular case. Further constraints exist as some matrices are intolerant to typical sample additives, such as buffer salts or some detergents, resulting in suppression or excessive degradation of the quality of spectra. Consequently, analysis efficiency is compromised by the lack of a universally applicable matrix, which may necessitate the comparison of spectra in possibly as many as three or four different matrices.

Cohen and Chait (1996) made a comprehensive study of the mass discrimination effects observed when using the ACH matrix for analysing complex mixtures of peptides

and proteins. Their findings suggested that, depending on whether visualisation of low-, medium- or high-molecular weight peptide material was required, particular matrix solution compositions may be more applicable than others. Additionally, it was found that varying the rate of matrix crystal growth also had a discriminating effect, with slow crystallisation (hours) favouring the observation of high-mass peptide components, rapid crystallisation (minutes) the observation of low-mass peptides, and a "dried drop" crystallisation process (seconds) exhibiting a strong pH-dependent mass discrimination effect.

Billeci and Stults (1993) used various matrix mixtures for MALDI-MS to characterise the tryptic maps created from the human growth hormone and human tissue plasminogen activator recombinant proteins, finding that carbohydrate-containing comatrices gave significant enhancement in the spectra of complex peptide mixtures over single-component matrices.

Protein identification by peptide mass fingerprinting has received considerable interest recently, undoubtedly a consequence of the ease with which experimental data can be obtained through mass spectrometry analysis (Cottrell, 1994). Protein separation using electrophoresis, specifically two-dimensional electrophoresis, allows the separation and resolution of potentially many hundreds of proteins. Subsequent membrane blotting and the enzymic digest of excised bands of interest, followed by MALDI-MS analysis of the peptide fragments generated, allows identification from the characteristic peptide mass fingerprint produced (Liang *et al.*, 1996). Such a procedural approach is now common place given the ease of access to protein databases, and as the impact of proteomic studies in the post-genomic era leads increasingly to the requirement of rapid protein identification before commitment to lengthy *de novo* sequencing regimes. Patterson (1996) investigated the identification of gel-separated proteins using MALDI-MS, and found for some proteins characterisation was possible from as little as 5pmol loaded on to the gel.

1.7.3.1 N-terminal sequencing by MALDI-MS

Direct sequence determination of proteins and peptides is traditionally achieved using automated Edman degradation, a process that sequentially removes one amino acid residue at a time from the N-terminus of a peptide. Phenylisothiocyanate (PITC) reacts

with the uncharged terminal amino group to form a phenylthiocarbamoyl derivative, which under mildly acidic conditions liberates a cyclic derivative of the terminal amino acid, leaving behind an intact peptide shortened by one amino acid. The released cyclic compound, a phenylthiohydantoin amino acid, is then identified by chromatographic procedures.

The recent emergence of MALDI-MS, coupled with the development of improved matrix materials and greater mass accuracy, has allowed protein and peptide sequencing to be approached in a novel way. Chait *et al.* (1993) described a new principle in protein sequencing, which combined multiple steps of wet chemistry with a final, single-step MALDI-MS read-out of the resulting amino acid "ladder" sequence.

The procedure involves the controlled generation of a set of peptide fragments, by performing stepwise degradations using PITC in the presence of small amounts of the chain terminating agent phenylisocyanate. The resulting mass spectrum contains molecular ions corresponding to each terminated polypeptide species present, with the mass difference between adjacent peaks corresponding to the molecular weight of the amino acid removed. Their order of occurrence in the data set therefore defines the sequence of amino acids in the original peptide chain. Peptide characterisation by mass differences additionally allows post-translational modifications to be identified.

This conceptually novel approach to protein sequencing was refined by the synthesis and development of a volatile isothiocyanate, trifluoroethylisothiocyanate (TFEITC) (Bartlet-Jones *et al.*, 1994). Using the TFEITC reagent, no complex washing procedures are required following each degradation cycle, as reagents and by-products are efficiently removed under vacuum eliminating extractive loss, thereby increasing potential sensitivity.

1.7.3.2 C-terminal sequencing by MALDI-MS

The use of carboxypeptidases to create C-terminally shortened peptides has been used as a sequencing method for many years. Liberated amino acids can be identified by amino acid analysis, or more recently by mass spectrometry of the resulting peptide mixture, with the mass differences between consecutive peaks in the spectrum identifying the amino acid released (Thiede, *et al.*, 1995; Patterson, *et al.*, 1995). Such an approach

may be necessary, given the commonplace occurrence of N-terminally blocked peptides, either by amino acid structural rearrangement or post-translational molecule addition.

Chapter 2

Effect of Manufacturing Conditions on the Concentrations of Free Amino Acids in Cheddar Cheese

2.1 Introduction

To date, little is known about the compounds that impart the typical flavour to Cheddar cheese. Further, the overall complexity of the cheese microflora means that contributions made to flavour by individual molecules is extremely difficult to interpret. Consequently, flavour development during cheese maturation remains relatively poorly understood. A range of chemical methods exist which allow the extent of cheese ripening to be assessed (McSweeney and Fox, 1993), of which proteolysis is generally accepted as being the most reliable indicator of maturation time.

Cheese flavour is normally associated with those components present in the water-soluble fraction of the cheese (McGugan *et al.*, 1979; Aston and Creamer, 1986). This fraction typically includes hydrophilic polypeptides, peptides and free amino acids. The concentration of free amino acids is considered to be a reliable indicator of both the extent of cheese ripening, and the rate of flavour production (Hickey *et al.*, 1983; Puchades *et al.*, 1989).

Law and Sharpe (1977) found no significant difference between the mean Cheddar flavour intensity scores of cheeses made using different microflora, despite recording increased free amino acid production. Their interpretation was that microbial enzymes are present only to produce the correct conditions for non-microbial reactions to proceed, and that amino acids are intermediate products in the production of certain aromatic compounds.

The free amino acid levels in conventional and hydrolysed lactone (HL) Cheddar cheese were determined by Weaver *et al.* (1978) at 28 day intervals over eight months of ripening using an amino acid analyser. Pre-hydrolysis of the milk disaccharide, lactose, provides an immediate source of simple sugars, resulting in the accelerated growth of bacteria and a faster rate of ripening of the cheese. Typically, a 3–4 month old HL Cheddar cheese was very similar in all aspects to a 6-, 7- or 8-month-old

conventionally manufactured Cheddar cheese. The average increases in total free amino acid concentrations were from 500μg g^{-1} to 9,967μg g^{-1} and 14,564μg g^{-1} for the conventional and HL Cheddar cheese, respectively. Valine, tyrosine, phenylalanine, glutamate, leucine and lysine accounted for approximately 80% of the total free amino acids at all stages of ripening.

Following their conversion to volatile n-heptafluorobutyryl isobutyl derivatives, Wood *et al.* (1985) determined free amino acid levels in an eight month old Cheddar cheese using capillary column gas-liquid chromatography. Overall, the results compared favourably with Weaver *et al.* (1978), with the amino acids glutamate, leucine, valine, lysine and phenylalanine having the highest concentrations.

Laleye *et al.* (1987) also assessed Cheddar cheese quality by quantifying heptafluorobutyric anhydride-derivatised free amino acids by gas-liquid chromatography. In grade 1 aged cheese, the glutamate content was found to increase steadily throughout the eight-month ripening period, in agreement with Weaver *et al.* (1978).

Considerable research has focussed on the proteolysis of cheese, especially with regard to the separation and characterisation of peptides formed during the ripening period. The contribution of such identified peptides to cheese flavour is relatively unknown, although many of them are very hydrophobic, and consequently potentially bitter tasting. In contrast, there have been relatively few reports on the complete free amino acid profiling of cheese (McSweeney and Fox, 1993). Amino acids have characteristic tastes, and can be present in cheese at sufficiently high concentrations to contribute to at least its background savoury taste. Additionally, amino acids, while being the end products of proteolysis, can be further degraded to a range of sapid and aromatic compounds. Such amino acid catabolism involves their decarboxylation, deamination, transamination and desulphuration, with subsequent conversion of the resulting compounds.

Therefore, as an alternative approach, this chapter will describe the quantification of free amino acids released during the maturation of Cheddar cheese manufactured under different processing conditions. Amino acid concentrations were determined in Cheddar cheese made from low-fat, high heat-treated, pH-adjusted milk at 3 and 6 months of maturation. The amino acid levels in Cheddar cheese manufactured from standard pH (6.4) or reduced pH (6.0) milk at regular time intervals over a 246 day ripening period

were also measured. To aid interpretation of the changes in amino acid concentrations occurring during ripening of the cheese, ANOVA and the empirical multivariate statistical technique of Principal Component Analysis was used to analyse the results obtained as described by Muir and Hunter (1992).

2.2 Materials and methods

2.2.1 Low-fat, high heat-treated, pH-adjusted milk Cheddar cheese

Bulk herd milk from the Hannah Research Institute farm was used in all experiments, standardised to a casein-to-fat ratio of 2.05. Sufficient heat treatment of the milk to denature whey proteins was applied in an APV Junior plate heat exchanger, modified to facilitate heat treatment of the milk at 90^{0}C for 60sec. The period of heat treatment was controlled by insertion of a tube of appropriate length in the holding section. Control samples were pasteurised at 73^{0}C$\pm$1.0^{0}C for 16sec in a high temperature short time (HTST) plate pasteuriser (Junior Paraflow, APV).

Low-fat Cheddar cheese was manufactured in 45 litre pilot scale vats, using procedures previously described by Banks *et al.* (1984). The milk was warmed to 30^{0}C and the pH adjusted to 5.8, 6.0, 6.2 and 6.4 in the vats using hydrochloric acid. Four vats of cheese were manufactured in each of four duplicate trials, using either pasteurised or heat-treated milk. A direct vat inoculation (DVI) starter culture (MaO11, Texel) was added, and a recombinant DNA pure chymosin (Maxiren, Gist-Brocades) was used at either 100% or 50% of the recommended level (17.7g/100L of milk). Chymosin from calf rennet exists in three isomeric forms - A, B and C. Maxiren contains only the A isomer, although differences in their specificities have not been well characterised to date (Teuber, 1990).

A total of thirty two vats of cheese were manufactured on duplication of each set of processing variables. The cheese was matured at 8–10^{0}C for up to 6 months, and proteolysis was monitored in one set of duplicates.

2.2.2 Standard and reduced pH milk Cheddar cheese

Bulk herd milk from the Hannah Research Institute farm was used, pasteurised at 73^{0}C$\pm$1.0^{0}C for 16sec in a HTST plate pasteuriser (Junior Paraflow, APV). Cheese

manufacture was in 45 litre pilot scale vats using conditions previously described by Banks *et al.* (1984), with the milk either at the standard pH6.4 or reduced pH6.0. A DVI starter culture (MaO11, Texel), and a recombinant DNA pure chymosin (Maxiren, Gist-Brocades) was used at the recommended level (17.7g/100L of milk). The cheese was matured at 8–10^{0}C for a total of 246 days.

2.2.3 Reagents

Sodium acetate trihydrate (analytical grade), acetonitrile (HPLC grade solvent) and chloroform (high purity grade) were purchased from Fisher Scientific UK Ltd., Bishop Meadow Road, Loughborough, Leics., LE11 ORG.

Methanol (HPLC grade solvent) was purchased from Prolabo, Liverpool Road, Eccles, Manchester, M30 7RT.

Phenylisothiocyanate (PITC; protein sequencing grade), amino acid standard solution (AA-S-18) and amino acid standards glutamine, asparagine, cysteine and nor-leucine were purchased from the Sigma-Aldrich Company Ltd., Fancy Road, Poole, Dorset, BH12 4QH.

Triethylamine for amino acid derivatisation (protein sequencing analysis grade) and HPLC separation (amino acid analysis grade) was purchased from Fluka Chemicals, The Old Brickyard, New Road, Gillingham, Dorset, SP8 4JL.

All water used was of HPLC grade, prepared on site using a CD plus deioniser from USF Ltd., Harforde Court, John Tate Road, Hertford, SG13 7NW.

2.2.4 Extraction of the free amino acids

Samples of the Cheddar cheese, removed at regular time intervals during their ripening period, were finely grated, freeze dried to constant dry weight, and stored at -20^{0}C prior to further analysis. Each lyophilised cheese sample was homogenised in a pestle and mortar, and 50mg extracted in 750μL of a 2:1 (v/v) solution of chloroform:methanol in 1.5mL eppendorf vials for 1h using an orbital Vibrax® VXR shaker (Harwalker and Elliott, 1971). Additional whirlimixer agitation was applied to aid initial sample solubilisation, at 30min and after shaking for 1h. Following separation of the phases by addition of 250μL of water, the solution was whirlimixed, then microcentrifuged (MSE Microcentaur) for 2.5min at 13,000rpm. The aqueous methanolic

top layer was transferred by pasteur pipette to a clean eppendorf vial, from which residual chloroform was removed following recentrifugation. Aliquots of the aqueous methanolic fractions were removed, lyophilised and stored at -20^{0}C prior to PITC derivatisation of the extracted amino acids.

2.2.5 Phenylisothiocyanate derivatisation of the amino acids

The lyophilised aqueous methanolic samples and amino acid standards were solubilised in 25μL of 50% aqueous ethanol, to which was then added 50μL coupling buffer (acetonitrile:triethylamine:water, 7:2:1 v/v/v) and 5μL PITC. Following whirlimixing and microcentrifugation, the solutions were left for a minimum of 20min at room temperature to allow the derivatisation reaction to proceed to completion.

To prevent possible 'bubbling' and loss of sample on lyophilisation, samples were first spun in a 'Gyrovap' centrifugal evaporator (V. A. Howe and Co. Ltd.) at 30^{0}C before placement in the freeze drier. Lyophilised samples were stored at -20^{0}C prior to reversed phase-high performance liquid chromatography (RP-HPLC) separation of the phenylthiocarbamyl (PTC) amino acid derivatives.

2.2.6 Chromatographic separation of the derivatised amino acids

Separation of the PTC amino acids was performed by RP-HPLC on a Spherisorb ODS-2 column (5μm, 250x4.6mm i.d.; Jones Chromatography, New Road, Hengoed, Mid Glamorgan, CF8 8AU), protected by a 30x4.6mm i.d. guard column containing the same stationary phase, using an automated Spectraphysics HPLC system.

The PTC amino acids were eluted essentially as described by Ebert (1986). Buffer A consisted of 50mM sodium acetate plus triethylamine (2.75mL L^{-1}), adjusted to pH6.4 by the addition of phosphoric acid, and filtered through a 0.2μm cellulose acetate filter (Sartorius Limited, Blenheim Road, Epsom, Surrey, KT19 9BR). Buffer B consisted of buffer A, acetonitrile and methanol (5:4:1 v/v/v).

Immediately prior to RP-HPLC separation, the PTC amino acid derivatives were solubilised in 50% aqueous acetonitrile, and then diluted with buffer A (1:9 v/v), a solution in which the PTC amino acids are stable for at least 24 hours. Following solubilisation the solutions were microcentrifuged for 2.5min at 13,000rpm, and filtered through 0.2μm filters (Sartorius Limited) before injection onto the column.

2.2.7 Statistical analysis

Estimates of the treatment effects on the levels of amino acids were obtained by ANOVA (balanced design) using the Minitab statistical package (Version 11, State College, 16801–3008, USA). For the low-fat Cheddar cheese, a model was fitted with terms for pH, heat treatment, rennet level, age and the interactions of pH x age, pH x heat, pH x rennet, age x heat, age x rennet, heat x rennet and age x heat x rennet.

Further probing of the changes in amino acid composition was made using multivariate analysis. Principal Component Analysis (PCA) (Piggott, 1988) in the correlation matrix was used to simplify data interpretation. This analysis was carried out using Minitab (Version 11).

2.3 Results and discussion

2.3.1 Low-fat, high heat-treated, pH-adjusted milk Cheddar cheese

During manufacture, conditions of scald and stir were manipulated to minimise gross compositional differences in cheese produced from high heat-treated or pasteurised milk. Table 2.1 shows there was little difference in the moisture, fat, protein, fat-in-dry-matter and moisture-in-non-fat-solid levels between such samples.

The final pH of the cheese was influenced by heat treatment. Salt retention in the curd was also influenced by heat treatment, with cheese manufactured from the high heat-treated milk having increased levels of salt-in-moisture. While differences in gross composition of the cheese were minimal, calcium levels in the curd were effectively altered by pH, with cheese produced from milk at the lower pH having significantly lower levels of total calcium in the curd. Improved yields of up to 12% were evident using the higher heat treatment, and were associated with a highly significant increase in the level of crude protein retention in the curd.

Changes in the concentration of nineteen amino acids in the low-fat, high heat-treated, pH-adjusted Cheddar cheese, determined as their phenylthiocarbamyl derivatives by RP-HPLC at 3 and 6 months of maturation, are shown in Appendix II, Tables II.1–II.4 and Figures II.1-II.8. Of the twenty common amino acids, only tryptophan was not detected, probably due to its low concentration in casein.

TABLE 2.1: Estimated mean effects of processing variables on low-fat Cheddar cheese composition.

Treatment	Level	Moisture	Fat	Protein	pH	FDM[a]	MNFS[b]	S/M[c]	Calcium (mg/100g cheese)	Crude protein retention (% in curd)
pH	5.8	48.3	14.8	30.9	5.18	28.7	56.7	4.31	739.4	78.8
	6.0	48.5	14.8	30.8	5.18	29.1	56.9	3.97	807.3	79.2
	6.2	46.2	15.2	32.1	5.20	28.3	54.5	4.36	979.8	78.9
	6.4	47.2	14.7	32.1	5.23	27.8	55.3	4.08	1054.8	80.0
Heat	72°C/16sec	47.8	15.3	31.0	5.09	29.3	56.4	3.50	942.9	75.2
	90°C/60sec	47.3	14.5	31.9	5.30	27.6	55.3	4.86	847.7	83.3
Rennet	50%	47.7	14.8	31.7	5.22	28.5	55.9	4.21	796.4	79.5
	100%	47.4	14.9	31.2	5.18	28.4	55.7	4.15	994.2	79.0
Significance of Treatment[d]										
pH		*	NS	**	NS	**	**	NS	**	NS
Heat		NS	***	**	***	***	NS	***	NS	***
Rennet		NS	NS	NS	NS	NS	NS	NS	**	NS

[a] Fat-in-dry-matter.
[b] Moisture-in-non-fat-solid.
[c] Salt-in-moisture.
[d] NS = Not Significant, $p>0.05$; * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$.

The results shown in Table 2.2 indicate highly significant effects from the various treatments on amino acid compositions of the samples. Amino acid composition was greatly influenced by age, and the pH and severity of heat treatment of the milk. Reducing the rennet level also influenced amino acid composition, but to a lesser extent.

As the number of experimental variables increased, it became more difficult to efficiently extract important information from the data. Therefore, Principal Component Analysis was used to examine possible relationships between the determined amino acid concentrations and the manufacturing conditions of the Cheddar cheese. If experimental variables are correlated, then PCA makes it possible to highlight and summarise the important information in fewer dimensions than there are original variables.

The variance accounted for by the first three principal component dimensions was 74.3%, 7.5% and 6.6%, respectively (88.4% in total). Inspection of the vector loadings, and the correlation coefficients of the scores for the cheese samples obtained from the experimental amino acid concentrations, facilitated interpretation of the dimensions of the PCA.

Figure 2.1 shows the vector loadings for scores on the first three Principal Components for the nineteen amino acids. A vector loading greater than +/-0.25 was considered significant. The first Principal Component showed no discrimination, with all amino acids other than phenylalanine, possessing vector loadings of an essentially similar negative magnitude, and shows a universal relationship to quantity or concentration. The amino acids phenylalanine and arginine made the largest contributions to the second Principal Component, with minor contributions to the dimension coming from cysteine, serine and threonine. For the third Principal Component, cysteine provided the greatest contribution, with arginine, methionine, proline and serine making minor contributions to the dimension.

Changes to the amino acid concentrations in the low-fat, high heat-treated, pH-adjusted Cheddar cheese for scores on the first two Principal Components are shown in Figure 2.2. At three months of maturation, the cheese samples fell in to a coherent cluster, which by six months of maturation had moved progressively within the amino acid concentration space map. Cheese samples 3h▼ and 6h▼, and 3h◆ and 6h◆ were obvious outliers from these two clusters.

TABLE 2.2: Analysis of variance for amino acid concentrations in low-fat, high heat-treated, pH-adjusted Cheddar cheese.

	Amino Acid[a]																		
Treatment[b]	**Ala**	**Arg**	**Asn**	**Asp**	**Cys**	**Gln**	**Glu**	**Gly**	**His**	**Ile**	**Leu**	**Lys**	**Met**	**Phe**	**Pro**	**Ser**	**Thr**	**Tyr**	**Val**
pH	***	*	***	**	NS	***	***	***	**	***	***	*	**	NS	***	**	*	***	***
Age	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	*	***	***
Heat	***	***	***	***	***	**	***	***	NS	NS	NS	*	***	*	NS	***	NS	NS	NS
Rennet	NS	**	NS	*	NS	*	NS	*	NS	NS	***	NS	NS	**	NS	**	NS	***	*

[a] Determined as phenylthiocarbamyl-derivatised free amino acids by RP-HPLC.

[b] NS = Not Significant, $p>0.05$; * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$.

FIGURE 2.1: Simplification of the effect of high heat treatment and pH adjustment on amino acid concentrations in low-fat Cheddar cheese.

Analysis of changes to the amino acid concentrations over 6 months of maturation by Principal Component Analysis; vector loadings for scores on first three Principal Components.
(+/-0.25 significance level; --------).

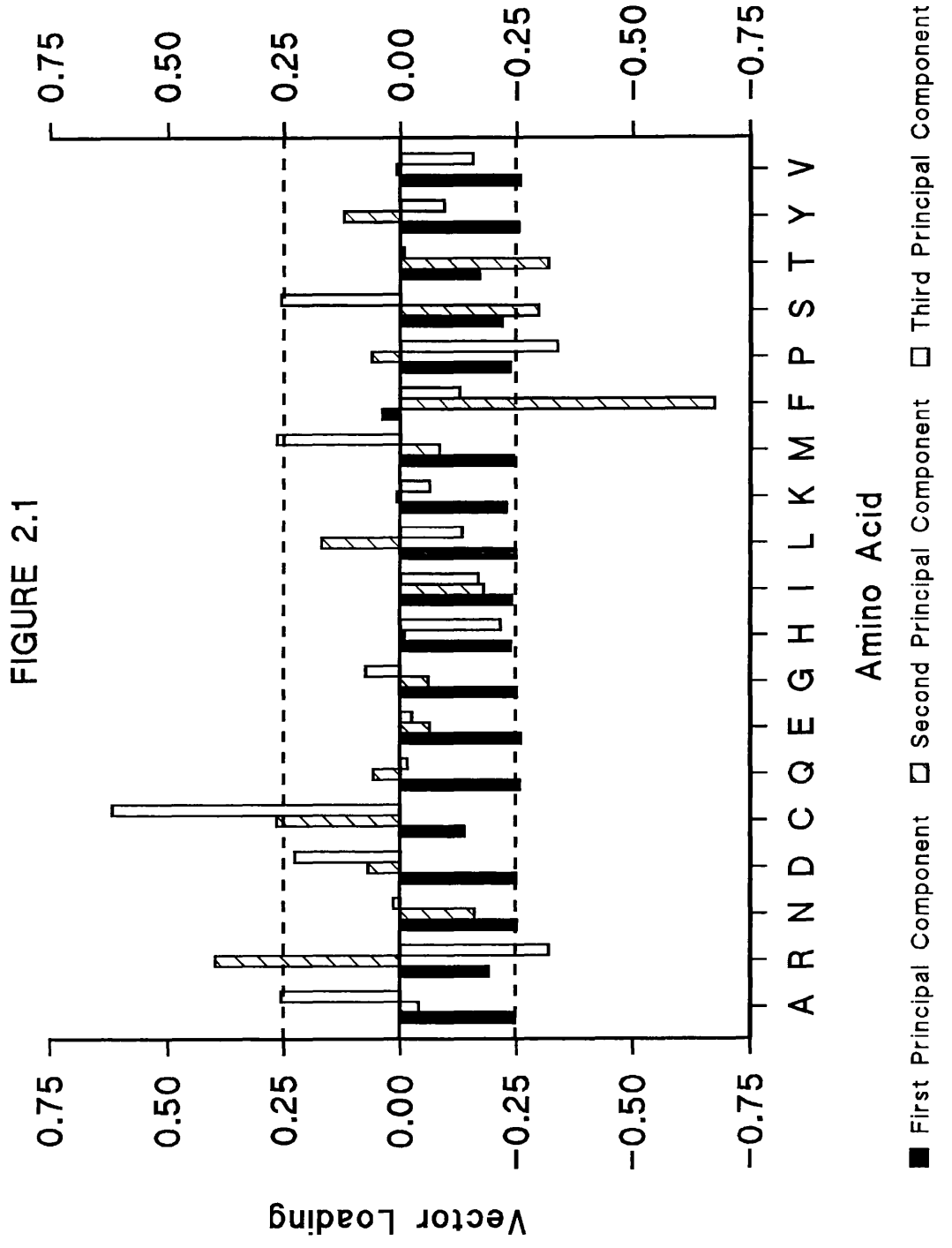


FIGURE 2.1
Vector Loading
0.75
0.50
0.25
0.00
−0.25
−0.50
−0.75
A R N D C Q E G H I L K M F P S T Y V
Amino Acid
First Principal Component
Second Principal Component
Third Principal Component

Figure 2.2 clearly illustrates the separation of the Cheddar cheese based on their maturity, and additionally shows a heat treatment and pH dependent effect. At either 3 or 6 months of maturation cheese manufactured from milk processed by the high heat treatment regime (90^0C/60sec) lay further to the right on the space map, compared to similarly aged cheese samples manufactured from milk heated under pasteurisation conditions (72^0C/16sec). Decreasing the milk pH from 6.4 to 5.8 had the overall effect of enhancing this progressive movement within the two clusters. Examination of the amino acid concentrations (Appendix II, Tables II.1–II.4) shows that, in general, reducing the pH of the milk at cheese manufacture resulted in decreased levels of amino acids.

Changes to the amino acid concentrations in the Cheddar cheese for scores on the second and third Principal Components are shown in Figure 2.3. At 3 months of maturation the cheese samples fell within two coherent clusters, the members of which were determined by which of the two heating regimes had been applied. By 6 months of maturation these two clusters had moved progressively within the amino acid concentration space map, maintaining their relative isolation. Cheese samples 6h▼ and 6h◆ were obvious outliers from these observations.

Figure 2.3 clearly illustrates the separation of the Cheddar cheese based on both their maturity and heat treatment. At either 3 or 6 months of maturation Cheddar cheese manufactured from milk processed by the two heat treatment regimes lay within their own discrete clusters. Cheddar cheese manufactured from milk treated at 90^0C/60sec lay further to the left on the space map, compared to similarly aged cheese manufactured from milk heated under pasteurisation conditions (72^0C/16sec). Additionally, Figure 2.3 showed a pH dependent effect, as increasing the milk pH from 5.8 to 6.4 had the effect of generally enhancing this leftward progressive movement. Examination of the amino acid concentrations (Appendix II, Tables II.1–II.4) shows that increasing the temperature and time of heating of the milk is associated with an overall lowering of the amino acid concentrations.

Tables 2.3–2.5 show the influence of treatment effects on the concentrations of the eight key amino acids identified in Figure 2.1 as contributing to the first, second and third Principal Components, respectively. The variation in concentration of these amino acids in Cheddar cheese manufactured under the different heat treatment regimes, pH values and rennet levels are shown in Figures 2.4–2.7. Maturity had a significant effect

FIGURE 2.2: Simplification of the effect of high heat treatment and pH adjustment on amino acid concentrations in low-fat Cheddar cheese.

Analysis of changes to the amino acid concentrations over 6 months of maturation by Principal Component Analysis; cheese scores on first and second Principal Components are shown, together with a standard error ellipse. Samples are coded by heat treatment, pH and time of maturation.

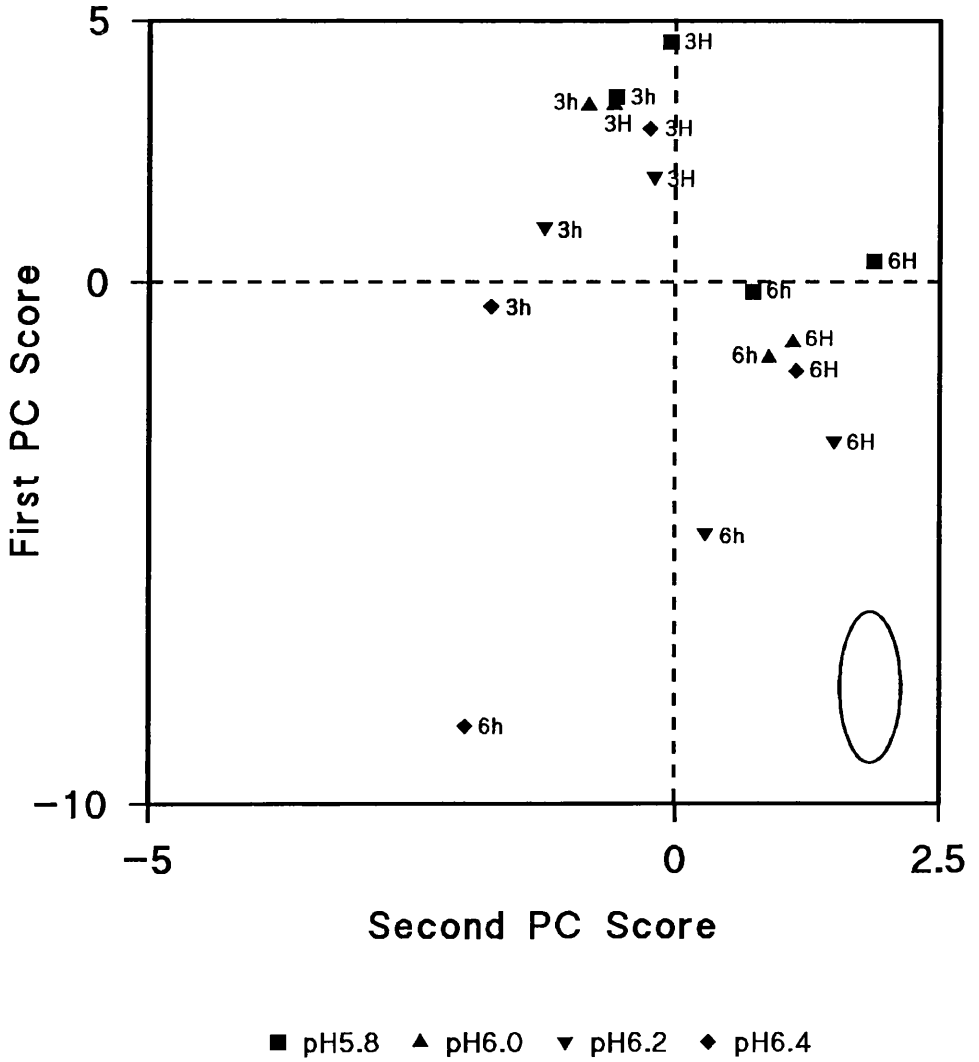
3 = 3 months maturation.

6 = 6 months maturation.

h = 72^0C/16sec heat treatment.

H = 90^0C/60sec heat treatment.

FIGURE 2.2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0
−10
First PC Score
−5
0
2.5
Second PC Score
3H
3h
3h
3H
3H
3H
3h
3h
6H
6h
6H
6h
6H
6H
6H
6h
6h
pH5.8
pH6.0
pH6.2
pH6.4

FIGURE 2.3: Simplification of the effect of high heat treatment and pH adjustment on amino acid concentrations in low-fat Cheddar cheese.

Analysis of changes to the amino acid concentrations over 6 months of maturation by Principal Component Analysis; cheese scores on second and third Principal Components are shown, together with a standard error ellipse. Samples are coded by heat treatment, pH and time of maturation.

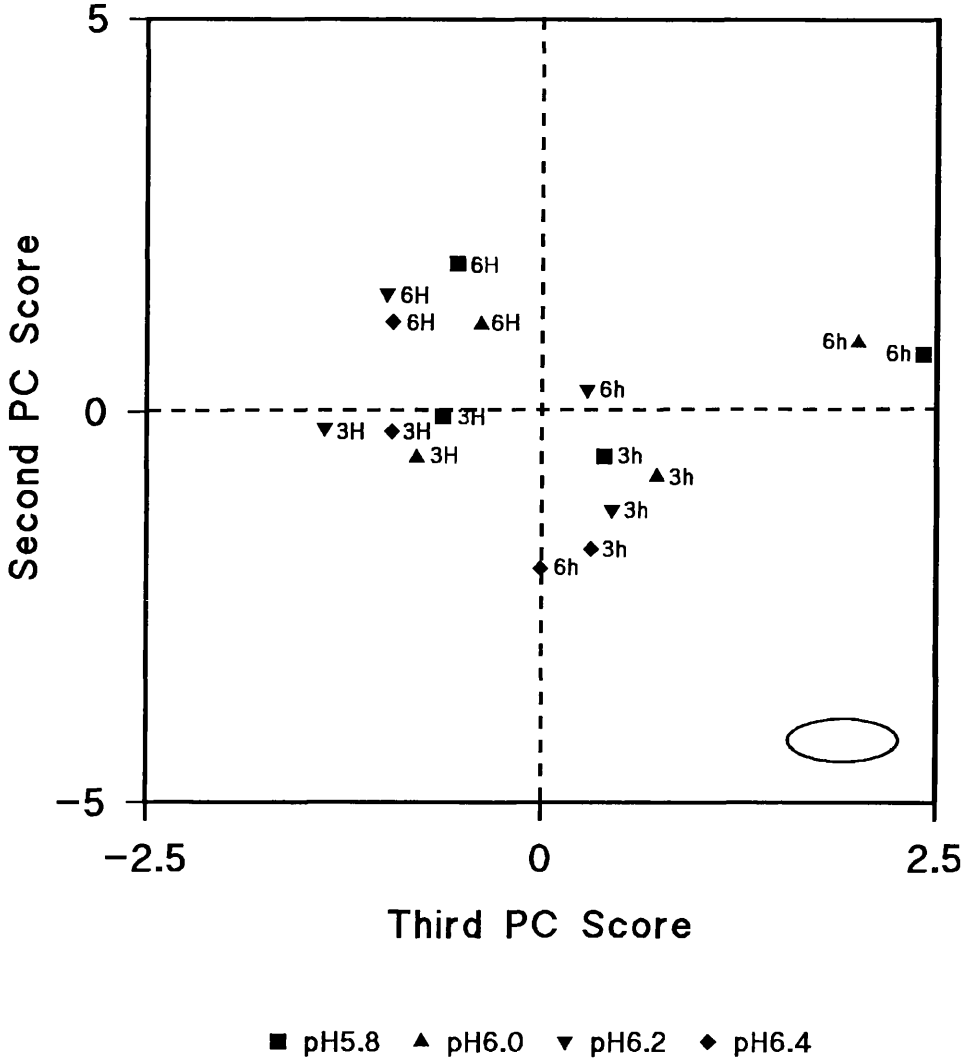
3 = 3 months maturation.

6 = 6 months maturation.

h = 72^0C/16sec heat treatment.

H = 90^0C/60sec heat treatment.

FIGURE 2.3


5
0
−5
−2.5
0
2.5
Second PC Score
Third PC Score
6H
6H
6H
6H
6h
6h
6h
3H
3H
3H
3H
3h
3h
3h
3h
6h
■ pH5.8 ▲ pH6.0 ▼ pH6.2 ◆ pH6.4

on the concentrations of all eight of these important amino acids, with their levels increasing between 3 and 6 months of ripening. Additionally, heat treatment, pH adjustment, rennet level, and the combined treatment effects of pH x heat and heat x rennet also had a significant effect on the levels of the majority of these amino acids.

Figures 2.4–2.7 allow a comparison of the effect of heat treatment on free amino acid production in Cheddar cheese manufactured from milk possessing equivalent levels of added rennet. Comparison of the two heat treatments, at either 3 or 6 months maturation, showed the concentrations of the eight key amino acids to be universally lower in the 90^{0}C/60sec heat-treated milk Cheddar cheese. Reference to Appendix II, Tables II.1–II.4 shows that with few exceptions this is the case for all nineteen amino acids measured.

Figures 2.8 and 2.9 illustrate the combined influence of pH adjustment and heat treatment on the mean concentrations of these eight key amino acids after 6 months of maturation. They show that raising the pH from 5.8 to 6.4 generally resulted in higher amino acid levels, with the 72^{0}C/16sec heat treatment producing higher concentrations than the 90^{0}C/60sec heat treatment regime. The influence of pH adjustment and heat treatment on the other amino acids is shown in Appendix II, Figures II.9–II.11.

Figures 2.10 and 2.11 illustrate the combined influence of heat treatment and rennet level on the mean concentrations of the eight key amino acids after 6 months of maturation. For the 90^{0}C/60sec heat treatment regime, changing the rennet level from 50 to 100% resulted in increased amino acid levels. However, while Cheddar cheese manufactured from the 72^{0}C/16sec heat-treated milk had higher concentration levels at the 50% rennet level compared to the 90^{0}C/60sec heat treatment regime, increasing it to 100% generally resulted in these levels decreasing. The influence of heat treatment and rennet level on the other amino acids is shown in Appendix II, Figures II.12–II.14.

Table 2.6 provides a summary of the influence of treatment effects on amino acid concentrations in low-fat Cheddar cheese over the 6 month maturation period.

Heating milk to temperatures higher than those normally used for pasteurisation (i.e. 72^{0}C/16sec) has generally not been favoured for Cheddar production. Excessive heating causes changes, including the interaction of β-lactoglobulin with κ-casein (Hindle and Wheelock, 1970a and b), resulting in slower coagulation, impaired syneresis and weaker curds. However, Banks *et al.* (1987) showed that the casein of extensively heated

milk is susceptible to rennet action if the pH of the milk is decreased prior to renneting. While subsequent sensory analysis of the resulting Cheddar cheese indicated a bitter off-flavour, this was attributed to the acidification process rather than the consequence of the whey protein incorporation. This chapter has shown that heat-treated, pH-adjusted milk used in Cheddar cheese manufacture is associated with lower levels of free amino acids. Therefore, an alternative interpretation could be that the heat-induced whey protein interactions with the casein establish a different proteolytic pathway, resulting in the formation of different peptides. This could possibly be caused by the unavailability of susceptible peptide bonds to cleavage, due to steric hindrance resulting from the close proximity of the attached whey protein.

Acidification of the milk has been shown to increase chymosin retention in the curd (Creamer *et al.*, 1985), resulting in increased proteolysis. In particular a more extensive breakdown of the α_{s1}-casein is observed, which may be related to the production of bitter off-flavours. Banks (1988) found it possible to eliminate this flavour defect in Cheddar cheese made from milk containing heat-denatured whey protein, by reducing the level of rennet used in cheese manufacture. However, the combined action of the modifying treatments was found to prevent the development of a typical Cheddar flavour.

The experimental results clearly showed that Cheddar cheese manufactured from high heat-treated milk had lower amino acid concentrations on reducing the rennet level from 100 to 50%. Generally, increased proteolysis leads to the production of more peptides, and ultimately higher levels of free amino acids. Reducing the rennet level by half may actually prevent adequate proteolysis occurring. Therefore, while eliminating the production of bitter off-flavours, it may reduce proteolysis to such an extent that creation of the peptide profile defining a typical Cheddar flavour is not then possible.

TABLE 2.3: Influence of treatment effects on amino acid concentrations in low fat Cheddar cheese contributing to the first Principal Component.

Treatment	Level	Amino Acid		
		Alanine	Cysteine	Phenylalanine
	Code	Ala	Cys	Phe
	Loading	-0.25	-0.14	0.04
pH	5.8	1.78	0.43	4.07
	6.0	2.14	0.42	4.25
	6.2	2.52	0.38	4.25
	6.4	2.72	0.37	4.38
Age	3 Months	1.60	0.28	4.91
	6 Months	2.98	0.52	3.56
Heat	72^0C/16sec	2.78	0.48	4.48
	90^0C/60sec	1.80	0.32	4.00
Rennet	50%	2.21	0.41	3.81
	100%	2.36	0.39	4.66
Significance of Treatment[a]				
pH		***	NS	NS
Age		***	***	***
Heat		***	***	*
Rennet		NS	NS	**
pH x age		*	NS	NS
pH x heat		**	NS	*
pH x rennet		NS	NS	NS
Age x heat		***	NS	NS
Age x rennet		NS	NS	NS
Heat x rennet		***	NS	NS
Age x heat x rennet		***	NS	NS

[a] NS = Not Significant, $p>0.05$; * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$.

TABLE 2.4: Influence of treatment effects on amino acid concentrations in low fat Cheddar cheese contributing to the second Principal Component.

Treatment	Level	Amino Acid				
		Arginine	Cysteine	Phenylalanine	Serine	Threonine
	Code	Arg	Cys	Phe	Ser	Thr
	Loading	0.40	0.27	-0.67	-0.30	-0.32
pH	5.8	1.64	0.43	4.07	1.51	5.68
	6.0	1.54	0.42	4.25	1.88	6.50
	6.2	1.95	0.38	4.25	1.93	7.15
	6.4	1.73	0.37	4.38	2.41	10.37
Age	3 Months	1.24	0.28	4.91	1.49	6.21
	6 Months	2.18	0.52	3.56	2.37	8.64
Heat	72^{0}C/16sec	1.48	0.48	4.48	2.49	8.44
	90^{0}C/60sec	1.94	0.32	4.00	1.38	6.41
Rennet	50%	1.56	0.41	3.81	1.69	6.91
	100%	1.87	0.39	4.66	2.18	7.94
Significance of Treatment[a]						
pH		*	NS	NS	**	*
Age		***	***	***	***	*
Heat		***	***	*	***	NS
Rennet		**	NS	**	**	NS
pH x age		NS	NS	NS	NS	NS
pH x heat		NS	NS	*	**	NS
pH x rennet		NS	NS	NS	NS	NS
Age x heat		NS	NS	NS	NS	NS
Age x rennet		NS	NS	NS	NS	NS
Heat x rennet		***	NS	NS	*	NS
Age x heat x rennet		NS	NS	NS	NS	NS

[a] NS = Not Significant, $p>0.05$; * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$.

TABLE 2.5: Influence of treatment effects on amino acid concentrations in low fat Cheddar cheese contributing to the third Principal Component.

Treatment	Level	Amino Acid				
		Arginine	Cysteine	Methionine	Proline	Serine
	Code	Arg	Cys	Met	Pro	Ser
	Loading	-0.32	0.62	0.27	-0.34	0.26
pH	5.8	1.64	0.43	0.60	2.16	1.51
	6.0	1.54	0.42	0.75	2.74	1.88
	6.2	1.95	0.38	0.96	4.07	1.93
	6.4	1.73	0.37	1.01	4.80	2.41
Age	3 Months	1.24	0.28	0.57	2.36	1.49
	6 Months	2.18	0.52	1.10	4.53	2.37
Heat	72^0C/16sec	1.48	0.48	1.04	3.22	2.49
	90^0C/60sec	1.94	0.32	0.62	3.67	1.38
Rennet	50%	1.56	0.41	0.84	3.27	1.69
	100%	1.87	0.39	0.82	3.62	2.18
Significance of Treatment[a]						
pH		*	NS	**	***	**
Age		***	***	***	***	***
Heat		***	***	***	NS	***
Rennet		**	NS	NS	NS	**
pH x age		NS	NS	NS	NS	NS
pH x heat		NS	NS	*	*	**
pH x rennet		NS	NS	NS	NS	NS
Age x heat		NS	NS	NS	NS	NS
Age x rennet		NS	NS	NS	NS	NS
Heat x rennet		***	NS	***	**	*
Age x heat x rennet		NS	NS	NS	NS	NS

[a] NS = Not Significant, $p>0.05$; * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$.

FIGURE 2.4: Concentrations of the eight key amino acids identified by Principal Component Analysis in heat-treated pH-adjusted low-fat Cheddar cheese at 3 months maturation.

(A) 72^0C/16sec heat-treated, 100% Maxiren.

(B) 90^0C/60sec heat-treated, 100% Maxiren.

Ala = Alanine.

Arg = Arginine.

Cys = Cysteine.

Met = Methionine.

Phe = Phenylalanine.

Pro = Proline.

Ser = Serine.

Thr = Threonine.

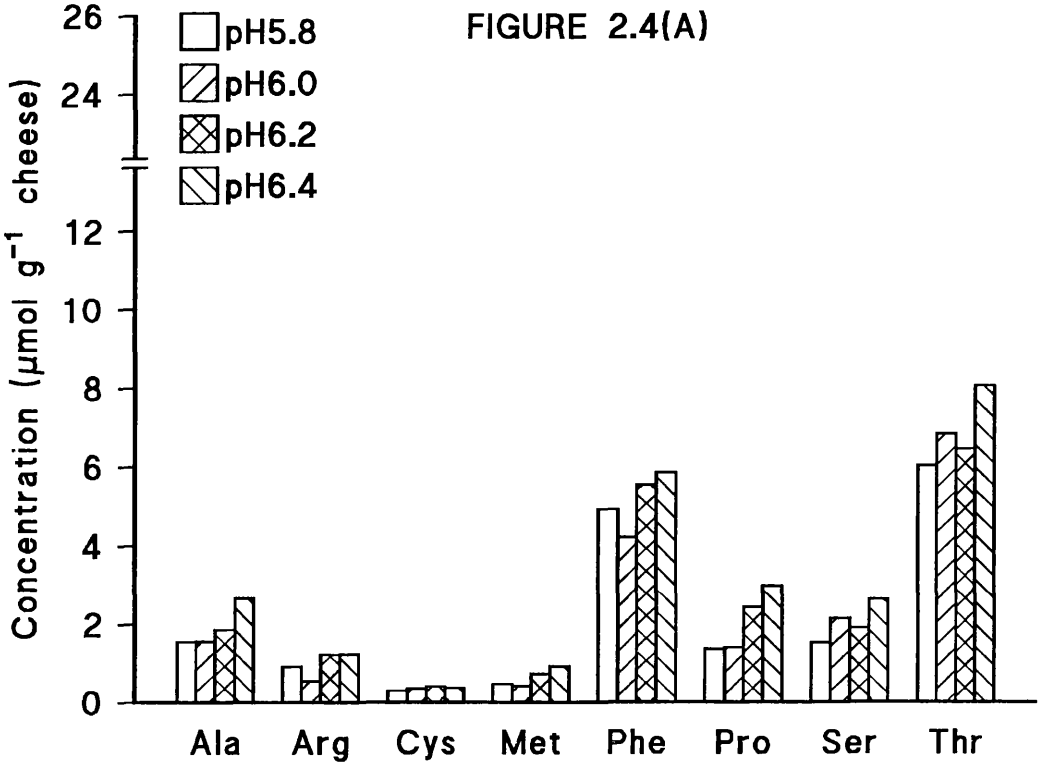


FIGURE 2.4(A)

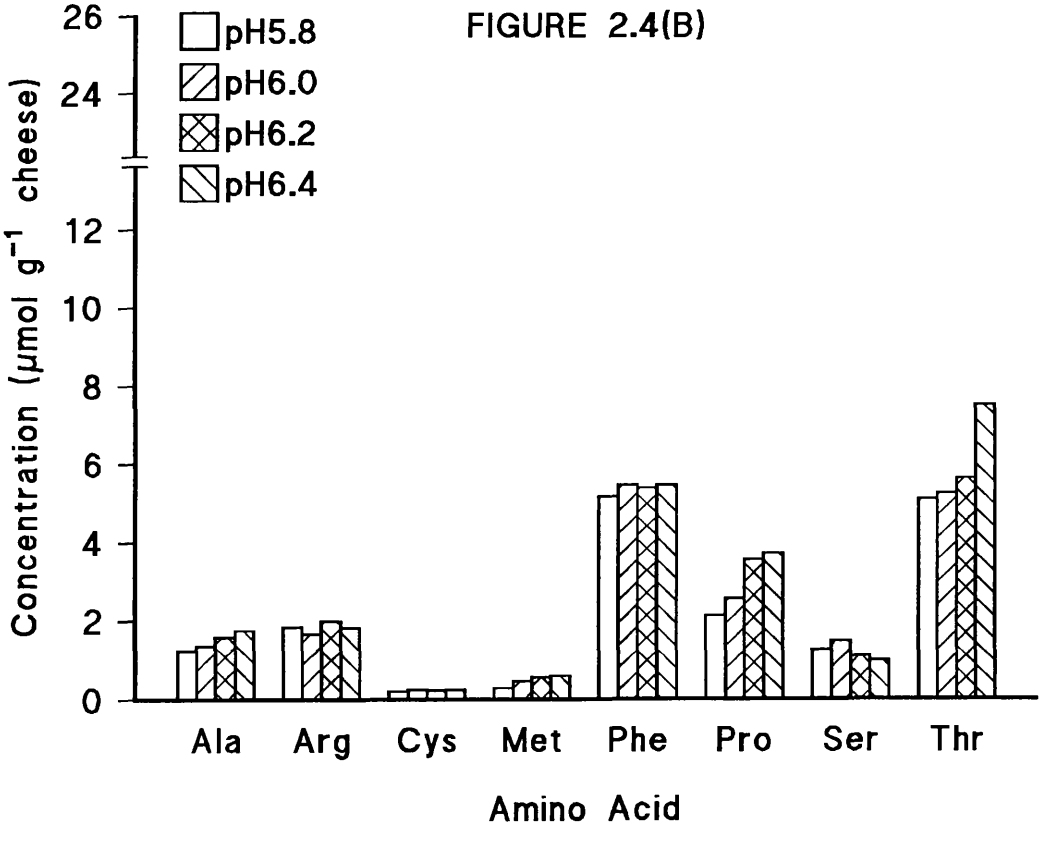


FIGURE 2.4(B)

FIGURE 2.5: Concentrations of the eight key amino acids identified by Principal Component Analysis in heat-treated pH-adjusted low-fat Cheddar cheese at 3 months maturation.

(A) 72^0C/16sec heat-treated, 50% Maxiren.

(B) 90^0C/60sec heat-treated, 50% Maxiren.

Ala = Alanine.

Arg = Arginine.

Cys = Cysteine.

Met = Methionine.

Phe = Phenylalanine.

Pro = Proline.

Ser = Serine.

Thr = Threonine.

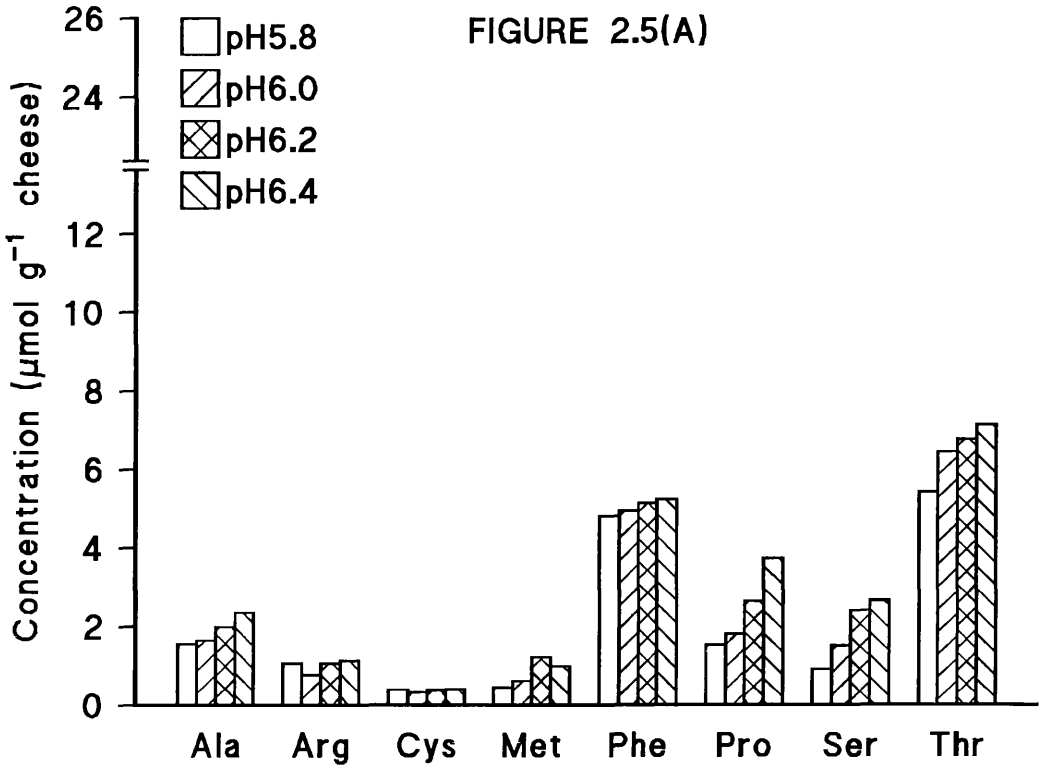


FIGURE 2.5(A)

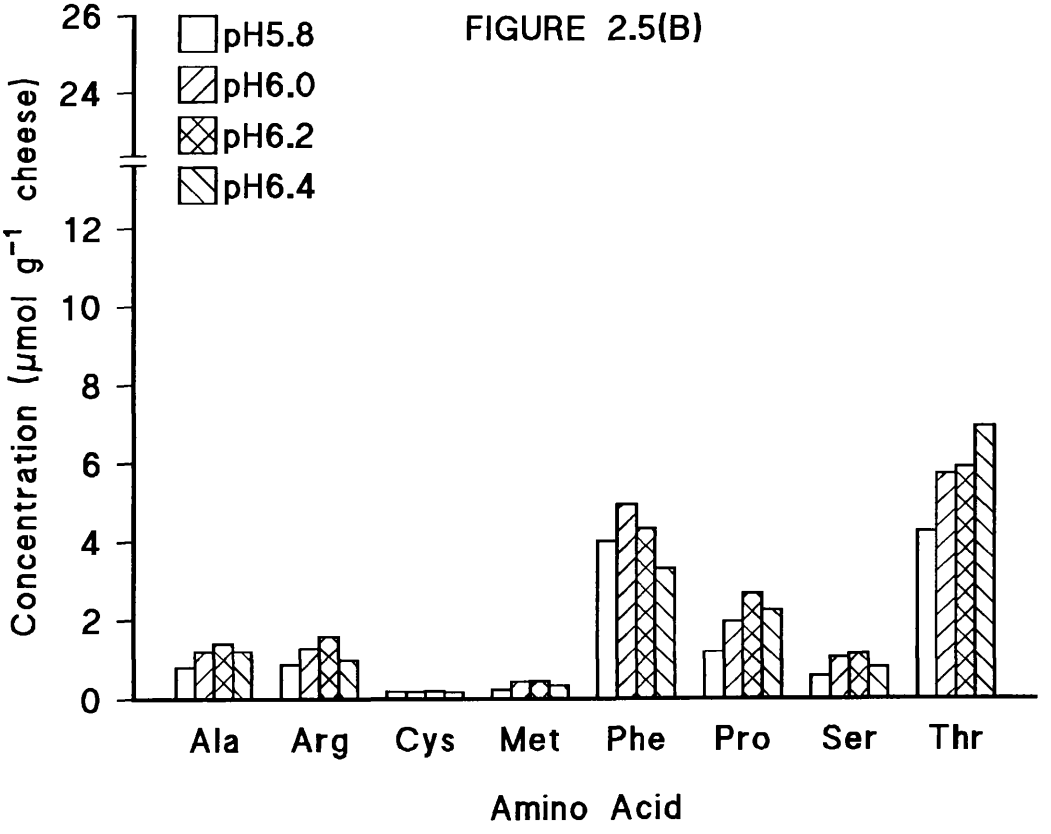


FIGURE 2.5(B)

FIGURE 2.6: Concentrations of the eight key amino acids identified by Principal Component Analysis in heat-treated pH-adjusted low-fat Cheddar cheese at 6 months maturation.

(A) 72^0C/16sec heat-treated, 100% Maxiren.

(B) 90^0C/60sec heat-treated, 100% Maxiren.

Ala = Alanine.

Arg = Arginine.

Cys = Cysteine.

Met = Methionine.

Phe = Phenylalanine.

Pro = Proline.

Ser = Serine.

Thr = Threonine.

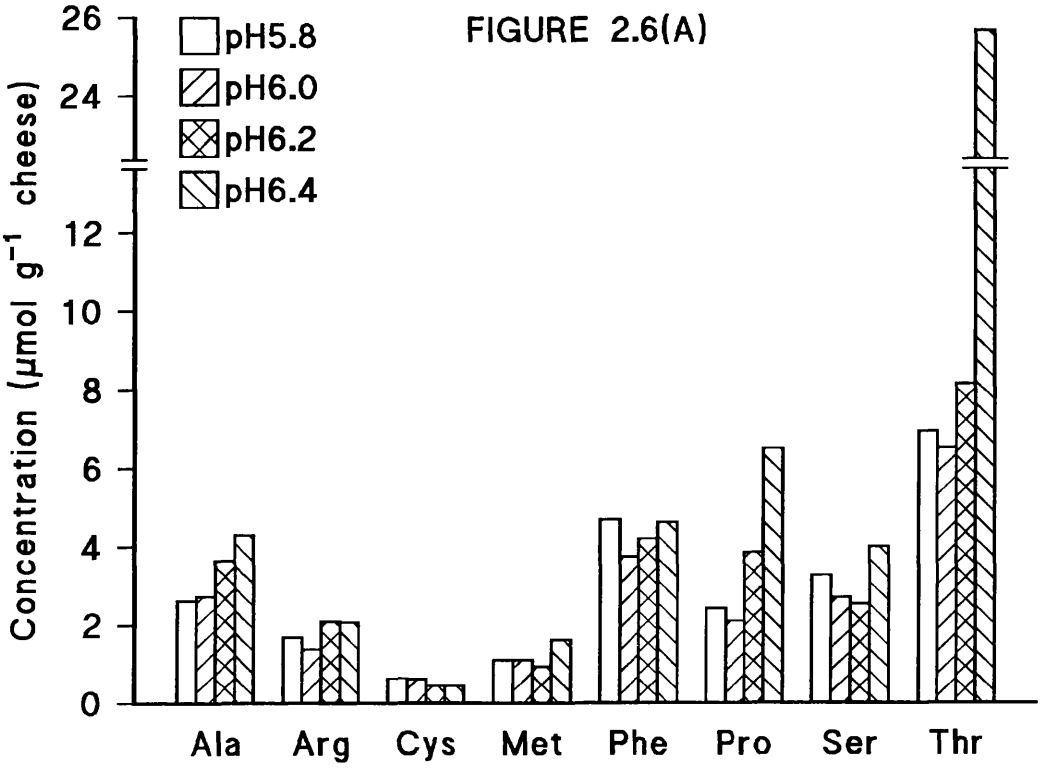


FIGURE 2.6(A)

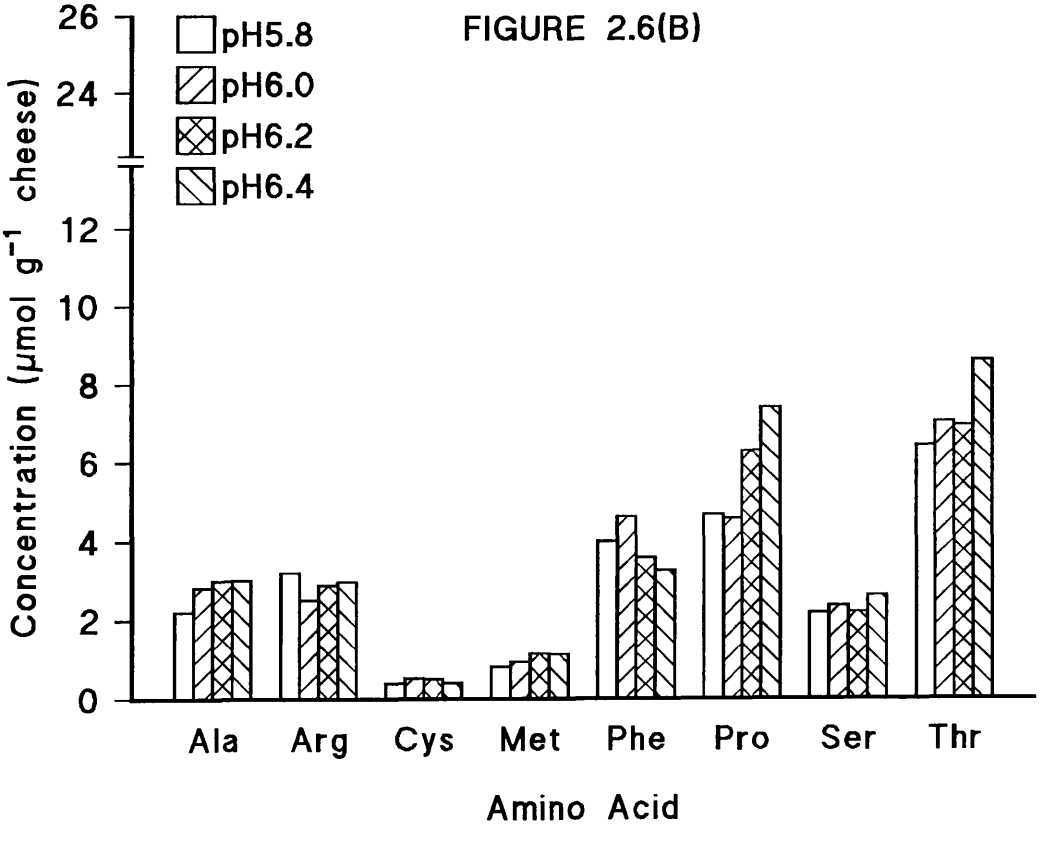


FIGURE 2.6(B)

FIGURE 2.7: Concentrations of the eight key amino acids identified by Principal Component Analysis in heat-treated pH-adjusted low-fat Cheddar cheese at 6 months maturation.

(A) 72^0C/16sec heat-treated, 50% Maxiren.

(B) 90^0C/60sec heat-treated, 50% Maxiren.

Ala = Alanine.

Arg = Arginine.

Cys = Cysteine.

Met = Methionine.

Phe = Phenylalanine.

Pro = Proline.

Ser = Serine.

Thr = Threonine.

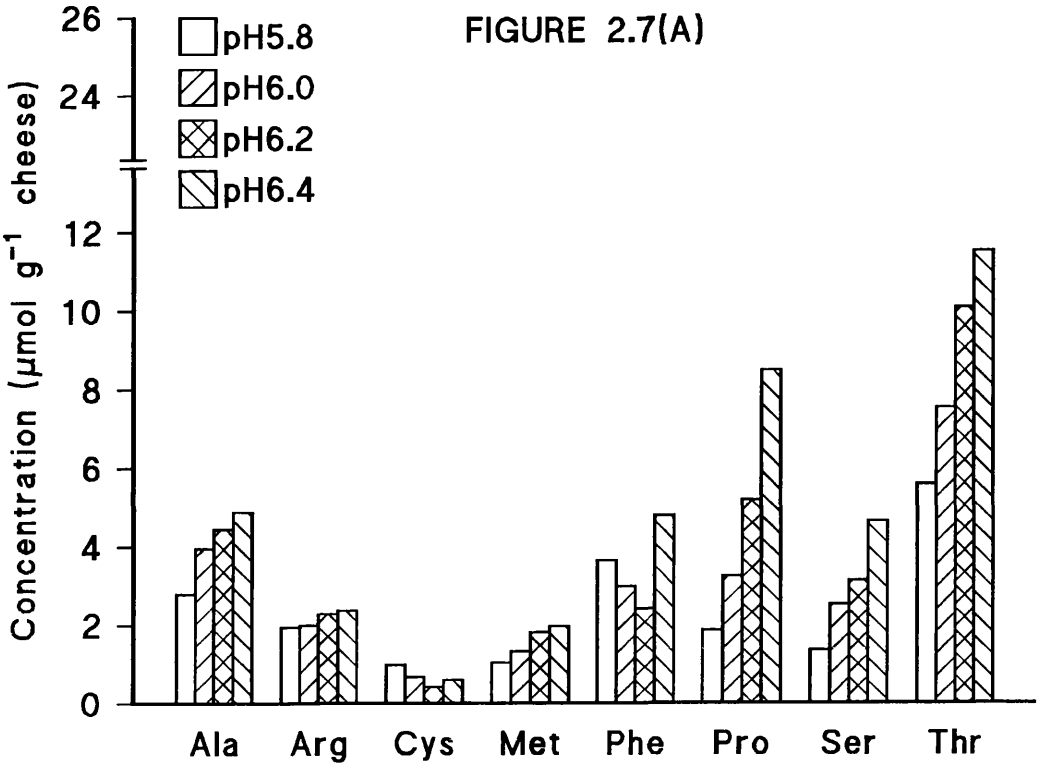


FIGURE 2.7(A)

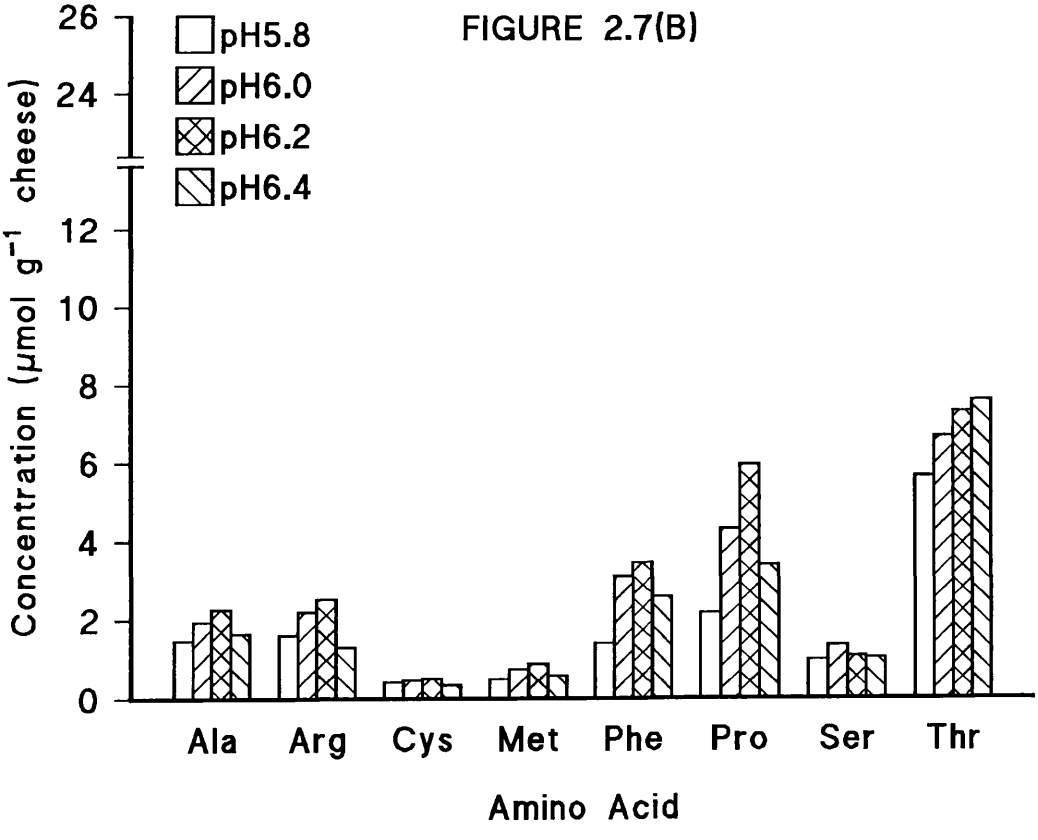


FIGURE 2.7(B)

FIGURE 2.8: Influence of the combined effects of pH adjustment and heat treatment on amino acid concentrations in low-fat Cheddar cheese.

Analysis of changes to the mean amino acid concentrations over 6 months of maturation by Principal Component Analysis.

(A) Alanine.

(B) Arginine.

(C) Cysteine.

(D) Methionine.

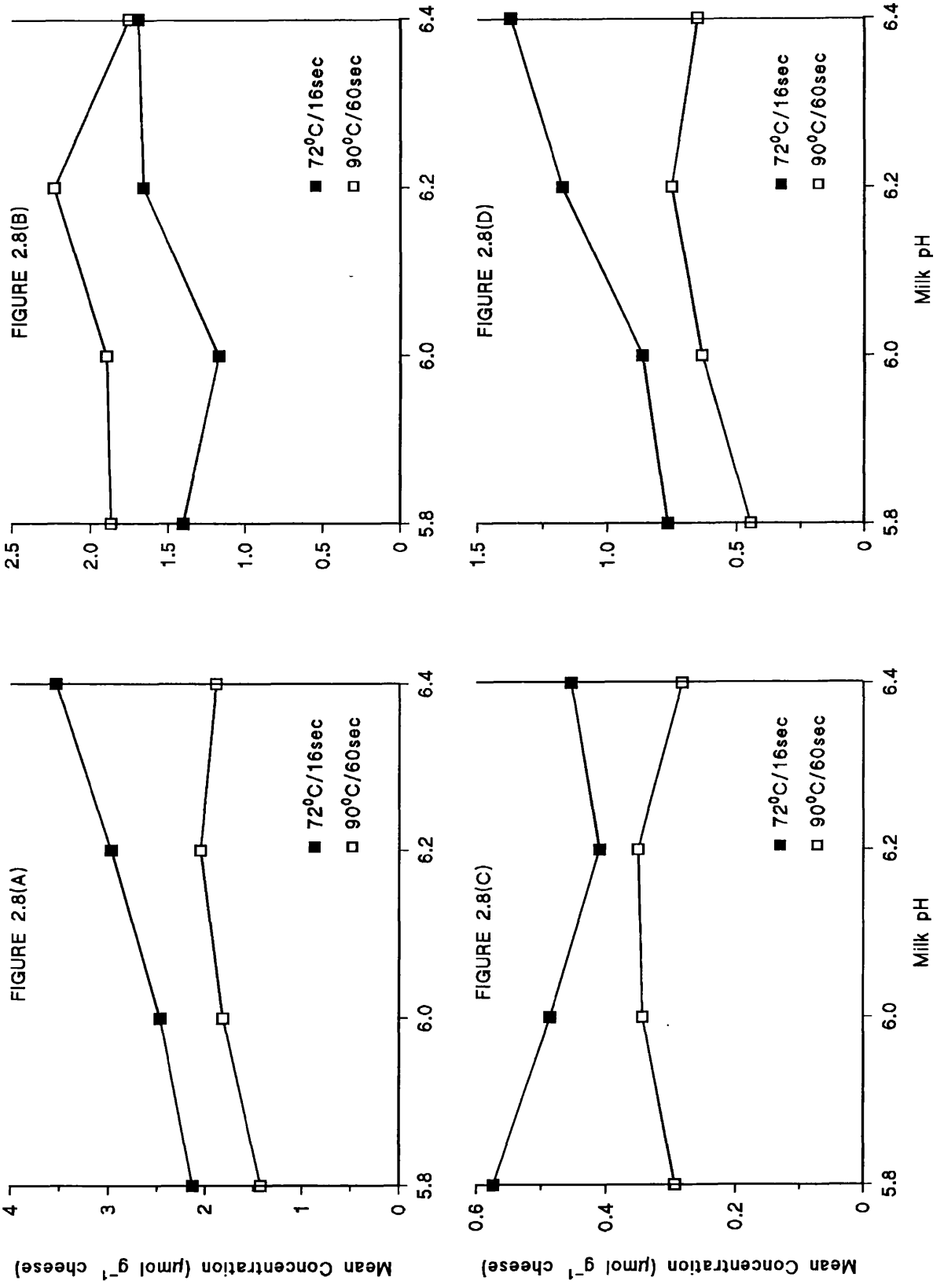


FIGURE 2.8(A)
FIGURE 2.8(B)
FIGURE 2.8(C)
FIGURE 2.8(D)
■ 72°C/16sec
□ 90°C/60sec
Mean Concentration (μmol g⁻¹ cheese)
Milk pH
0
1
2
3
4
0.5
1.0
1.5
2.0
2.5
0.2
0.4
0.6
5.8
6.0
6.2
6.4

FIGURE 2.9: Influence of the combined effects of pH adjustment and heat treatment on amino acid concentrations in low-fat Cheddar cheese.

Analysis of changes to the mean amino acid concentrations over 6 months of maturation by Principal Component Analysis.

(A) Phenylalanine.

(B) Proline.

(C) Serine.

(D) Threonine.

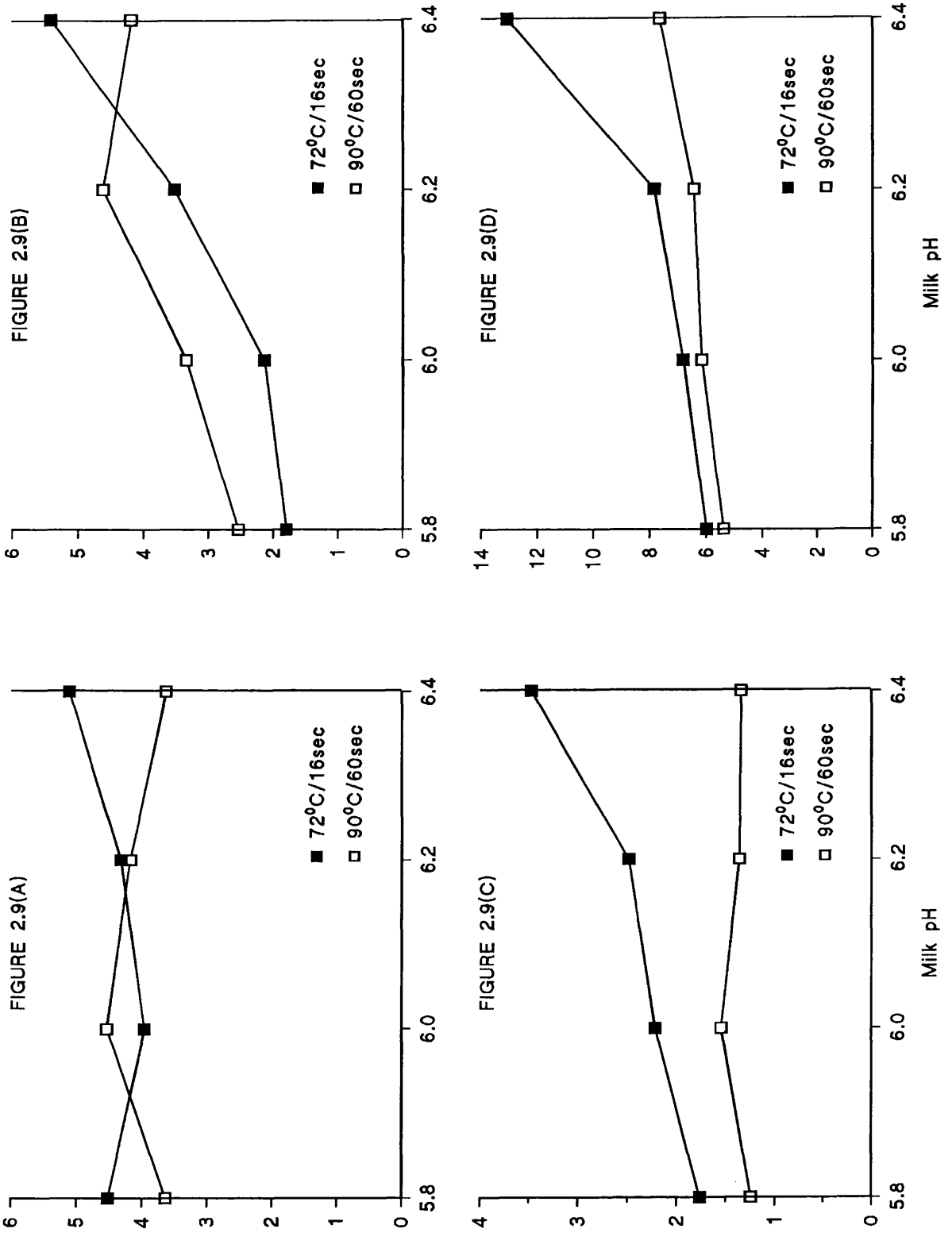


FIGURE 2.9(A)
FIGURE 2.9(B)
FIGURE 2.9(C)
FIGURE 2.9(D)
■ 72°C/16sec
□ 90°C/60sec
Mean Concentration (μmol g⁻¹ cheese)
Milk pH
0
1
2
3
4
5
6
8
10
12
14
5.8
6.0
6.2
6.4

FIGURE 2.10: Influence of the combined effects of rennet level and heat treatment on amino acid concentrations in low-fat Cheddar cheese.

Analysis of changes to the mean amino acid concentrations over 6 months of maturation by Principal Component Analysis.

(A) Alanine.

(B) Arginine.

(C) Cysteine.

(D) Methionine.

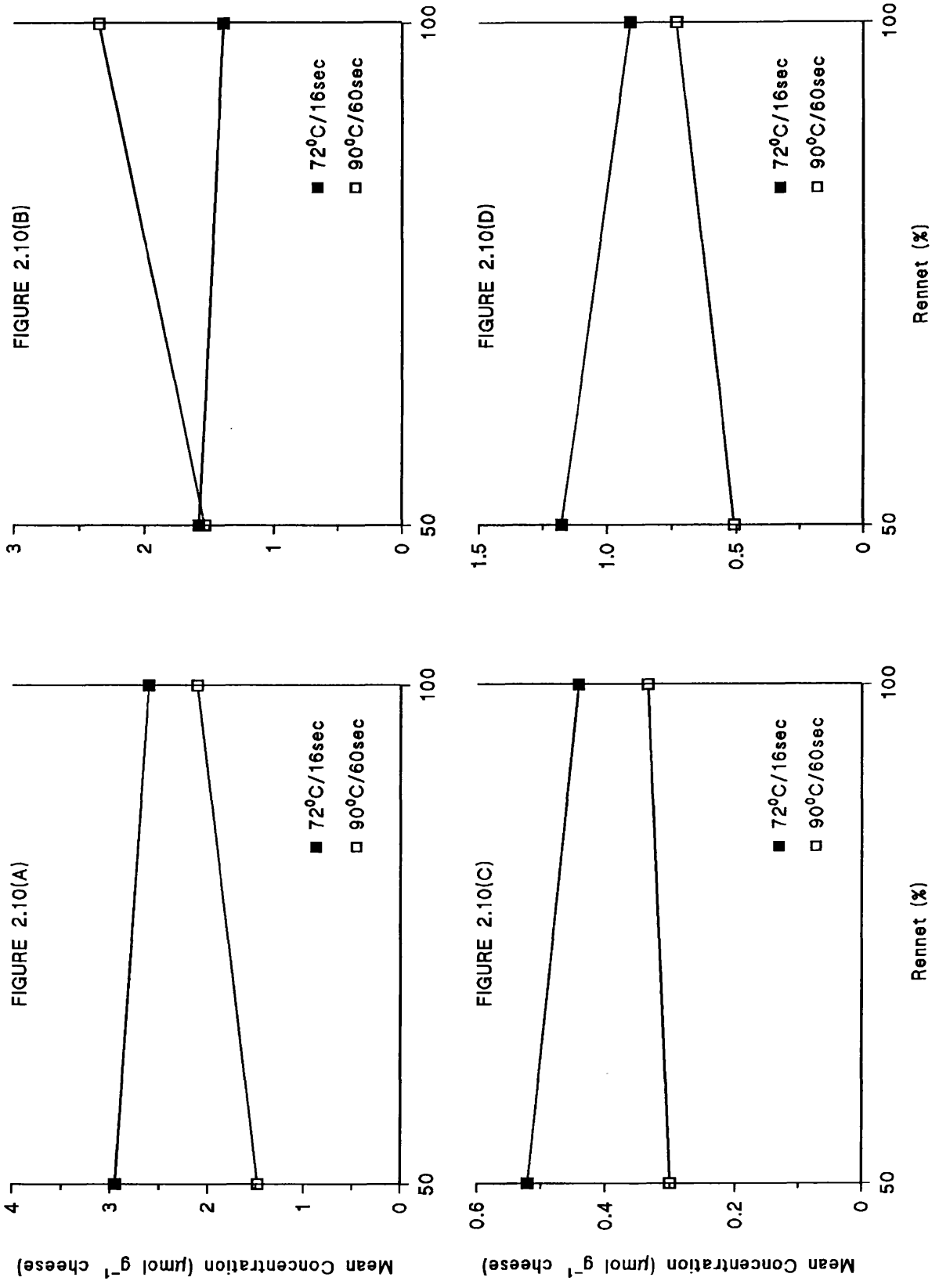


FIGURE 2.10(A)
FIGURE 2.10(B)
FIGURE 2.10(C)
FIGURE 2.10(D)
■ 72°C/16sec
□ 90°C/60sec
Mean Concentration (μmol g⁻¹ cheese)
Rennet (%)
0
1
2
3
4
0.2
0.4
0.6
0.5
1.0
1.5
50
100

FIGURE 2.11: Influence of the combined effects of rennet level and heat treatment on amino acid concentrations in low-fat Cheddar cheese.

Analysis of changes to the mean amino acid concentrations over 6 months of maturation by Principal Component Analysis.

(A) Phenylalanine.

(B) Proline.

(C) Serine.

(D) Threonine.

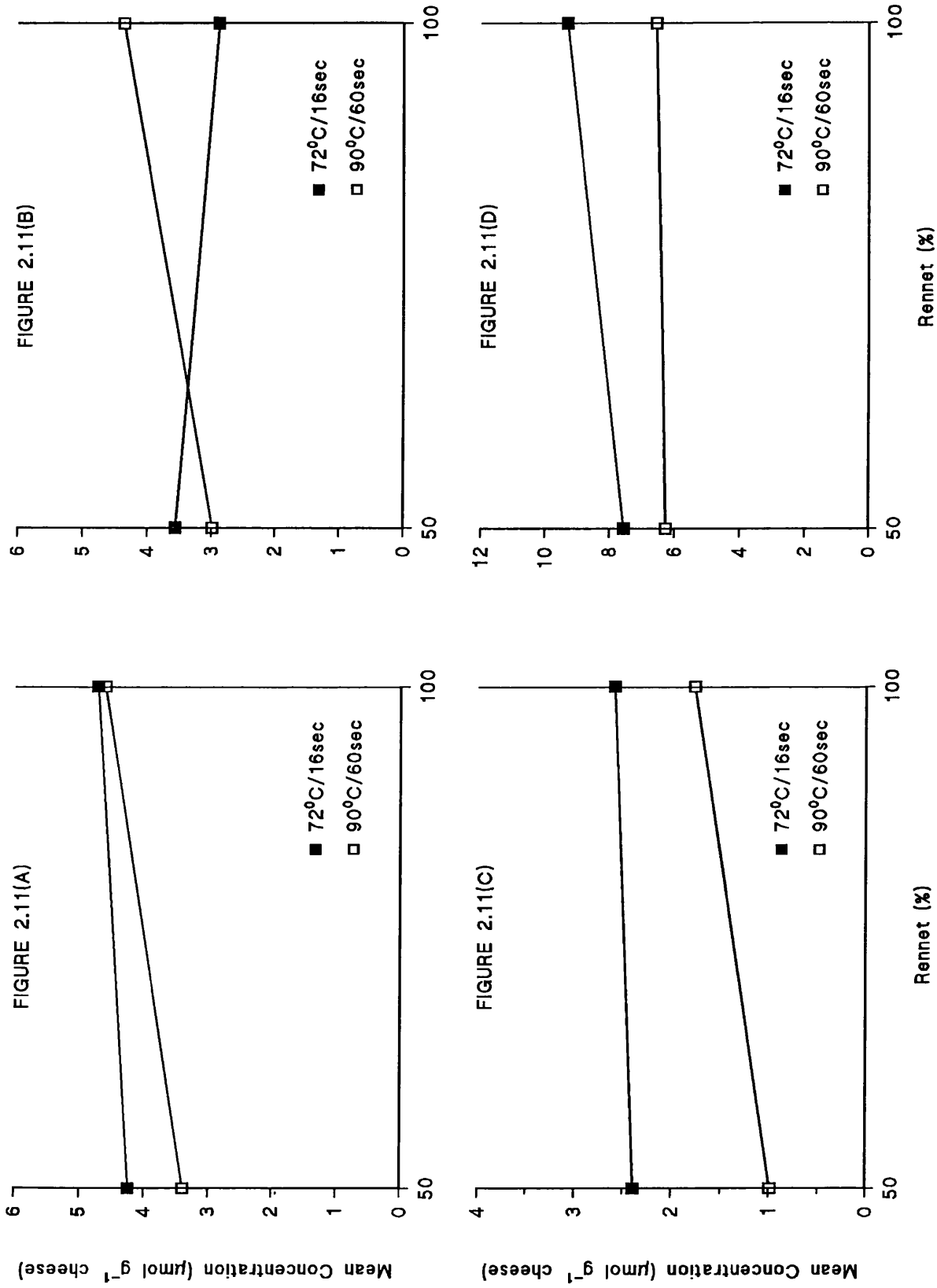


FIGURE 2.11(A)
FIGURE 2.11(B)
FIGURE 2.11(C)
FIGURE 2.11(D)
■ 72°C/16sec
□ 90°C/60sec
Mean Concentration (μmol g⁻¹ cheese)
Rennet (%)
0
1
2
3
4
5
6
50
100

TABLE 2.6: Summary of the influence of treatment effects on amino acid concentrations in low-fat Cheddar cheese up to 6 months maturation.

	Direction of significant effects			
Amino Acid	pH	Age	Heat	Rennet
Ala	↑	↑	↓	NS
Arg	↑/↓	↑	↑	↑
Asn	↑	↑	↓	NS
Asp	↑	↑	↓	↑
Cys	NS	↑	↓	NS
Gln	↑	↑	↓	↑
Glu	↑	↑	↓	NS
Gly	↑	↑	↓	↑
His	↑	↑	NS	NS
Ile	↑	↑	NS	NS
Leu	↑	↑	NS	↑
Lys	↑	↑	↓	NS
Met	↑	↑	↓	NS
Phe	NS	↓	↓	↑
Pro	↑	↑	NS	NS
Ser	↑	↑	↓	↑
Thr	↑	↑	NS	NS
Tyr	↑	↑	NS	↑
Val	↑	↑	NS	↑

↑ = Increased; ↓ = Decreased; NS = Not Significant.

2.3.2 Standard and reduced pH milk Cheddar cheese

The change in concentration of eighteen amino acids in Cheddar cheese manufactured from milk either at the standard or reduced pH, determined as their phenylthiocarbamyl derivatives by RP-HPLC at regular time intervals over a 246 day maturation period, are shown in Appendix II, Tables II.5–II.6 and Figures II.15–II.20. Of the twenty common amino acids, only tryptophan and cysteine were not detected, probably due to their low concentration in casein.

The results shown in Table 2.7 indicate highly significant effects of the age and pH treatment on amino acid composition of the samples. Amino acid composition was greatly influenced by the age of the cheese, while reducing the pH of the milk at manufacture also influenced amino acid composition, although to a lesser extent.

The variance accounted for by the first two principal component dimensions was 95.5%, and 2.3% respectively (97.8% in total). Inspection of the vector loadings, and the correlation coefficients of the scores for the cheese samples obtained from the experimental amino acid concentrations, facilitated interpretation of the dimensions of the PCA.

Figure 2.12 shows the vector loadings for scores on the first two Principal Components for the eighteen amino acids. A vector loading greater than +/-0.25 was considered significant. The first Principal Component showed no discrimination, with all amino acids possessing vector loadings of an essentially similar negative magnitude, and shows a universal relationship to quantity or concentration. Four amino acids made notably significant contributions to the second Principal Component; isoleucine and proline made a positive contribution, while phenylalanine and tyrosine a negative contribution.

Changes to the amino acid concentrations in the standard and reduced pH milk Cheddar cheese for scores on the first two Principal Components are shown in Figure 2.13. The cheese samples can be seen to follow two similar, yet distinctly different lines, which at approximately day 66 of maturation were subject to a noticeable direction change.

Figure 2.14 shows the changes in concentration of these four key amino acids identified by PCA over the 246 days of maturation. Isoleucine and proline concentrations were higher throughout the ripening period in the standard pH milk Cheddar cheese. In

TABLE 2.7: Analysis of variance for amino acid concentrations in pH adjusted milk Cheddar cheese.

Amino Acid[a]	pH	Age
Ala	NS[b]	***
Arg	*	***
Asn/Ser	NS	***
Asp	*	***
Gln	NS	***
Glu	NS	***
Gly	NS	***
His	NS	***
Ile	**	***
Leu	*	***
Lys	**	***
Met	**	***
Phe	***	***
Pro	*	***
Thr	**	***
Tyr	NS	***
Val	NS	***

[a] Determined as phenylthiocarbamyl-derivatised free amino acids by RP-HPLC.

[b] NS = Not Significant, $p>0.05$; * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$.

contrast, the phenylalanine and tyrosine concentration levels were higher overall in the reduced pH milk cheese. Isoleucine and proline are two of the most expressed amino acids in the caseins, while the abundance of phenylalanine and tyrosine by comparison is relatively limited. Consequently, it is highly significant that Figure 2.15, a plot of the (Ile+Pro)–(Phe+Tyr) concentration value against maturation time, essentially mirrors Figure 2.13 when turned clockwise through 90^0.

It is of further interest to note that the next pair of amino acids making a positive and negative contribution to the second Principal Component are histidine, lysine and arginine, leucine, respectively. Their changes in concentration over the 246 days of maturation are shown in Appendix II, Figures II.15–II.20, and can be seen to essentially mirror the trends observed for the four previous key amino acids. A plot of the (His+Lys)–(Arg+Leu) concentration value against maturation time is shown in Figure 2.16, and although similar is noticeably different to Figure 2.13 on subsequent 90^0C clockwise rotation. This provides further evidence of the discriminating power of Principal Component Analysis, strongly suggesting isoleucine, proline; phenylalanine, tyrosine are indeed the amino acids being most affected by the changes in milk pH prior to cheese manufacture.

Chapter 3 provides evidence of the differences in individual amino acid concentrations being closely associated with differences in the peptide precursor supply. The two individual and extremely different experiments discussed in some detail in this chapter would appear to bear this hypothesis out. Their different processing and manufacturing variables lead to different free amino acid profiles, which are presumably the result of variations in initial casein degradation and subsequent peptide formation.

These results suggest that monitoring the changes in concentration of free amino acids during Cheddar cheese ripening would be a useful diagnostic tool in assessing the degree of maturity, and possible deviations from an intended ripening pathway. Such an approach has previously been proposed with respect to monitoring Cheddar cheese ripening, although using only the determination of free glutamate levels (Fritsch *et al.*, 1992). Therefore, the effect of modifying processing variables in order to accelerate the ripening time of cheese varieties could be monitored and assessed in a more quantitative and objective way than currently possible. Adherence, or changes, to an already measured and characterised amino acid profile, could theoretically allow the possible

effects of these manufacturing variables to be assessed. This would certainly be easier than recourse to the more complicated interpretation of qualitative changes in peptide chromatogram profiles during maturation.

FIGURE 2.12: Simplification of the effect of standard and reduced pH milk on amino acid concentrations in Cheddar cheese.

Analysis of changes to the amino acid concentrations over 246 days of maturation by Principal Component Analysis; vector loadings for scores on first two Principal Components.
(+/-0.25 significance level; --------).

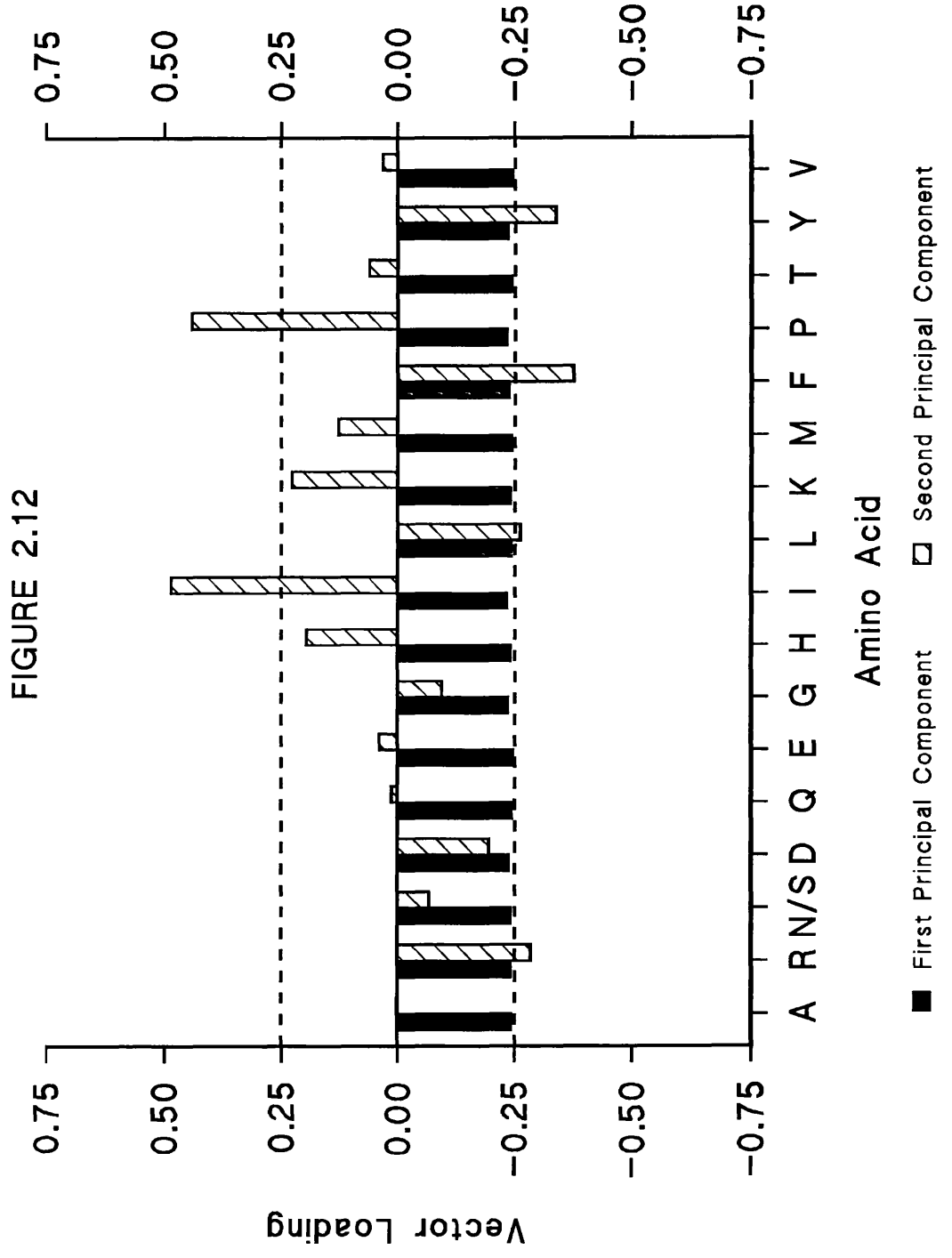


FIGURE 2.12
Vector Loading
0.75
0.50
0.25
0.00
-0.25
-0.50
-0.75
A R N/S D Q E G H I L K M F P T Y V
Amino Acid
First Principal Component
Second Principal Component

FIGURE 2.13: Simplification of the effect of standard and reduced pH milk on amino acid concentrations in Cheddar cheese.

Analysis of changes to the amino acid concentrations over 246 days of maturation by Principal Component Analysis; cheese scores on first and second Principal Components are shown.

8 = Day 8 of maturation.

66 = Day 66 of maturation.

246 = Day 246 of maturation.

FIGURE 2.13

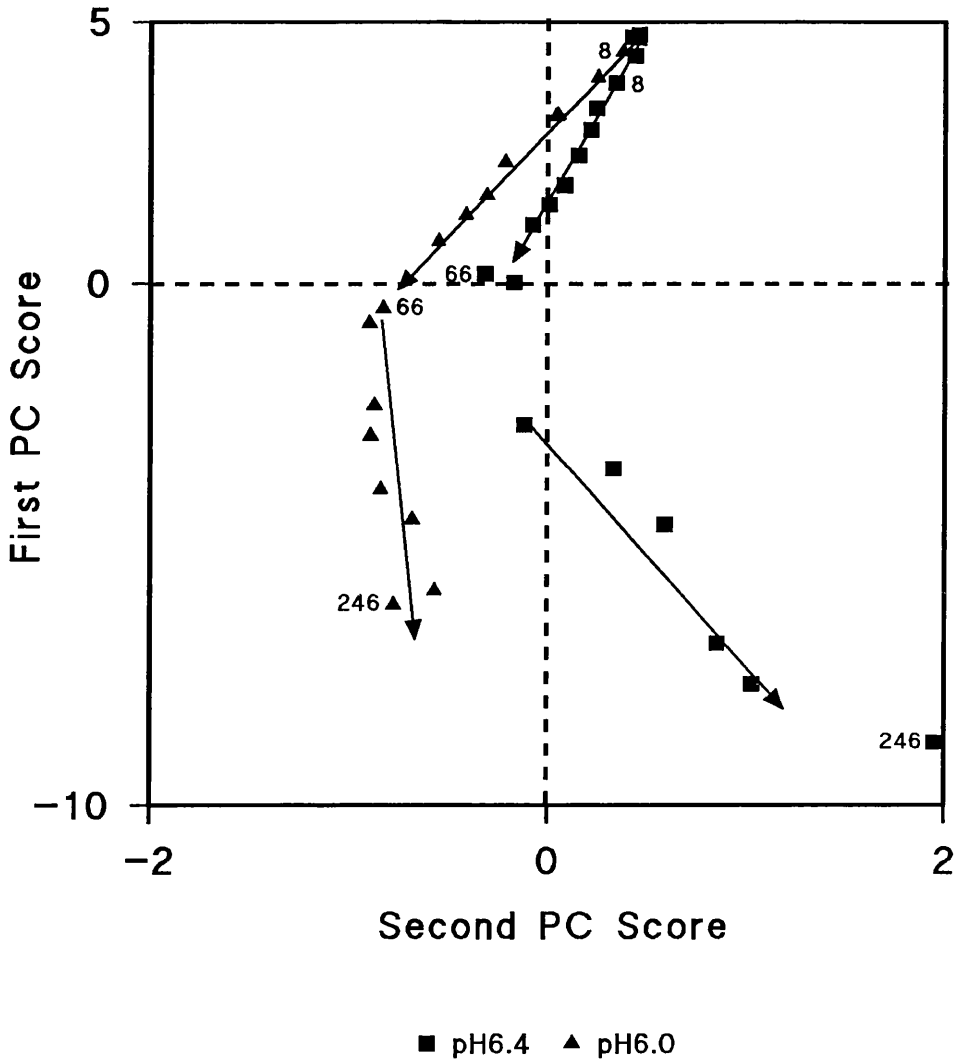

5
0
−10
First PC Score
−2
0
2
Second PC Score
8
8
66
66
246
246
pH6.4
pH6.0

FIGURE 2.14: Changes in concentration of the four key amino acids identified by Principal Component Analysis in standard pH and reduced pH milk Cheddar cheese over 246 days of maturation.

(A) Isoleucine.

(B) Proline.

(C) Phenylalanine.

(D) Tyrosine.

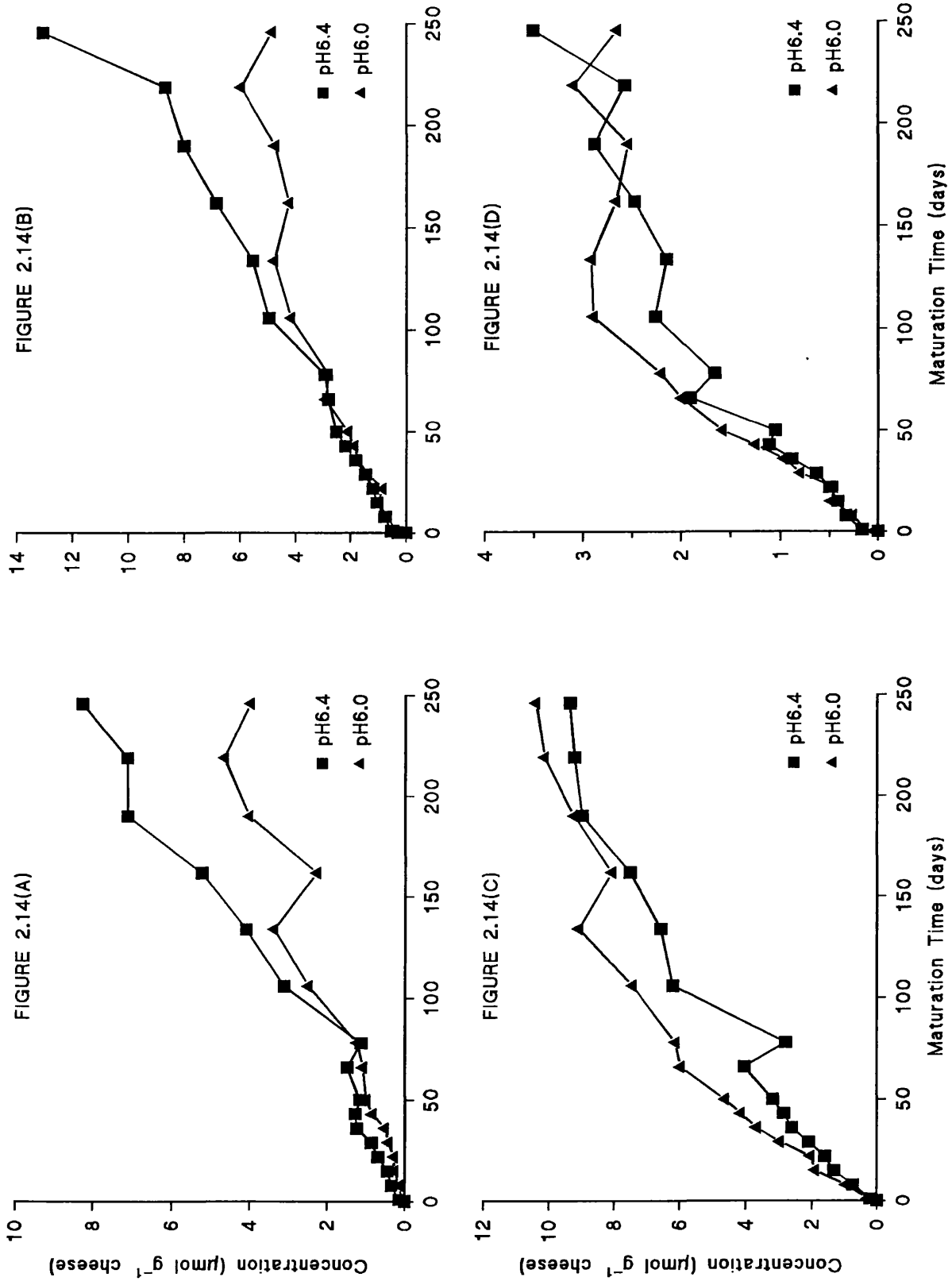


FIGURE 2.14(A)
FIGURE 2.14(B)
FIGURE 2.14(C)
FIGURE 2.14(D)
pH6.4
pH6.0
Concentration (μmol g⁻¹ cheese)
Maturation Time (days)

FIGURE 2.15: The effect of standard and reduced pH milk on the (Ile+Pro)-(Phe+Tyr) amino acid concentration value in Cheddar cheese over 246 days of maturation.

Ile = Isoleucine.

Pro = Proline.

Phe = Phenylalanine.

Tyr = Tyrosine.

FIGURE 2.15

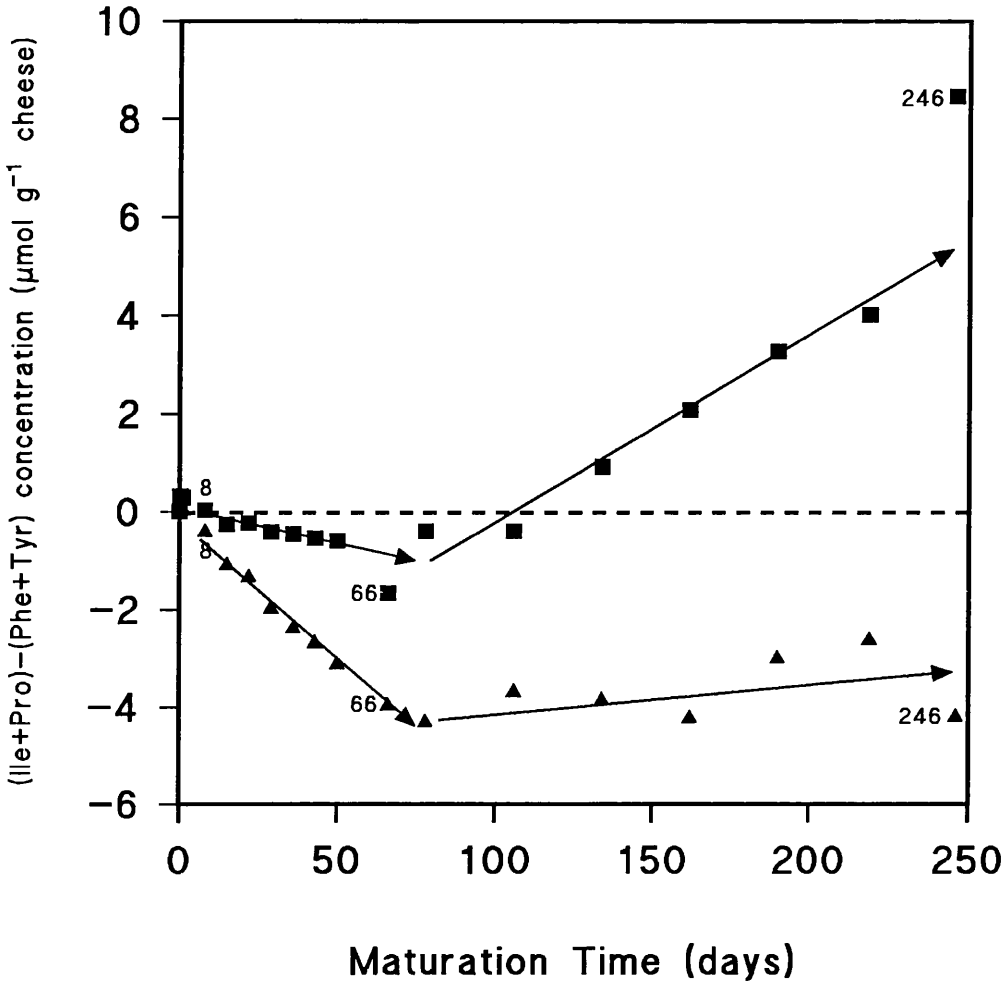

10
8
6
4
2
0
−2
−4
−6
0
50
100
150
200
250
246
8
66
66
246
(Ile+Pro)−(Phe+Tyr) concentration (µmol g^{-1} cheese)
Maturation Time (days)
pH6.4
pH6.0

FIGURE 2.16: The effect of standard and reduced pH milk on the (His+Lys)-(Arg+Leu) amino acid concentration value in Cheddar cheese over 246 days of maturation.

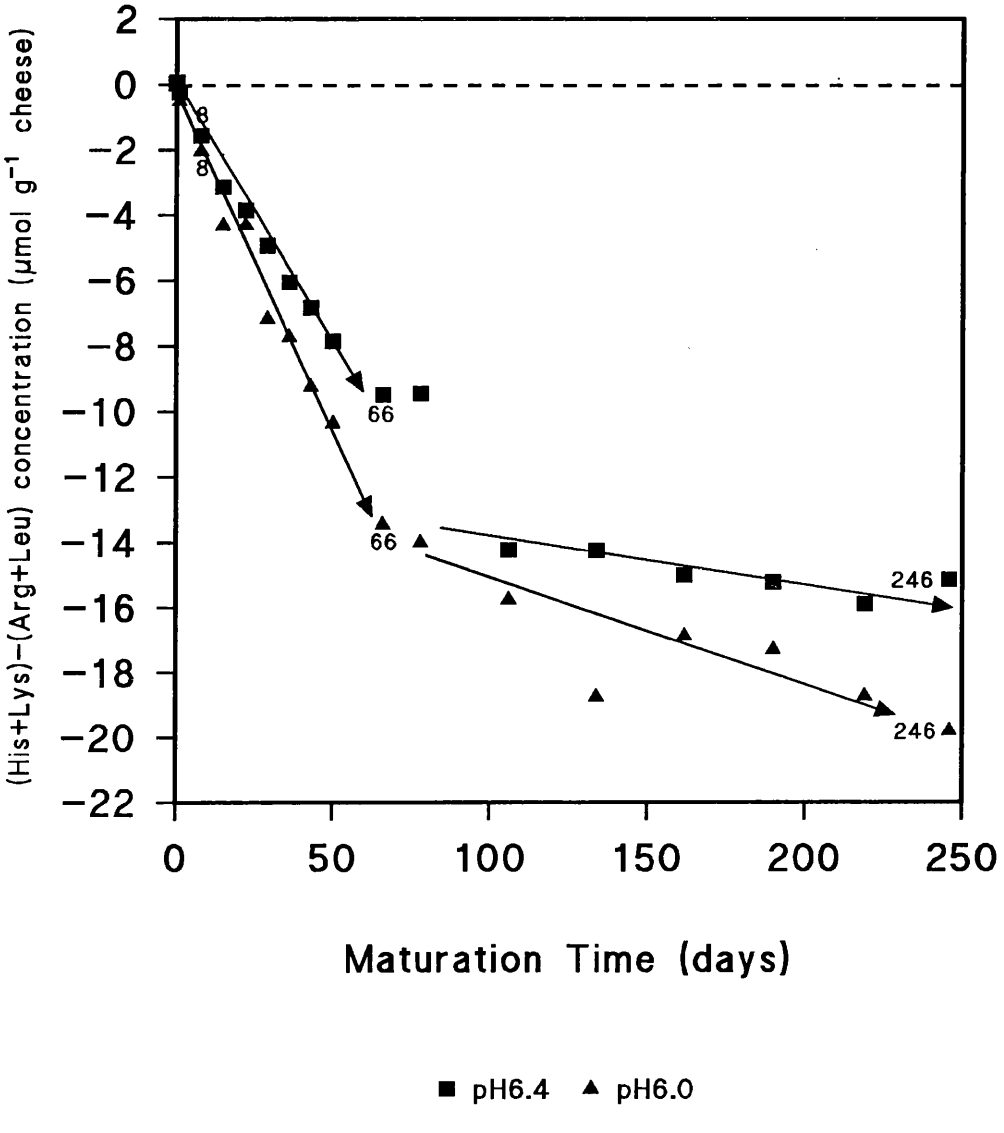
His = Histidine.

Lys = Lysine.

Arg = Arginine.

Leu = Leucine.

FIGURE 2.16


(His+Lys)−(Arg+Leu) concentration (µmol g⁻¹ cheese)
2
0
−2
−4
−6
−8
−10
−12
−14
−16
−18
−20
−22
0
50
100
150
200
250
Maturation Time (days)
8
8
66
66
246
246
■ pH6.4 ▲ pH6.0

Chapter 3

Reversed Phase-High Performance Liquid Chromatography Analysis of the Effect of Manufacturing Conditions on the Levels of Water-Soluble Peptides in Cheddar Cheese

3.1 Introduction

Reversed phase-high performance liquid chromatography (RP-HPLC) is a useful technique for the analysis and purification of proteins and peptides. Primarily because of the resolution achieved by RP-HPLC, it is now used routinely to separate peptide fragments from enzymatic digests, in the separation and purification of natural and synthetic peptides, and in purifying proteins for characterisation.

The application of RP-HPLC to the investigation of proteolysis during cheese ripening, especially the analysis and comparison of cheese manufactured under different processing conditions, is now widely reported. Following chemical fractionation of the cheese, the chromatographic separation of the peptide material generates profiles, which can provide valuable information about the maturity of the cheese. Additionally, such chromatograms provide a characteristic 'fingerprint' of a particular cheese variety (Gonzalez de Llano *et al.*, 1995).

Cliffe *et al.* (1993) used gel filtration chromatography to fractionate the water-soluble extract of a mature Cheddar cheese, and then determined the different flavours present in the collected fractions. Subsequent peptide profiling by RP-HPLC showed that those fractions possessing a bitter taste were composed largely of material that eluted late on the reversed phase column, and were believed to be composed of hydrophobic peptides. In contrast, those fractions possessing a savoury flavour had a much greater proportion of early eluting material, believed to be composed of the more hydrophilic peptides and free amino acids.

Altemueller and Rosenberg (1996) investigated proteolysis during the maturation of full-fat and low-fat Cheddar cheese by RP-HPLC separation of the pH4.6 water-soluble nitrogen fractions of the cheese. Separated compounds were divided into four molecular weight ranges, and it was found that the number of peaks and the amount of nitrogen compounds separated increased significantly both within-

and between-varieties with ripening time. The proportion of the cheese nitrogen fraction soluble in water at pH4.6 has been suggested as a possible ripening index (McSweeney and Fox, 1993), with the components present providing detailed quantitative information on the type and extent of proteolytic activities occurring during ripening.

Successful acceleration of cheese ripening requires chromatograms of the water-soluble peptides present to be similar to those of the traditional cheese, the only difference being in their relative rates of formation. Cliffe and Law (1991) investigated peptide production in accelerated-ripened Cheddar cheese through a time-course study using RP-HPLC. Cheddar cheese manufactured using standard commercial rennet and a single starter strain (*Streptococcus cremoris* NCDO 924) was split into four portions before salting. The following additions were then made to the four experimental samples using cheese making salt as the carrier:

1. Neutrase.
2. "Accelase" - a starter peptidase extract from *S. lactis* NCDO 712.
3. Neutrase and "Accelase".
4. Salt only (control).

Analysis by polyacrylamide gel electrophoresis showed very little difference in the casein breakdown pattern of the four cheeses at sixteen weeks of maturation. However, in contrast the RP-HPLC analysis showed large differences in the peptides present in the water-soluble nitrogen fractions of the cheese containing the mixture of neutrase and "Accelase" enzymes, compared to the other three cheeses.

Semi-liquid slurries of Cheddar cheese curd were used by Cliffe and Law (1990) to investigate enzyme-accelerated cheese ripening. The addition of a neutral proteinase from *Bacillus subtilis* was found to create bitter off-flavours. These subsequently disappeared following the further addition of an intracellular peptidase extract from *S. lactis* NCDO 712, resulting in the restoration of a normal Cheddar flavour. Addition of the *S. lactis* peptidase extract caused a dramatic reduction in height of a group of late eluting RP-HPLC peaks, believed to be hydrophobic in nature and containing bitter tasting peptides. At the same time there was a corresponding

increase in the amount of early eluting peaks, presumed to be the resultant breakdown products of these bitter peptides.

This chapter describes the RP-HPLC profiling of the water-soluble peptides present in Cheddar cheese manufactured from standard pH (6.4) and reduced pH (6.0) milk over a 246 day ripening period. Analysis of the resultant chromatograms showed that one region in particular qualitatively represented the area of greatest difference between the water-soluble extracts from the two types of cheese, regardless of the time of maturation. The peptide components from this region were then further separated on a modified gradient to allow a more detailed comparison, with some of the peptides present characterised by mass spectrometry (Chapter 4).

Finally, a statistical model derived using Partial Least Squares Regression was assessed for predicting individual amino acid concentrations from the chromatogram profiles.

3.2 Materials and methods

3.2.1 Standard and reduced pH milk Cheddar cheese

Cheddar cheese was manufactured from milk at either the standard pH or reduced pH as previously described in section 2.2.2.

3.2.2 Reagents

Trifluoroacetic acid (TFA, anhydrous) was purchased from Sigma Chemical Company Ltd., Fancy Road, Poole, Dorset, BH17 7BR.

Acetonitrile (far UV, HPLC grade solvent) was purchased from Fisher Scientific UK Ltd., Bishop Meadow Road, Loughborough, Leics., LE11 ORG.

All water used was of HPLC grade, prepared on site using a CD plus deioniser from USF Ltd., John Tate Road, Hertford, SG13 7NW.

3.2.3 Extraction of the water-soluble cheese peptides

Samples of the standard and reduced pH milk Cheddar cheese, removed at regular time intervals during their 246 day ripening period, were finely grated,

freeze-dried to constant dry weight, and water-soluble extracts prepared as illustrated in Figure 3.1.

Lyophilised grated cheese equivalent to 5g 'wet weight' was homogenised for 2min in 10mL of deionised water using an Ultra Turrax T25 high-speed mixer emulsifier (Janke and Kunkel GmbH and Co., Germany). The resulting cheese "slurry" was then incubated in a water bath at 40^{0}C for 30min to allow temperature equilibration, followed by a further 1h incubation with regular stirring.

Homogenised cheese slurries were then centrifuged (16,000rpm, 30min, 8^{0}C), the uppermost fat layer removed with a spatula, and the supernatant decanted and recentrifuged. Recovered supernatants were diluted 1:1 (v/v) with deionised water in round bottom flasks, and frozen in cardice/ethanol before lyophilisation and storage at -20^{0}C prior to chromatographic separation.

3.2.4 Chromatographic separation of the water-soluble cheese peptides

Separation of the water-soluble cheese peptides was performed by RP-HPLC on an APEX WP ODS (C_{18}) column (7μm, 250x4.6mm i.d.; Jones Chromatography, New Road, Hengoed, Mid Glamorgan, CF8 8AU), using an automated Spectraphysics HPLC system.

Lyophilised water-soluble extracts were dissolved in 1.5mL eppendorf vials at 5mg mL^{-1} in HPLC grade water using an orbital Vibrax® VXR shaker. Additional whirlimixer agitation was applied to aid initial sample solubilisation, at 30min, and after shaking for 1h. Following solubilisation, the samples were microcentrifuged (MSE Microcentaur) for 2.5min at 13,000rpm, then filtered through 0.2μm filters (Sartorius Limited, Blenheim Road, Epsom, Surrey, KT19 9BR) before injection onto the column.

Peptides were eluted using a linear gradient of 0–75% buffer B over 50min. Buffer A consisted of 0.1% TFA in water and buffer B of 0.1% TFA in 90% acetonitrile/water (v/v). Flow rate was 1.50mL min^{-1}, column temperature was 46^{0}C and peak detection was at 214nm.

A modified gradient was then applied to further separate the peptides eluting in the chromatograms between the buffer B gradient of 30–48% (20–32min). Qualitatively this region represented the area of greatest difference between the

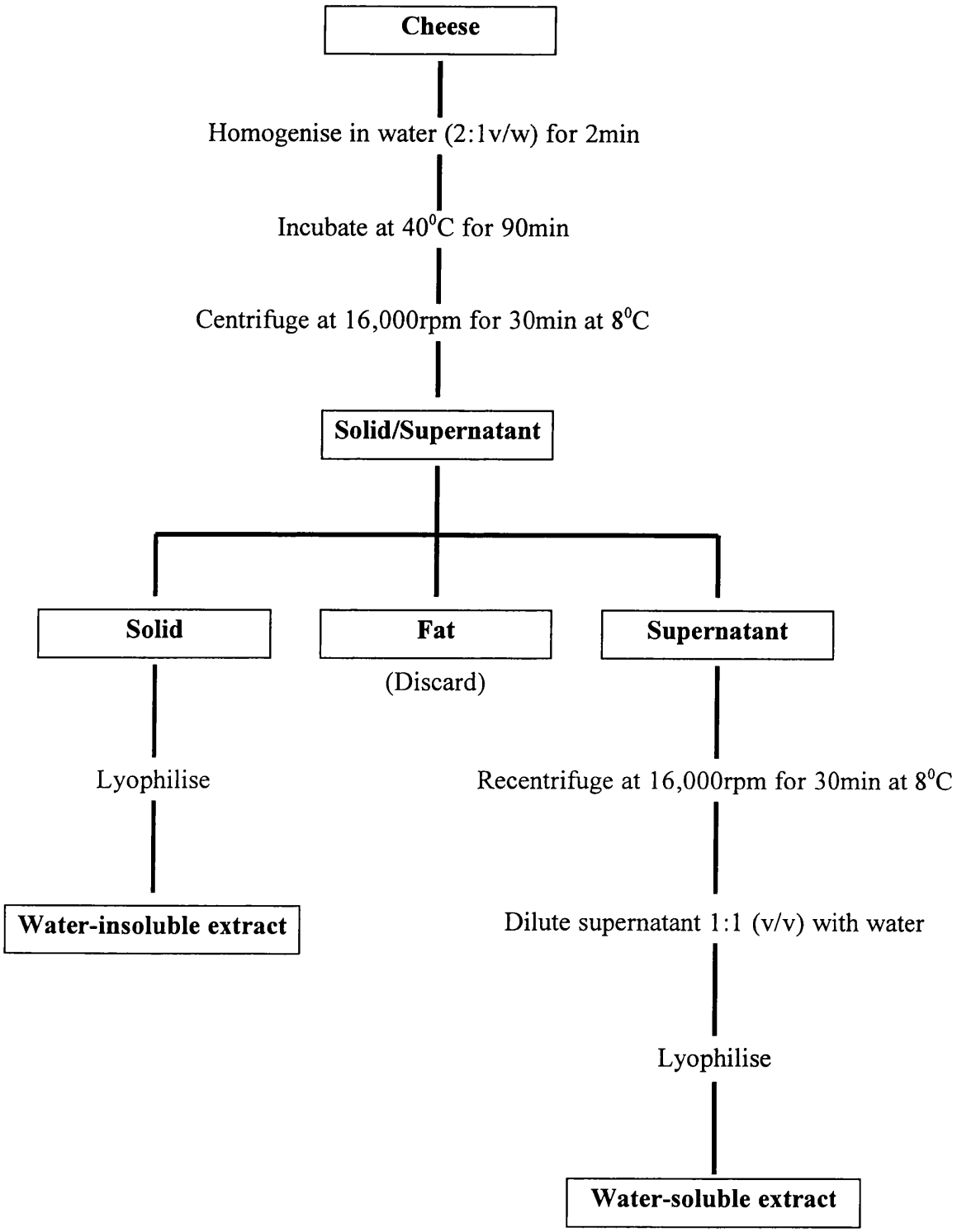


FIGURE 3.1: Schematic outline of the experimental procedure used for water fractionating Cheddar cheese.

water-soluble extracts of the two cheeses at any particular time of maturation. Lyophilised extracts were dissolved in HPLC grade water at 10mg mL^{-1}, and the peptides separated using buffer B held at 24% from 0–5min, followed by a linear gradient to 52% buffer B from 5–50min.

3.2.5 Statistical analysis

Changes in chromatogram peak areas during ripening are unlikely to be reliable indicators of quantitative changes in individual peptide concentrations. Analysis by mass spectrometry (Chapter 4) showed that even sharp chromatography peaks, seemingly representing individual peptides, often contained several different components. The information contained in the peptide profiles is however extremely useful. By dividing the modified gradient chromatograms into segments equal to 150sec elutions from 5–45min, and normalising each segment area to a percentage of the total chromatogram area, a characteristic "fingerprint" was derived for each profile. Example chromatograms treated in this way are shown in Figure 3.2(A) and (B).

The normalised percentage sizes of the 17 segment areas from the peptide chromatograms of the standard and reduced pH milk Cheddar cheese at days 1, 22, 78, 190 and 246 of maturation were determined. These maturation times covered the periods of major change in the free amino acid composition in the cheese, as determined by Principal Component Analysis (Chapter 2). The data formed a matrix of 10 rows (cheese samples) by 17 columns (segment areas). An analogous matrix composed of 10 rows (cheese samples) by 17 columns (amino acid concentrations) was also created. These matrices form Tables III.1 and III.2 in Appendix III.

The relationship between peptide precursor supply and amino acid composition was then investigated by evaluating the potential of the peptide chromatograms for predicting individual amino acid concentrations. For this purpose, a model was derived using the multivariate technique of Partial Least Squares Regression (PLS2; Unscrambler 5.3, Camo AS, Norway). The statistical model was fitted to the experimental data for maturation time x segment area and maturation time x individual amino acid concentration.

FIGURE 3.2: RP-HPLC modified gradient chromatograms of water-soluble extracts from standard pH and reduced pH Cheddar cheese at day 78 of maturation.

Each chromatogram was divided into seventeen equal segment areas of 150sec from 5–45min, and normalised to a percentage of the total chromatogram area.

(A) Water-soluble extract from standard pH Cheddar cheese, dissolved in water (final concentration 10mg mL^{-1}), and eluted with a modified gradient of 0.1% TFA in 90% acetonitrile/water (v/v) as described in the text.

(B) Water-soluble extract from reduced pH Cheddar cheese, dissolved in water (final concentration 10mg mL^{-1}), and eluted with a modified gradient of 0.1% TFA in 90% acetonitrile/water (v/v) as described in the text.

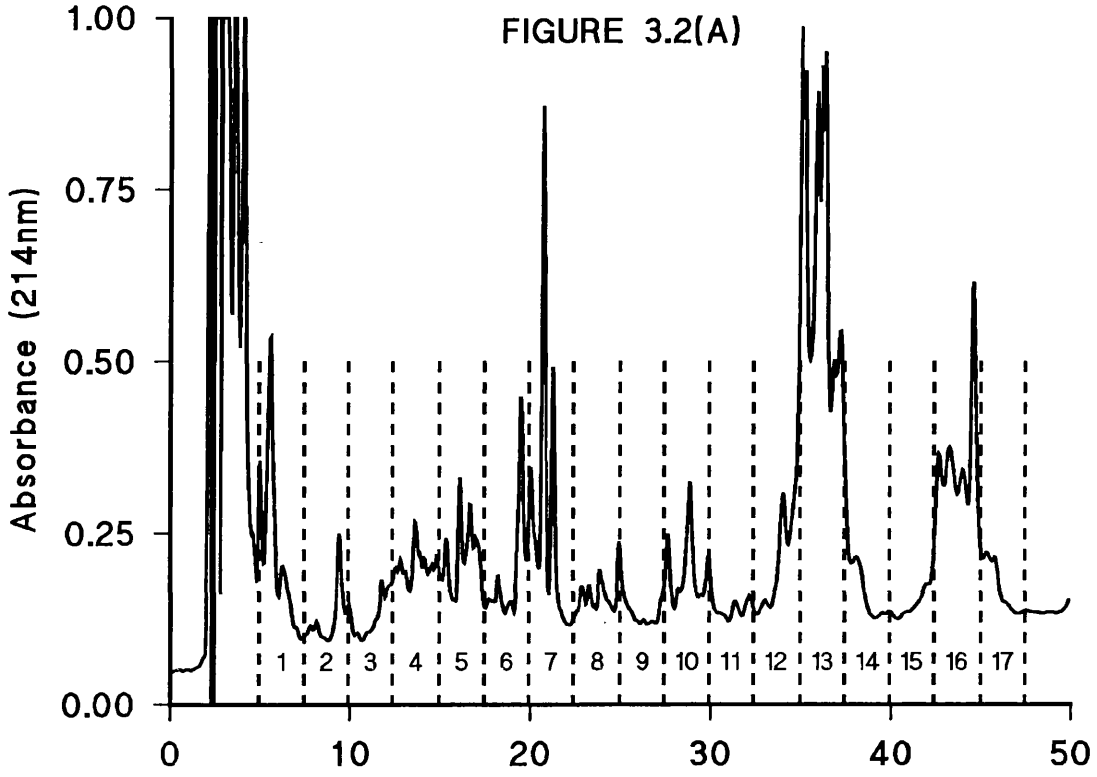


FIGURE 3.2(A)
Absorbance (214nm)
1.00
0.75
0.50
0.25
0.00
0
10
20
30
40
50
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

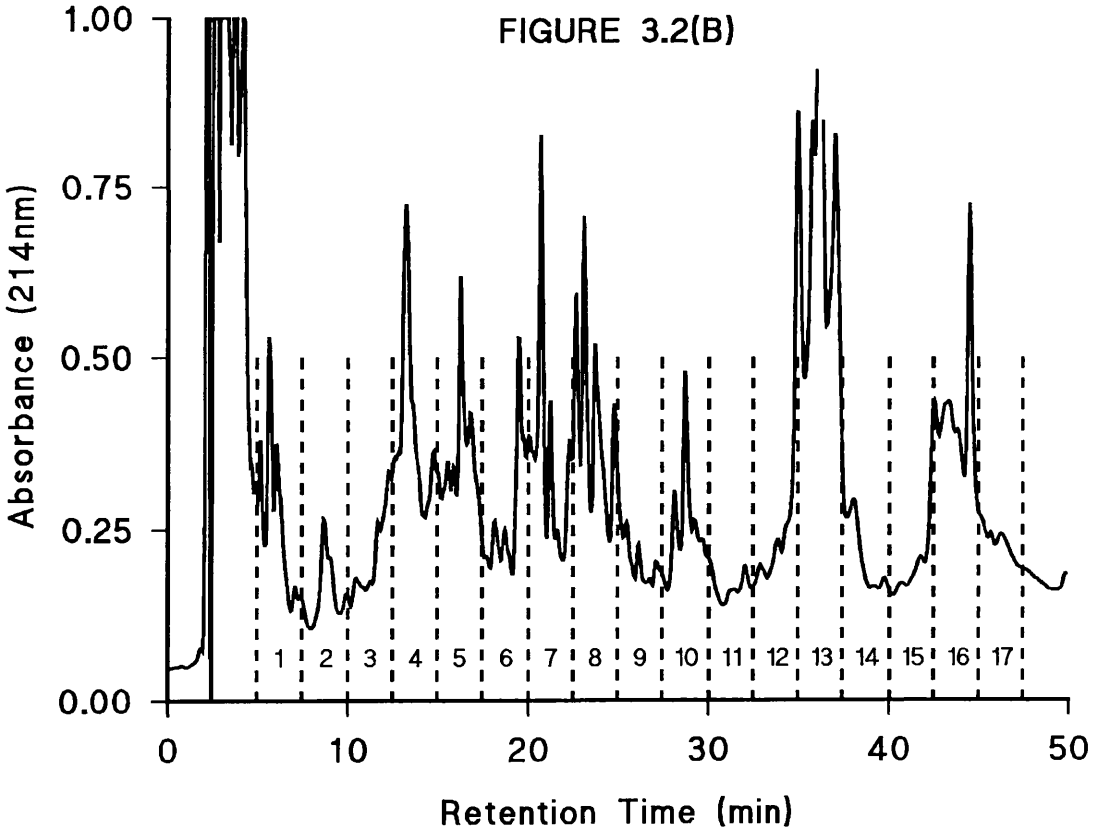


FIGURE 3.2(B)
Absorbance (214nm)
1.00
0.75
0.50
0.25
0.00
0
10
20
30
40
50
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
Retention Time (min)

3.3 Results and discussion

The RP-HPLC chromatograms of the water-soluble extracts from the standard and reduced pH Cheddar cheese at days 1, 22, 78, 190 and 246 of maturation are shown in Figures 3.3–3.7(A) and (B). Their respective modified gradient chromatograms are shown in Figures 3.3–3.7(a) and (b). Chromatograms of the water-soluble extracts at other maturation times are shown in Appendix III, Figures III.1–III.15.

The chromatograms of the water-soluble extracts from the reduced pH Cheddar cheese were qualitatively and quantitatively significantly different to those obtained from the standard pH Cheddar cheese over the 246 days of maturation. Such differences in the peptide composition between the two Cheddar cheeses could be the result of different proteolytic activities, or the consequence of a similar but overall faster proteolysis at the reduced pH.

Chapter 2 described the changes in the free amino acid concentrations in the Cheddar cheeses during ripening. Of the factors associated with these changes the most probable causes are:

1. Differences in the peptide precursor supply.
2. Differences in aminopeptidase activities.
3. Differential rates of transformation of released amino acids into flavour compounds.

A statistical model for predicting the amino acid concentrations from the water-soluble peptide chromatograms was derived using Partial Least Squares Regression. Examination of the residual variance after cross-validation suggested that a four factor model explaining 66.5% of the x-variance and 93.6% of the y-variance was optimal. The sample scores on PLS dimension 1 and 2 are shown in Figure 3.8(A), which shows a clear segregation of the water-soluble extracts based on both pH and maturity.

Figure 3.8(B) shows the corresponding factor loadings for scores on the first two PLS dimensions for the seventeen segment areas. A factor loading greater than +/-0.25 was considered significant. The first score shows segment areas 12, 13 and

16 contribute to PLS dimension 1, while segment areas 4, 8, 13 and 16 contribute to PLS dimension 2.

The changes in area of these important chromatogram segments with respect to pH adjustment and ripening time are shown in Figures 3.9 and 3.10. Corresponding changes in the other segment areas are shown in Appendix III, Figures III.16–III.19. Segment areas 4 and 8 were largest in the water-soluble extracts from the reduced pH Cheddar cheese throughout maturation, while areas 12, 13 and 16 were overall lower during ripening. All segment areas, apart from 12, either reached and maintained a plateau level, or subsequently proceeded to decrease in size as the ripening time increased. This relative rate of degradation varied between the two Cheddar cheeses.

Table 3.1 shows the variance explained after cross-validation, and summarises the success of the model for predicting individual amino acid concentrations from the RP-HPLC chromatograms of the water-soluble extracts. Thirteen amino acids were very well predicted by the model (variance explained $> 90\%$), alanine and glycine were well predicted ($> 85\%$), while proline and tyrosine were predicted with less certainty ($< 80\%$).

In conclusion, the result implies that the observed variations in individual amino acid concentrations between the standard pH and reduced pH milk Cheddar cheeses are closely associated with differences in the peptide precursor supply. Consequently, differences in aminopeptidase activities, and differential rates of transformation of released amino acids into flavour compounds are likely to be of more limited importance.

FIGURE 3.3: RP-HPLC chromatograms of water-soluble extracts from standard pH and reduced pH Cheddar cheese at day 1 of maturation.

(A) Water-soluble extract from standard pH Cheddar cheese, dissolved in water (final concentration 5mg mL^{-1}), and eluted with a gradient of 0.1 % TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

(a) Water-soluble extract from standard pH Cheddar cheese, dissolved in water (final concentration 10mg mL^{-1}), and eluted with a modified gradient of 0.1 % TFA in 90 % acetonitrile/water (v/v) (buffer B; --------) as described in the text.

(B) Water-soluble extract from reduced pH Cheddar cheese, dissolved in water (final concentration 5mg mL^{-1}), and eluted with a gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

(b) Water-soluble extract from reduced pH Cheddar cheese, dissolved in water (final concentration 10mg mL^{-1}), and eluted with a modified gradient of 0.1 % TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

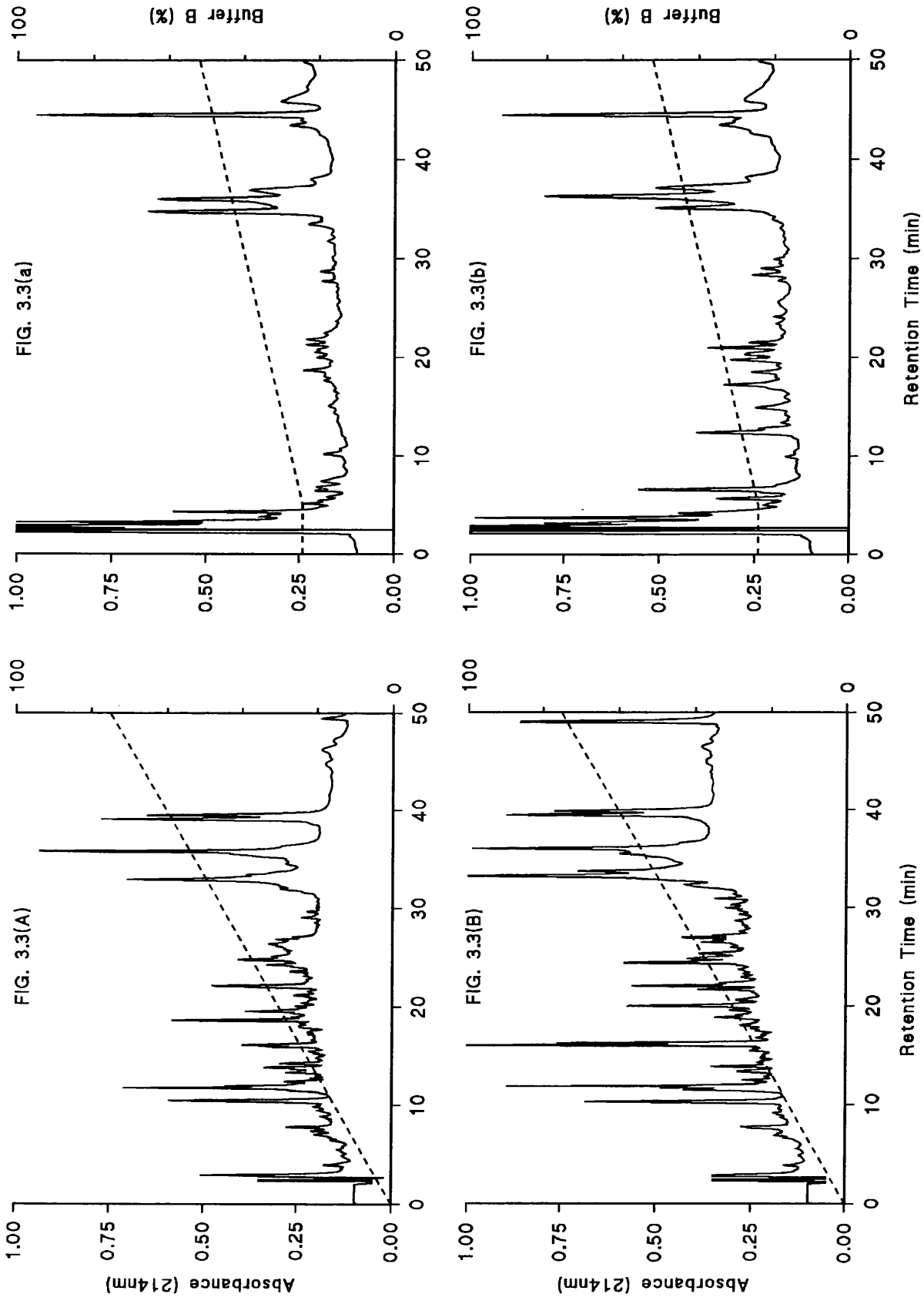


FIG. 3.3(A)
FIG. 3.3(B)
FIG. 3.3(a)
FIG. 3.3(b)
Absorbance (214nm)
Buffer B (%)
Retention Time (min)
1.00
0.75
0.50
0.25
0.00
100
0
10
20
30
40
50

FIGURE 3.4: RP-HPLC chromatograms of water-soluble extracts from standard pH and reduced pH Cheddar cheese at day 22 of maturation.

(A) Water-soluble extract from standard pH Cheddar cheese, dissolved in water (final concentration 5mg mL^{-1}), and eluted with a gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

(a) Water-soluble extract from standard pH Cheddar cheese, dissolved in water (final concentration 10mg mL^{-1}), and eluted with a modified gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

(B) Water-soluble extract from reduced pH Cheddar cheese, dissolved in water (final concentration 5mg mL^{-1}), and eluted with a gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

(b) Water-soluble extract from reduced pH Cheddar cheese, dissolved in water (final concentration 10mg mL^{-1}), and eluted with a modified gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

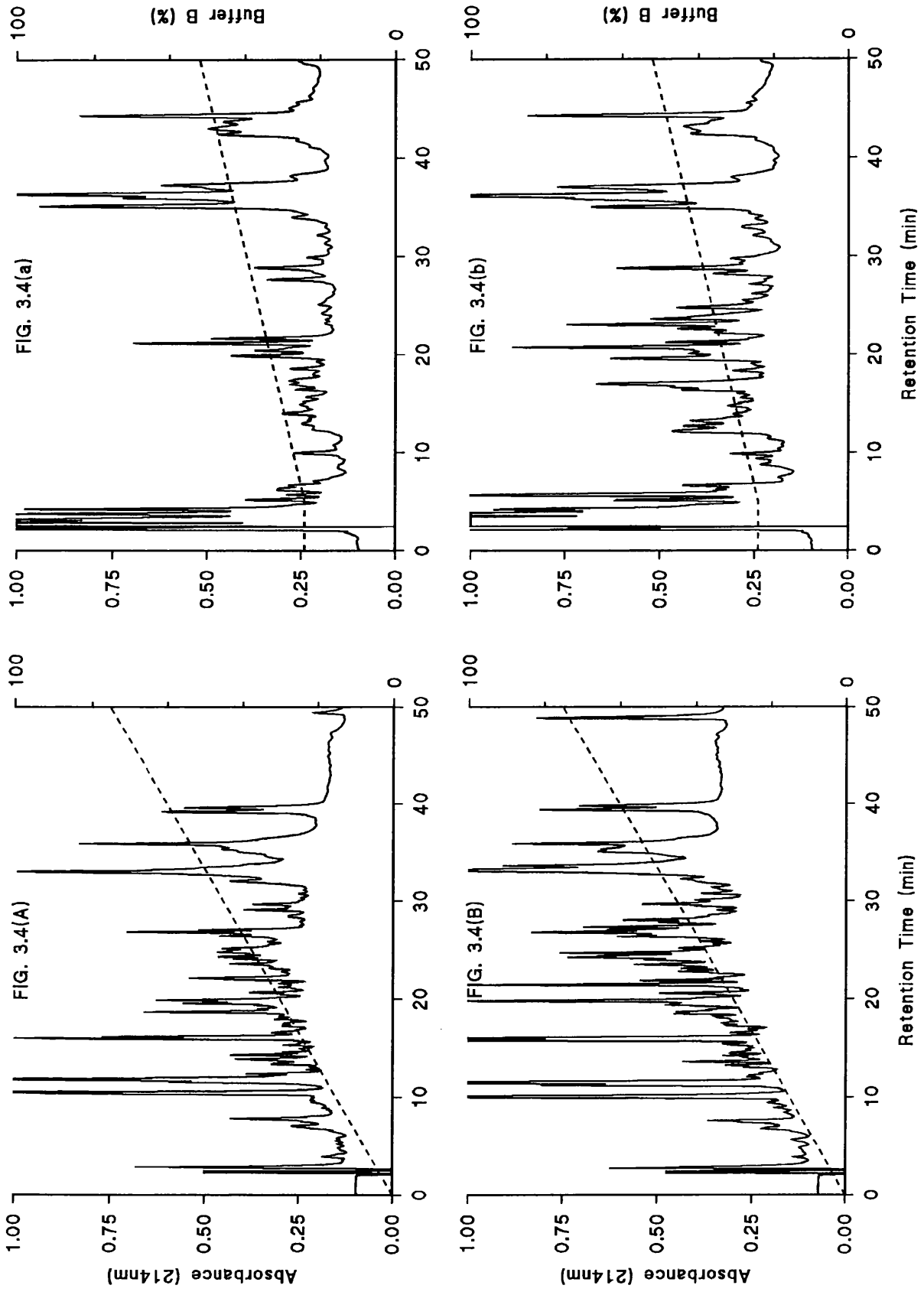


FIG. 3.4(A)
FIG. 3.4(B)
FIG. 3.4(a)
FIG. 3.4(b)
Absorbance (214nm)
Buffer B (%)
Retention Time (min)
1.00
0.75
0.50
0.25
0.00
100
0
50
40
30
20
10
0

FIGURE 3.5: RP-HPLC chromatograms of water-soluble extracts from standard pH and reduced pH Cheddar cheese at day 78 of maturation.

(A) Water-soluble extract from standard pH Cheddar cheese, dissolved in water (final concentration 5mg mL^{-1}), and eluted with a gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

(a) Water-soluble extract from standard pH Cheddar cheese, dissolved in water (final concentration 10mg mL^{-1}), and eluted with a modified gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

(B) Water-soluble extract from reduced pH Cheddar cheese, dissolved in water (final concentration 5mg mL^{-1}), and eluted with a gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

(b) Water-soluble extract from reduced pH Cheddar cheese, dissolved in water (final concentration 10mg mL^{-1}), and eluted with a modified gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

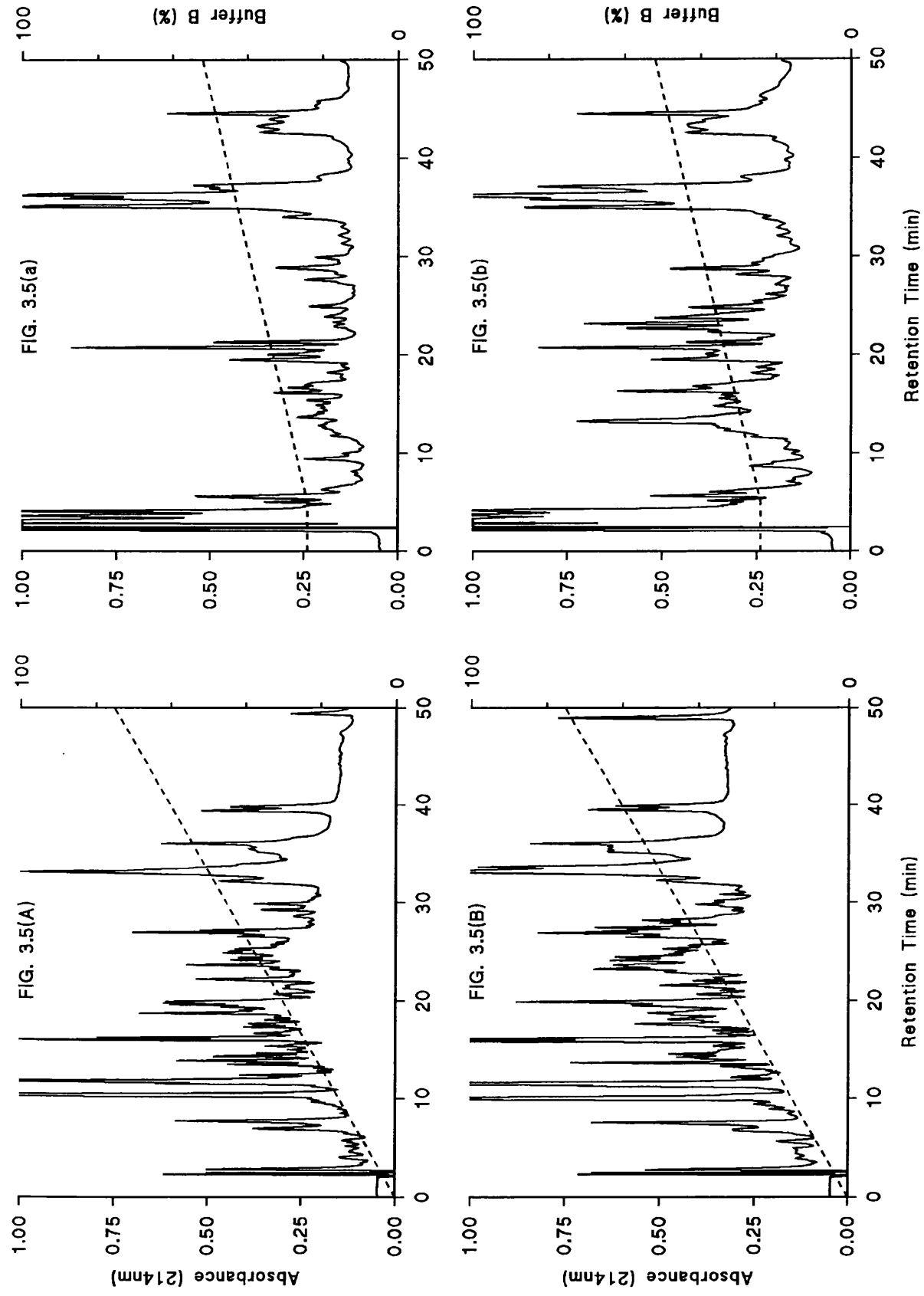


FIG. 3.5(A)
FIG. 3.5(B)
FIG. 3.5(a)
FIG. 3.5(b)
Absorbance (214nm)
Buffer B (%)
Retention Time (min)
1.00
0.75
0.50
0.25
0.00
100
0
10
20
30
40
50

FIGURE 3.6: RP-HPLC chromatograms of water-soluble extracts from standard pH and reduced pH Cheddar cheese at day 190 of maturation.

(A) Water-soluble extract from standard pH Cheddar cheese, dissolved in water (final concentration 5mg mL^{-1}), and eluted with a gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

(a) Water-soluble extract from standard pH Cheddar cheese, dissolved in water (final concentration 10mg mL^{-1}), and eluted with a modified gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

(B) Water-soluble extract from reduced pH Cheddar cheese, dissolved in water (final concentration 5mg mL^{-1}), and eluted with a gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

(b) Water-soluble extract from reduced pH Cheddar cheese, dissolved in water (final concentration 10mg mL^{-1}), and eluted with a modified gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

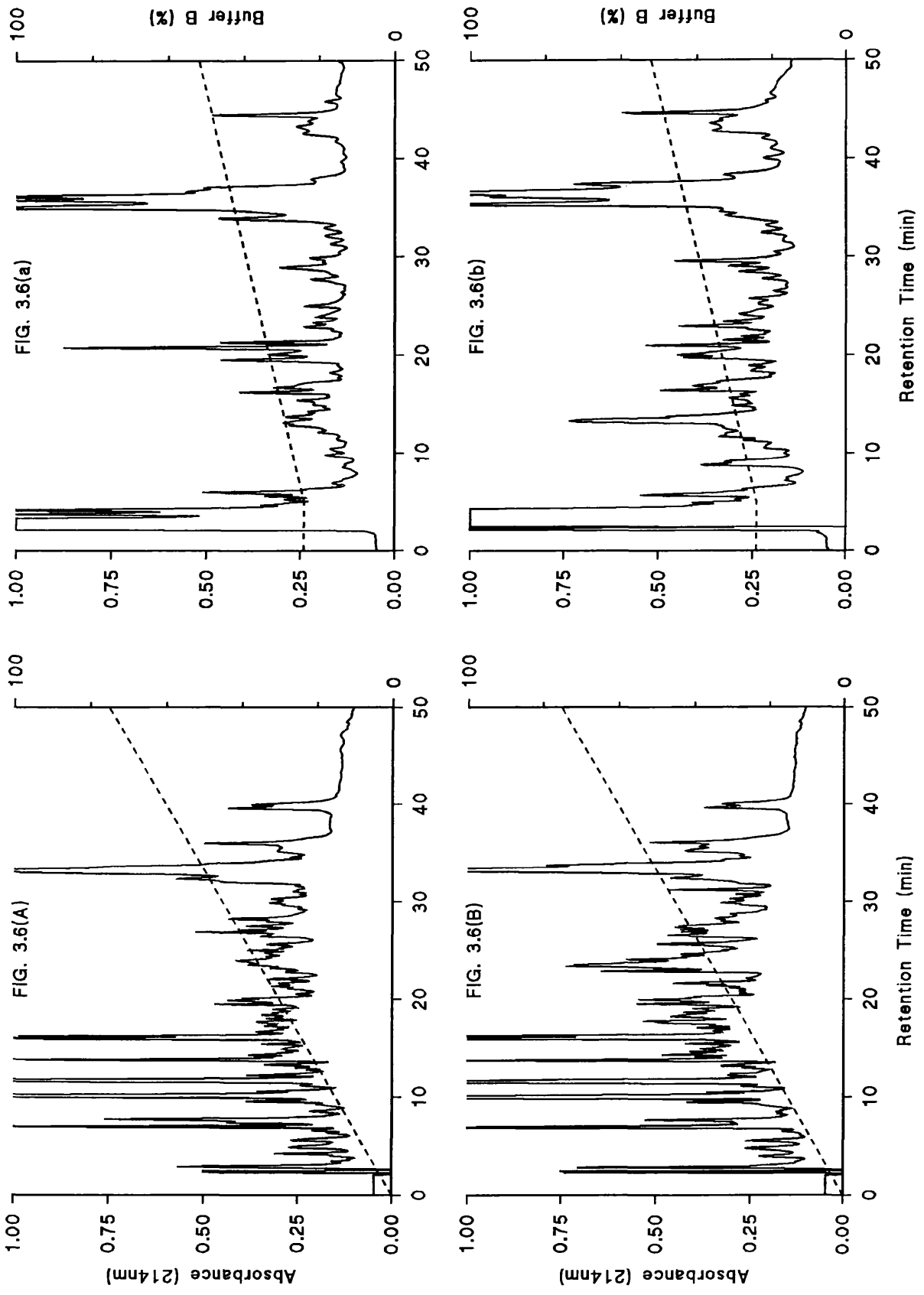


FIG. 3.6(A)
FIG. 3.6(B)
FIG. 3.6(a)
FIG. 3.6(b)
Absorbance (214nm)
Buffer B (%)
Retention Time (min)
1.00
0.75
0.50
0.25
0.00
100
0
50
40
30
20
10
0

FIGURE 3.7: RP-HPLC chromatograms of water-soluble extracts from standard pH and reduced pH Cheddar cheese at day 246 of maturation.

(A) Water-soluble extract from standard pH Cheddar cheese, dissolved in water (final concentration 5mg mL^{-1}), and eluted with a gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

(a) Water-soluble extract from standard pH Cheddar cheese, dissolved in water (final concentration 10mg mL^{-1}), and eluted with a modified gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

(B) Water-soluble extract from reduced pH Cheddar cheese, dissolved in water (final concentration 5mg mL^{-1}), and eluted with a gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

(b) Water-soluble extract from reduced pH Cheddar cheese, dissolved in water (final concentration 10mg mL^{-1}), and eluted with a modified gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

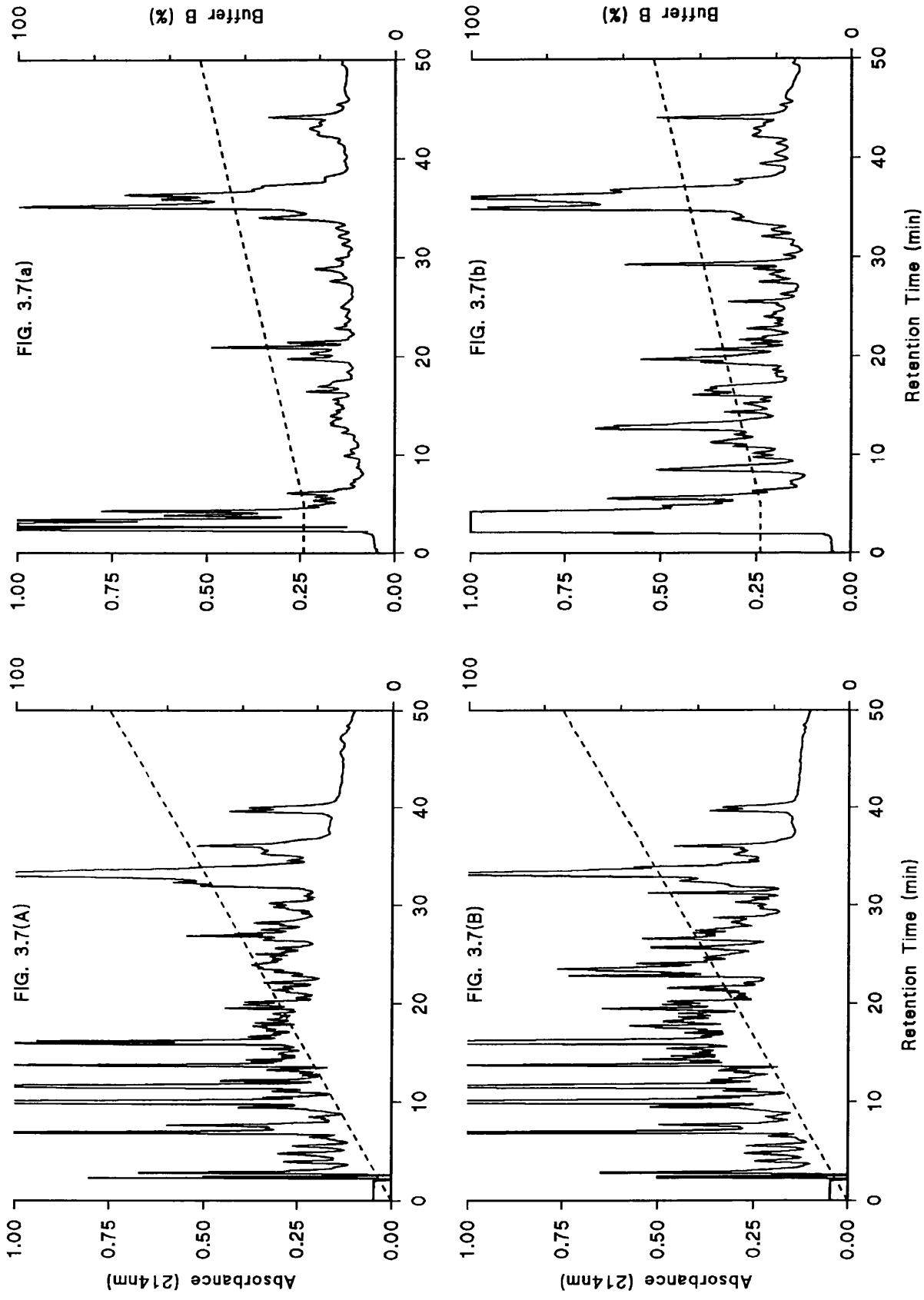


FIG. 3.7(A)
FIG. 3.7(B)
FIG. 3.7(a)
FIG. 3.7(b)
Absorbance (214nm)
Buffer B (%)
Retention Time (min)
1.00
0.75
0.50
0.25
0.00
100
0
10
20
30
40
50

FIGURE 3.8: Simplification of the effect of standard pH and reduced pH milk on Cheddar cheese water-soluble extracts.

Analysis of changes to the modified gradient chromatogram segment areas over 246 days of maturation by Partial Least Squares Regression.

(A) Cheese scores on first and second PLS dimensions over 246 days of maturation.

(B) Factor loadings for scores on first two PLS dimensions.
(+/-0.25 significance level; --------)

FIGURE 3.8(A)

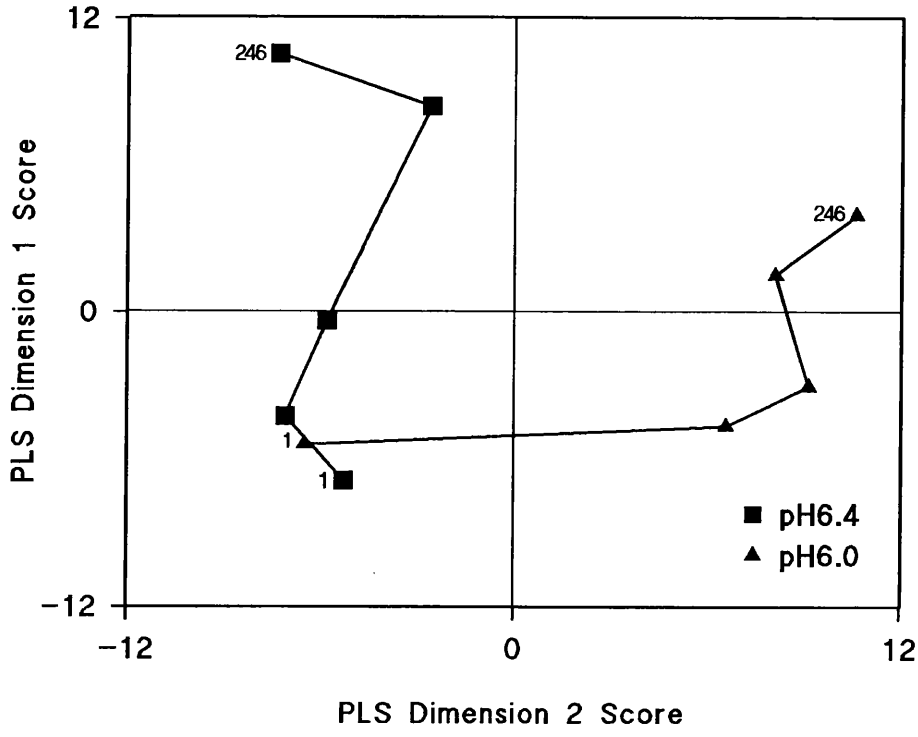


FIGURE 3.8(B)

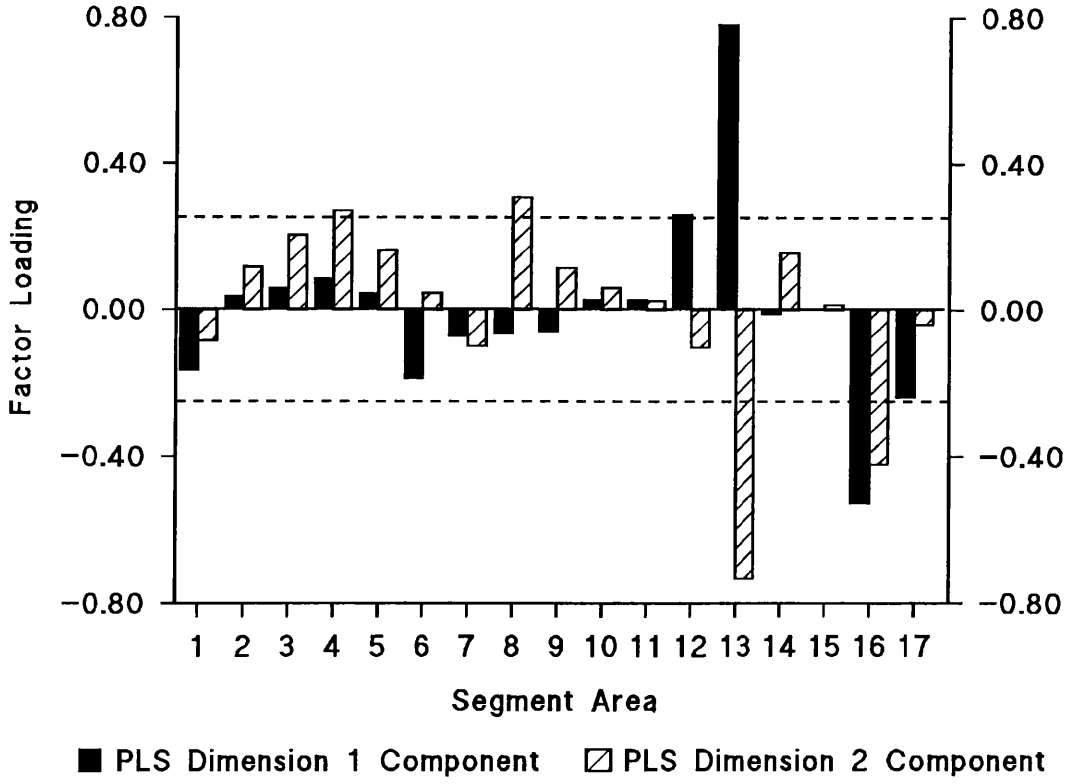


FIGURE 3.9: Changes in the key modified gradient water-soluble extract RP-HPLC chromatogram segment areas identified by Partial Least Squares Regression, in standard pH and reduced pH Cheddar cheese over 246 days of maturation.

(A) Segment area 4.

(B) Segment area 8.

(C) Segment area 12.

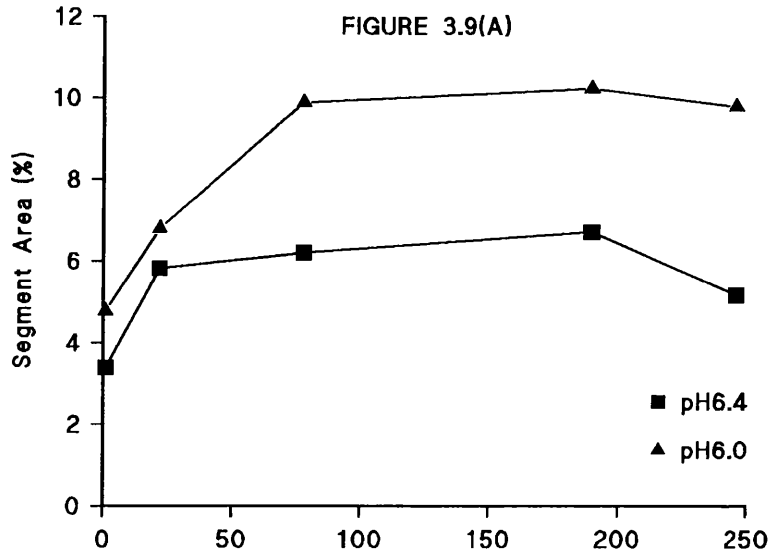


FIGURE 3.9(A)
Segment Area (%)
12
10
8
6
4
2
0
0
50
100
150
200
250
pH6.4
pH6.0

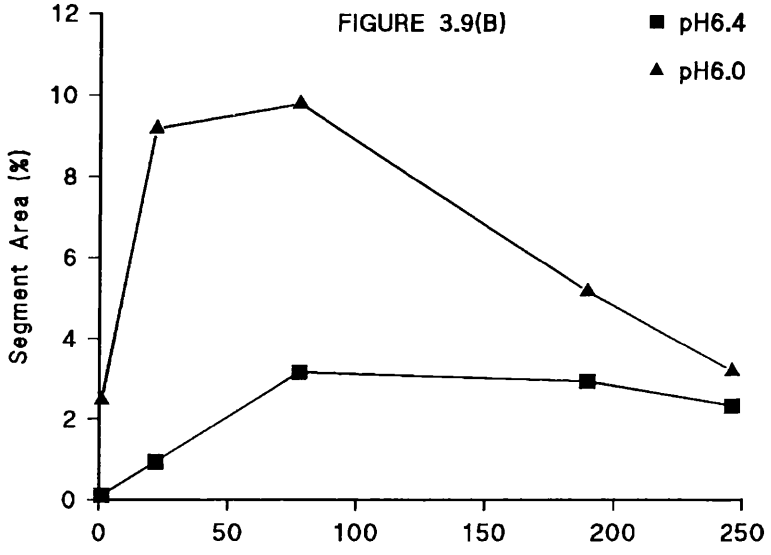


FIGURE 3.9(B)
Segment Area (%)
12
10
8
6
4
2
0
0
50
100
150
200
250
pH6.4
pH6.0

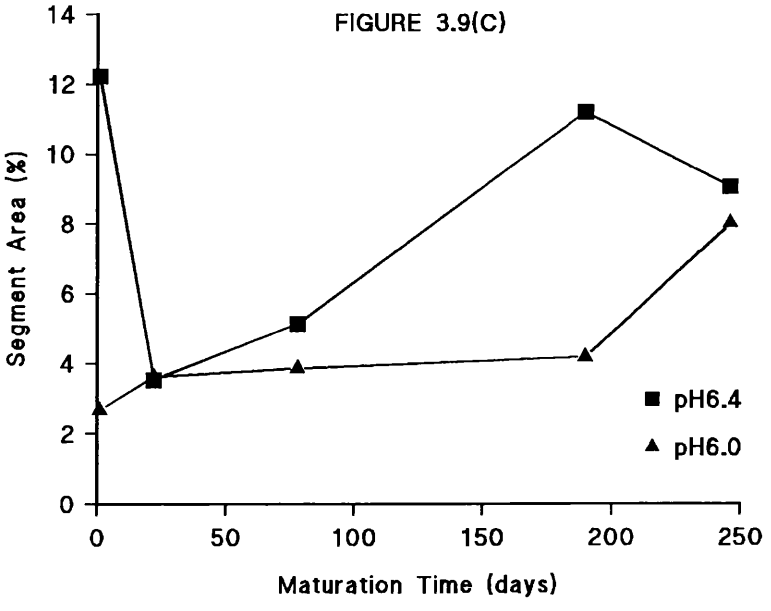


FIGURE 3.9(C)
Segment Area (%)
14
12
10
8
6
4
2
0
0
50
100
150
200
250
Maturation Time (days)
pH6.4
pH6.0

FIGURE 3.10: Changes in the key modified gradient water-soluble extract RP-HPLC chromatogram segment areas identified by Partial Least Squares Regression, in standard pH and reduced pH Cheddar cheese over 246 days of maturation.

(A) Segment area 13.

(B) Segment area 16.

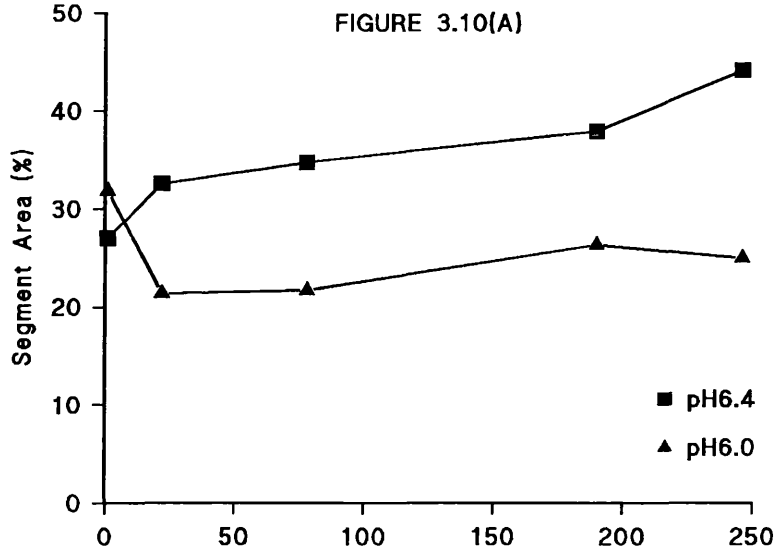


FIGURE 3.10(A)
Segment Area (%)
50
40
30
20
10
0
0
50
100
150
200
250
pH6.4
pH6.0

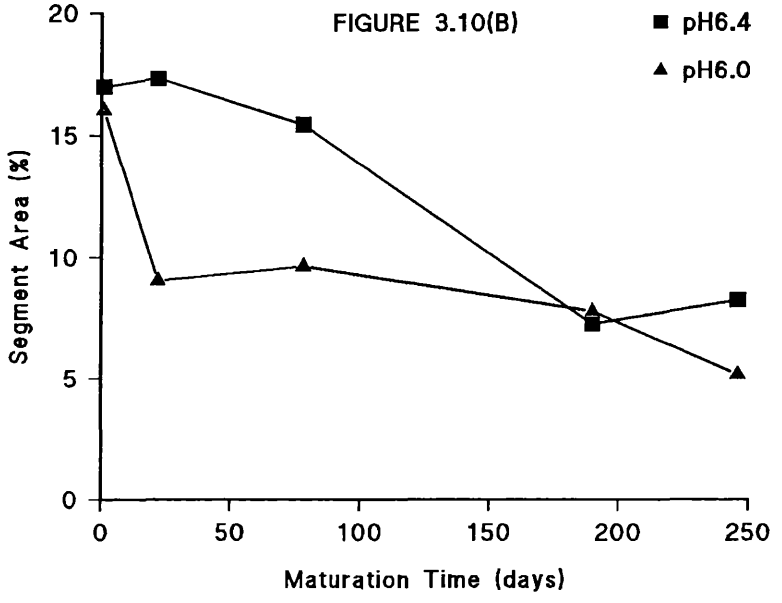


FIGURE 3.10(B)
pH6.4
pH6.0
Segment Area (%)
20
15
10
5
0
0
50
100
150
200
250
Maturation Time (days)

TABLE 3.1: Partial Least Squares Regression for the prediction of amino acid concentrations from standard and reduced pH milk Cheddar cheese water-soluble extract RP-HPLC chromatograms; variance explained by the model (validation variance - four factors).

Amino Acid	Variance (%)	Amino Acid	Variance (%)
Ala	89.4	Leu	94.8
Arg	93.9	Lys	97.1
Asn/Ser	94.2	Met	97.3
Asp	93.6	Phe	94.5
Gln	94.5	Pro	79.4
Glu	97.1	Thr	96.6
Gly	86.1	Tyr	78.8
His	93.8	Val	96.7
Ile	92.7	Overall	93.6

Chapter 4

Characterisation of Water-Soluble Peptides from Cheddar Cheese by Mass Spectrometry

4.1 Introduction

The development of flavour and texture during the cheese maturation process is the result of three primary biochemical processes; glycolysis, lipolysis and proteolysis. Proteolysis is the most extensive of these phenomena, the products of which, being non-volatile, contribute greatly to the cheese flavour (Fox, 1989). Proteolytic agents are derived from a number of sources; the milk, coagulant, starter and non-starter lactic acid bacteria and, in certain cheese varieties, deliberately added secondary microflora. The casein proteins are therefore exposed to a range of proteolytic enzymes, resulting in their hydrolysis to polypeptides, peptides and ultimately free amino acids.

There has been much interest in recent years in identifying the peptides produced during cheese proteolysis, and relating their presence to the action of specific proteases (Fox *et al.*, 1994). Usually, following separation by column chromatography or on polyacrylamide gels, such peptides are identified by Edman degradation gas-phase sequencing, combined with amino acid analysis. This chapter describes an alternative approach to peptide characterisation, in which matrix assisted laser desorption/ionisation-mass spectrometry (MALDI-MS) was used to obtain the molecular mass of the peptides, and to determine their N-terminal sequence.

MALDI mass spectrometry was first described by Karas and Hillenkamp (1988), and is now an established technique for the analysis of a wide range of biomolecules. During the MALDI process, the analyte under investigation, embedded in the crystal lattice of a suitable matrix compound, is irradiated with pulses of laser light. The incident laser energy allows the transfer of essentially non-volatile molecules into the gas phase with little or no fragmentation (Hillenkamp and Karas, 1990).

Protein sequencing by mass spectrometry was approached in a new way by Chait *et al.* (1993), who described an N-terminal 'protein ladder sequence' methodology. The procedure involved the controlled generation from a polypeptide chain of a family of

peptide fragments, each fragment differing from the next by a single amino acid residue. A one step read out of the resulting protein ladder sequence by MALDI-MS then allows each residue to be identified from the mass difference between successive peaks. The isobaric amino acids leucine and isoleucine cannot, however, be distinguished using this sequencing procedure.

This sequencing principle was refined by the synthesis of a volatile isothiocyanate reagent, trifluoroethylisothiocyanate (TFEITC), allowing greater sensitivity as reagents and by-products are efficiently removed under vacuum, thus eliminating extractive loss (Bartlet-Jones *et al.*, 1994). Figure 4.1(A) shows the steps involved in performing a single TFEITC degradation cycle. Figure 4.1(B) illustrates the principle of TFEITC mass spectrometry sequencing, and shows a ladder sequence through five degradation cycles of FSH-Peptide A, with the mass difference between adjacent peaks identifying the residue removed. The degradation was performed using a total of 270pmol of starting peptide, with the spectrum obtained from one tenth of the final pool of material.

This chapter will describe the MALDI-MS characterisation of peptides formed during the maturation of Cheddar cheese. Sixteen peptides were chromatographically separated from a water-soluble extract of a one year-old mature Cheddar cheese and identified. Twenty peptides, chromatographically separated from water-soluble extracts of Cheddar cheese manufactured from standard pH and reduced pH milk at day 22 of ripening, were also characterised using mass spectrometry.

4.2 Materials and methods

4.2.1 Mature Cheddar cheese

The cheese investigated was a one year-old commercial mature Cheddar cheese (moisture 35.84%, salt-in-moisture 4.74%, pH5.33).

4.2.2 Standard and reduced pH milk Cheddar cheese

Cheddar cheese was manufactured from milk at either the standard pH or reduced pH as previously described in section 2.2.2.

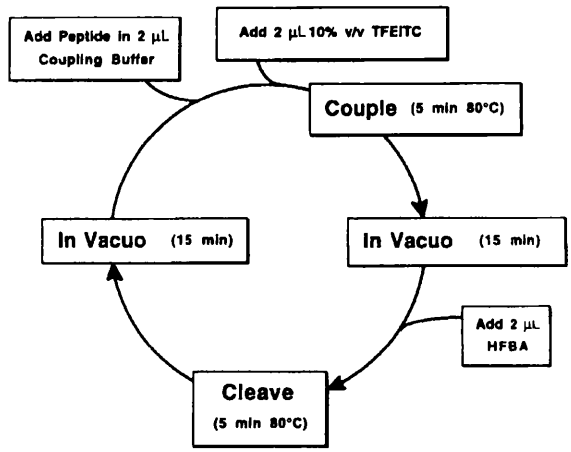


FIGURE 4.1(A): Flow diagram of the steps involved in a single TFEITC degradation cycle. The nested set of peptides is generated by adding equal aliquots of fresh peptide to each cycle, and driving both the coupling and cleavage chemistry towards completion. Excess reagent, coupling base and cleavage acid (anhydrous heptafluorobutyric acid; HFBA) are removed each cycle by drying *in vacuo*. From Bartlet-Jones *et al.*, 1994.

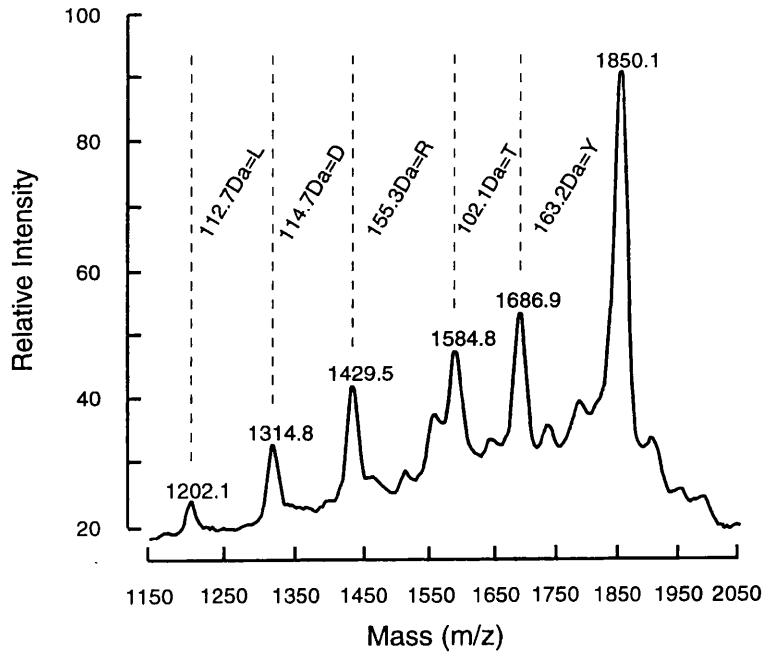


FIGURE 4.1(B): A ladder sequence through five TFEITC degradation cycles of FSH-Peptide A. (Sequence = YTRDLVYRDPARPNI, $[M+H]^+$ = 1850.1Da)

4.2.3 Reagents

Bis-tris propane (1,3-bis[tris(Hydroxymethyl)methylamino]propane), 2-mercaptoethanol and trifluoroacetic acid (TFA, anhydrous) were purchased from Sigma Chemical Company Ltd., Fancy Road, Poole, Dorset, BH17 7BR.

Urea and acetonitrile (far UV, HPLC grade solvent) were purchased from Fisher Scientific UK Ltd., Bishop Meadow Road, Loughborough, Leics., LE11 ORG.

Alpha-cyano-4-hydroxycinnamic acid (ACH) was purchased from Aldrich Chemical Company, New Road, Gillingham, Dorset, SP8 4JL.

Mass spectrometry peptide standards, kemptide, bradykinin and substance P, were purchased from Sigma Chemical Company Ltd. FSH-Peptide A and PDE-3 were chemically synthesised at the Hannah Research Institute.

Coupling buffer and TFEITC protein sequencing reagent were gifts provided by the Protein Sequencing Laboratory, Imperial Cancer Research Fund, PO Box 123, 44 Lincoln's Inn Fields, London, WC2A 3PX.

All water used was deionised or of high performance liquid chromatography (HPLC) grade, prepared on site using a CD plus deioniser from USF Ltd., John Tate Road, Hertford, SG13 7NW.

4.2.4 Extraction of the water-soluble cheese peptides

A sample of the one year old mature Cheddar cheese was removed, finely grated, and freeze dried to constant dry weight prior to water fractionation. Lyophilised grated cheese equivalent to 50g 'wet weight' was homogenised for 5min in 100mL of deionised water using a mixer emulsifier (Silverson Machines Ltd., Waterside, Chesham, Bucks.). The resulting cheese "slurry" was then incubated in a water bath at 40^{0}C for 1h with occasional stirring.

Samples of the standard and reduced pH milk Cheddar cheese, removed at regular time intervals during their 246 day ripening period, were finely grated, freeze dried to constant dry weight, and water-soluble extracts prepared as previously described in section 3.2.3. Lyophilised supernatants were stored at -20^{0}C prior to chromatographic separation and MALDI-MS analysis.

4.2.5 Chromatographic separation of the water-soluble cheese peptides

Separation of the water-soluble cheese peptides was performed by reversed phase-high performance liquid chromatography (RP-HPLC), on an APEX WP ODS (C_{18}) column (7μm, 250x4.6mm i.d.; Jones Chromatography, New Road, Hengoed, Mid Glamorgan, CF8 8AU), using an automated Spectraphysics HPLC system.

4.2.5.1 Mature Cheddar cheese

Samples of the lyophilised mature Cheddar cheese water-soluble extract were dissolved at 10mg mL^{-1} in dissociation buffer (20mM bis-tris propane, 4M urea and 0.24% 2-mercaptoethanol, pH7.0; Visser *et al.*, 1986b). The dissolved samples were then filtered through 0.2μm filters (Sartorius Ltd., Blenheim Road, Epsom, Surrey, KT19 9BR), and 1mL aliquots injected onto the column.

Peptides were eluted using a linear gradient of 0–60% buffer B over 50min. Buffer A consisted of 0.1% TFA in 10% acetonitrile/water (v/v) and buffer B of 0.1% TFA in 90% acetonitrile/water (v/v). Flow rate was 1.50mL min^{-1}, column temperature was 36^{0}C and peak detection was at 230nm. Peak fractions were collected manually, lyophilised and stored at -20^{0}C prior to MALDI-MS analysis.

Some peak fractions, determined by MALDI-MS to be complex mixtures, were rechromatographed using an APEX WP Butyl (C_4) column (7μm, 250x4.6mm i.d.). Peptides were eluted using linear gradients of 0.1% TFA in 10% acetonitrile/water (v/v) and 0.1% TFA in 90% acetonitrile/water (v/v), optimised to achieve the best separation of individual components. Flow rate was 1.50mL min^{-1}, column temperature 36^{0}C and peak detection was at 205nm.

4.2.5.2 Standard and reduced pH milk Cheddar cheese

Lyophilised water-soluble extracts from standard pH and reduced pH Cheddar cheese at day 22 of maturation were dissolved in water at 5mg mL^{-1}, 10mg mL^{-1}, or 40mg mL^{-1} for subsequent peak fraction collection, and chromatographically separated as previously described in section 3.2.4. Peak fractions were collected manually, lyophilised and stored at -20^{0}C prior to MALDI-MS analysis.

4.2.6 Mass spectrometry

Mass spectrometry was performed on a LASERMAT 2000 MALDI time-of-flight mass spectrometer (Thermo Bioanalysis Ltd., Paradise, Hemel Hempstead, Herts, HP2 4TG). Sample desorption was achieved using a 337nm nitrogen laser, acceleration voltage was 20kV and peptide spectra were obtained by summing either 50 or 100 laser pulses at a fixed position on the target slide.

Lyophilised peak fractions collected from the one year old mature Cheddar cheese water-soluble extract were dissolved in 10μL of water. The aqueous solution (2μL) was then mixed with 2μL of matrix solution (1% w/v ACH in 70% aqueous acetonitrile + 0.1% (v/v) TFA), and 1μL of the mixture pipetted on to a target slide and allowed to air dry (approximately 5min).

Lyophilised peak fractions collected from the standard and reduced pH Cheddar cheese water-soluble extracts at day 22 of maturation were dissolved in 20μL of 50% aqueous acetonitrile + 0.1% (v/v) TFA. Aliquots (0.5μL) were spotted onto a LASERMAT 2000 autosampler target strip, allowed to air dry (approximately 5min), then overlaid with 0.5μL of the ACH matrix solution.

Mass accuracy was improved by using standard peptide calibrants, kemptide (M_w=771.9Da), bradykinin (M_w=1060.2Da), substance P (M_w=1347.6Da), FSH-Peptide A (M_w=1849.1Da) and PDE-3 (M_w=2533.8Da), each dissolved in water to a final concentration of 16.2pmol per 0.3μl aliquot. Typically, accuracies better than 0.05% were achieved by 'sandwiching' the peptide of interest between two internal calibrants of similar masses.

Peptide mass searching of the different caseins was performed using the Protein Abacus software program, (Lighthouse data, version 2.0.2, Thermo Bioanalysis Ltd.). The primary structures of the caseins and their variants were taken from Swaisgood (1992).

4.2.7 N-terminal sequencing

N-terminal sequencing was carried out essentially as described by Bartlet-Jones *et al.* (1994). However, in some cases, rather than creating a nested set of ragged end peptides to be analysed as a ladder sequence in a one step read out, peptide spectra were obtained after each degradation cycle. This experimental approach of visualising

successive mass changes allows the sequencing of overlapping peptide mixtures, which would otherwise be too complicated to interpret.

Peptides were additionally analysed by mass spectrometry after each initial coupling reaction with the TFEITC reagent. This was performed in order to determine the number of lysine residues present, owing to the concomitant modification of the ε-amino group on lysine residue side chains by the TFEITC reagent giving perceived mass increases of 141Da. This coupled peptide mass determination also allowed the identification of any N-terminal lysine residues, which would otherwise give a perceived mass loss on cleavage of 128.17Da, creating ambiguity with glutamine (128.13Da).

To maintain equal concentrations of peptide per 0.3μl aliquot pipetted, proportional reductions were made to the volume of 50% aqueous acetonitrile + 0.1% (v/v) TFA in which each cycle product was subsequently dissolved in. Mass accuracy was enhanced by either using internal calibrants, or by calibrating the LASERMAT 2000 mass spectrometer to the mass range of the peptide and its cleavage products on the preceding slide.

Three cycles of N-terminal sequencing, in conjunction with the mass of the peptide, were sufficient to identify which casein fragment each of the peptides analysed represented.

4.3 Results and discussion

4.3.1 Mature Cheddar cheese

The C_{18} RP-HPLC chromatogram of the water-soluble extract from the one year old mature Cheddar cheese is shown in Figure 4.2, with the peak fractions collected for mass spectrometry analysis numbered 1 to 15. Fractions 1, 5, 8, 10 and 14 contained only one peptide, with the remaining fractions consisting of multiple peptide components, as determined by MALDI-MS. Fraction 15 in particular was a complex mixture of many low- and medium-molecular weight peptides, the cumulative absorbance of all of these components giving rise to the appearance of a single large peak.

Sixteen peptides were characterised, of which eight were derived from α_{s1}-casein, one from α_{s2}-casein and seven from β-casein (Tables 4.1 and 4.2). In most cases the accuracy of mass measurement between successive cycle products gave mass errors of

FIGURE 4.2: RP-HPLC chromatogram of a water-soluble extract from mature Cheddar cheese.

Water-soluble extract from a one year old mature Cheddar cheese, dissolved in dissociation buffer (final concentration 10mg mL^{-1}), and eluted with a gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

Numbered peak fractions were collected and their peptides identified by MALDI-MS N-terminal sequencing (Tables 4.1 and 4.2).

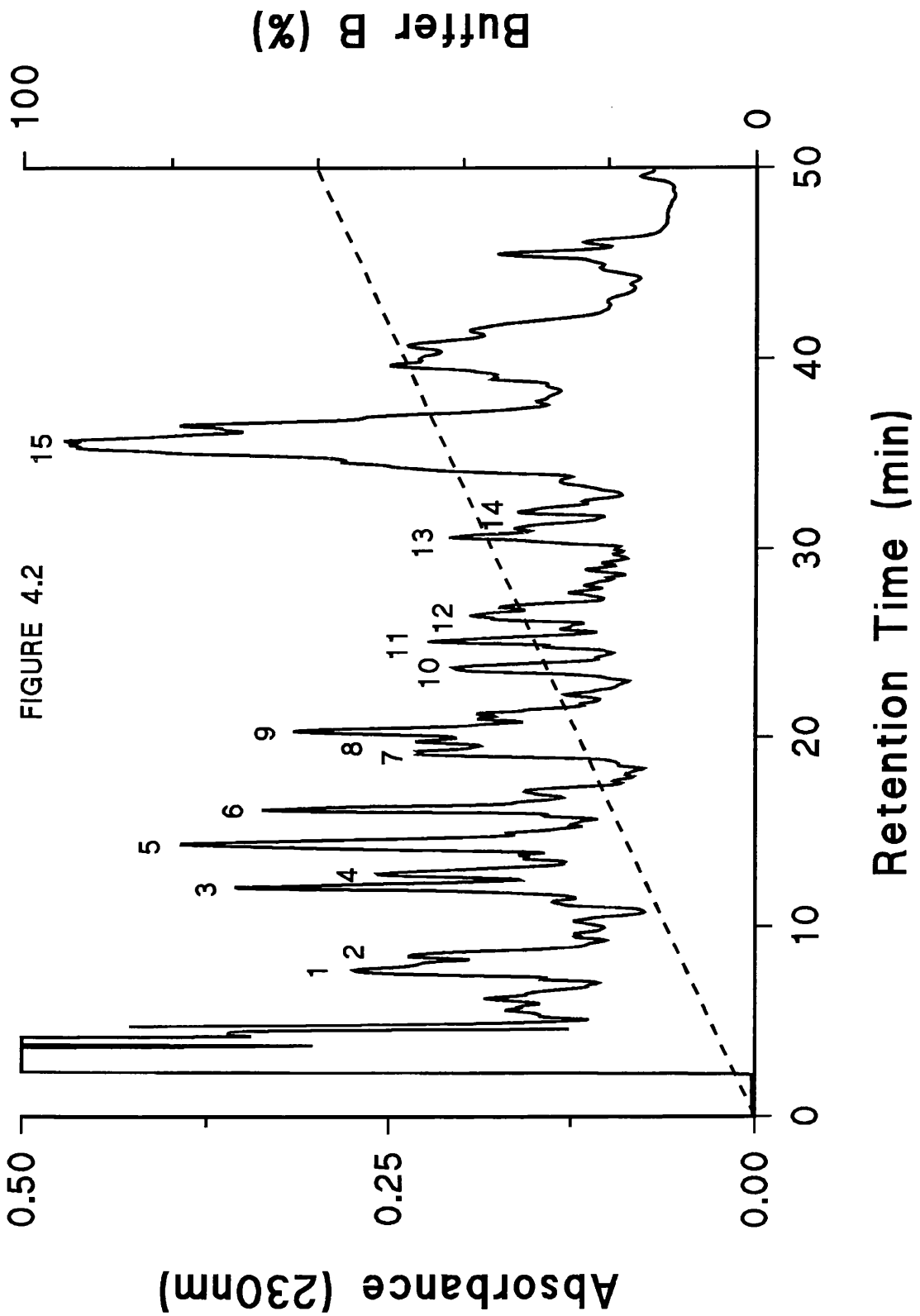

Buffer B (%)
100
0
50
40
30
20
10
0
Retention Time (min)
0.50
0.25
0.00
Absorbance (230nm)
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15

FIGURE 4.2

TABLE 4.1: MALDI-MS sequencing data for the α_s-casein water-soluble peptides characterised from the one year old mature Cheddar cheese.

Peak No	Peptide Mass	Mass Found	Error (%)	N-Terminal Sequencing: Coupled Peptide	1st Cycle	2nd Cycle	3rd Cycle	# Lys	Peptide Sequence
1	1535.8	1536.3	0.03	1959.5	1662.4 Arg	1565.6 Pro	1294.9 Lys	2	α_{s1}-Casein f(1–13)
2	1664.9	1665.9	0.06	2088.1	1792.5 Arg	1694.5 Pro	1424.8 Lys	2	α_{s1}-Casein f(1–14)
11	1246.4	1246.0	0.03	1388.9	1118.8 Glu	1018.5 Val	905.3 Leu	0	α_{s1}-Casein f(14–23)
9	905.0	905.0	0.00	1045.5	791.4 Asn	662.4 Glu	547.6 Asn	0	α_{s1}-Casein f(17–23)
9	661.8	661.3	0.07	802.9	547.2 Asn	434.4 Leu	321.2 Leu	0	α_{s1}-Casein f(19–23)
7	805.9	805.4	0.06	[a]969.4	658.8 Phe	560.3 Val	489.7 Ala	0	α_{s1}-Casein f(24–30)
4	978.1	976.9	0.12	1117.7	863.1 Asp	762.6 Val	665.9 Pro	0	α_{s1}-Casein f(85–92)
11	1566.8	1567.1	0.02	1709.5	1467.4 Val	[b]1373.7 Pro	1282.3 Ser	0	α_{s1}-Casein f(86–98)
4	920.0	919.1	0.10	1201.1	931.6 Glu	661.4 Lys	548.4 Asn	1	α_{s2}-Casein f(157–163)

[a] Increase in coupled peptide mass probably due to Na^+.
[b] 2nd cycle product of β-casein f(192–206) partially obscured 2nd cycle product of α_{s1}-casein f(86–98), resulting in observed inaccuracies.

TABLE 4.2: MALDI-MS sequencing data for the β-casein water-soluble peptides characterised from the one year old mature Cheddar cheese.

				N-Terminal Sequencing					
Peak No	Peptide Mass	Mass Found	Error (%)	Coupled Peptide	1st Cycle	2nd Cycle	3rd Cycle	# Lys	Peptide Sequence
6	997.2	996.8	0.04	1279.2	1008.5 Leu	896.5 Gln	781.4 Asp	1	β-Casein f(45–52)
4	755.9	755.1	0.11	1038.4	780.1 Asp	511.3 Lys	399.0 Ile	1	β-Casein f(47–52)
11	1669.0	1669.5	0.03	1811.9	1557.2 Leu	1377.6 Tyr	1266.3 Gln	0	β-Casein f(192–206)
10	1555.8	1556.6	0.05	1697.0	1376.0 Tyr	1264.3 Gln	1136.0 Glu	0	β-Casein f(193–206)
13	1782.1	1782.7	0.03	1925.2	1602.2 Tyr	1494.0 Gln	1363.4 Glu	0	β-Casein f(193–208)
14	1881.3	1882.3	0.05	2028.1	1702.1 Tyr	1591.0 Gln	1459.2 Glu	0	β-Casein f(193–209)
9	1038.3	1038.3	0.00	1180.2	939.1 Val	826.1 Leu	769.3 Gly	0	β-Casein f(197–206)

$\pm$1.0Da, with mass difference uncertainties reduced by comparing measured differences on either side of the residue peak in question (Chait *et al.*, 1993).

When analysed by MALDI-MS, fraction 4 gave signals for three peptides of mass 755.1, 919.1 and 976.9Da, which were subsequently identified as β-casein f(47–52), α_{s2}-casein f(157–163) and α_{s1}-casein f(85–92), respectively. Since these peptides all possessed very similar molecular weights a one step ladder readout after three degradation cycles would have been very complicated, perhaps even impossible to follow with confidence. The sequence interpretation of their overlapping spectrums was made possible however by the MALDI-MS visualisation of successive mass changes. Such an approach eliminated the need to further separate the fraction by C_4 RP-HPLC, substantially decreasing analysis time and preserving sample.

A similar approach was attempted for fraction 11, whose MALDI-MS spectrum gave strong peptide signals at 1246.0, 1494.6, 1567.1, 1638.8 and 1669.5Da. However, when applying successive cycle visualisation the peptides of mass 1246.0Da and 1669.5Da gave signals that could be followed, while the signals from peptides of mass 1494.6Da and 1567.1Da were suppressed and lost. Interestingly, the 1638.8Da signal remained present and unchanged throughout the sequencing cycles, suggesting that this peptide may possibly be N-terminally blocked.

Selective suppression of signal is often seen when attempting mass spectrometric analysis of complex peptide mixtures (Billeci and Stults, 1993), and following its isolation by C_4 RP-HPLC, peptide 1567.1Da was successfully sequenced through three residues, allowing its identification as α_{s1}-casein f(86–98). The three peptides identified from fraction 9, α_{s1}-casein f(17–23), α_{s1}-casein f(19–23) and β-casein f(197–206) (Tables 4.1 and 4.2), were separated by C_4 RP-HPLC prior to sequencing.

The apparent loss of mass spectrometer signals when applying TFEITC sequencing chemistry is likely to be the result of decreased peptide solubility, especially with those peptides containing predominantly hydrophobic residues. Additionally, the progressive adsorption, and consequent loss of peptide material on to the vessel walls during repeated drying *in vacuo*, could cause the elimination of weaker peptide signals, especially those samples having an initially low concentration. Obtaining a higher concentration of peptide before sequencing may reduce the signal weakening, as the level of suppression increases with loss of residues.

Signal suppression is unlikely to be caused by a peptide possessing phosphorylated residues; such a modification should actually aid solubilisation. Bartlet-Jones (personal communication) has sequenced through a phosphorylated tyrosine residue, and has visualised synthetic peptides possessing one or more phosphorylated residues by MALDI-MS, with only a slight reduction in signal intensity. Peptide sequencing by mass differences is an ideal way to establish the existence and position of secondary modifications.

Residual trimethylamine or the conjugate TFA salt has the potential to exert a negative buffering effect on the sequencing chemistry, as well as causing significant suppression of signal in the mass spectrometer. It is therefore important to ensure removal of all base reagent following each coupling step, so that salt formation with the cleavage acid is kept to a minimum.

Partial blockage to further degradation was observed when a glutamine residue was exposed at the N-terminus, resulting from its concomitant cyclisation under acid conditions to a pyrrolidonecarboxylyl (pyroglutamyl) residue. The preceding residue removed by acid cleavage was then perceived to have a mass 17Da higher than its theoretical weight, due to the release of an ammonia molecule during this cyclic rearrangement (Allen, 1989). This was observed for β-casein f(45–52), f(192–206), f(193–206), f(193–208) and f(193–209).

Three peptides, α_{s1}-casein f(1–13), β-casein f(45–52) and β-casein f(193–208), were sequenced by automated Edman degradation using a Model 477A Pulsed-Liquid Phase Protein/Peptide Sequencer (Applied Biosystems Inc.), at the National Food Biotechnology Centre, University College, Cork, Ireland. Each peptide was analysed through five cycles, with liberated amino acids detected as their phenylthiohydantoin derivatives by an on-line Model 120A Analyser (Applied Biosystems Inc.). The results confirmed the MALDI mass spectrometry sequence interpretations.

This study was undertaken to demonstrate and optimise the application of TFEITC mass spectrometry sequencing to the investigation of proteolysis in cheese, and as a consequence the number of peptides characterised was not exhaustive. However, the data obtained do allow comparisons to be made with regard to peptides identified by other investigators.

In cheese, the primary site of chymosin hydrolysis of α_{s1}-casein is the peptide bond Phe_{23}-Phe_{24}, resulting in the release of the small water-soluble peptide α_{s1}-casein f(1–23) early in the maturation process (McSweeney *et al.*, 1993b). Under conditions prevailing in cheese (viz. pH5.2 and about 4% NaCl in-the-moisture), the purified P_I-type and P_{III}-type free proteinases of *Lactococcus lactis* subsp. *cremoris* show optimum specificity to peptide bonds Gln_{13}-Glu_{14} and Leu_{16}-Asn_{17} in the α_{s1}-casein f(1–23), respectively (Exterkate and Alting, 1993). Additionally, α_{s1}-casein f(1–23) on incubation with whole cells of *Lactococcus lactis* subsp. *cremoris* strain AM_1 (P_{III}-type proteinase), showed a buildup of f(1–13) and f(1–14), with NaCl stimulating their formation at pH5.2. Such proteinase activity could account for the α_{s1}-casein f(1–13), f(1–14), f(14–23) and f(17–23) identified, with aminopeptidase hydrolysis of the latter fragment creating f(19–23).

The majority of the β-casein peptides identified originated from the C-terminal end of the protein, specifically between residues Leu_{192} and Val_{209}. Three β-casein peptides, f(193–209), f(193–208) and f(193–206), have an N-terminus consistent with the known proteolytic cleavage of peptide bond Leu_{192}-Tyr_{193} by chymosin (Visser and Slangen, 1977), or the P_{III}-type lactococcal proteinase (Reid *et al.*, 1991b). Additionally, β-casein f(193–208) would require carboxypeptidase activity for formation, with f(193–206) possibly being formed by lactococcal proteinase cleavage at Ile_{207}-Ile_{208} (Monnet *et al.*, 1986; Juillard *et al.*, 1995), followed by carboxypeptidase hydrolysis. However, there are no reports of carboxypeptidase activity in lactococcal subsp., although this activity has been reported in lactobacilli (Atlan *et al.*, 1993), the principal non-starter lactic acid bacteria in Cheddar cheese.

The peptide β-casein f(47–52), could be formed by the hydrolysis of bonds Gln_{46}-Asp_{47} and Phe_{52}-Ala_{53} by the *Lactococcus lactis* subsp. *cremoris* AM_1 P_{III}-type proteinase, and β-casein f(45–52) by the lactococcal proteinase cleavage of bond Asp_{43}-Glu_{44} (Visser *et al.*, 1991a) and aminopeptidase activity. Singh *et al.* (1995), have recently reported the identification of β-casein f(47–52) in a commercial mature Cheddar cheese (approximately 9 months old), made using a pair of *Lactococcus lactis* subsp. *cremoris* strains.

While it appears that α_{s2}-casein f(157–163) has not previously been observed, its formation could be the result of chymosin hydrolysis of bond Phe_{163}-Leu_{164} (McSweeney *et al.*, 1994), and lactococcal proteinase activity at peptide bond Glu_{156}-Glu_{157}, which exhibits the characteristics of a primary cleavage site for such an enzyme.

4.3.2 Standard and reduced pH milk Cheddar cheese

The RP-HPLC profiles of the water-soluble extracts from standard pH and reduced pH Cheddar cheese at day 22 of maturation are shown in Figure 4.3(A) and (B). Their respective modified gradient chromatograms are shown in Figure 4.3(a) and (b). It can be seen that the water-soluble extract from the reduced pH Cheddar cheese was qualitatively and quantitatively significantly different to that from the standard pH Cheddar cheese. Chromatograms of the water-soluble extracts at other maturation times are shown in Appendix III, Figures III.1–III.15.

A total of 38 and 47 peak fractions were collected from the modified gradient chromatograms of the standard and reduced pH Cheddar cheese water-soluble extracts respectively. All the peak fractions recovered were analysed by MALDI-MS, to determine the number and molecular weight of peptide components present in each.

Only peptides from peak fractions collected from the reduced pH cheese water-soluble extract were characterised by MALDI-MS TFEITC N-terminal sequencing. Peptides from the standard pH cheese water-soluble extract were identified subsequently, by comparing their molecular mass and/or RP-HPLC retention time with those peptides identified in the reduced pH cheese water-soluble extract peak fractions. The water-soluble peak fractions containing characterised peptides are shown in Figure 4.4(A) and (B). For ease of comparison, and to highlight the qualitative differences and similarities between the two water-soluble extracts, peak fractions containing the same peptides are similarly labelled.

Twenty peptides were characterised from the first 21 numbered peak fractions collected from the reduced pH cheese water-soluble extract. Ten peptides were derived from α_{s1}-casein and ten from β-casein (Tables 4.3 and 4.4). Mass spectrometry signals from peak fractions 2, 4, 5, 17 and 20 were lost during the sequencing analysis, preventing their unequivocal identification. This was almost certainly the result of signal suppression or low peptide concentrations, as previously discussed in section 4.3.1.

FIGURE 4.3: RP-HPLC chromatograms of water-soluble extracts from standard pH and reduced pH Cheddar cheese at day 22 of maturation.

(A) Water-soluble extract from standard pH Cheddar cheese, dissolved in water (final concentration 5mg mL^{-1}), and eluted with a gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

(a) Water-soluble extract from standard pH Cheddar cheese, dissolved in water (final concentration 10mg mL^{-1}), and eluted with a modified gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

(B) Water-soluble extract from reduced pH Cheddar cheese, dissolved in water (final concentration 5mg mL^{-1}), and eluted with a gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

(b) Water-soluble extract from reduced pH Cheddar cheese, dissolved in water (final concentration 10mg mL^{-1}), and eluted with a modified gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

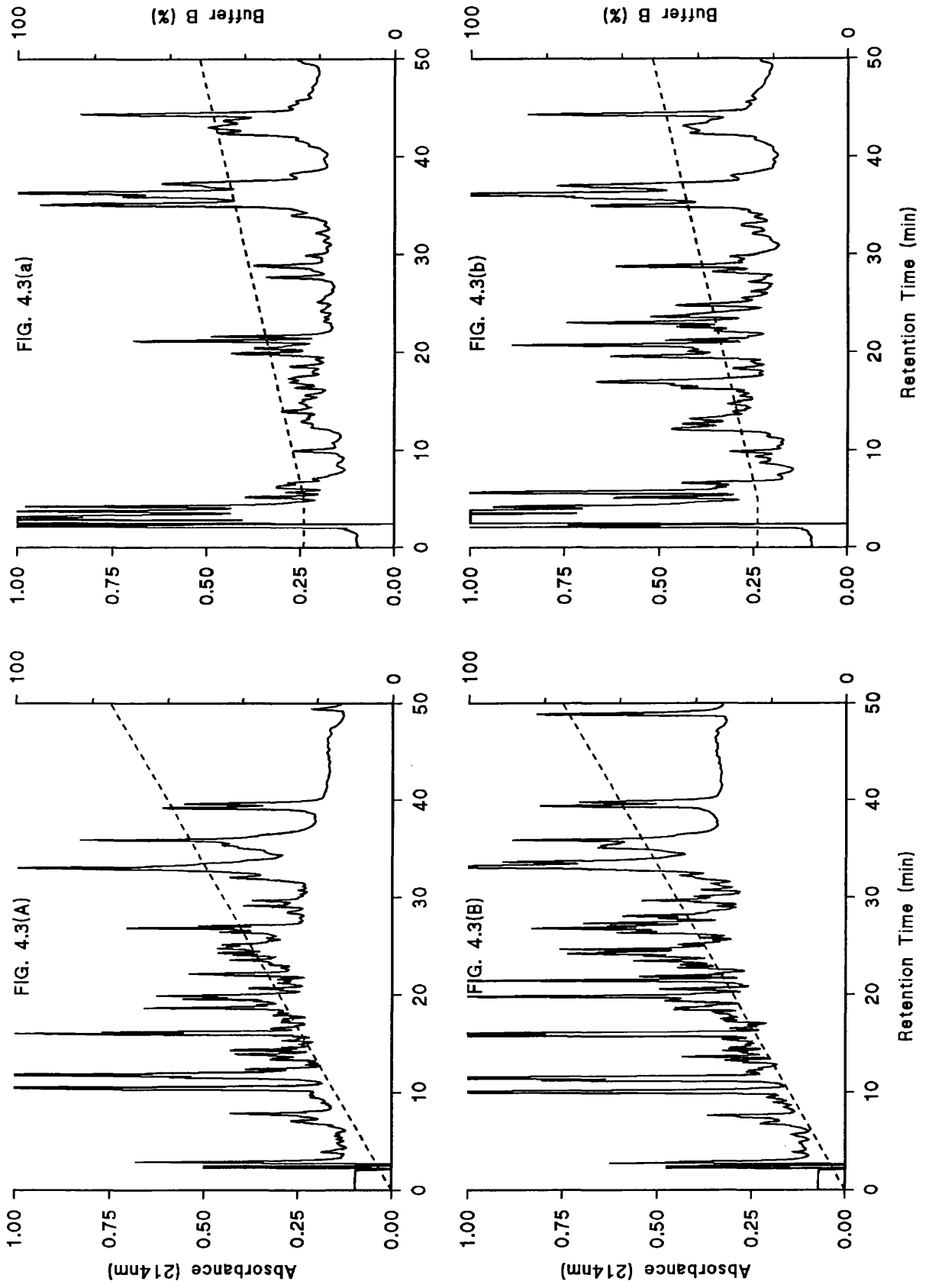


FIG. 4.3(A)
FIG. 4.3(B)
FIG. 4.3(a)
FIG. 4.3(b)
Absorbance (214nm)
Buffer B (%)
Retention Time (min)
1.00
0.75
0.50
0.25
0.00
100
0
10
20
30
40
50

FIGURE 4.4: RP-HPLC modified gradient chromatograms of water-soluble extracts from reduced pH and standard pH Cheddar cheese at day 22 of maturation.

(A) Water-soluble extract from reduced pH Cheddar cheese, dissolved in water (final concentration 40mg mL^{-1}), and eluted with a modified gradient of 0.1% TFA in 90% acetonitrile/water (v/v) as described in the text.

Numbered peak fractions were collected and their constituent peptides identified by MALDI-MS N-terminal sequencing (Tables 4.3 and 4.4).

(B) Water-soluble extract from standard pH Cheddar cheese, dissolved in water (final concentration 40mg mL^{-1}), and eluted with a modified gradient of 0.1% TFA in 90% acetonitrile/water (v/v) as described in the text.

Numbered peaks identify those fractions containing peptides common to the reduced pH Cheddar cheese water-soluble extract.

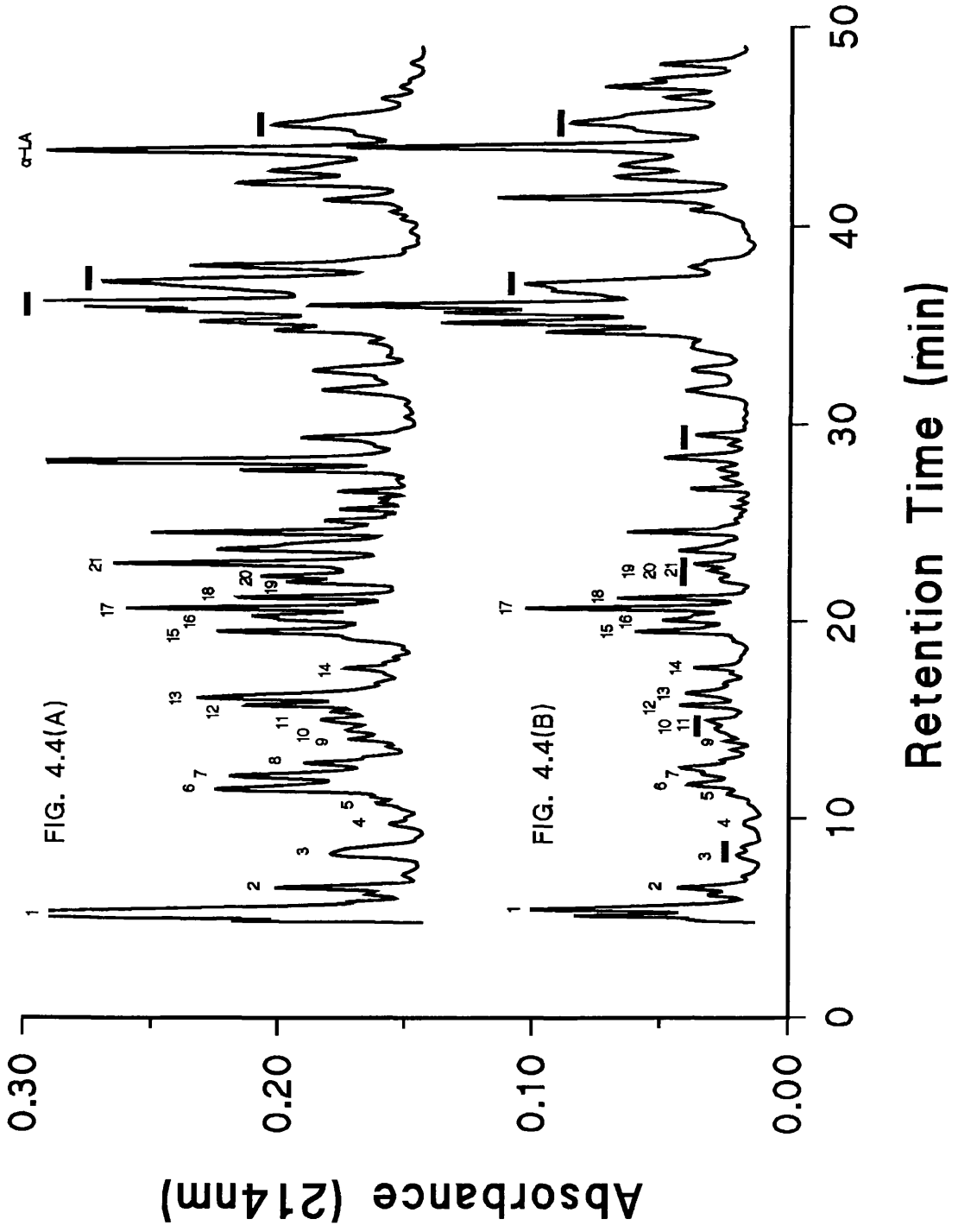

Absorbance (214nm)
0.30
0.20
0.10
0.00
0
10
20
30
40
50
Retention Time (min)
FIG. 4.4(A)
FIG. 4.4(B)
α-LA

TABLE 4.3: MALDI-MS sequencing data for the α_{s1}-casein water-soluble peptides characterised from the reduced pH Cheddar cheese at day 22 of maturation.

				N-Terminal Sequencing					
Peak No	Peptide Mass	Mass Found	Error (%)	Coupled Peptide	1st Cycle	2nd Cycle	3rd Cycle	# Lys	Peptide Sequence
1	1877.2	1875.6	0.09	2298.6	2002.5 Arg	1906.2 Pro	1635.4 Lys	2	α_{s1}-Casein f(1–16)
3	2347.7	2345.5	0.09	2771.6	2474.9 Arg	2377.9 Pro	2108.4 Lys	2	α_{s1}-Casein f(1–20)
19	2764.2	2762.9	0.05	No Signal	2892.3 Arg	2791.7 Pro	2524.1 Lys	2	α_{s1}-Casein f(1–23)
14	1117.3	1117.7	0.04	1257.7	1018.3 Val	905.1 Leu	790.3 Asn	0	α_{s1}-Casein f(15–23)
6	905.0	904.3	0.08	1045.9	791.6 Asn	660.8 Glu	546.7 Asn	0	α_{s1}-Casein f(17–23)
7	790.9	790.6	0.04	930.5	660.9 Glu	546.7 Asn	433.9 Leu	0	α_{s1}-Casein f(18–23)
16	1681.9	1682.0	0.01	1822.1	1566.6 Asp	1467.2 Val	1368.8 Pro	0	α_{s1}-Casein f(85–98)
12	1283.4	1283.1	0.02	1425.2	1154.4 Glu	999.4 Arg	835.6 Tyr	0	α_{s1}-Casein f(89–98)
8	2320.6	2321.9	0.06	2889.4	2479.0 Lys	2209.5 Lys	2045.4 Tyr	3	α_{s1}-Casein f(102–120)
8	3240.7	3242.2	0.05	3951.2	3538.5 Lys	3269.5 Lys	3106.9 Tyr	4	α_{s1}-Casein f(102–128)

TABLE 4.4: MALDI-MS sequencing data for the β-casein water-soluble peptides characterised from the reduced pH Cheddar cheese at day 22 of maturation.

Peak No	Peptide Mass	Mass Found	Error (%)	N-Terminal Sequencing				# Lys	Peptide Sequence
				Coupled Peptide	1st Cycle	2nd Cycle	3rd Cycle		
21	2641.1	2635.4	0.22	2777.0	2522.0 Leu	2425.3 Val	2264.8 Tyr	0	β-Casein f(58–81)
13	1555.8	1554.9	0.06	1697.0	1376.0 Tyr	1264.1 Gln	1135.3 Glu	0	β-Casein f(193–206)
21	1669.0	1666.0	0.18	1809.4	1487.4 Tyr	Blocked	Blocked	0	β-Casein f(193–207)
9	1392.6	1392.6	0.00	1534.5	1263.2 Gln	1134.1 Glu	1037.7 Pro	0	β-Casein f(194–206)
18	1505.8	1505.4	0.03	1647.0	1376.9 Gln	1247.6 Glu	1149.1 Pro	0	β-Casein f(194–207)
10	1264.5	1263.7	0.06	1404.5	1135.4 Glu	1038.3 Pro	938.6 Val	0	β-Casein f(195–206)
19	1377.7	1377.8	0.01	1519.5	1248.7 Glu	1151.3 Pro	1051.5 Val	0	β-Casein f(195–207)
6	1038.3	1038.6	0.03	1181.5	940.2 Val	826.0 Leu	769.5 Gly	0	β-Casein f(197–206)
15	1151.4	1150.8	0.05	1293.1	1052.6 Val	941.4 Leu	883.3 Gly	0	β-Casein f(197–207)
21	1151.4	1149.1	0.20	1291.2	1092.2 Gly	995.3 Pro	896.2 Val	0	β-Casein f(199–209)

Lowering the pH of the milk used in cheese manufacture to 6.0 increases the retention of chymosin in the curd matrix (Creamer *et al.*, 1985), leading to enhanced hydrolysis of the α_{s1}-casein Phe_{23}-Phe_{24} peptide bond. Consequently, there is a resultant increase in the production of α_{s1}-casein f(1–23) and the α_{s1}-I polypeptide f(24–199), and peptide material derived from these fragments through their subsequent degradation by other enzyme systems.

This effect can be clearly observed in Figure 4.4, where all peak fractions containing α_{s1}-casein peptides (viz. 1, 3, 6, 7, 8, 12, 16 and 19) are qualitatively larger in the reduced pH, rather than the standard pH cheese water-soluble extract, with the sole exception of peak fraction 14 which is of a comparable size in both. These observations are substantiated by capillary electrophoresis studies of the degradation of α_{s1}-casein and the α_{s1}-I polypeptide, both of which were found to be hydrolysed at a faster rate in the reduced pH milk Cheddar cheese (Chapter 5).

The primary chymosin cleavage site in β-casein is at peptide bond Leu_{192}-Tyr_{193} (Visser and Slangen, 1977; Carles and Ribadeau-Dumas, 1984), the hydrolysis of which releases the C-terminal water-soluble peptide, β-casein f(193–209). Increasing chymosin retention in the curd matrix by lowering the pH of the cheese milk to 6.0 could also be expected to result in the greater hydrolysis of the Leu_{192}-Tyr_{193} bond. Consequently, there would be a resultant increase in the production of the β-casein f(193–209), and peptide material derived from it through subsequent degradation by other enzymes. Most peak fractions containing β-casein peptides (viz. 6, 9, 13, 15, 19 and 21) were qualitatively larger in the case of the reduced pH than the standard pH cheese water-soluble extract. Exceptions are peak fractions 10 and 18, which were of a comparable size in both extracts (Figure 4.4).

Comparison of the peptides characterised in this study with peptides identified in model systems and in other cheese samples, and relating their formation to known enzyme specificities, provides for an interesting discussion.

Four of the α_{s1}-casein peptides identified, f(1–23), f(15–23), f(17–23) and f(18–23), possess a C-terminus consistent with the well-documented cleavage of the Phe_{23}-Phe_{24} peptide bond by chymosin. Exterkate and Alting (1993) showed that the purified P_{III}-type free proteinase of *Lactococcus lactis* subsp. *cremoris*, under conditions prevailing in cheese (viz. pH5.2 and about 4% NaCl in-the-moisture), cleaved the peptide

bonds Gln_{13}-Glu_{14}, Leu_{16}-Asn_{17} and Asn_{17}-Glu_{18} in the α_{s1}-casein f(1–23). Additionally, the P_I-type free proteinase under cheese conditions gave enhanced cleavage of peptide bond Gln_{13}-Glu_{14}, while the splitting of those bonds specific for the P_{III}-type proteinase, Leu_{16}-Asn_{17} and Asn_{17}-Glu_{18}, also increased. These three peptide bonds were also cleaved on incubation of α_{s1}-casein f(1–23) with whole lactococcal cells at pH5.2 in the presence of NaCl. Hydrolysis of the Leu_{16}-Asn_{17} peptide bond by the cell envelope proteinase could account for the formation of α_{s1}-casein f(17–23) and f(1–16). Proteinase cleavage of peptide bond Asn_{17}-Glu_{18}, or aminopeptidase action on the α_{s1}-casein f(17–23), could create α_{s1}-casein f(18–23), with proteinase hydrolysis of Gln_{13}-Glu_{14}, followed by aminopeptidase activity producing the peptide α_{s1}-casein f(15–23).

Four of the cheese peptides characterised were derived from the central region of the α_{s1}-casein protein molecule. α_{s1}-Casein f(85–98) and f(89–98) possess a C-terminus consistent with the observed *in vitro* cleavage of the peptide bond Leu_{98}-Leu_{99} in the α_{s1}-I polypeptide by chymosin under cheese conditions (Exterkate *et al.*, 1995), and upon incubation of α_{s1}-casein with chymosin (Pahkala *et al.*, 1989). Cleavage of bonds Glu_{84}-Asp_{85} and Ser_{88}-Glu_{89}, to form the α_{s1}-casein f(85–98) and f(89–98) peptides, have been observed under simulated cheese conditions, following the incubation of chymosin generated α_{s1}-casein f(24–98) with both purified P_I-type cell envelope proteinase and whole cells from *Lactococcus lactis* strain HP (Exterkate *et al.*, 1995), and upon digestion of α_{s1}-casein with the partially purified P_{III}-type proteinase from *Lactococcus lactis* subsp. *cremoris* SK11 (Reid *et al.*, 1991a).

α_{s1}-Casein f(102–120) and f(102–128) have an N-terminus consistent with the chymosin cleavage of peptide bond Leu_{101}-Lys_{102} (McSweeney *et al.*, 1993b; Exterkate *et al.*, 1995). Further chymosin hydrolysis of the peptide bond His_{128}-Ala_{129} (Pahkala *et al.*, 1989; Exterkate *et al.*, 1995) would create the α_{s1}-casein f(102–128). Cleavage of peptide bond His_{121}-Ser_{122} was observed by the partially purified P_{III}-type proteinase from *Lactococcus lactis* subsp. *cremoris* SK11 (Reid *et al.*, 1991a), which, followed by carboxypeptidase digestion, could lead to the formation of α_{s1}-casein f(102–120).

Apart from β-casein f(58–81), all of the peptides characterised as being formed from β-casein were derived from the C-terminal region of the molecule, between residues Leu_{192} and Val_{209}. Two β-casein peptides, f(193–207) and f(193–206), have an

N-terminus consistent with the known proteolytic cleavage of bond Leu_{192}-Tyr_{193} by chymosin, or the P_{III}-type lactococcal proteinase (Reid *et al.*, 1991b). Formation of β-casein f(193–207) would require either additional carboxypeptidase activity, or cleavage of peptide bond Ile_{207}-Ile_{208} by lactococcal proteinase (Monnet *et al.*, 1986; Juillard *et al.*, 1995), with β-casein f(193–206) perhaps being formed from further carboxypeptidase digestion. Singh *et al.* (1995), have recently reported the identification of β-casein f(193–206) in a commercial mature Cheddar cheese (approximately 9 months old) made using a pair of *Lactococcus lactis* subsp. *cremoris* strains.

While there is no report of carboxypeptidase activity in lactococcal subsp., this activity has however been detected in lactobacilli (Atlan *et al.*, 1993), the principal non-starter lactic acid bacteria in Cheddar cheese.

The six β-casein peptides, f(194–206), f(194–207), f(195–206), f(195–207), f(197–206) and f(197–207), were most probably created by aminopeptidase activity. Aminopeptidases are well characterised in lactococcal species (Pritchard and Coolbear, 1993; Tan *et al.*, 1993a), and have important roles in generating essential amino acids for cell growth.

4.4 Summary

A number of fundamental advantages of using MALDI mass spectrometry for peptide identification have been demonstrated. Identification of peptides was possible after only three sequencing cycles, as the mass weight determination eliminates the need to sequence fully a peptide in order to determine its size and hence absolute identity. Peptides composed of, or ultimately comprising after sequencing, only a few residues are not subject to the washout problems associated with gas phase sequencers - one of the peptides identified in this study was only five amino acids in length.

The potential sensitivity of the TFEITC ladder sequencing protocol was investigated by Bartlet-Jones *et al.* (1994). For peptides possessing readily ionisable residues in their primary structure, sensitivity in the low femtomole range is quite possible, while in working practice 50-100 femtomoles would be reasonable. This compares favourably with Edman degradation sequencing, the operating concentrations of which are normally in the low picomole range.

Multiple peptide samples can be processed simultaneously, allowing very high sample throughput. One degradation cycle of TFEITC sequencing chemistry is typically of the order of 40min, and by allowing multiple sample sequencing, many more residues can be identified than that possible by Edman degradation during a similar length of time.

The number of peptides common to both the mature one year old Cheddar cheese and the pH adjusted milk Cheddar cheeses at day 22 of maturation was small, viz. α_{s1}-casein f(17-23), β-casein f(193–206) and β-casein f(197–206). However, a large number differed only by a single amino acid residue, or possessed the same N- or C-terminus. The total number of peptides generated by proteolysis during cheese maturation is considerable, and this observation suggests that flavour and texture development in cheese is likely to be highly dependent on the relative concentrations and proportions of certain peptides. The identification and quantification of such key peptides would certainly contribute greatly to understanding the molecular mechanisms involved in the creation of quality cheese.

Since the primary structures of the milk proteins are very well characterised, MALDI mass spectrometry and TFEITC ladder sequencing is an ideal partnership with which to identify peptide products formed during proteolysis in cheese.

Chapter 5

Casein Degradation in Cheddar Cheese

5.1 Introduction

The development of cheese flavour and texture is highly dependent on the rate and extent of proteolysis during ripening. Manipulation of the manufacturing conditions, by heating the milk, adjusting the pH on renneting or varying the starter cultures used, alters the kinetics and nature of this proteolysis, changing the properties of the mature cheese. Information on the extent and course of proteolysis occurring during the maturation period is therefore extremely important, if control of the quality of the final product is to be achieved.

Initial chymosin hydrolysis of α_{s1}-casein peptide bond Phe_{23}-Phe_{24}, creating the small water-soluble peptide α_{s1}-casein f(1–23) and the water-insoluble polypeptide α_{s1}-I-casein f(24–199), is well characterised and leads to a general weakening of the casein network (Lawrence *et al.*, 1987). Subsequent hydrolysis of β-casein, and the further degradation of α_{s1}-casein and the α_{s1}-I polypeptide, results in the release of peptides and free amino acids, the contribution of which to flavour and texture development has been the subject of much investigation.

Milk caseins have traditionally been fractionated by low-pressure column techniques, using materials such as DEAE-cellulose (Davies and Law, 1977), or hydroxyapatite (Donnelly, 1977). Alkylation of whole casein by reaction with cysteamine and cystamine, followed by fast protein liquid chromatography (FPLC) separation, was found by Davies and Law (1987) to give quantitatively good agreement for α_{s1}-, α_{s2}-, β- and γ_2- plus γ_3-caseins with that obtained by other methods, including ion-exchange chromatography on DEAE-cellulose.

Visser *et al.* (1986b) used high performance liquid chromatography (HPLC) to compare bovine casein separations on hydroxyapatite, anion-exchange and reversed phase columns. Whole casein separations were carried out on a semi-preparative scale, with collected fractions being characterised by starch-gel electrophoresis at alkaline pH. For analytical peptide mapping of complex mixtures, or the further isolation of individual

components, the application of anion-exchange followed by reversed phase-high performance liquid chromatography (RP-HPLC) was considered to offer the best means of separation.

A RP-HPLC method for the simultaneous identification of various casein variants, and the whey proteins α-lactalbumin, β-lactoglobulin A and β-lactoglobulin B was described by Visser *et al.* (1991b). From the protein separations and resolution obtained it was concluded that, except for α_{s2}-casein, quantification should be possible, provided that standards were included in each series of analyses and standardisation of the experimental conditions was made.

Christensen *et al.* (1989) used RP-HPLC to determine the extent of proteolysis in Danbo and Havarti cheese manufactured under different processing conditions. Using a C_{18} column they achieved separation of α_{s1}-, α_{s2}-, and β-casein, and additionally resolved the α_{s1}-I polypeptide, enabling the hydrolysis of this initial degradation product of α_{s1}-casein to be followed.

Because ion-exchange FPLC had been used to separate and quantify individual casein fractions in milk (Davies and Law, 1987), its application was assessed for quantifying the various casein levels present in the water-insoluble extracts from Cheddar cheese (Calvo *et al.*, 1992). Over a twelve week ripening period quantification of the α_{s1}- and para-κ-casein, and an estimate of the α_{s2}-casein levels, was achieved using cation-exchange chromatography, while β-casein was quantified by anion-exchange chromatography.

Changes in the levels of casein during maturation of cheese made from pasteurised milk, or milk heated to higher temperatures so as to denature and incorporate whey proteins into the curd, was investigated using a combination of ion-exchange and reversed phase chromatography by Leaver *et al.* (1993). Application of both cation- and anion-exchange chromatography was necessary in order to analyse fully the casein levels in the water-insoluble extracts, with cheese manufactured from the higher heat treated milk showing faster degradation of both α_{s1}- and β-casein.

Recently, capillary zone electrophoresis (CZE) has been used to determine the composition of whole casein and casein fractions, and to monitor the chymosin degradation of purified caseins (Kristiansen *et al.*, 1994). CZE employs the separation mechanisms of conventional electrophoresis in a capillary format, allowing separated

components to be identified in a matter of minutes by on-line detection, eliminating the need for time consuming gel staining. With only nanolitres of sample consumed during each analysis, hundreds of CZE runs can be performed from the microlitre sample volumes typical of HPLC injections.

This chapter will describe the application of CZE in quantifying the changes to casein levels in Cheddar cheese manufactured from standard pH (6.4) and reduced pH (6.0) milk over a 246 day ripening period. Additionally, the separation and identification of the α_{s1}-I-casein f(24–199) allowed quantitative changes to this important polypeptide to be monitored in the two types of cheese.

Finally, a statistical model derived using Partial Least Squares Regression was assessed for predicting the RP-HPLC water-soluble peptide chromatogram segment peak areas from the measured changes in the casein levels.

5.2 Materials and methods

5.2.1 Standard and reduced pH milk Cheddar cheese

Cheddar cheese was manufactured from milk at either the standard pH or reduced pH as previously described in section 2.2.2.

5.2.2 Reagents

Bis-tris propane (1,3-bis[tris(Hydroxymethyl)methylamino]propane), calcium chloride, dipotassium hydrogen orthophosphate trihydrate, lauryl sulfate sodium salt (sodium dodecyl sulfate (SDS), approx. 99%), 2-mercaptoethanol, recombinant rennet (chymosin, E.C. 3.4.23.4), sodium azide, trifluoroacetic acid (TFA, anhydrous) and tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl, Trizma® hydrochloride) were purchased from Sigma Chemical Company Ltd., Fancy Road, Poole, Dorset, BH17 7BR.

Analytical grade diaminoethanetetraacetic acid disodium salt (EDTA), glacial acetic acid, glycerol, methanol and acetonitrile (far UV, HPLC grade solvent) were purchased from Fisher Scientific UK Ltd., Bishop Meadow Road, Loughborough, Leics., LE11 ORG.

The PhastSystem electrophoresis apparatus, PhastGels, SDS buffer strips and PhastGel Blue R tablets (Coomassie R 350 stain) were purchased from Pharmacia Biotech, 23 Grosvenor Road, St. Albans, Herts, AL1 3AW.

Alpha-cyano-4-hydroxycinnamic acid (ACH) was purchased from Aldrich Chemical Company, New Road, Gillingham, Dorset, SP8 4JL.

Mass spectrometry peptide standards, kemptide, bradykinin, substance P and insulin chain B (oxidised) were purchased from Sigma Chemical Company Ltd. FSH-Peptide A and PDE-3 were chemically synthesised at the Hannah Research Institute.

Phosphate buffer (100mM, containing polymer modifier) and electrophoresis grade urea were purchased from Bio-Rad Laboratories Ltd., Bio-Rad House, Maylands Avenue, Hemel Hempstead, Herts, HP2 7TD.

Sequencing grade modified trypsin was purchased from Promega Ltd., Delta House, Enterprise Road, Chilworth Research Centre, Southampton, SO16 7NS.

All water used was deionised or of HPLC grade, prepared on site using a CD plus deioniser from USF Ltd., John Tate Road, Hertford, SG13 7NW.

5.2.3 Extraction of the water-insoluble cheese peptides

Samples of the standard and reduced pH Cheddar cheese, removed at regular time intervals during their 246 day ripening period, were finely grated, freeze dried to constant dry weight, and water-insoluble extracts prepared using the fractionation procedure described in section 3.2.3.

Following decanting of the supernatant, the residual water-insoluble extract was recovered, lyophilised to constant dry weight, and ground to homogeneity in a pestle and mortar. Lyophilised extracts were stored at -20^{0}C, prior to analysis by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and capillary zone electrophoresis.

5.2.4 Mass spectrometry

Mass spectrometry was performed on a LASERMAT 2000 matrix assisted laser desorption/ionisation (MALDI) time-of-flight mass spectrometer (Thermo Bioanalysis Ltd., Paradise, Hemel Hempstead, Herts, HP2 4TG) as described in section 4.2.6.

Mass accuracy was improved by using standard peptide calibrants, kemptide (M_w=771.9Da), bradykinin (M_w=1060.2Da), substance P (M_w=1347.6Da), FSH-Peptide A (M_w=1849.1Da), PDE-3 (M_w=2533.8Da) and insulin chain B (oxidised) (M_w=3495.9Da), with the LASERMAT 2000 mass spectrometer calibrated to the mass range of the peptide or peptides to be analysed on the preceding slide.

5.2.5 Preparation and characterisation of the α_{s1}-casein f(24–199)

Lyophilised α_{s1}-casein, purified by Hi-Load S cation-exchange chromatography and stored at -20^{0}C, was dissolved at 10mg mL^{-1} in 100mM phosphate buffer (pH6.5) containing 0.05% (w/v) sodium azide. Chymosin was added at 0.015CU mL^{-1}, and 25μL aliquots of the digestion mixture were automatically injected and separated by RP-HPLC every hour over a 24h incubation period.

Chromatographic separation was performed on an APEX WP ODS (C_{18}) column (7μm, 250x4.6mm i.d.; Jones Chromatography, New Road, Hengoed, Mid Glamorgan, CF8 8AU), using an automated Spectraphysics HPLC system. Elution was achieved using a linear gradient of 35–55% buffer B over 50min. Buffer A consisted of 0.1% TFA in water and buffer B of 0.1% TFA in 90% acetonitrile/water (v/v). Flow rate was 1.50mL min^{-1}, column temperature was 46^{0}C and peak detection was at 230nm. Peak fractions were collected manually, lyophilised and stored at -20^{0}C prior to MALDI-MS analysis.

Peak fractions requiring characterisation by MALDI-MS tryptic peptide mapping were resuspended in dilution buffer (25mM Tris-HCl, 1mM calcium chloride, pH7.6), and trypsin added to give a protease:protein ratio of between 1:100 to 1:20 (w/w). At regular time intervals an assessment of the extent of trypsinolysis was made by MALDI-MS. Aliquots (1μL) of the digestion mixture were pipetted onto a LASERMAT 2000 target slide, allowed to air dry (approximately 5min), then overlaid with 1μL of a saturated solution of ACH matrix in 70% aqueous acetonitrile + 0.1% (v/v) TFA.

5.2.6 SDS-PAGE

5.2.6.1 Sample preparation

Lyophilised water-insoluble extracts were dissolved at 10mg mL^{-1} in a stock SDS buffer solution (20% SDS, 2-mercaptoethanol, 10mM Tris-HCl/1mM EDTA (pH8.0), 0.05% bromophenol blue (2.5:1:15.25:1.25 v/v/v/v)). The dissolved samples were then denatured by heating them in a boiling water bath for 5min.

5.2.6.2 Sample separation

A maximum of 12 x 0.3μL samples were analysed on Pharmacia 20% homogeneous PhastGels using SDS buffer strips. The PhastSystem was used in accordance with the manufacturer's instructions, and run at 250 volts, 15^{0}C for 97 volthours.

5.2.6.3 PhastGel development

Gels were developed by staining in a 0.1% solution of Coomassie blue for 8min at 50^{0}C, then destained for a total of 23min at 50^{0}C, using a solution of methanol, water and glacial acetic acid (3:6:1 v/v/v), with three changes of buffer. If necessary, background staining was further reduced by immersing the gels in destain solution for approximately 30min, or until acceptably clear.

After destaining, gels were preserved by incubating them in a solution of 5% glycerol and 10% glacial acetic acid at 50^{0}C for 7.5min.

5.2.7 Capillary zone electrophoresis

5.2.7.1 Sample preparation

Lyophilised water-insoluble extracts were dissolved, with continuous shaking for 1h at room temperature using an orbital Vibrax® VXR shaker, to 10mg mL^{-1} in 1.5mL eppendorf vials in denaturing buffer (40mM bis-tris propane, 8M electrophoresis grade urea (pH7.0), containing 5μL mL^{-1} 2-mercaptoethanol). Following solubilisation, the samples were microcentrifuged (MSE Microcentaur) for 2.5min at 13,000rpm, and an aliquot removed for analysis by capillary zone electrophoresis.

Skimmed bovine milk was diluted 5-fold in the same denaturing buffer, and used as a reference standard. The α_{s1}-I-casein previously prepared (section 5.2.5) was added to the milk sample to determine its retention time, allowing its identification in the water-insoluble extracts.

5.2.7.2 Sample separation

Separation was performed using a Biofocus 2000 capillary electrophoresis instrument (Bio-Rad Laboratories Ltd., Bio-Rad House, Maylands Avenue, Hemel Hempstead, Herts, HP2 7TD), essentially as described by Kanning *et al.* (1993).

Proteins were separated on a coated 36cm x 50μm i.d. fused-silica capillary (Bio-Rad Laboratories Ltd.) at 20^{0}C in 100mM phosphate buffer containing polymer modifier (pH2.5), to which had been added 8M electrophoresis grade urea to prevent the reformation of casein micelles. Injections were performed using the pressure injection mode (10 psi*sec), with UV detection at 200nm.

5.2.8 Statistical analysis

Using the strategy described in section 3.2.5, changes in the CZE electrophoretogram peak areas of the α_{s1}-casein, α_{s1}-I-casein, para-κ-casein and β-casein variants over the 246 day ripening period, were used to predict the calculated water-soluble peptide RP-HPLC chromatogram segment areas.

Following standardisation of the recovered water-insoluble extracts to 1g "wet" weight Cheddar cheese, normalised casein peak areas were calculated from the integrated electrophoretograms of water-insoluble extracts at days 1, 22, 78, 190 and 246 of maturation (Tables IV.1 and IV.2, Appendix IV). The data formed a matrix of 10 rows (cheese samples) by 6 columns (normalised individual casein peak areas), and forms Table IV.3 in Appendix IV.

The relationship between the rate and extent of casein degradation and water-soluble peptide formation was then investigated by evaluating the potential of the casein electrophoretograms for predicting the peptide RP-HPLC chromatogram segment areas. A model was derived using the multivariate technique of Partial Least Squares Regression (PLS2; Unscrambler 5.3, Camo AS, Norway). The statistical model was

fitted to the experimental data for maturation time x casein peak area (Table IV.3, Appendix IV) and maturation time x peptide segment area (Table III.1, Appendix III).

5.3 Results and discussion

5.3.1 Analysis of the water-insoluble extracts by SDS-PAGE

The SDS-PAGE patterns of the water-insoluble extracts from the standard pH and reduced pH Cheddar cheese up to days 78 and 246 of maturation are shown in Figure 5.1(A) and (B) and Figure 5.2(A) and (B), respectively.

The electrophoretic pattern of the water-insoluble extracts from the reduced pH Cheddar cheese appeared to be more complex at any particular time of maturation, suggesting a greater, and possibly slightly differing type of, proteolysis than that occurring in the standard pH cheese. As seen in Figure 5.1(A) and (B), qualitatively the degradation of α_{s1}-casein was greatest in the reduced pH cheese, as by day 78 of maturation the Coomassie blue staining of the α_{s1}-casein band was significantly reduced in comparison to the α_{s1}-casein band from the standard pH cheese.

The proposed identities of some of the water-insoluble extract gel bands are indicated in Figure 5.1. Given the difficulties and potential inaccuracy of densitometric scanning, no attempt was made to quantify the changes in these, and other casein levels, from the gels. However, capillary zone electrophoresis was additionally used to separate the water-insoluble extracts, enabling accurate quantification of the caseins and some of their initial degradation products.

5.3.2 Analysis of the water-insoluble extracts by CZE

Example electrophoretograms showing the separation of milk and cheese proteins by CZE are shown in Figure 5.3(A) and (B) and Figure 5.4(A) and (B) respectively.

Good separation and resolution of α-lactalbumin, β-lactoglobulin and the caseins, including the major β-casein phenotypes, was achieved for the milk. As expected α-lactalbumin and β-lactoglobulin were not detected in the water-insoluble extracts of the cheese, as these proteins are lost at whey drainage. The β-casein B variant is a minor component of bovine milk, and migrates with, or close to, κ-casein because of their similar retention times. However, the hydrolysis of κ-casein peptide bond Phe_{105}-Met_{106}

FIGURE 5.1: SDS-PAGE analysis of water-insoluble extracts from standard pH and reduced pH Cheddar cheese up to day 78 of maturation.

(A) Water-insoluble extracts from standard pH Cheddar cheese, prepared as described in the text. Track 1 contains casein and whey protein standards. Tracks 2–12 contain water-insoluble extracts prepared from cheese at curd at whey off and 1, 8, 15, 22, 29, 36, 43, 50, 66 and 78 days maturation.

(B) Water-insoluble extracts from reduced pH Cheddar cheese, prepared as described in the text. Track 1 contains casein and whey protein standards. Tracks 2–12 contain water-insoluble extracts prepared from cheese at curd at whey off and 1, 8, 15, 22, 29, 36, 43, 50, 66 and 78 days maturation.

α-La	- α-Lactalbumin.
β-Lg	- β-Lactoglobulin.
κ	- κ-Casein.
β	- β-Casein.
α_{s1}	- α_{s1}-Casein.
α_{s2}	- α_{s2}-Casein.
High M_w WP	- High molecular weight whey proteins.
γ	- γ-Caseins.
Para-κ	- Para-κ-casein.
α_{s1}-I	- α_{s1}-I-Casein.

FIGURE 5.1(A)

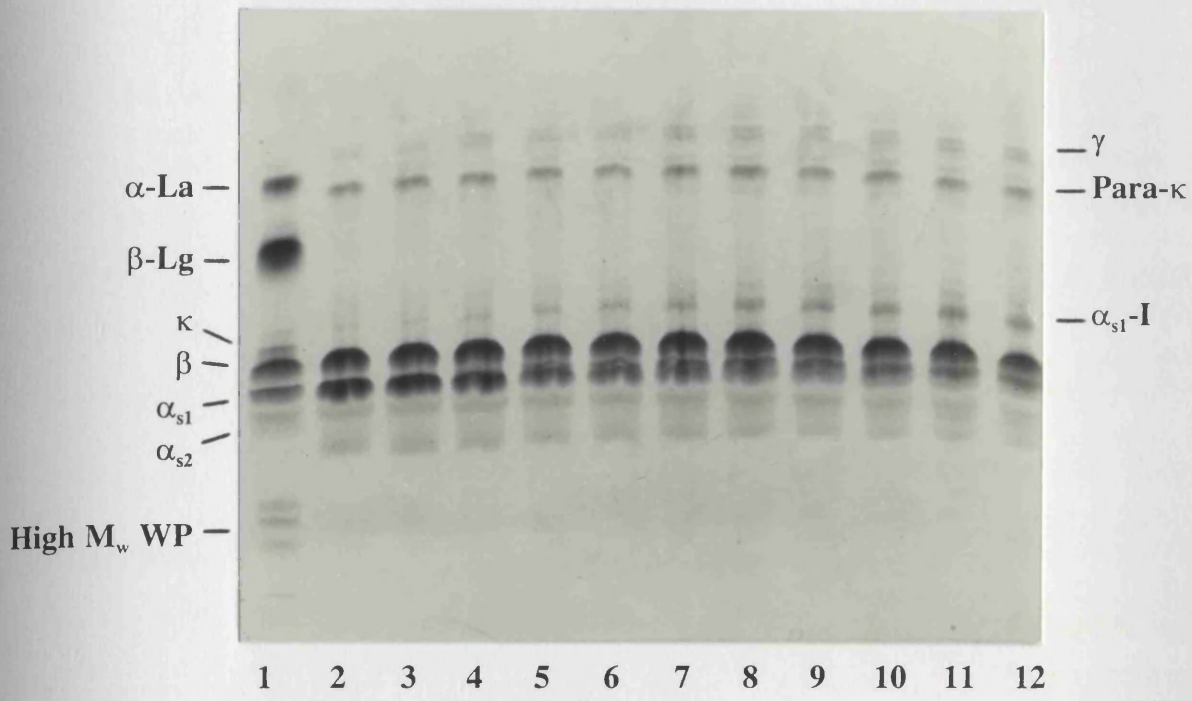


FIGURE 5.1(B)

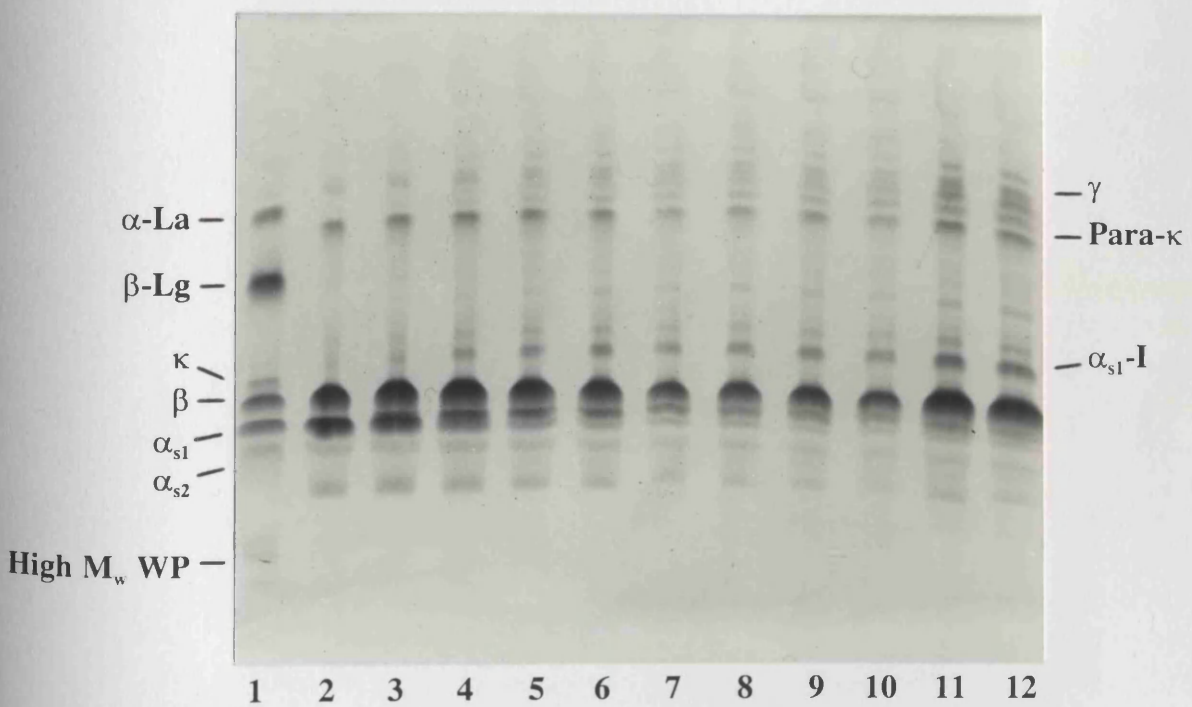


FIGURE 5.2: SDS-PAGE analysis of water-insoluble extracts from standard pH and reduced pH Cheddar cheese up to day 246 of maturation.

(A) Water-insoluble extracts from standard pH Cheddar cheese, prepared as described in the text. Tracks 1 and 12 contain casein and whey protein standards. Tracks 2–11 contain water-insoluble extracts prepared from cheese at 1, 22, 50, 78, 106, 134, 162, 190, 219 and 246 days maturation.

(B) Water-insoluble extracts from reduced pH Cheddar cheese, prepared as described in the text. Tracks 1 and 12 contain casein and whey protein standards. Tracks 2–11 contain water-insoluble extracts prepared from cheese at 1, 22, 50, 78, 106, 134, 162, 190, 219 and 246 days maturation.

α-La - α-Lactalbumin.
β-Lg - β-Lactoglobulin.
κ - κ-Casein.
β - β-Casein.
α_{s1} - α_{s1}-Casein.
α_{s2} - α_{s2}-Casein.
High M_w WP - High molecular weight whey proteins.

FIGURE 5.2(A)

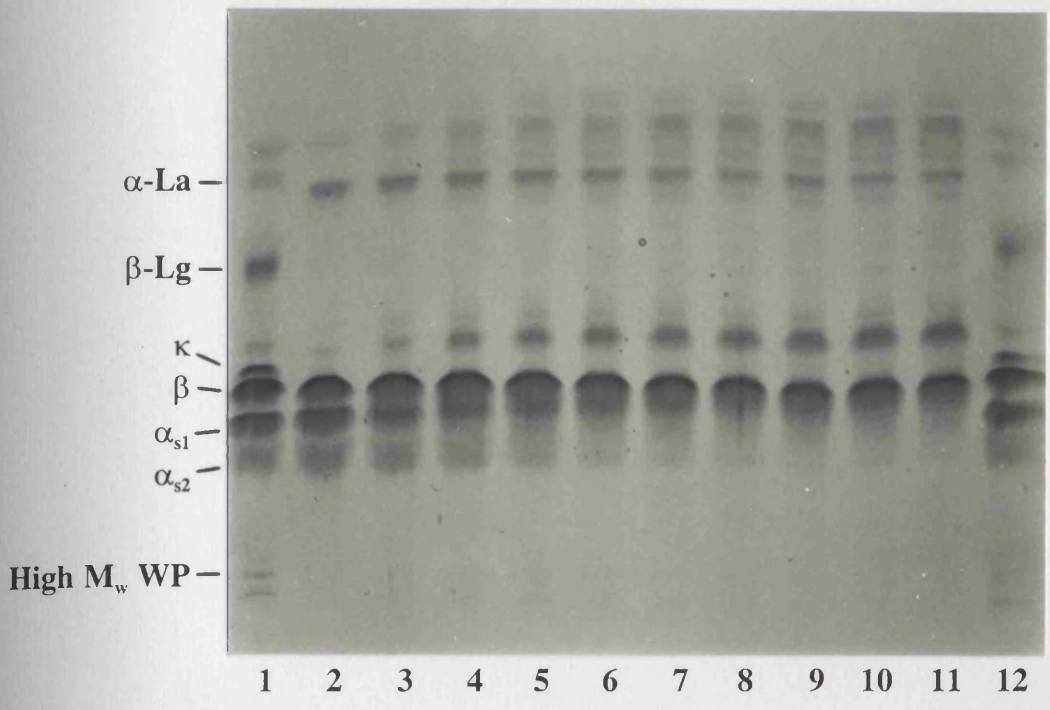


FIGURE 5.2(B)

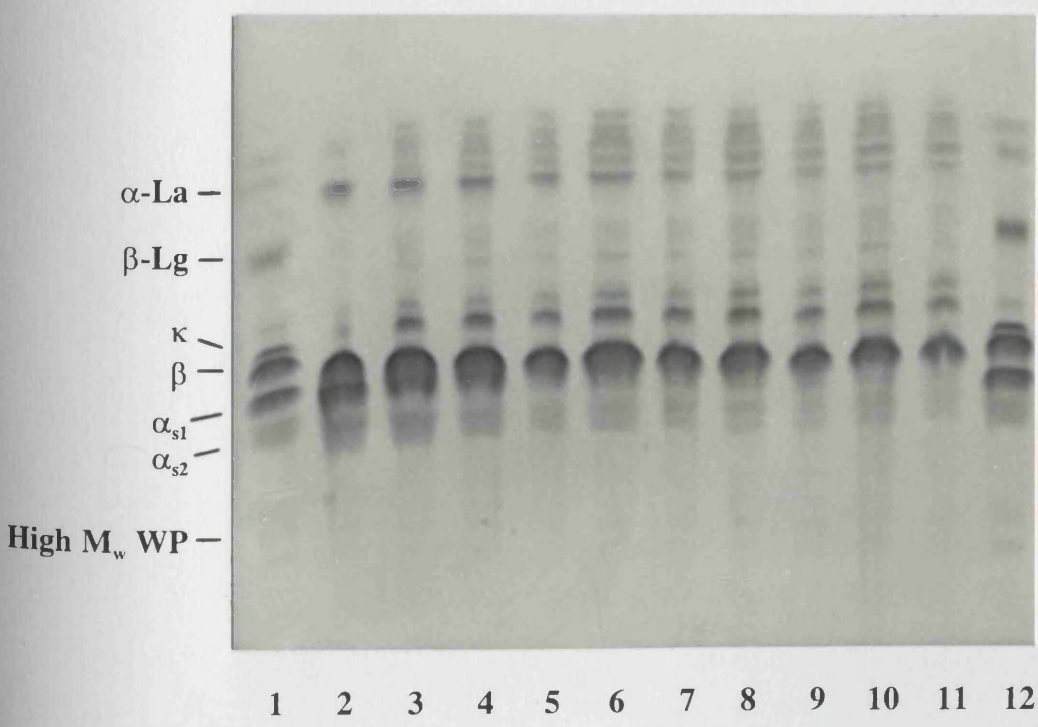


FIGURE 5.3: Electrophoretograms showing the CZE separation of skimmed bovine milk and skimmed bovine milk with α_{s1}-I-casein added.

(A) Skimmed bovine milk, diluted in denaturing buffer as described in the text.

(B) Skimmed bovine milk with α_{s1}-I-casein added, diluted in denaturing buffer as described in the text.

α-La - α-Lactalbumin.

β-Lg - β-Lactoglobulin.

α_{s1} - α_{s1}-Casein.

α_{s1}-I - α_{s1}-I-Casein.

α_{s2} - α_{s2}-Casein.

β^{A1} - β-Casein A1 variant.

β^{A2} - β-Casein A2 variant.

κ - κ-Casein.

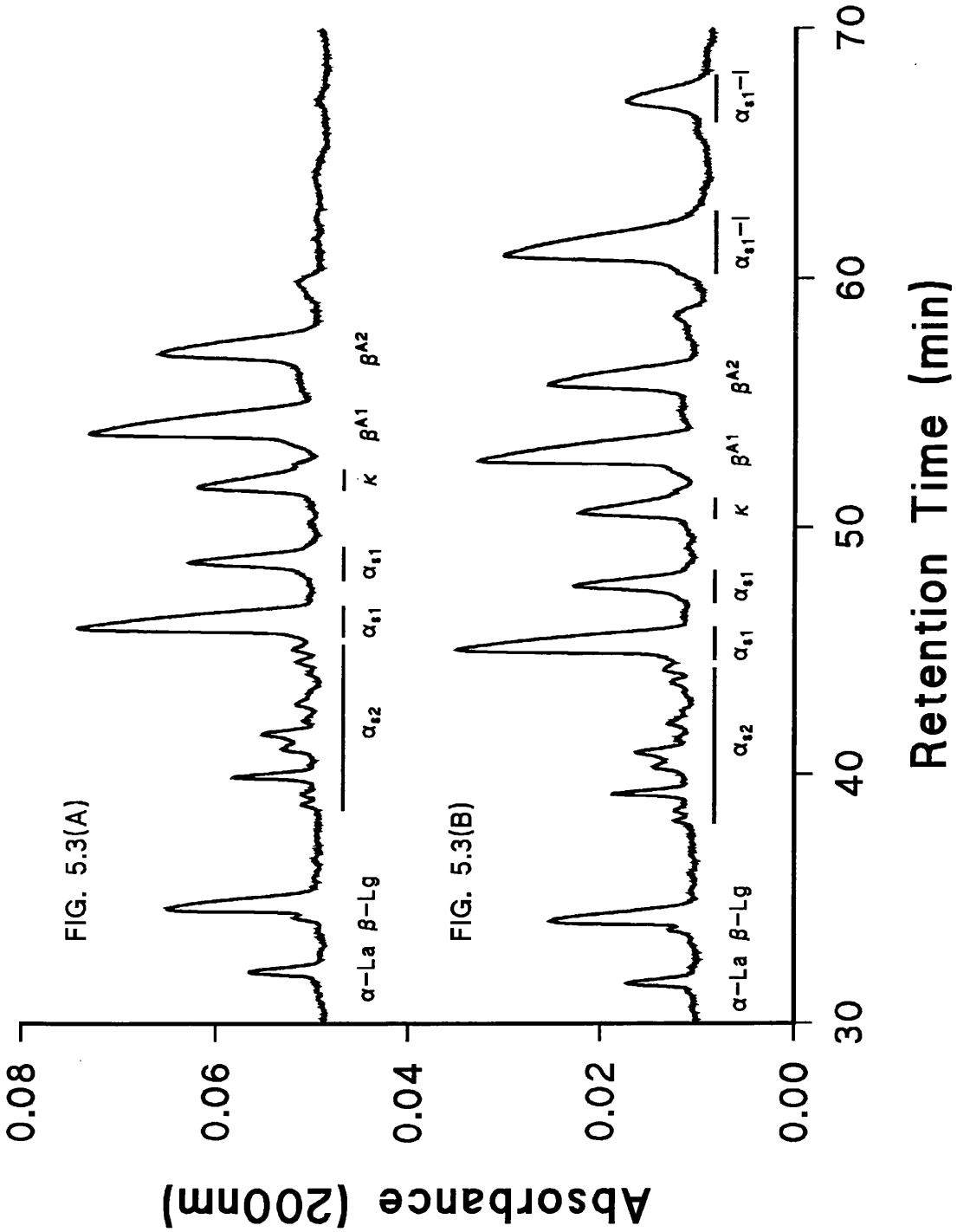


FIG. 5.3(A)
FIG. 5.3(B)
α-La β-Lg
α_{s2} α_{s1} α_{s1} κ β^{A1} β^{A2}
α_{s1}-I
Absorbance (200nm)
0.00
0.02
0.04
0.06
0.08
30
40
50
60
70
Retention Time (min)

FIGURE 5.4: Electrophoretograms showing the CZE separation of water-insoluble extracts from reduced pH Cheddar cheese at days 1 and 78 of maturation.

(A) Water-insoluble extract from reduced pH Cheddar cheese at day 1 of maturation, prepared as described in the text.

(B) Water-insoluble extract from reduced pH Cheddar cheese at day 78 of maturation, prepared as described in the text.

α_{s1} - α_{s1}-Casein.

α_{s1}-I - α_{s1}-I-Casein.

α_{s2} - α_{s2}-Casein.

β^{A1} - β-Casein A1 variant.

β^{A2} - β-Casein A2 variant.

β^{B} - β-Casein B variant.

Para-κ - Para-κ-casein.

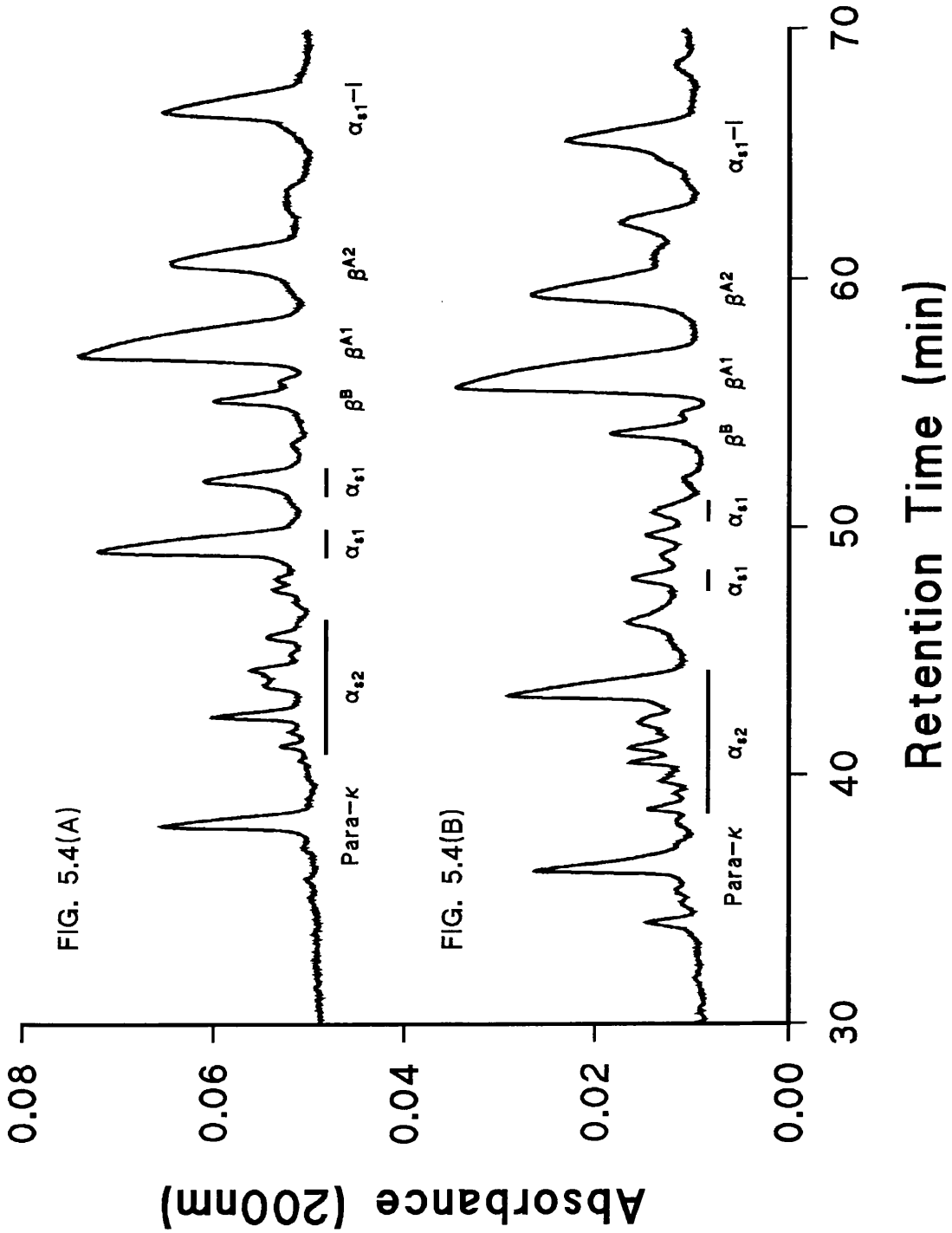


FIG. 5.4(A)
FIG. 5.4(B)
Para-κ
α_{s2}
α_{s1}
α_{s1}
β^{B}
β^{A1}
β^{A2}
α_{s1}-I
Absorbance (200nm)
0.00
0.02
0.04
0.06
0.08
30
40
50
60
70
Retention Time (min)

on renneting of milk by chymosin creates the new fast running peak of para-κ-casein, thereby allowing visualisation of the β-casein B variant peak (Figure 5.4).

Because capillary electrophoresis is an analytical technique it was not possible to collect peak fractions for further characterisation. Therefore, the α_{s1}-I-casein f(24–199) was prepared by the limited chymosin degradation of α_{s1}-casein and, following its characterisation by MALDI-MS, added as a 'spike' to milk samples, allowing a determination of its retention time and subsequent identification in the cheese water-insoluble extracts.

Figure 5.5 shows the peak fractions created by the limited chymosin digestion of α_{s1}-casein. These peak fractions were collected manually, lyophilised, and characterised either directly by MALDI-MS, or by MALDI-MS tryptic peptide mapping.

Fraction 1 was identified as α_{s1}-casein f(1–23) directly from its MALDI-MS weight (theoretical mass = 2764.2Da; determined mass = 2762.5Da).

Fractions 2 and 3 were characterised by trypsinolysis. Trypsin is a serine protease, which specifically cleaves on the carboxylic side of lysine and arginine residues, except when proline is the adjacent amino acid, which renders the peptide bond almost completely resistant to cleavage. Figure 5.6 and Table 5.1 show the tryptic peptides resulting from the complete theoretical trypsinolysis of α_{s1}-casein variant B, the predominant variant in western cattle. The peptides were identified using the Protein Abacus software program, version 2.0.2 (Lighthouse data, Thermo Bioanalysis Ltd.), and the primary structure of α_{s1}-casein variant B was taken from Swaisgood (1992).

Figure 5.7 shows the MALDI-MS tryptic peptide map of fraction 3. The eight numbered peaks (1–8) were identified from their mass weights, with their peptide identities listed in Table 5.2, confirming fraction 3 as α_{s1}-casein. Removal of the N-terminal 23 residues of α_{s1}-casein by chymosin cleavage at peptide bond Phe_{23}-Phe_{24}, forming α_{s1}-I-casein, eliminates three potential trypsin cleavage sites, producing a characteristically different tryptic peptide map. Figure 5.8 shows the MALDI-MS tryptic peptide map of fraction 2. The eight numbered peaks (1–8) were identified from their mass weights, with their peptide identities listed in Table 5.3, confirming fraction 2 as the α_{s1}-I polypeptide. For the purposes of direct comparison of tryptic peptides the same amino acid residue numbering system has been kept for α_{s1}-I as for α_{s1}-casein, therefore the N-terminal residue of α_{s1}-I has been kept as residue 24 as in α_{s1}-casein (Figure 5.6).

FIGURE 5.5: RP-HPLC chromatograms of α_{s1}-casein and degradation products resulting from the limited chymosin digestion of α_{s1}-casein.

(A) Purified α_{s1}-casein, dissolved in phosphate buffer (final concentration 10mg mL^{-1}), and eluted with a gradient of 0.1% TFA in 90% acetonitrile/water (v/v) as described in the text.

(B) Degradation products resulting from the limited chymosin digestion of α_{s1}-casein after a 24h incubation. Purified α_{s1}-casein, dissolved in phosphate buffer (final concentration 10mg mL^{-1}), and products of its chymosin digestion were eluted with a gradient of 0.1% TFA in 90% acetonitrile/water (v/v) as described in the text.

Peak fractions 1, 2 and 3 were collected and characterised either directly by MALDI-MS or MALDI-MS tryptic peptide mapping.

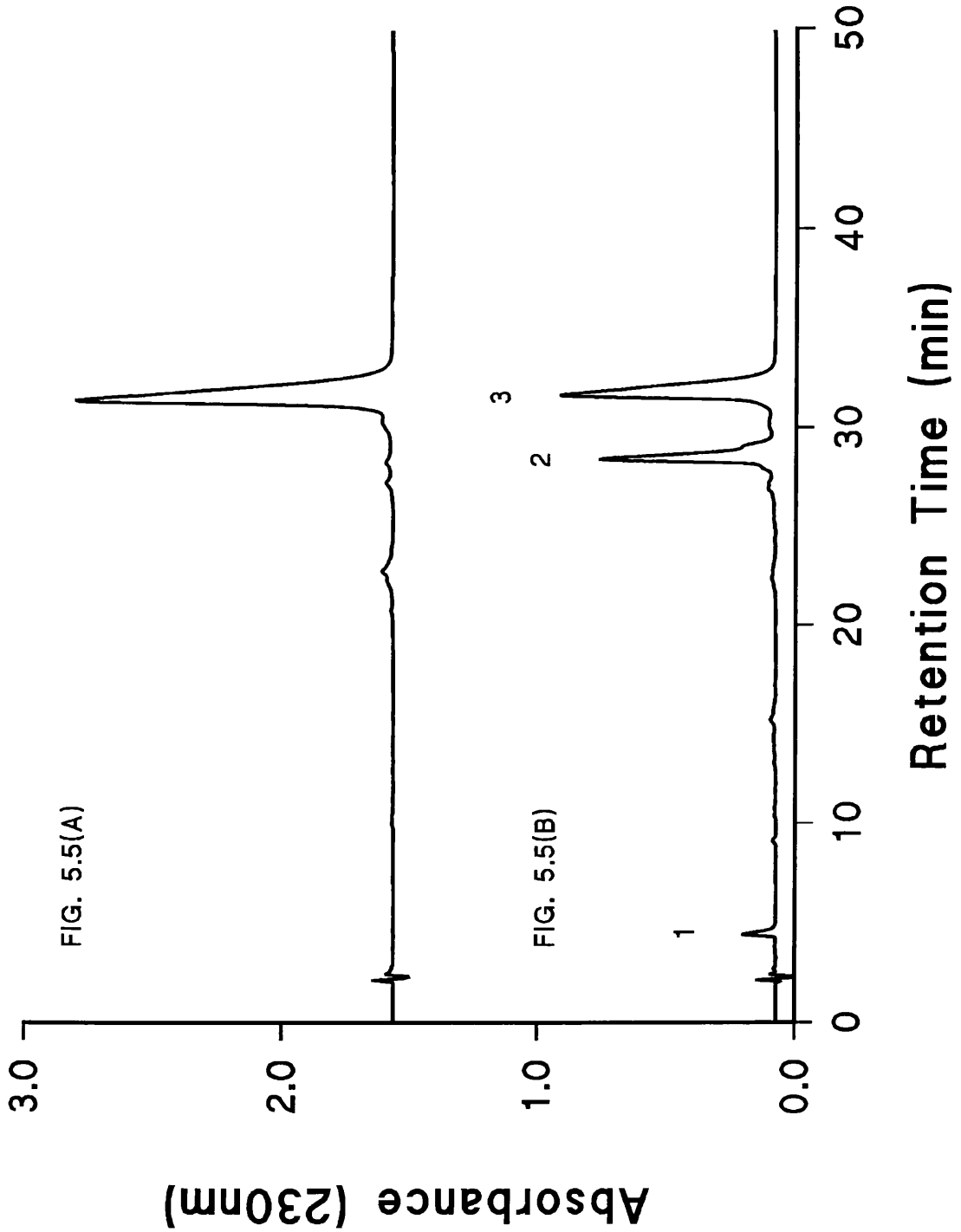

Absorbance (230nm)
3.0
2.0
1.0
0.0
0
10
20
30
40
50
Retention Time (min)
FIG. 5.5(A)
FIG. 5.5(B)
1
2
3

FIGURE 5.6: Primary structure of α_{s1}-casein variant B with potential trypsin cleavage sites indicated.

Primary structure of α_{s1}-casein variant B from Swaisgood (1992).

Sites of post-translational phosphorylation underlined and in italics.

↓ = Potential trypsin cleavage site.

TABLE 5.1: Tryptic peptides from α_{s1}-casein variant B.

Peptide	Mass (Da)	Sequence
1–3	399.5	RPK
4–7	493.6	HPIK
8–22	1760.0	HQGLPQEVLNENLLR
23–34	1384.6	FFVAPFPEVFGK
35–36	275.3	EK
37–42	688.8	VNELSK
43–58	1925.8	DIGSESTEDQAMEDIK
59–79	2716.4	QMEAESISSSSEEIVPNSVEQK
80–83	524.6	HIQK
84–90	830.9	EDVPSER
91–100	1267.5	YLGYLEQLLR
101–102	259.4	LK
103–103	146.2	K
104–105	309.4	YK
106–119	1659.7	VPQLEIVPNSAEER
120–124	614.8	LHSMK
125–132	910.0	EGIHAQQK
133–151	2316.7	EPMIGVNQELAYFYPELFR
152–193	4718.1	QFYQLDAYPS.....NPIGSENSEK
194–199	747.9	TTMPLW

FIGURE 5.7: MALDI-MS tryptic peptide map of fraction 3.

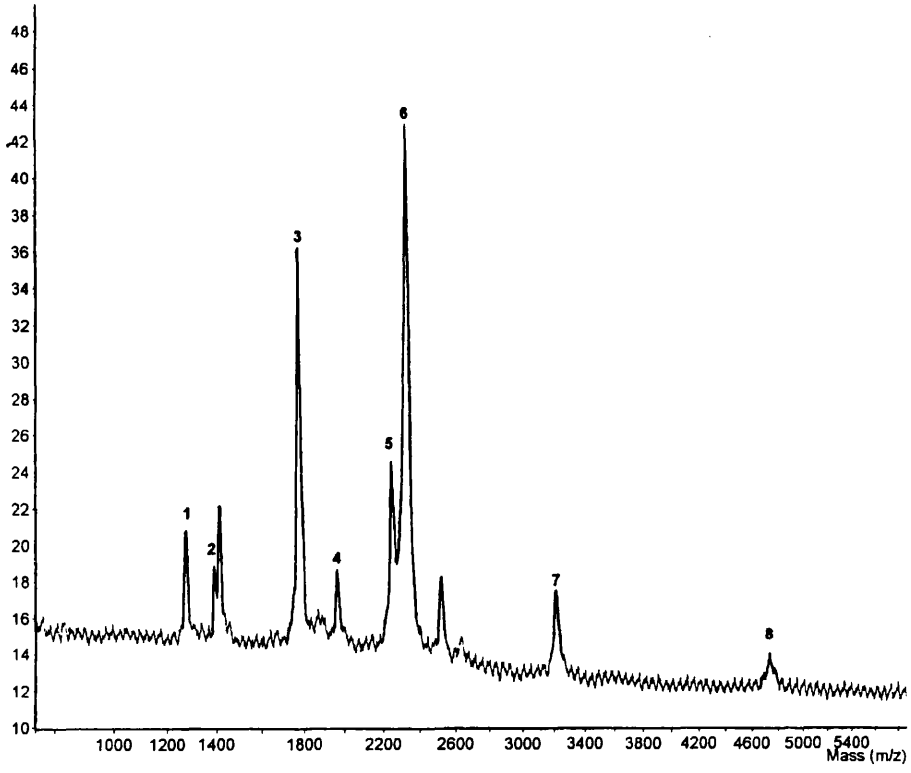


TABLE 5.2: Identity of tryptic peptides from fraction 3.

Peak No.	Peptide	Peptide Mass (Da)	Mass Found (Da)
1	91–100	1267.5	1270.1
2	23–34	1384.6	1386.8
3	8–22	1760.0	1762.2
4	104–119	1951.1	1956.9
5	4–22	2235.6	2238.1
6	133–151	2316.7	2317.5
7	125–151	3208.7	3208.9
8	152–193	4718.1	4720.6

FIGURE 5.8: MALDI-MS tryptic peptide map of fraction 2

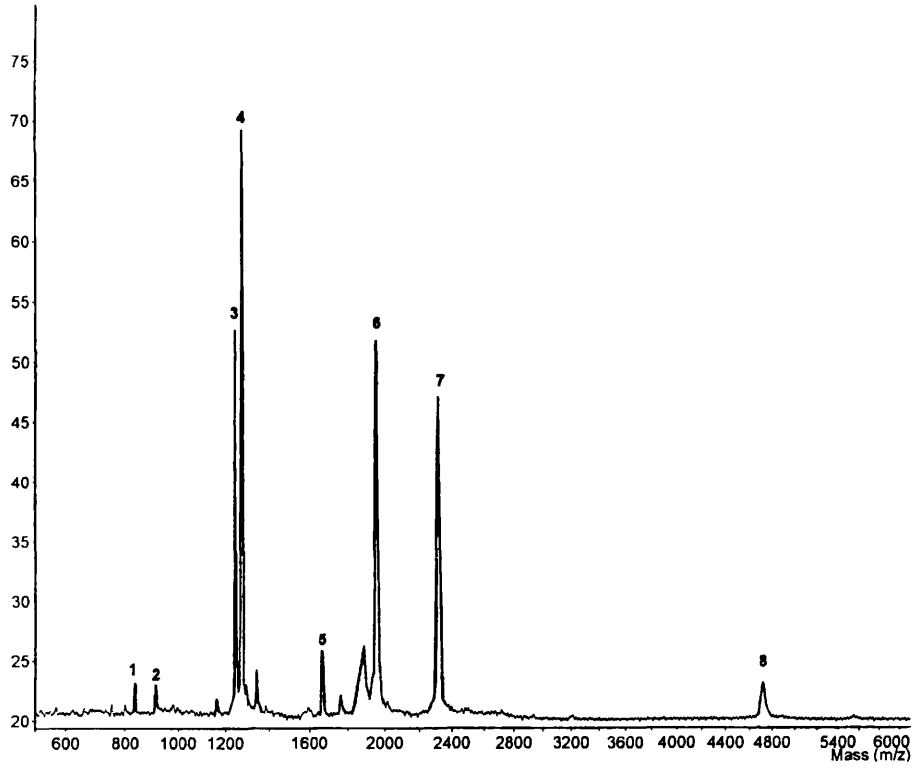


TABLE 5.3: Identity of tryptic peptides from fraction 2

Peak No.	Peptide[a]	Peptide Mass (Da)	Mass Found (Da)
1	84–90	830.9	830.6
2	125–132	910.0	909.3
3	24–34	1237.5	1235.9
4	91–100	1267.5	1265.1
5	106–119	1659.7	1657.4
6	104–119	1951.1	1947.9
7	133–151	2316.7	2311.2
8	152–193	4718.1	4708.8

[a] Amino acid residue numbering as for α_{s1}-casein variant B (Figure 5.6).

Two α_{s1}-casein and two α_{s1}-I peaks were detected at pH2.5, and although not previously seen by either HPLC or FPLC separations, this phenomenon has been observed for all Hannah herd bulk milks and in the milks of individual animals analysed by CZE. Although the reason for this remains unknown, initial degradation of the α_{s1}-casein peaks during cheese ripening is mirrored by proportional increases in the two α_{s1}-I peaks, and may possibly be due to differences in the degree of α_{s1}-casein phosphorylation.

Because of continuous proteolytic degradation within cheese, the amount of water-insoluble extract material recovered decreases as the maturation time increases. Therefore, the integrated electrophoretogram peak areas were standardised to account for the fact that all water-insoluble extracts had been dissolved at 10mg solids mL^{-1}. The results are shown in Tables IV.1 and IV.2 in Appendix IV.

In cheese the primary site of chymosin hydrolysis of α_{s1}-casein is the Phe_{23}-Phe_{24} peptide bond (McSweeney *et al.*, 1993b). Its cleavage results in the release of the small water-soluble peptide α_{s1}-casein f(1–23) early in the maturation process, with the concomitant formation of the water-insoluble α_{s1}-I-casein f(24–199).

Figure 5.9(A) shows the quantitative changes in the levels of α_{s1}-casein in standard pH and reduced pH milk Cheddar cheese over the 246 day ripening period. Up to day 22 of maturation the rate of degradation of α_{s1}-casein was approximately 50% faster in the reduced pH cheese than in the standard pH cheese. After this time the rate of degradation began to level out, until at approximately 134 days of maturation the concentration of intact α_{s1}-casein remaining in each cheese was essentially equal and of negligible concentration.

Quantitative changes in the level of the α_{s1}-I-casein in the standard and reduced pH cheese are shown in Figure 5.9(B). Up to day 15 of maturation there was a significantly faster, and quantitatively greater production of α_{s1}-I in the reduced pH cheese. Between days 22 and 50 of maturation the α_{s1}-I concentrations in each cheese reached an equivalent plateau level, indicating that its rate of formation was equal to its rate of degradation. However, beyond day 50 there was an abrupt and continuous decline in the level of α_{s1}-I in the reduced pH cheese, until by day 246 of maturation only a residual amount remained. By comparison the decline of α_{s1}-I in the standard pH cheese was much less pronounced and began later, after approximately day 106 of maturation,

so that by day 246 the level of α_{s1}-I was still some 15 times greater than that remaining in the reduced pH cheese.

Gel electrophoretic methods, including SDS-PAGE, have been used previously to investigate the hydrolysis of casein during cheese ripening. Visser and De Groot-Mostert (1977) investigated the contribution of enzymes from rennet, starter bacteria and milk to proteolysis in Gouda cheese using quantitative polyacrylamide gel electrophoresis. They concluded that rennet, in the concentration as present in Gouda cheese, is responsible for the almost complete degradation of α_{s1}-casein and for the partial hydrolysis of β-casein during the first month of ripening.

McSweeney *et al.* (1993a) manufactured Cheddar cheese from raw milk, pasteurised milk (72^{0}C, 15sec), or milk produced from skimmed milk microfiltered to reduce the total bacterial count and then mixed with pasteurised cream (72^{0}C, 30sec). Despite these processing differences, analysis of the cheeses and their water-soluble extracts by urea-PAGE showed them to be indistinguishable up to 3 months of ripening, although slight differences were apparent at 6 months.

Such observations show that the extent of casein degradation is primarily related to the concentration of chymosin added to the milk, and subsequently retained in the cheese curd. More subtle proteolytic effects are the result of further degradation of the chymosin- (or plasmin-) released polypeptides by the proteinase and peptidase activities of starter and non-starter bacteria.

Lowering the pH of the milk used in cheese manufacture to 6.0 increases retention of chymosin in the curd (Creamer *et al.*, 1985), leading to enhanced cleavage of the α_{s1}-casein peptide bond Phe_{23}-Phe_{24}. The result is an increase in the production of α_{s1}-casein f(1–23), α_{s1}-I polypeptide, and of peptide material derived from these fragments through their subsequent degradation by other enzyme systems. Faster degradation of α_{s1}-casein and β-casein has also been reported in cheeses made from milk heated to higher temperatures in order to denature and incorporate whey proteins into the curd (Leaver *et al.*, 1993). However, these whey protein-containing cheeses were found to develop a bitter flavour, rather than the full flavour found in equivalent cheese made from pasteurised milk.

Cheese texture is primarily determined by its pH and the ratio of intact casein to moisture. During the first 7–14 days of ripening the rubbery texture of the young cheese

curd is transformed, as the network of caseins is weakened by chymosin hydrolysis of the Phe_{23}-Phe_{24} peptide bond of α_{s1}-casein creating the α_{s1}-I polypeptide (Lawrence *et al.*, 1987). Differences in the textural attributes of mouthcoating, rubberness and crumbliness were detected in sensory analysis between the standard pH and reduced pH milk Cheddar cheese (Chapter 6).

Figure 5.9(C) shows the level of para-κ-casein in the reduced pH cheese decreased at a slow and constant rate right up to day 246 of maturation. In contrast, the para-κ-casein level in the standard pH cheese decreased at a faster rate, although beyond day 78 of maturation its concentration increased, returning approximately to its starting concentration, at which level it remained for the remainder of the ripening period. This apparent increase was most probably the result of other polypeptide material generated during proteolysis running at the same retention time as the para-κ-casein on CZE. Studies by Leaver *et al.* (1993) show an initially comparable decrease in para-κ-casein levels in cheese made from pasteurised and heat-treated milk by ion-exchange FPLC, although they only determined relative concentrations up to week 12 of ripening.

The levels of β^{A1}-, β^{A2}- and β^{B}-casein variants in the standard and reduced pH Cheddar cheeses are shown in Figure 5.10(A), (B) and (C), respectively. Their initial concentrations reflect their relative genetic polymorphism frequency distribution in the Hannah herd. Over the 246 days of maturation 60–70% of the β-casein in both cheeses was hydrolysed at an essentially similar rate. Complete degradation was not achieved, although some unhydrolysed β-casein remains at the end of ripening in all cheese varieties, except a few in which an internal mold is present (Lawrence *et al.*, 1987).

The level of NaCl present in the cheese environment promotes aggregation of the β-casein molecules, resulting in enzyme inaccessibility to some of its chymosin-sensitive bonds. Consequently, α_{s1}-casein degradation in cheese is much more extensive than β-casein, which is predominantly hydrolysed by plasmin resulting in the formation of the γ-caseins.

A model for predicting the water-soluble peptide RP-HPLC chromatogram segment peak areas, from changes in the casein levels determined by CZE over the 246 day ripening period, was derived from Partial Least Squares Regression. The variance explained by the optimum model using PLS2 regression is shown in Table 5.4. However, in contrast to the strong association found previously between the prediction of amino

acid concentrations from water-soluble peptide chromatogram segment areas (section 3.3), no useful predictions were established in this case.

This result suggests that the observed differences in the water-soluble peptide profiles from the standard pH and reduced pH milk Cheddar cheese are consequences of differences in both the nature and activity of proteolysis. Given the reported differences in retention of protease in cheese curd associated with changes in pH (Creamer *et al.*, 1985), this would not be an unreasonable conclusion. However, this result does substantiate the previous deduction regarding the water-soluble peptide chromatograms, that it is the supply of precursor peptides that dictates the free amino acid profile in cheese, with differences in aminopeptidase activity making a smaller contribution.

5.4 Summary

This study has shown the potential of using capillary zone electrophoresis to follow casein degradation in cheese. Resolution of all caseins was achieved in a single run using one type of capillary, in contrast to ion-exchange FPLC chromatography which requires casein fractionation on both cation- and anion-exchange columns. Additionally, large quantities of expensive solvents and time-consuming buffer preparations are eliminated with the nanoscale injection volumes required.

Casein quantification is more accurate than that which can be achieved by densitometric scanning of electrophoresis gels, where variations in the intensity of band staining and the dependence on being able to accurately integrate the peak areas for each of the components must be considered. Differences in the relative casein concentration levels determined between the standard and reduced pH milk Cheddar cheeses, allow quantification of proteolysis to be made which would have been impossible from the equivalent SDS-PAGE gels.

This study has shown capillary zone electrophoresis to be an inexpensive and rapid technique, ideally suited for the quantification and comparison of proteolysis in cheese manufactured under a range of different processing conditions.

FIGURE 5.9: Capillary zone electrophoresis analysis of the changes in the levels of caseins in water-insoluble extracts from standard pH and reduced pH Cheddar cheese up to 246 days of maturation.

(A) α_{s1}-Casein.

(B) α_{s1}-I-Casein.

(C) Para-κ-casein.

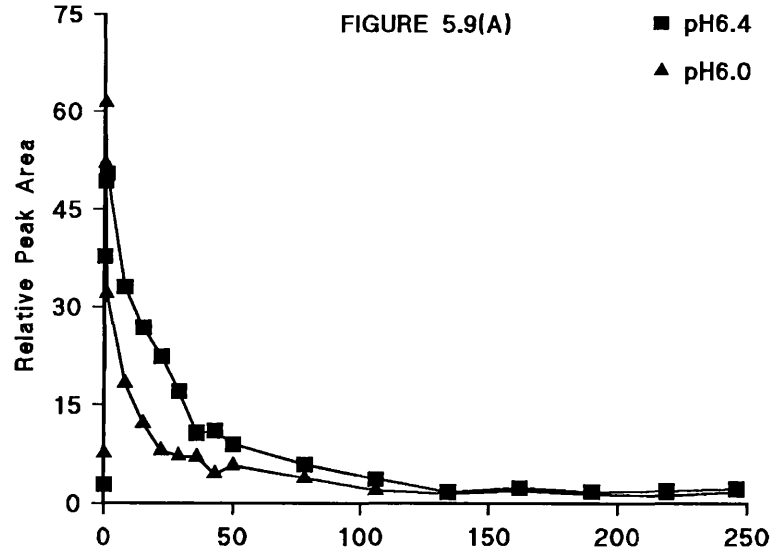


FIGURE 5.9(A)
pH6.4
pH6.0
Relative Peak Area
75
60
45
30
15
0
0
50
100
150
200
250

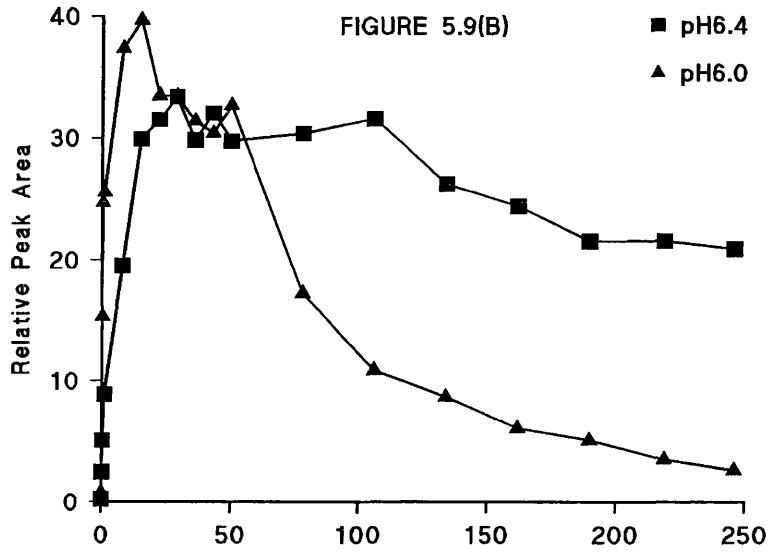


FIGURE 5.9(B)
pH6.4
pH6.0
Relative Peak Area
40
30
20
10
0
0
50
100
150
200
250

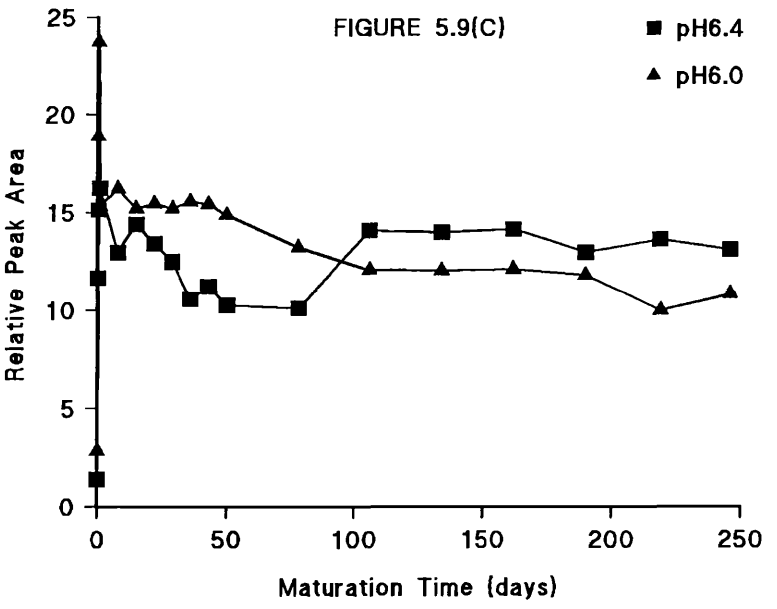


FIGURE 5.9(C)
pH6.4
pH6.0
Relative Peak Area
25
20
15
10
5
0
0
50
100
150
200
250
Maturation Time (days)

FIGURE 5.10: Capillary zone electrophoresis analysis of the changes in the levels of caseins in water-insoluble extracts from standard pH and reduced pH Cheddar cheese up to 246 days of maturation.

(A) β^{A1}-Casein.

(B) β^{A2}-Casein.

(C) β^{B}-Casein.

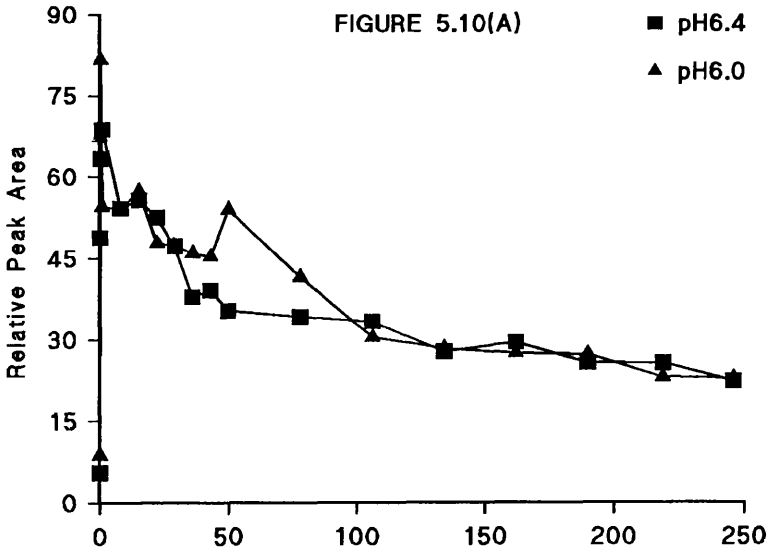


FIGURE 5.10(A)
pH6.4
pH6.0
Relative Peak Area
90
75
60
45
30
15
0
0
50
100
150
200
250

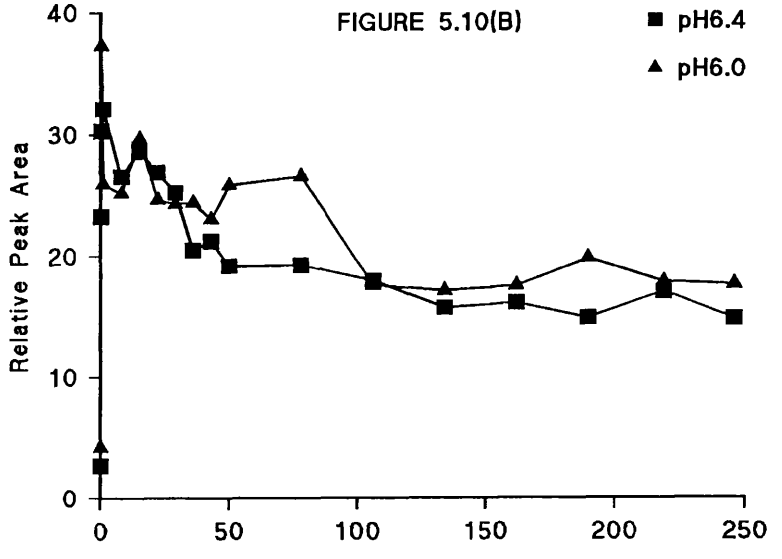


FIGURE 5.10(B)
pH6.4
pH6.0
Relative Peak Area
40
30
20
10
0
0
50
100
150
200
250

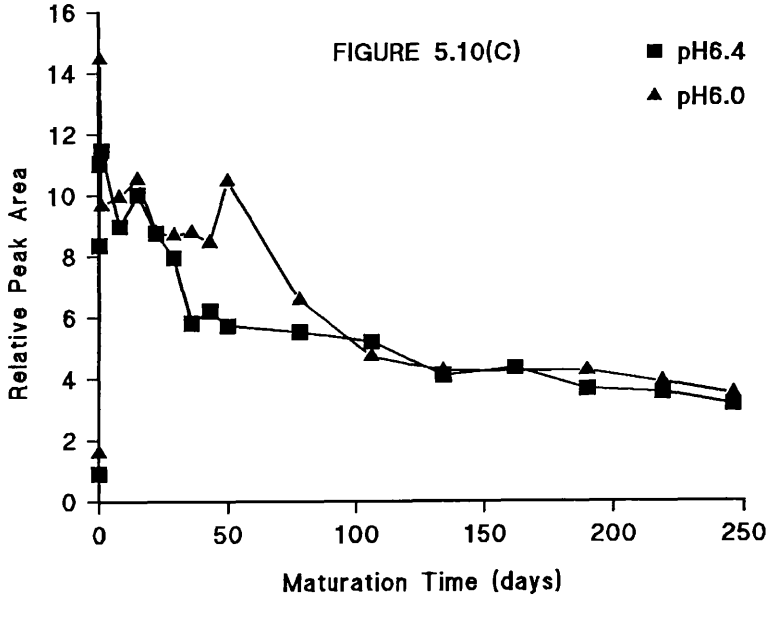


FIGURE 5.10(C)
pH6.4
pH6.0
Relative Peak Area
16
14
12
10
8
6
4
2
0
0
50
100
150
200
250
Maturation Time (days)

TABLE 5.4: PLS2 regression for the prediction of water-soluble peptide RP-HPLC chromatogram segment peak areas resulting from the proteolysis of individual caseins in standard pH and reduced pH Cheddar cheese; variance explained by the model (cross-validated; optimum number of factors in parenthesis).

Segment	Variance (%)	Segment	Variance (%)
1	49.5 (2)	10	No Fit
2	9.8 (1)	11	17.1 (1)
3	11.7 (1)	12	No Fit
4	33.4 (1)	13	No Fit
5	24.8 (1)	14	No Fit
6	26.9 (1)	15	No Fit
7	9.8 (1)	16	64.8 (1)
8	No Fit[a]	17	57.2 (1)
9	No Fit	Total	< 10.0

[a] After cross-validation the model was found to be inadequate.

Chapter 6

Discussion

6.1 Discussion

The purpose of this chapter is to bring together the results of the previously described experiments on the low-fat, high heat-treated, pH-adjusted Cheddar cheese, and the standard and reduced pH milk Cheddar cheese, and discuss the overall conclusions of the work to understanding the significance of the protein degradation observed.

The flavour and texture of cheese are their most important attributes, with the former being the subject of intensive research effort over many years. However, despite extensive study of the flavour of several cheese varieties, few definite conclusions have been reached, a situation made even more problematic by the lack of definition of what a typical flavour should be.

Cheese ripening is essentially an enzymatic process. The enzymes responsible are either freely available, or located within living cells and released upon subsequent lysis. Proteolysis is therefore generally considered the most complex and important biochemical event occurring during the maturation of most cheese varieties, the products of which range from large polypeptides, through medium and small peptides, to ultimately free amino acids. Consequently, a range of experimental approaches, instrumental techniques and statistical analyses were necessarily employed to examine and interpret fully the quantitative and qualitative changes of these various components during the cheese ripening period.

As discussed in Chapter 1 proteolytic enzymes in cheese originate from a variety of sources, including the milk, coagulant, starter and non-starter bacteria, and considerable research on model systems have identified the principal peptide bonds hydrolysed by each within the casein protein. Following the chymosin hydrolysis of peptide bond Phe_{105}-Met_{106} in κ-casein, the enzyme is then responsible for the primary degradation of α_{s1}-casein, initially at peptide bond Phe_{23}-Phe_{24}, and later at a number of other sites (McSweeney *et al.*, 1993b).

Recently Exterkate *et al.* (1997) have shown the selectivity of chymosin on α_{s1}-casein in Gouda cheese, as observed in solution, is affected by the cheese environment. The explanation proposed involved a specific structural arrangement believed to occur in the cheese, which exclusively affected the accessibility of that part of the protein containing the peptide bonds Trp_{164}-Tyr_{165}, Leu_{156}-Asp_{157} and Leu_{149}-Phe_{150}, known to be highly susceptible to cleavage in solution. This result would suggest that extrapolation of the results of *in vitro* studies to possible events occurring *in vivo* must be treated with some degree of caution.

Lowering the pH of cheese milk to 6.0 increases the retention of chymosin in the curd matrix (Creamer *et al.*, 1985), leading to enhanced hydrolysis of the α_{s1}-casein Phe_{23}-Phe_{24} peptide bond, with a resultant increase in the production of α_{s1}-casein f(1–23), the α_{s1}-I-polypeptide, and peptides derived from them through their subsequent degradation by other enzyme systems. This effect can be observed in Chapter 4, Figure 4.4, where all but one of the peak fractions containing α_{s1}-casein peptides were qualitatively larger in the case of the reduced pH than the standard pH cheese water-soluble extract. Moreover, capillary electrophoretograms of the water-insoluble extracts indicate differential rates of degradation of α_{s1}-casein, and its primary hydrolysis product, the α_{s1}-I-polypeptide f(24–199) during maturation (Chapter 5).

The primary chymosin cleavage site in β-casein is peptide bond Leu_{192}-Tyr_{193} (Visser and Slangen, 1977; Carles and Ribadeau-Dumas, 1984), hydrolysis of which releases the C-terminal water-soluble peptide, β-casein f(193–209), which has been identified as a potential source of bitter-tasting peptides. Increasing chymosin retention in the curd matrix by lowering the pH of the milk used in cheese manufacture to 6.0, would reasonably be expected to result in greater hydrolysis of the Leu_{192}-Tyr_{193} bond, with a subsequent increase in the production of the β-casein f(193–209) and peptide material derived from it through subsequent degradation by other enzymes. Chapter 4, Figure 4.4, showed that most peak fractions containing β-casein peptides were qualitatively larger in the case of the pH6.0 than the pH6.4 cheese water-soluble extract. However, capillary electrophoresis studies of the degradation of the β-casein variants showed them to be hydrolysed at essentially similar rates in the two types of Cheddar cheese over the 246 day maturation period (Chapter 5, Figure 5.10). Consequently, the differences in the water-soluble peptide chromatogram profiles described in Chapter 3

could reasonably be explained by the variations in the extent of α_{s1}-casein degradation alone. This is perhaps not totally surprising, given the fact that most β-casein degradation is affected by the endogenous milk protein plasmin (Fox *et al.*, 1994).

The estimated mean effects of processing variables on the sensory character of the low-fat cheese (ANOVA; Minitab 11) are shown in Table 6.1, with bitter flavour highest in cheese manufactured at pH5.8 and 6.0 (Table 6.2). Although the values are not exceptionally high for a low-fat Cheddar, they are certainly considerably higher than similar cheeses made at pH6.2 and 6.4.

These data relate well with the bitter scores from the full-fat, standard and reduced pH milk Cheddar cheese at 3 months maturation (Table 6.4), the values of which suggest that although there may be an effect of pH on bitterness it is not significant. For the low-fat cheese there was more experimental replication, enabling the identification of significant effects to be more readily observed.

Incorporation of whey protein through a high heat-treatment regime did not increase bitterness in the low-fat cheese, but rather decreased it (Table 6.2). Therefore, while whey protein-containing cheeses have been found to develop a bitter flavour, rather than the full flavour found in equivalent cheese made from pasteurised milk (Leaver *et al.*, 1993), this result suggests milk pH also has an effect. The salt-in-moisture content of cheese with whey protein incorporated was high (Table 2.1), due to the syneretic properties of the curd changing on high heat-treatment, and high levels of salt-in-moisture have been found to minimise bitterness (Banks *et al.*, 1993).

The exceptionally high levels of bitterness that would have been expected on acidification of the cheese milk, particularly in a low-fat cheese, were not observed. This may in part be due to the fact that a pure chymosin rather than a calf rennet was used, although, it is not known how pH manipulation would affect pepsin retention in the curd or the development of bitterness. Another factor that may be important is that calf rennet contains three genetic variants of chymosin - A, B and C. Maxiren contains only the A variant (Teuber, 1990).

Changes in texture observed in the low-fat cheese experiment (Table 6.3) were also seen in the standard and reduced pH milk Cheddar (Table 6.4). Mouthcoating properties improved with pH reduction, and crumbliness increased both with lowering pH and whey protein incorporation. Such observations are in agreement with Creamer

TABLE 6.1: Influence of treatment effects on the sensory character of low-fat Cheddar cheese.

	Processing Variable			
Sensory Attribute	pH	Heat Treatment	Rennet Level	Age
Aroma				
Cheddar Intensity	[a]NS	NS	NS	*
Creamy	NS	NS	NS	NS
Sulphur	NS	*	NS	**
Fruity	NS	NS	NS	NS
Nutty	NS	NS	NS	NS
Rancid	NS	*	NS	*
Flavour				
Cheddar	NS	***	NS	NS
Creamy	NS	NS	NS	NS
Acid	***	***	NS	*
Sulphur	NS	*	NS	*
Fruity	NS	NS	NS	NS
Nutty	NS	NS	NS	NS
Rancid	NS	NS	NS	**
Bitter	***	***	**	**
Cowy	NS	NS	NS	NS
Salt	NS	NS	NS	NS
Texture				
Firmness	*	NS	***	*
Crumbliness	**	***	NS	NS
Pastiness	**	NS	***	***
Graininess	NS	NS	NS	NS
Mouthcoating	*	NS	NS	***
Acceptability	NS	**	NS	**

[a] NS = Not Significant, $p>0.05$; * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$.

TABLE 6.2: Estimated mean effects of processing treatments on flavour attributes in low-fat Cheddar cheese.

Treatment	Level	Flavour Attribute			
		Cheddar	Acid	Sulphur	Bitter
pH	5.8	38.6	22.8	9.2	16.4
	6.0	37.7	22.3	8.9	14.9
	6.2	38.5	15.4	9.8	6.5
	6.4	35.9	13.0	8.0	5.6
Heat	72°C/16sec	40.2	21.5	10.0	14.5
	90°C/60sec	35.1	15.2	8.0	7.2
Rennet	50%	38.3	17.5	9.3	8.8
	100%	37.0	19.2	8.6	12.8
Age	3 Months	36.8	16.6	7.8	9.2
	6 Months	38.5	20.1	10.2	12.5

TABLE 6.3: Estimated mean effects of processing treatments on texture attributes in low-fat Cheddar cheese.

Treatment	Level	Texture Attribute			
		Firmness	Crumbliness	Pastiness	Mouthcoating
pH	5.8	54.6	39.7	27.4	34.9
	6.0	54.2	43.2	26.4	35.2
	6.2	58.8	43.2	20.9	31.1
	6.4	56.4	34.9	27.3	30.7
Heat	72^{0}C/16sec	56.2	43.5	26.1	34.1
	90^{0}C/60sec	55.8	37.0	24.9	31.9
Rennet	50%	58.0	41.5	22.3	31.9
	100%	53.9	39.0	28.8	34.0
Age	3 Months	57.4	39.8	22.9	30.6
	6 Months	54.6	40.7	28.1	35.4

TABLE 6.4: Influence of milk pH on the sensory character of full-fat Cheddar cheese.

	3 Months				6 Months			
	pH6.4	pH6.0	±	Sig.	pH6.4	pH6.0	±	Sig.
Aroma								
Cheddar Intensity	32.1	29.8	2.7	[a]NS	43.6	42.8	3.6	NS
Creamy	30.1	36.8	3.6	NS	47.0	52.5	6.3	NS
Sulphur/Eggy	2.4	2.9	1.1	NS	6.9	6.8	2.8	NS
Fruity	6.1	6.1	1.5	NS	14.3	16.4	4.6	NS
Rancid	0.0	0.0	0.0	NS	0.0	0.0	0.0	NS
Acid	5.9	4.9	1.6	NS	11.9	8.9	4.0	NS
Musty	0.1	0.2	0.2	NS	2.1	0.0	1.0	NS
Pungent	5.6	6.0	1.9	NS	12.9	7.0	4.2	NS
Manurial	0.1	0.0	0.1	NS	0.0	0.0	0.0	NS
Flavour								
Cheddar Intensity	48.3	52.9	2.1	NS	53.5	59.5	4.5	NS
Creamy	45.5	50.0	4.5	NS	62.0	62.9	6.9	NS
Acid	14.0	21.8	2.8	0.050	24.2	35.3	6.0	NS
Sulphur/Eggy	6.4	3.7	1.5	NS	9.3	7.8	3.0	NS
Fruity	11.5	9.8	2.4	NS	19.4	12.8	5.2	NS
Rancid	0.0	0.3	0.2	NS	0.0	0.0	0.0	NS
Bitter	8.8	12.5	2.9	NS	12.1	13.0	5.4	NS
Manurial	0.0	0.0	0.0	NS	1.6	2.5	2.1	NS
Salty	24.3	34.5	3.1	0.050	41.8	44.0	4.4	NS
Other	5.4	5.3	2.6	NS	1.4	8.9	4.5	NS
Texture								
Firmness	65.5	68.9	2.6	NS	45.5	53.2	4.7	NS
Rubberness	28.5	20.9	3.7	NS	44.2	13.1	4.3	0.001
Crumbliness	20.5	21.0	3.2	NS	16.1	31.2	4.6	0.050
Graininess	1.4	0.1	0.5	NS	1.3	0.3	0.8	NS
Mouthcoating	34.0	45.3	3.4	0.050	49.7	53.1	6.1	NS
Maturity	45.6	49.8	2.4	NS	48.0	55.6	4.0	NS
Acceptability	59.8	63.0	3.7	NS	69.9	74.4	5.2	NS

[a] NS - Not Significant.

et al. (1985), who reported that throughout maturation the texture of Cheddar cheeses made from acidified milk was more crumbly, with the force required to fracture them being lower.

It has been reported previously that there is a relationship between the starter strains, flavour intensity and concentration of amino acids in both Cheddar and Gouda cheeses (Amantea *et al.*, 1986; Visser, 1977). Additionally, while the proteolytic activities of rennet and endogenous milk proteinases contribute to the appearance of amino acids, the greatest source is via the proteolytic activities of starters (Fox, 1989). The findings of Chapter 3 strongly suggest that variations in amino acid concentrations are closely associated with differences in the peptide precursor supply. The extent and type of peptide formation is almost certainly directed by the initial casein degradation, the products of which are subsequently hydrolysed by the microbial starter enzymes, thereby determining the type and extent of amino acids released.

As discussed in Chapter 1, the bitterness of a peptide is determined by its primary structure - the series of amino acid residues defining its sequence - and various studies have shown a correlation between perceived bitterness and a peptide's calculated hydrophobicity. Table 6.5 shows the average hydrophobicity values for the cheese peptides characterised from the standard and reduced pH milk Cheddar cheese study, confirming many of the β-casein C-terminal peptides as potentially bitter.

Crow *et al.* (1993) reviewed the contribution of starters to cheese ripening, suggesting their importance in continuing the long-term ripening of cheese was a function of both their high biocatalytic activity, and variation in the level and distribution of the ripening enzyme activities. Exterkate and Alting (1995) confirmed the significance of starter peptidases to cheese ripening, by studying their role in the initial proteolytic events leading to amino acid formation in Gouda cheese. It was found that cell envelope proteinase (CEP) degradation of the α_{s1}-casein f(1–23) peptide proceeded as previously observed *in vitro* with cells under cheese conditions, and that the proteinase is essential not only for the efficient conversion of this particular peptide, but of water-soluble peptides in general into free amino acids. Significantly, the importance of chymosin in the initial degradation process was confirmed, with the proteinase being involved essentially in the secondary proteolysis events. It seems highly probable therefore, that

TABLE 6.5: Average hydrophobicity values for the cheese peptides characterised.

Peak No.	# Residues	Peptide Sequence	Hydrophobicity[a] ($H\phi_{ave}$ cal/mol)
1	16	α_{s1}-Casein f(1–16)	
		RPKHPIKHQGLPQEVL...NEN	1287.5
3	20	α_{s1}-Casein f(1–20)	
		RPKHPIKHQGLPQEVLNENL...LRF	1120.0
19	23	α_{s1}-Casein f(1–23)	
		RPKHPIKHQGLPQEVLNENLLRF...FVA	1193.5
14	9	α_{s1}-Casein f(15–23)	
		PQE...**VLNENLLRF**...FVA	1127.8
6	7	α_{s1}-Casein f(17–23)	
		EVL...**NENLLRF**...FVA	978.6
7	6	α_{s1}-Casein f(18–23)	
		VLN...**ENLLRF**...FVA	1141.7
16	14	α_{s1}-Casein f(85–98)	
		QKE...**DVPSERYLGYLEQL**...LRL	1039.3
12	10	α_{s1}-Casein f(89–98)	
		VPS...**ERYLGYLEQL**...LRL	1075.0
8	19	α_{s1}-Casein f(102–120)	
		LRL...**KKYKVPQLEIVPNSAEERL**...HSM	1184.2
8	27	α_{s1}-Casein f(102–128)	
		LRL...**KKYKVPQLEIVPNSAEERLHSMKEGIH**...AQQ	1072.2
21	24	β-Casein f(58–81)	
		TQS...**LVYPFPGPIHNSLPQNIPPLTQTP**...VVV	1533.3
13	14	β-Casein f(193–206)	
		FLL...**YQEPVLGPVRGPFP**...IIV	1482.1
21	15	β-Casein f(193–207)	
		FLL...**YQEPVLGPVRGPFPI**...IV	1580.0
9	13	β-Casein f(194–206)	
		LLY...**QEPVLGPVRGPFP**...IIV	1419.2
18	14	β-Casein f(194–207)	
		LLY...**QEPVLGPVRGPFPI**...IV	1528.6
10	12	β-Casein f(195–206)	
		LYQ...**EPVLGPVRGPFP**...IIV	1537.5
19	13	β-Casein f(195–207)	
		LYQ...**EPVLGPVRGPFPI**...IV	1646.2
6	10	β-Casein f(197–206)	
		QEP...**VLGPVRGPFP**...IIV	1585.0
15	11	β-Casein f(197–207)	
		QEP...**VLGPVRGPFPI**...IV	1709.1
21	11	β-Casein f(199–209)	
		PVL...**GPVRGPFPIIV**	1813.6

[a] Based on the Hϕ values of amino acid residue side chains by Bigelow and Channon (1976).

any changes in the free amino acid concentration distribution would necessarily be a function of variations in the peptide precursor supply.

Lane and Fox (1996) assessed the contribution of starter and adjunct lactobacilli to proteolysis in Cheddar cheese during ripening. While the coagulant was responsible for production of most of the water-soluble nitrogen, the starter enzymes were confirmed as the major contributors to the production of small peptides and free amino acids. Addition of lactobacilli to starter-free cheeses caused a significant increase in proteolysis. This increase was however not as apparent when the adjunct was added to starter-containing cheese, presumably due to lack of available substrate for the competing enzymes.

Two-dimensional high performance liquid chromatography was used by Lagerwerf *et al.* (1995) to compare Cheddar cheeses produced using recombinant chymosin and traditional calf rennet. Protein breakdown in cheese is traditionally monitored by gel electrophoresis or reversed phase chromatography, neither of which sufficiently resolves the complex peptide mixtures which is so characteristic of mature cheeses. The resolving power of a two-dimensional system results in a considerably higher peak separating capacity, thus providing a more detailed peptide map. Such an experimental approach would benefit the analysis of qualitative changes in peptide material, and ease the interpretation of the effect of processing variables on protein degradation in general.

It is now generally accepted that no one component alone determines typical flavour production in Cheddar cheese. Therefore, using a two-dimensional separation approach it should be possible to readily identify key peptides, as following adequate separation the concentrations or peak areas of such peptides could be easily monitored to assess the impact on cheese flavour and texture development under different manufacturing conditions. Such an approach would not be dissimilar to that proposed in Chapter 2 with regard to monitoring changes in the individual amino acid concentrations.

6.2 Further work

Continuation of the study into the effect of processing variables on protein degradation in Cheddar cheese could involve a number of aspects. Using matrix assisted laser desorption/ionisation-mass spectrometry, in combination with a modified Edman

degradation procedure, allowed the characterisation of chromatographically separated peptides. However, many interesting chromatographic peaks, which varied qualitatively between the reduced and standard pH Cheddar cheese water-soluble extracts, were not identified due to lack of time. Simple comparison of peptide profiles of cheeses does not seem to be a pertinent approach, unless combined with the separating power of a 2-D chromatography system, and the identification of peptides subsequently determined as being unique. Alternatively, implementation of other mass spectrometric techniques may further enhance the detection and identification of peptides. In this regard electrospray ionisation tandem mass spectrometry would allow the direct in-line coupling of chromatography systems, and combine the simultaneous sequencing of separated peptides by collisionally induced dissociation. Such an approach has recently been demonstrated in the identification of the P_I-type proteinase digestion products of β-casein (Juillard *et al.*, 1995), and would undoubtedly lead to faster throughput and more accurate sample analysis.

The application of capillary electrophoresis to investigate changes in the caseins and major water-insoluble polypeptides during maturation proved extremely successful. Indeed, a number of recent publications have further demonstrated the successful application of capillary electrophoresis to studying bovine caseins and their primary hydrolysis products. Otte *et al.* (1997) separated casein fractions from milk and various cheeses, as well as isolating casein standards, by capillary electrophoresis under acidic conditions. They found the four major caseins in an acid precipitate from milk to be well separated, furthermore, in the α_{s1}-, and α_{s2}-caseins, and some genetic variants of β-casein, the extent of phosphorylation could be estimated.

Some of the most interesting results have come from the studies of the concentrations of free amino acids, determined in samples taken at regular time intervals during the ripening period. This approach, combined with the subsequent statistical analysis, provided a wealth of conclusive and illuminating data. Interestingly, there is little published information in the literature regarding changes in free amino acid concentrations in cheese, yet the analytical approach would appear to provide useful, quantifiable data. Deviations from the amino acid concentrations representative of traditionally manufactured Cheddar cheese would therefore appear to be an ideal way in which to assess the effect of changed processing variables, such as in assessing

accelerated ripening protocols for example. Additionally, such an experimental approach requires little in the way of sophisticated or dedicated instrumentation, thereby making it applicable to the majority of laboratories.

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TABLE I.1: Data for the calculation of the masses of proteins and peptides.

Amino Acid	Symbols	Monoisotopic mass (Da)	Average mass (Da)
Alanine	Ala A	71.0371	71.0788
Arginine	Arg R	156.1011	156.1876
Asparagine	Asn N	114.0429	114.1039
Aspartic Acid	Asp D	115.0269	115.0886
Cysteine	Cys C	103.0092	103.1448
Glutamic Acid	Glu E	129.0426	129.1155
Glutamine	Gln Q	128.0586	128.1308
Glycine	Gly G	57.0215	57.0520
Histidine	His H	137.0589	137.1412
Isoleucine	Ile I	113.0841	113.1595
Leucine	Leu L	113.0841	113.1595
Lysine	Lys K	128.0950	128.1742
Methionine	Met M	131.0405	131.1986
Phenylalanine	Phe F	147.0684	147.1766
Proline	Pro P	97.0528	97.1167
Serine	Ser S	87.0320	87.0782
Threonine	Thr T	101.0477	101.1051
Tryptophan	Trp W	186.0793	186.2133
Tyrosine	Tyr Y	163.0633	163.1760
Valine	Val V	99.0684	99.1326

The molecular mass of a normally terminated and post-translationally unmodified protein or peptide may be calculated by summing the masses of the appropriate amino acid residues and adding the masses of H and OH for the N- and C-termini respectively.

TABLE II.1: Amino acid concentrations in low-fat, pH-adjusted Cheddar cheese at 3 and 6 months maturation. (72^0C/16sec heat-treated, 100% Maxiren).

Amino	pH5.8		pH6.0		pH6.2		pH6.4	
Acid[a]	3M	6M	3M	6M	3M	6M	3M	6M
Ala	[b]1.541	2.615	1.557	2.724	1.857	3.638	2.660	4.289
Arg	0.910	1.686	0.540	1.380	1.222	2.084	1.231	2.066
Asn	6.947	8.125	6.336	8.255	8.687	11.051	10.314	12.338
Asp	0.608	1.450	0.602	1.356	0.695	1.339	0.855	2.043
Cys	0.291	0.633	0.350	0.605	0.408	0.440	0.371	0.442
Gln	2.690	5.174	3.004	5.763	3.642	6.079	4.677	8.946
Glu	6.026	10.554	3.798	8.220	9.637	13.866	11.205	18.916
Gly	1.685	2.768	1.395	2.759	2.360	4.185	3.228	4.463
His	0.298	0.471	0.217	0.500	0.457	0.466	0.368	1.020
Ile	0.369	0.779	0.425	0.583	0.859	1.888	1.409	3.136
Leu	9.602	15.291	8.366	13.889	11.806	18.637	13.414	20.558
Lys	1.586	2.802	1.591	3.027	2.760	4.772	1.265	6.576
Met	0.475	1.102	0.413	1.098	0.731	0.935	0.910	1.622
Phe	4.916	4.676	4.203	3.730	5.549	4.198	5.853	4.616
Pro	1.366	2.416	1.400	2.099	2.430	3.849	2.971	6.496
Ser	1.516	3.274	2.144	2.691	1.900	2.539	2.633	3.990
Thr	6.033	6.921	6.827	6.497	6.447	8.135	8.061	25.654
Tyr	0.840	1.404	0.905	2.017	1.258	2.256	1.523	2.874
Val	2.781	4.533	2.205	3.490	4.173	7.114	4.817	8.722

[a] Determined as phenylthiocarbamyl-derivatised free amino acids by RP-HPLC.

[b] Amino acid concentration levels in μmol g^{-1} Cheddar cheese.

TABLE II.2: Amino acid concentrations in low-fat, pH-adjusted Cheddar cheese at 3 and 6 months maturation. (72^0C/16sec heat-treated, 50% Maxiren).

Amino	pH5.8		pH6.0		pH6.2		pH6.4	
Acid[a]	3M	6M	3M	6M	3M	6M	3M	6M
Ala	[b]1.556	2.799	1.639	3.952	1.985	4.433	2.342	4.881
Arg	1.064	1.951	0.770	2.001	1.067	2.282	1.123	2.365
Asn	7.382	8.005	7.622	10.576	9.193	13.871	10.594	15.393
Asp	0.556	1.249	0.592	1.807	0.662	2.067	0.899	2.288
Cys	0.389	0.980	0.319	0.674	0.372	0.423	0.405	0.601
Gln	2.909	4.268	3.217	7.871	4.845	10.083	5.203	10.733
Glu	6.738	10.925	6.069	12.440	9.771	20.580	13.623	24.578
Gly	1.603	2.915	1.871	3.010	2.216	3.769	2.768	5.187
His	0.257	0.312	0.377	0.581	0.447	0.701	0.652	1.113
Ile	0.411	0.589	0.546	0.930	1.213	2.991	2.151	4.817
Leu	9.309	13.094	9.480	16.625	11.170	20.612	12.452	22.069
Lys	1.840	2.492	2.247	3.399	3.452	6.400	4.491	3.304
Met	0.443	1.040	0.621	1.324	1.221	1.813	0.995	1.964
Phe	4.795	3.648	4.923	2.990	5.112	2.422	5.214	4.797
Pro	1.516	1.865	1.799	3.245	2.633	5.184	3.718	8.485
Ser	0.907	1.364	1.507	2.526	2.386	3.135	2.642	4.656
Thr	5.412	5.596	6.425	7.542	6.749	10.087	7.116	11.516
Tyr	0.820	1.349	1.079	2.403	1.255	2.843	1.619	3.065
Val	2.835	4.095	2.888	4.887	3.961	8.429	5.158	10.319

[a] Determined as phenylthiocarbamyl-derivatised free amino acids by RP-HPLC.

[b] Amino acid concentration levels in μmol g^{-1} Cheddar cheese.

TABLE II.3: Amino acid concentrations in low-fat, pH-adjusted Cheddar cheese at 3 and 6 months maturation. (90^{0}C/60sec heat-treated, 100% Maxiren).

Amino	pH5.8		pH6.0		pH6.2		pH6.4	
Acid[a]	3M	6M	3M	6M	3M	6M	3M	6M
Ala	[b]1.231	2.198	1.338	2.810	1.580	2.986	1.739	3.004
Arg	1.839	3.192	1.646	2.504	1.984	2.870	1.812	2.959
Asn	6.592	9.014	7.390	9.492	8.634	10.684	8.901	11.586
Asp	0.489	1.088	0.456	1.441	0.509	1.655	0.584	1.717
Cys	0.201	0.385	0.233	0.511	0.216	0.501	0.237	0.399
Gln	2.855	6.339	3.159	7.540	4.024	9.241	4.271	9.650
Glu	6.447	12.021	7.467	13.337	9.138	15.982	9.404	17.264
Gly	1.461	2.665	1.858	2.562	2.489	3.073	2.364	3.778
His	0.251	0.591	0.365	0.513	0.693	0.768	0.515	1.083
Ile	0.640	1.385	0.680	1.556	1.042	2.179	1.524	2.377
Leu	11.619	19.726	12.737	19.728	13.754	20.268	13.375	21.191
Lys	1.491	3.861	1.843	3.331	2.662	4.705	2.620	5.506
Met	0.276	0.799	0.456	0.930	0.547	1.144	0.586	1.122
Phe	5.148	3.992	5.456	4.624	5.372	3.571	5.447	3.246
Pro	2.114	4.685	2.550	4.578	3.541	6.303	3.710	7.410
Ser	1.253	2.177	1.472	2.348	1.089	2.200	0.967	2.622
Thr	5.088	6.441	5.231	7.057	5.618	6.949	7.495	8.601
Tyr	1.136	2.516	1.243	2.594	1.373	2.729	1.484	2.916
Val	3.358	6.527	3.771	6.727	4.421	7.837	4.381	7.980

[a] Determined as phenylthiocarbamyl-derivatised free amino acids by RP-HPLC.

[b] Amino acid concentration levels in μmol g^{-1} Cheddar cheese.

TABLE II.4: Amino acid concentrations in low-fat, pH-adjusted Cheddar cheese at 3 and 6 months maturation. (90^{0}C/60sec heat-treated, 50% Maxiren).

Amino	pH5.8		pH6.0		pH6.2		pH6.4	
Acid[a]	3M	6M	3M	6M	3M	6M	3M	6M
Ala	[b]0.791	1.469	1.175	1.938	1.385	2.258	1.191	1.635
Arg	0.853	1.597	1.265	2.189	1.571	2.519	0.966	1.298
Asn	4.368	6.540	6.411	7.488	6.837	9.193	4.830	6.784
Asp	0.352	0.603	0.297	0.814	0.377	1.051	0.217	0.581
Cys	0.178	0.409	0.171	0.459	0.186	0.500	0.163	0.333
Gln	1.195	2.483	2.955	4.084	3.062	6.410	2.675	3.581
Glu	3.929	6.550	5.823	9.014	6.844	11.442	4.300	7.228
Gly	0.764	1.931	1.142	2.810	1.884	3.035	0.986	2.034
His	0.090	0.362	0.164	0.593	0.406	0.733	0.175	0.484
Ile	0.307	0.585	0.479	1.052	0.753	1.655	0.579	0.866
Leu	7.415	12.569	10.133	15.800	10.810	17.093	7.233	11.195
Lys	0.866	1.411	1.075	2.651	1.820	3.496	1.379	1.705
Met	0.216	0.488	0.422	0.727	0.437	0.879	0.326	0.573
Phe	4.014	1.400	4.946	3.099	4.328	3.451	3.290	2.576
Pro	1.171	2.165	1.946	4.308	2.665	5.948	2.242	3.395
Ser	0.571	0.978	1.036	1.340	1.108	1.063	0.781	1.026
Thr	4.257	5.662	5.725	6.673	5.896	7.316	6.947	7.598
Tyr	0.514	1.059	0.853	1.802	1.416	2.003	0.608	1.195
Val	1.855	3.544	2.829	5.055	3.277	6.044	1.901	3.283

[a] Determined as phenylthiocarbamyl-derivatised free amino acids by RP-HPLC.

[b] Amino acid concentration levels in μmol g^{-1} Cheddar cheese.

FIGURE II.1: Amino acid concentrations in low-fat, pH-adjusted Cheddar cheese at 3 months maturation.
(72^0C/16sec heat-treated, 100% Maxiren).

(A) Alanine, arginine, asparagine, aspartate, cysteine, glutamine.

(B) Glutamate, glycine, histidine, isoleucine, leucine, lysine.

(C) Methionine, phenylalanine, proline, serine, threonine, tyrosine, valine.

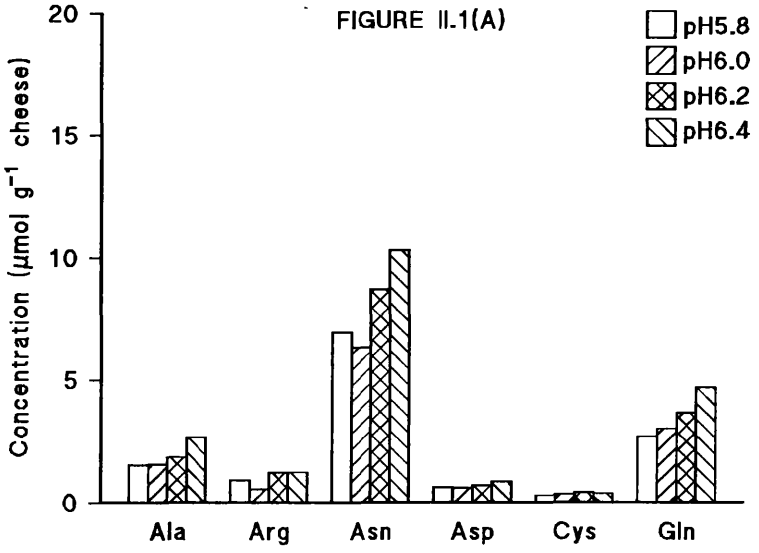
FIGURE II.1(A)
pH5.8
pH6.0
pH6.2
pH6.4
Concentration (µmol g^{-1} cheese)
20
15
10
5
0
Ala
Arg
Asn
Asp
Cys
Gln

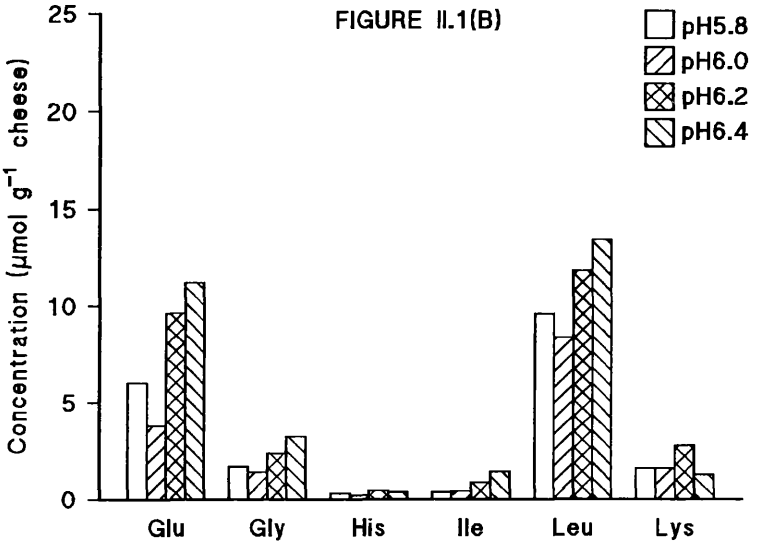
FIGURE II.1(B)
pH5.8
pH6.0
pH6.2
pH6.4
Concentration (µmol g^{-1} cheese)
25
20
15
10
5
0
Glu
Gly
His
Ile
Leu
Lys

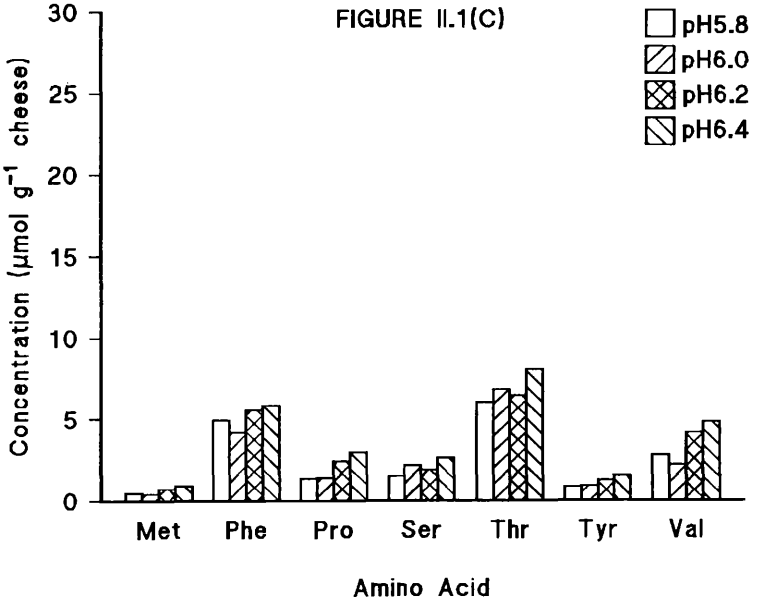
FIGURE II.1(C)
pH5.8
pH6.0
pH6.2
pH6.4
Concentration (µmol g^{-1} cheese)
30
25
20
15
10
5
0
Met
Phe
Pro
Ser
Thr
Tyr
Val
Amino Acid

FIGURE II.2: Amino acid concentrations in low-fat, pH-adjusted Cheddar cheese at 3 months maturation.
(72^0C/16sec heat-treated, 50% Maxiren).

(A) Alanine, arginine, asparagine, aspartate, cysteine, glutamine.

(B) Glutamate, glycine, histidine, isoleucine, leucine, lysine.

(C) Methionine, phenylalanine, proline, serine, threonine, tyrosine, valine.

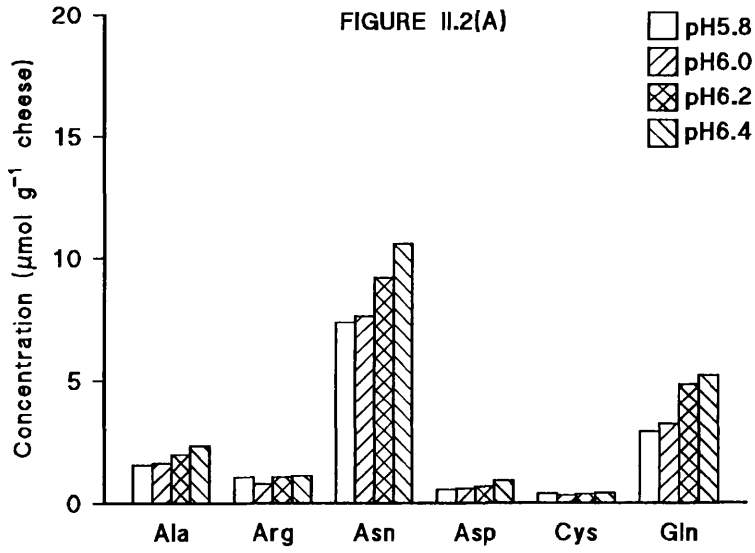
FIGURE II.2(A)
pH5.8
pH6.0
pH6.2
pH6.4
Concentration (µmol g⁻¹ cheese)
20
15
10
5
0
Ala
Arg
Asn
Asp
Cys
Gln

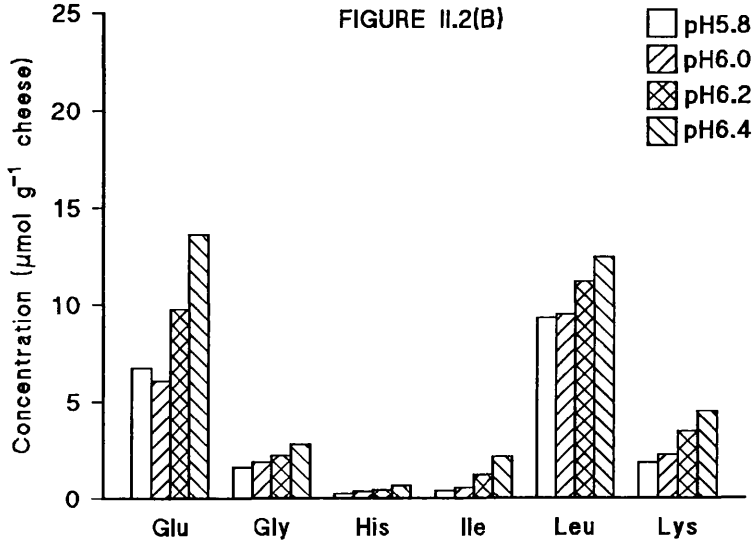
FIGURE II.2(B)
pH5.8
pH6.0
pH6.2
pH6.4
Concentration (µmol g⁻¹ cheese)
25
20
15
10
5
0
Glu
Gly
His
Ile
Leu
Lys

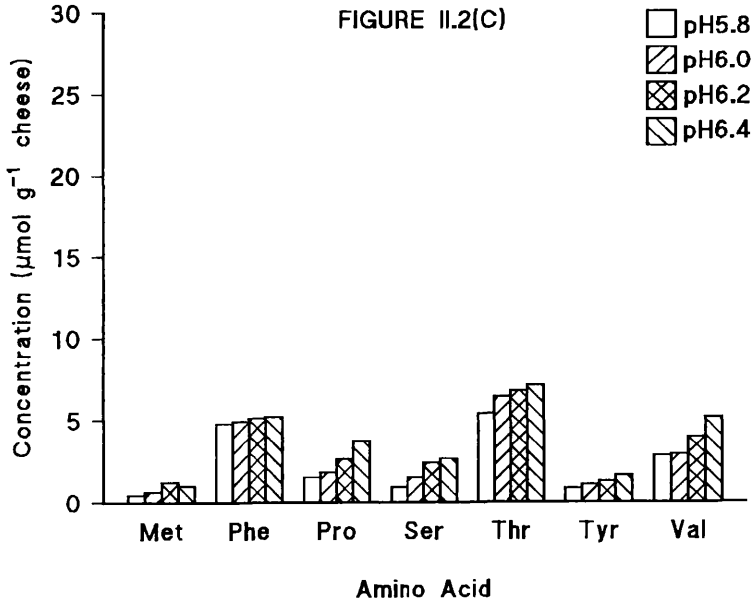
FIGURE II.2(C)
pH5.8
pH6.0
pH6.2
pH6.4
Concentration (µmol g⁻¹ cheese)
30
25
20
15
10
5
0
Met
Phe
Pro
Ser
Thr
Tyr
Val
Amino Acid

FIGURE II.3: Amino acid concentrations in low-fat, pH-adjusted Cheddar cheese at 3 months maturation.
(90^0C/60sec heat-treated, 100% Maxiren).

(A) Alanine, arginine, asparagine, aspartate, cysteine, glutamine.

(B) Glutamate, glycine, histidine, isoleucine, leucine, lysine.

(C) Methionine, phenylalanine, proline, serine, threonine, tyrosine, valine.

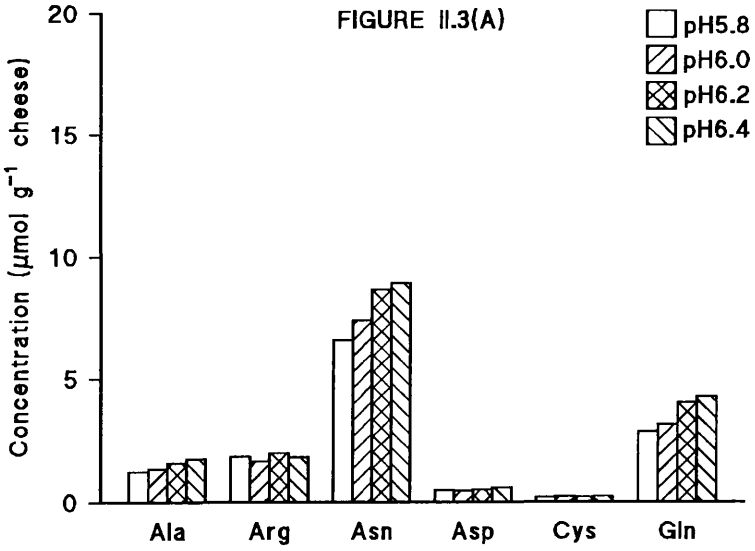
FIGURE II.3(A)
pH5.8
pH6.0
pH6.2
pH6.4
Concentration (µmol g⁻¹ cheese)
20
15
10
5
0
Ala
Arg
Asn
Asp
Cys
Gln

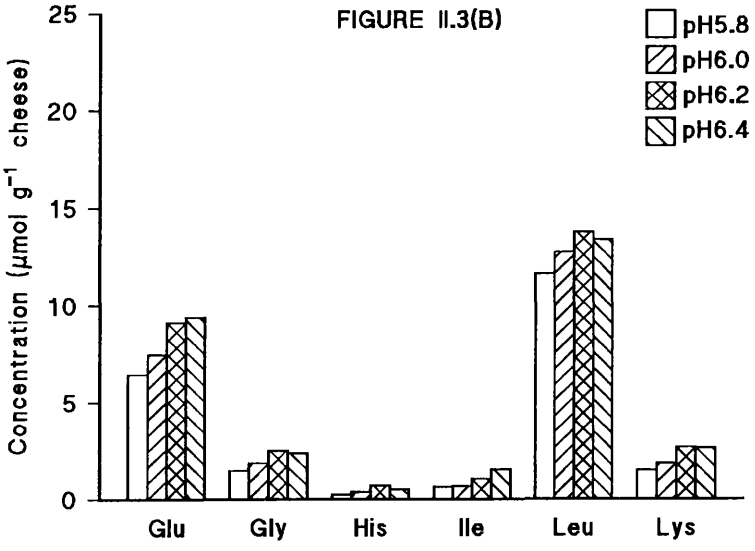
FIGURE II.3(B)
pH5.8
pH6.0
pH6.2
pH6.4
Concentration (µmol g⁻¹ cheese)
25
20
15
10
5
0
Glu
Gly
His
Ile
Leu
Lys

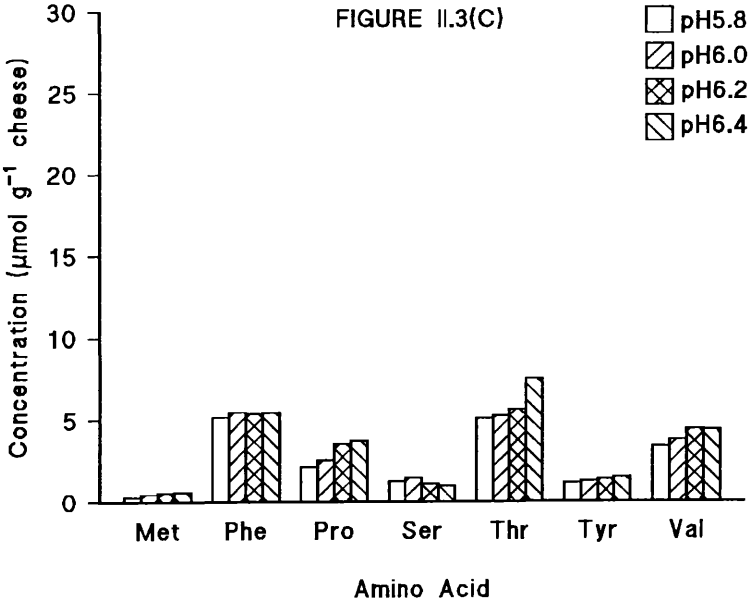
FIGURE II.3(C)
pH5.8
pH6.0
pH6.2
pH6.4
Concentration (µmol g⁻¹ cheese)
30
25
20
15
10
5
0
Met
Phe
Pro
Ser
Thr
Tyr
Val
Amino Acid

FIGURE II.4: Amino acid concentrations in low-fat, pH-adjusted Cheddar cheese at 3 months maturation.
(90^0C/60sec heat-treated, 50% Maxiren).

(A) Alanine, arginine, asparagine, aspartate, cysteine, glutamine.

(B) Glutamate, glycine, histidine, isoleucine, leucine, lysine.

(C) Methionine, phenylalanine, proline, serine, threonine, tyrosine, valine.

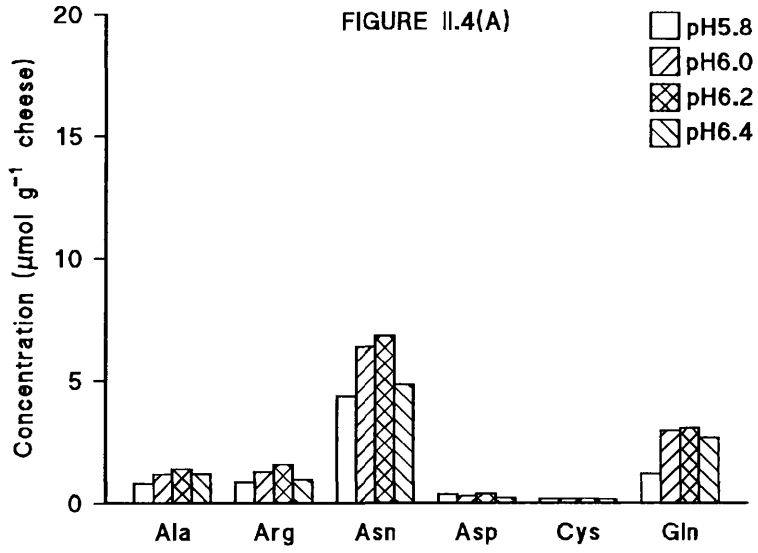
FIGURE II.4(A)
pH5.8
pH6.0
pH6.2
pH6.4
Concentration (µmol g⁻¹ cheese)
20
15
10
5
0
Ala
Arg
Asn
Asp
Cys
Gln

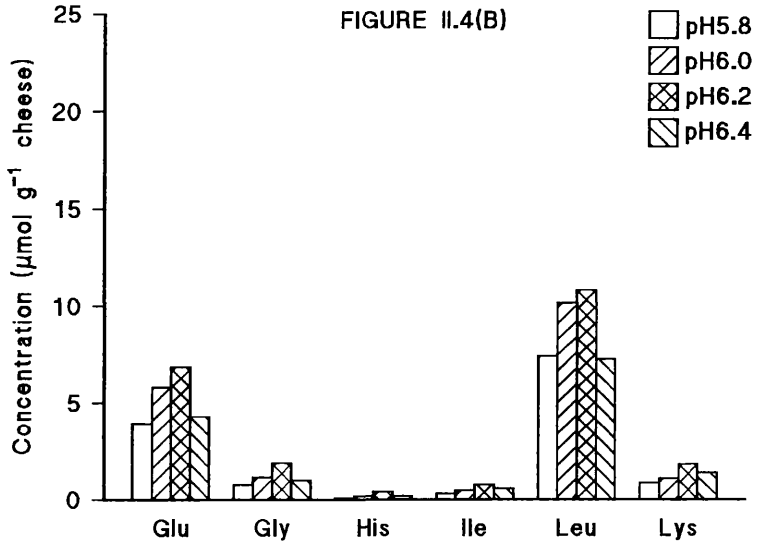
FIGURE II.4(B)
pH5.8
pH6.0
pH6.2
pH6.4
Concentration (µmol g⁻¹ cheese)
25
20
15
10
5
0
Glu
Gly
His
Ile
Leu
Lys

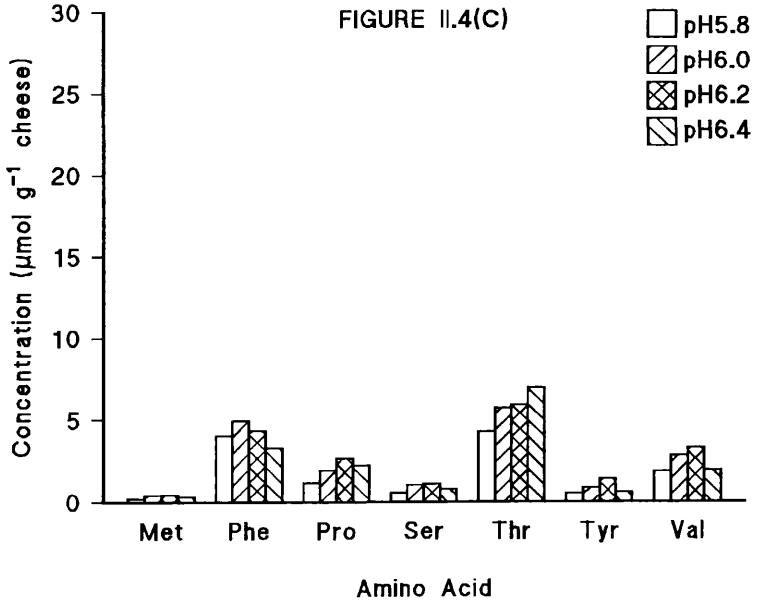
FIGURE II.4(C)
pH5.8
pH6.0
pH6.2
pH6.4
Concentration (µmol g⁻¹ cheese)
30
25
20
15
10
5
0
Met
Phe
Pro
Ser
Thr
Tyr
Val
Amino Acid

FIGURE II.5: Amino acid concentrations in low-fat, pH-adjusted Cheddar cheese at 6 months maturation.
(72^0C/16sec heat-treated, 100% Maxiren).

(A) Alanine, arginine, asparagine, aspartate, cysteine, glutamine.

(B) Glutamate, glycine, histidine, isoleucine, leucine, lysine.

(C) Methionine, phenylalanine, proline, serine, threonine, tyrosine, valine.

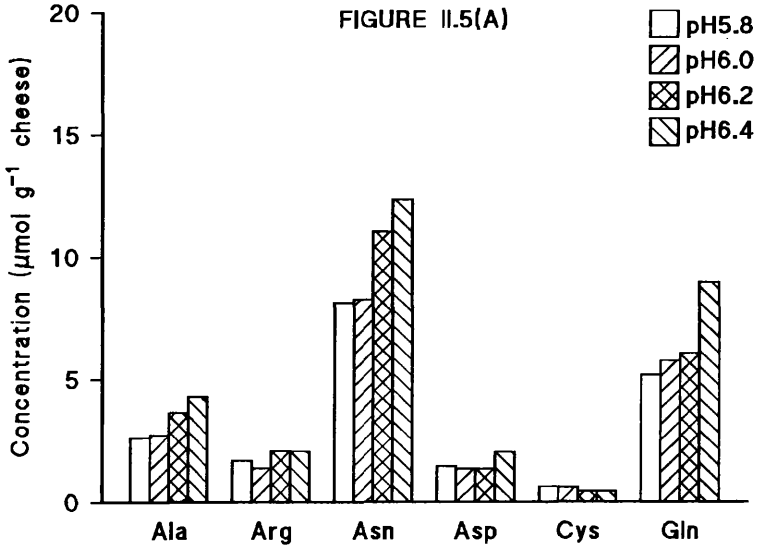
FIGURE II.5(A)
pH5.8
pH6.0
pH6.2
pH6.4
Concentration (µmol g⁻¹ cheese)
20
15
10
5
0
Ala
Arg
Asn
Asp
Cys
Gln

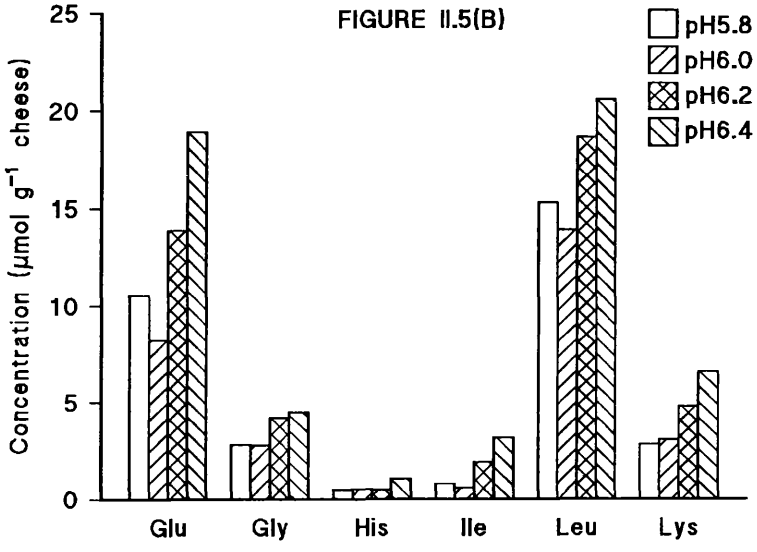
FIGURE II.5(B)
pH5.8
pH6.0
pH6.2
pH6.4
Concentration (µmol g⁻¹ cheese)
25
20
15
10
5
0
Glu
Gly
His
Ile
Leu
Lys

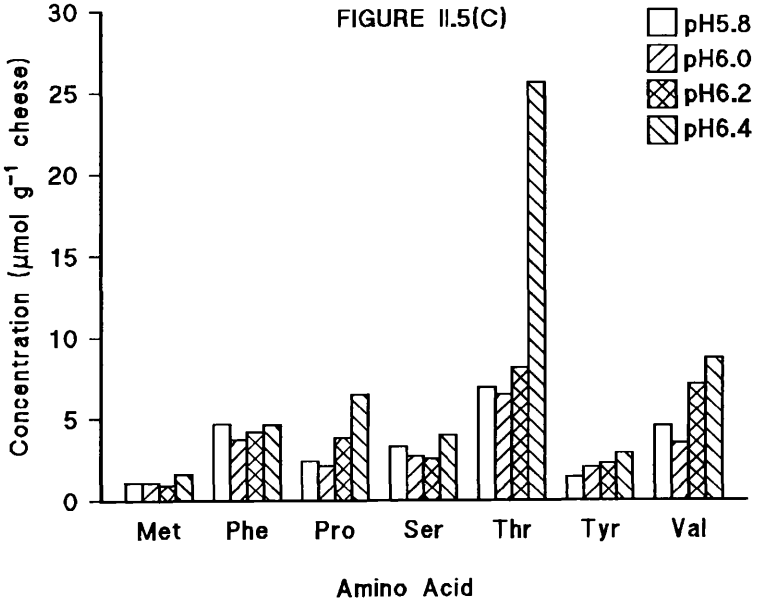
FIGURE II.5(C)
pH5.8
pH6.0
pH6.2
pH6.4
Concentration (µmol g⁻¹ cheese)
30
25
20
15
10
5
0
Met
Phe
Pro
Ser
Thr
Tyr
Val
Amino Acid

FIGURE II.6: Amino acid concentrations in low-fat, pH-adjusted Cheddar cheese at 6 months maturation.
(72^0C/16sec heat-treated, 50% Maxiren).

(A) Alanine, arginine, asparagine, aspartate, cysteine, glutamine.

(B) Glutamate, glycine, histidine, isoleucine, leucine, lysine.

(C) Methionine, phenylalanine, proline, serine, threonine, tyrosine, valine.

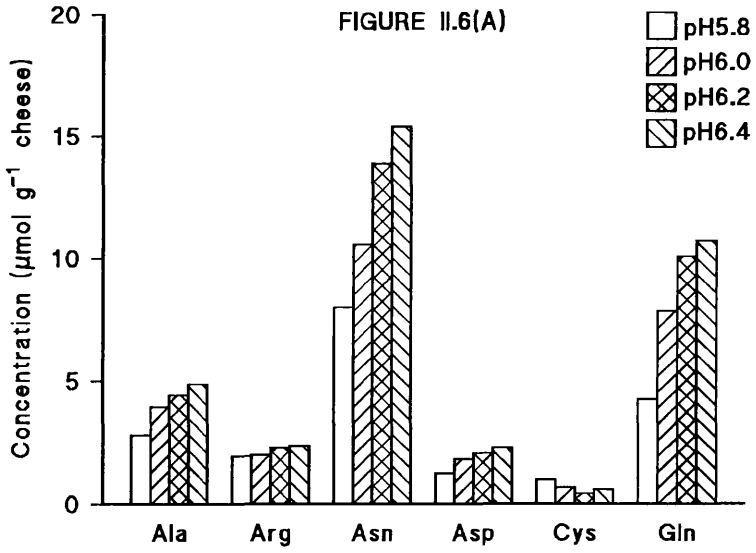
FIGURE II.6(A)
pH5.8
pH6.0
pH6.2
pH6.4
Concentration (µmol g⁻¹ cheese)
20
15
10
5
0
Ala
Arg
Asn
Asp
Cys
Gln

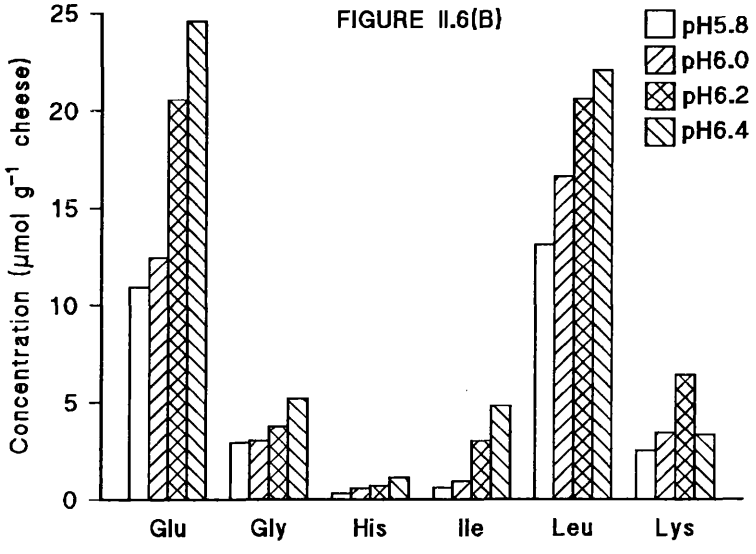
FIGURE II.6(B)
pH5.8
pH6.0
pH6.2
pH6.4
Concentration (µmol g⁻¹ cheese)
25
20
15
10
5
0
Glu
Gly
His
Ile
Leu
Lys

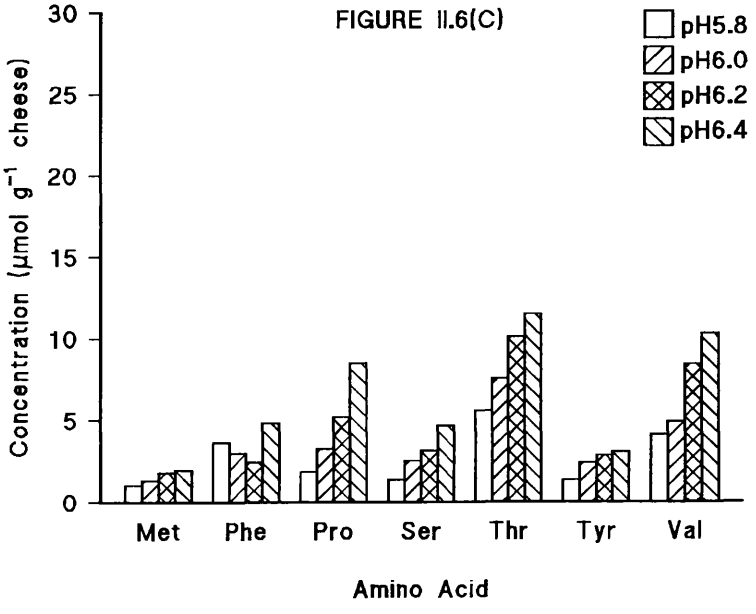
FIGURE II.6(C)
pH5.8
pH6.0
pH6.2
pH6.4
Concentration (µmol g⁻¹ cheese)
30
25
20
15
10
5
0
Met
Phe
Pro
Ser
Thr
Tyr
Val
Amino Acid

FIGURE II.7: Amino acid concentrations in low-fat, pH-adjusted Cheddar cheese at 6 months maturation.
(90^0C/60sec heat-treated, 100% Maxiren).

(A) Alanine, arginine, asparagine, aspartate, cysteine, glutamine.

(B) Glutamate, glycine, histidine, isoleucine, leucine, lysine.

(C) Methionine, phenylalanine, proline, serine, threonine, tyrosine, valine.

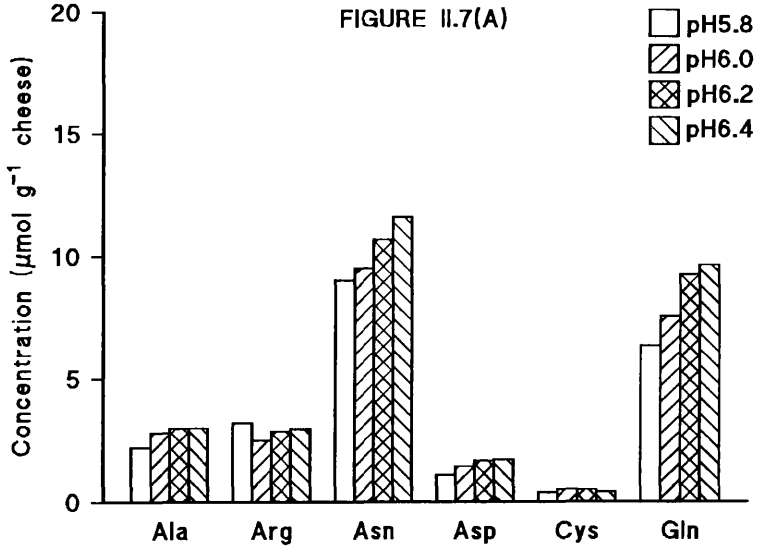
FIGURE II.7(A)
pH5.8
pH6.0
pH6.2
pH6.4
Concentration (µmol g^{-1} cheese)
20
15
10
5
0
Ala
Arg
Asn
Asp
Cys
Gln

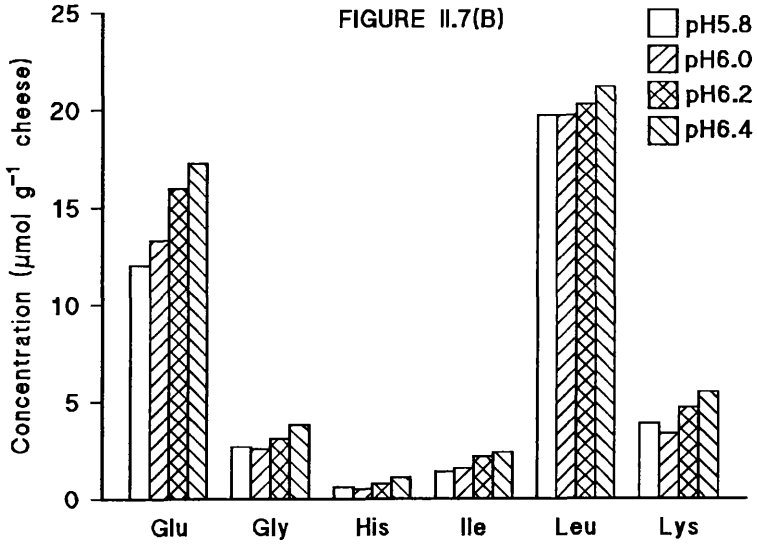
FIGURE II.7(B)
pH5.8
pH6.0
pH6.2
pH6.4
Concentration (µmol g^{-1} cheese)
25
20
15
10
5
0
Glu
Gly
His
Ile
Leu
Lys

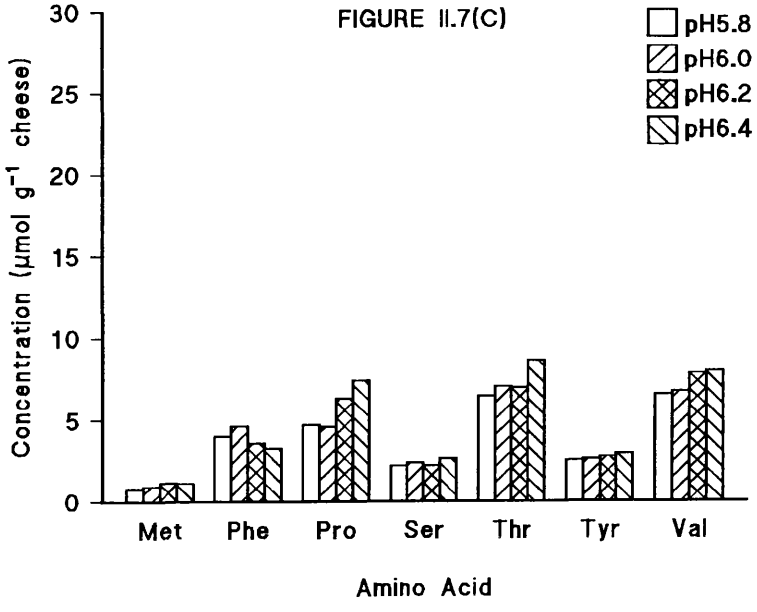
FIGURE II.7(C)
pH5.8
pH6.0
pH6.2
pH6.4
Concentration (µmol g^{-1} cheese)
30
25
20
15
10
5
0
Met
Phe
Pro
Ser
Thr
Tyr
Val
Amino Acid

FIGURE II.8: Amino acid concentrations in low-fat, pH-adjusted Cheddar cheese at 6 months maturation.
(90^0C/60sec heat-treated, 50% Maxiren).

(A) Alanine, arginine, asparagine, aspartate, cysteine, glutamine.

(B) Glutamate, glycine, histidine, isoleucine, leucine, lysine.

(C) Methionine, phenylalanine, proline, serine, threonine, tyrosine, valine.

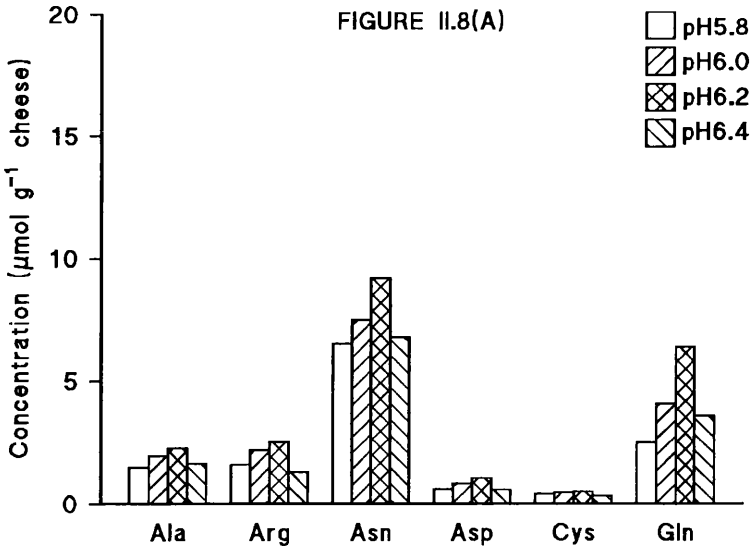
FIGURE II.8(A)
pH5.8
pH6.0
pH6.2
pH6.4
Concentration (μmol g⁻¹ cheese)
20
15
10
5
0
Ala
Arg
Asn
Asp
Cys
Gln

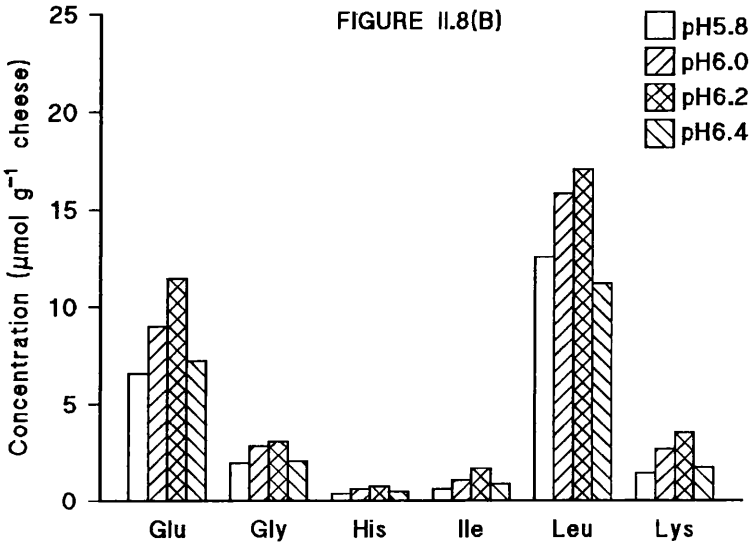
FIGURE II.8(B)
pH5.8
pH6.0
pH6.2
pH6.4
Concentration (μmol g⁻¹ cheese)
25
20
15
10
5
0
Glu
Gly
His
Ile
Leu
Lys

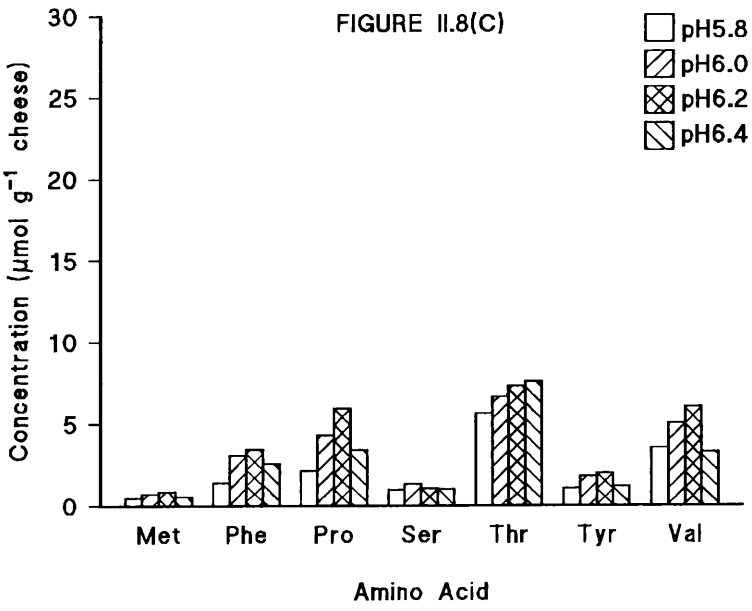
FIGURE II.8(C)
pH5.8
pH6.0
pH6.2
pH6.4
Concentration (μmol g⁻¹ cheese)
30
25
20
15
10
5
0
Met
Phe
Pro
Ser
Thr
Tyr
Val
Amino Acid

FIGURE II.9: Influence of the combined effects of pH adjustment and heat treatment on amino acid concentrations in low-fat Cheddar cheese.

Analysis of changes to the mean amino acid concentrations over 6 months of maturation by Principal Component Analysis.

(A) Asparagine.

(B) Aspartate.

(C) Glutamine.

(D) Glutamate.

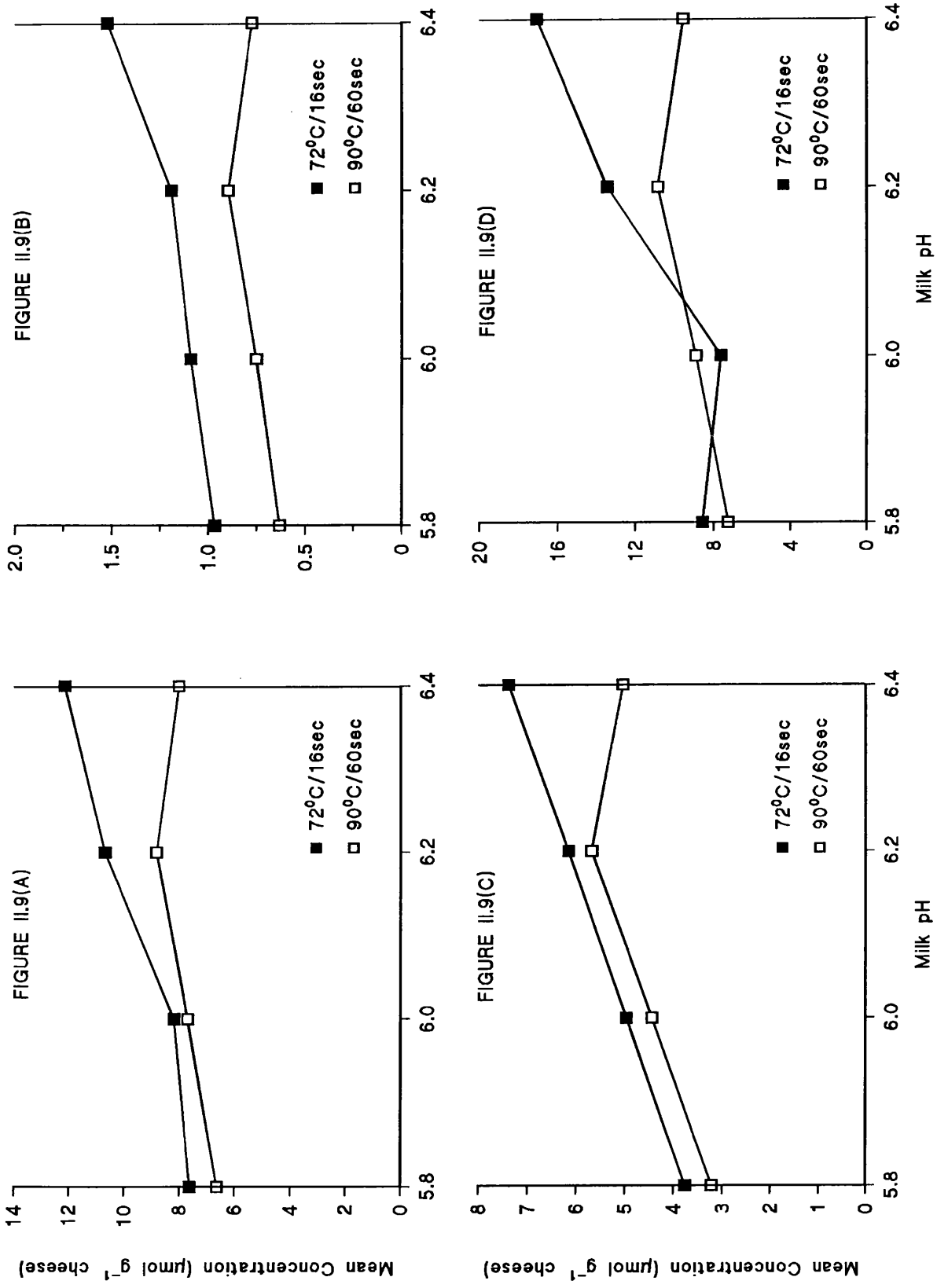
FIGURE II.9(A)
FIGURE II.9(B)
FIGURE II.9(C)
FIGURE II.9(D)
■ 72°C/16sec
□ 90°C/60sec
Mean Concentration (μmol g⁻¹ cheese)
Milk pH

FIGURE II.10: Influence of the combined effects of pH adjustment and heat treatment on amino acid concentrations in low-fat Cheddar cheese.

Analysis of changes to the mean amino acid concentrations over 6 months of maturation by Principal Component Analysis.

(A) Glycine.

(B) Histidine.

(C) Isoleucine.

(D) Leucine.

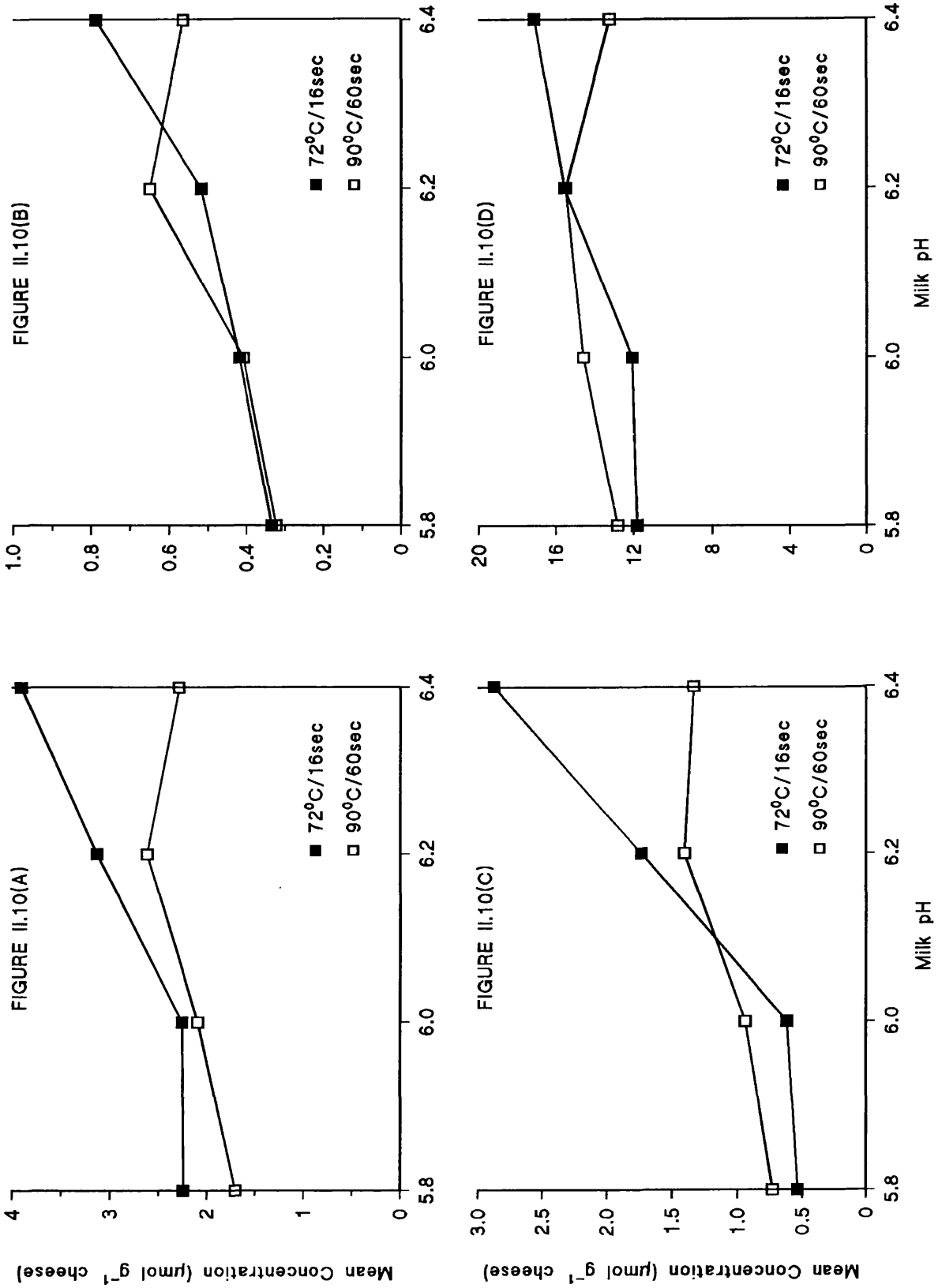
FIGURE II.10(A)
FIGURE II.10(B)
FIGURE II.10(C)
FIGURE II.10(D)
■ 72°C/16sec
□ 90°C/60sec
Mean Concentration (μmol g⁻¹ cheese)
Milk pH

FIGURE II.11: Influence of the combined effects of pH adjustment and heat treatment on amino acid concentrations in low-fat Cheddar cheese.

Analysis of changes to the mean amino acid concentrations over 6 months of maturation by Principal Component Analysis.

(A) Lysine.

(B) Tyrosine.

(C) Valine.

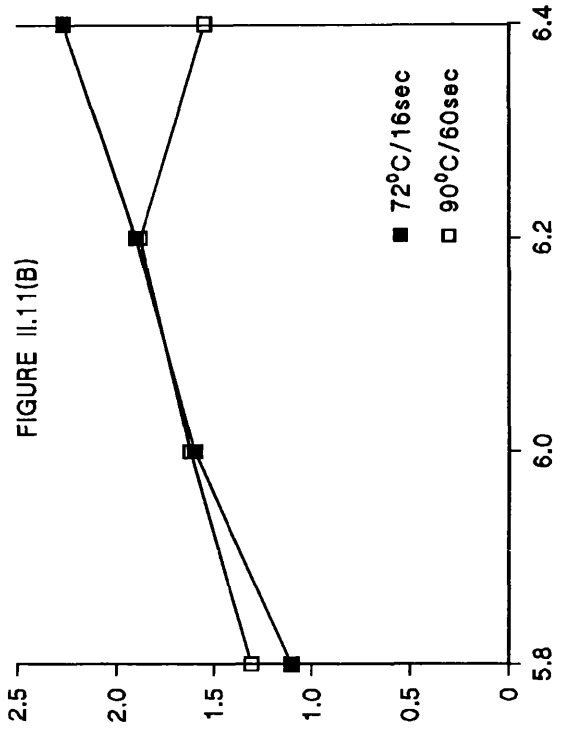
FIGURE II.11(B)
■ 72°C/16sec
□ 90°C/60sec
2.5
2.0
1.5
1.0
0.5
0
5.8
6.0
6.2
6.4

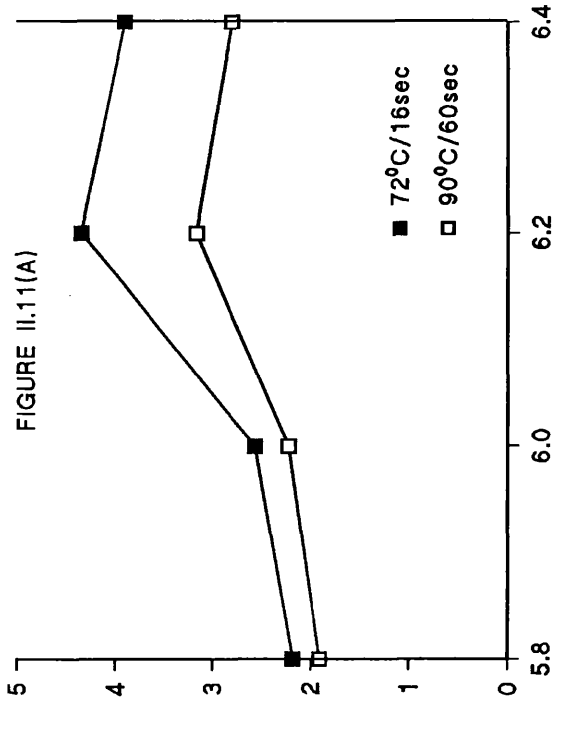
FIGURE II.11(A)
■ 72°C/16sec
□ 90°C/60sec
5
4
3
2
1
0
5.8
6.0
6.2
6.4
Mean Concentration (μmol g⁻¹ cheese)

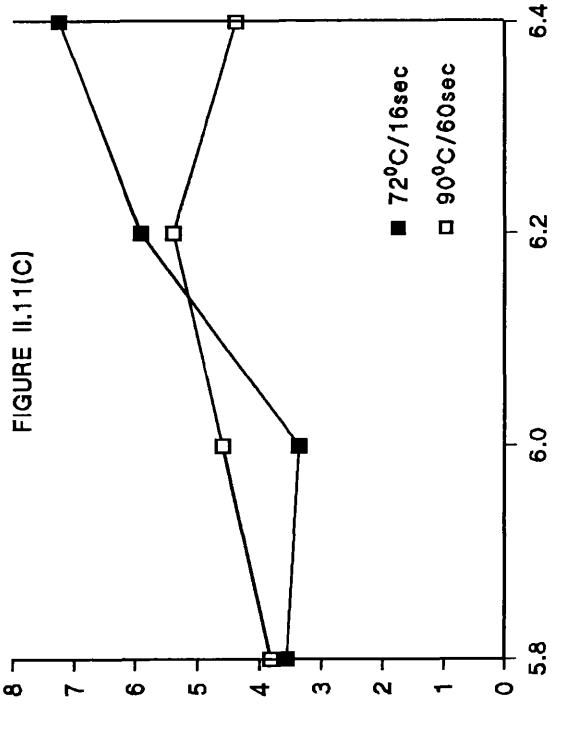
FIGURE II.11(C)
■ 72°C/16sec
□ 90°C/60sec
8
7
6
5
4
3
2
1
0
5.8
6.0
6.2
6.4
Milk pH
Mean Concentration (μmol g⁻¹ cheese)

FIGURE II.12: Influence of the combined effects of rennet level and heat treatment on amino acid concentrations in low-fat Cheddar cheese.

Analysis of changes to the mean amino acid concentrations over 6 months of maturation by Principal Component Analysis.

(A) Asparagine.

(B) Aspartate.

(C) Glutamine.

(D) Glutamate.

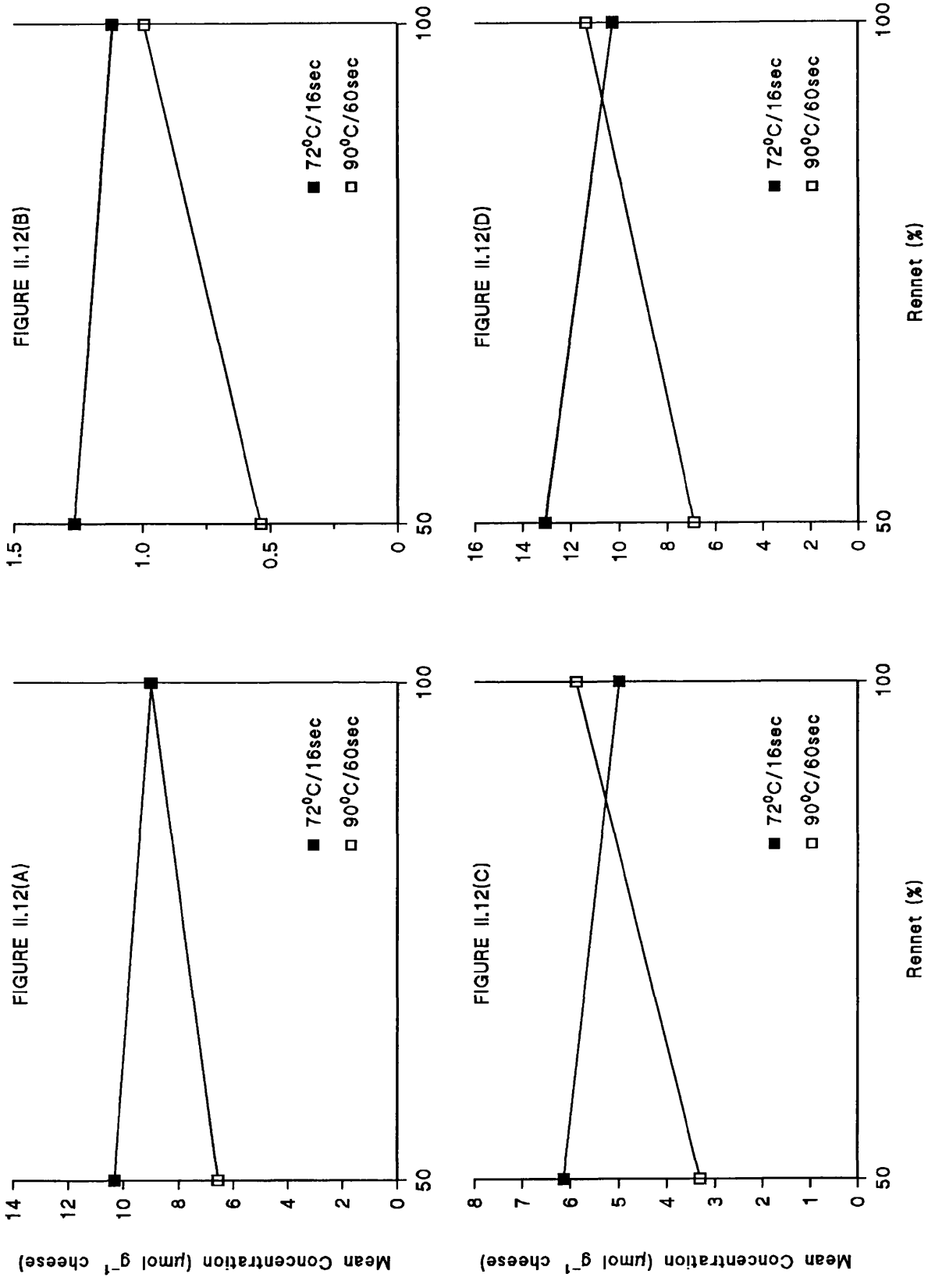
FIGURE II.12(A)
FIGURE II.12(B)
FIGURE II.12(C)
FIGURE II.12(D)
■ 72°C/16sec
□ 90°C/60sec
Mean Concentration (μmol g⁻¹ cheese)
Rennet (%)
0
2
4
6
8
10
12
14
0.5
1.0
1.5
1
3
5
7
16
50
100

FIGURE II.13: Influence of the combined effects of rennet level and heat treatment on amino acid concentrations in low-fat Cheddar cheese.

Analysis of changes to the mean amino acid concentrations over 6 months of maturation by Principal Component Analysis.

(A) Glycine.

(B) Histidine.

(C) Isoleucine.

(D) Leucine.

FIGURE II.13(A)
Mean Concentration (μmol g⁻¹ cheese)
Rennet (%)
■ 72°C/16sec
□ 90°C/60sec
0
1
2
3
4
50
100

FIGURE II.13(B)
■ 72°C/16sec
□ 90°C/60sec
0
0.2
0.4
0.6
0.8
50
100

FIGURE II.13(C)
Mean Concentration (μmol g⁻¹ cheese)
Rennet (%)
■ 72°C/16sec
□ 90°C/60sec
0
0.4
0.8
1.2
1.6
2.0
50
100

FIGURE II.13(D)
■ 72°C/16sec
□ 90°C/60sec
0
4
8
12
16
20
50
100

FIGURE II.14: Influence of the combined effects of rennet level and heat treatment on amino acid concentrations in low-fat Cheddar cheese.

Analysis of changes to the mean amino acid concentrations over 6 months of maturation by Principal Component Analysis.

(A) Lysine.

(B) Tyrosine.

(C) Valine.

FIGURE II.14(A)
Mean Concentration (μmol g⁻¹ cheese)
4
3
2
1
0
50
100
■ 72°C/16sec
□ 90°C/60sec

FIGURE II.14(B)
2.5
2.0
1.5
1.0
0.5
0
50
100
■ 72°C/16sec
□ 90°C/60sec

FIGURE II.14(C)
Mean Concentration (μmol g⁻¹ cheese)
7
6
5
4
3
2
1
0
50
100
Rennet (%)
■ 72°C/16sec
□ 90°C/60sec

TABLE II.5: Amino acid concentrations in standard pH milk Cheddar cheese up to 246 days of maturation.

Time	Amino Acid[a]																
(Days)	Ala	Arg	Asn/Ser	Asp	Gln	Glu	Gly	His	Ile	Leu	Lys	Met	Phe	Pro	Thr	Tyr	Val
0	[b]0.024	0.135	0.111	0.020	0.176	0.341	0.000	0.152	0.000	0.000	0.025	0.009	0.000	0.005	0.140	0.000	0.009
0.2	0.000	0.000	0.000	0.000	0.000	0.526	0.000	0.000	0.000	0.000	0.085	0.000	0.000	0.316	0.373	0.000	0.000
1	0.244	0.229	0.486	0.206	0.242	1.402	0.119	0.179	0.137	0.535	0.344	0.067	0.219	0.538	0.882	0.172	0.220
8	0.521	0.407	1.742	0.270	0.798	2.628	0.565	0.201	0.341	1.960	0.609	0.190	0.754	0.783	1.587	0.337	0.638
15	0.656	0.620	3.043	0.360	1.323	4.040	0.984	0.246	0.443	3.623	0.850	0.360	1.336	1.060	2.294	0.419	1.036
22	0.805	0.870	3.893	0.471	1.731	5.367	1.276	0.298	0.683	4.409	1.125	0.484	1.611	1.202	2.983	0.500	1.364
29	1.125	1.030	5.093	0.640	2.391	6.750	1.464	0.357	0.854	5.630	1.370	0.627	2.100	1.468	3.618	0.635	1.706
36	1.281	1.287	6.450	0.809	3.213	8.430	1.763	0.405	1.223	6.894	1.717	0.831	2.618	1.823	3.948	0.886	2.168
43	1.422	1.489	7.157	0.874	3.642	9.418	1.913	0.428	1.261	7.707	1.936	0.893	2.869	2.184	4.763	1.116	2.471
50	1.554	1.947	7.653	0.964	3.435	10.657	2.606	0.523	1.143	8.675	2.239	1.038	3.198	2.519	4.983	1.054	2.683
66	1.950	1.977	9.465	1.277	4.959	13.206	2.511	0.571	1.470	10.894	2.806	1.459	4.042	2.819	6.646	1.908	3.466
78	2.088	2.186	8.673	1.325	5.326	13.150	3.574	0.758	1.114	11.465	3.434	1.603	2.800	2.950	6.228	1.662	3.846
106	2.824	3.440	11.599	2.410	8.014	21.284	3.981	1.166	3.115	18.111	6.171	2.653	6.212	4.962	9.099	2.262	7.381
134	3.493	3.715	12.731	2.719	8.586	23.987	3.527	1.655	4.089	19.258	7.078	3.074	6.581	5.551	9.706	2.147	8.555
162	4.028	3.824	12.608	3.106	10.848	26.537	3.812	1.683	5.231	20.820	7.958	3.615	7.534	6.858	11.226	2.478	9.622
190	4.767	4.543	18.238	4.021	15.164	35.574	4.525	1.933	7.123	23.419	10.811	4.369	8.992	8.018	12.913	2.889	11.715
219	5.718	4.723	18.945	3.965	12.828	38.020	6.427	2.134	7.140	25.381	12.093	4.767	9.227	8.680	14.157	2.579	12.754
246	6.623	4.731	18.607	3.718	15.503	38.016	5.280	2.459	8.287	26.049	13.178	5.216	9.371	13.056	15.147	3.514	13.707

[a] Determined as phenylthiocarbamyl-derivatised free amino acids by RP-HPLC.

[b] Amino acid concentration levels in μmol g^{-1} Cheddar cheese.

TABLE II.6: Amino acid concentrations in reduced pH milk Cheddar cheese up to 246 days of maturation.

Time	Amino Acid[a]																
(Days)	Ala	Arg	Asn/Ser	Asp	Gln	Glu	Gly	His	Ile	Leu	Lys	Met	Phe	Pro	Thr	Tyr	Val
0	[b]0.053	0.167	0.132	0.044	0.188	0.370	0.000	0.188	0.000	0.000	0.016	0.014	0.000	0.041	0.201	0.000	0.014
0.2	0.112	0.000	0.091	0.122	0.000	0.483	0.000	0.089	0.034	0.101	0.059	0.026	0.055	0.337	0.561	0.052	0.084
1	0.240	0.202	0.496	0.183	0.202	1.223	0.073	0.108	0.067	0.585	0.176	0.033	0.296	0.391	0.877	0.138	0.241
8	0.460	0.409	1.891	0.238	0.780	2.338	0.467	0.164	0.139	2.128	0.332	0.113	0.964	0.672	1.674	0.265	0.644
15	0.715	0.775	3.549	0.332	1.637	4.186	1.030	0.253	0.270	4.529	0.713	0.264	1.907	1.026	2.441	0.488	1.266
22	0.826	0.686	3.742	0.320	1.645	4.125	1.057	0.274	0.281	4.552	0.653	0.243	2.036	0.865	2.721	0.454	1.290
29	1.046	1.287	5.212	0.597	2.938	6.545	1.529	0.395	0.425	7.387	1.102	0.475	2.982	1.372	3.294	0.799	2.125
36	1.305	1.464	6.633	0.844	3.231	8.427	2.252	0.562	0.512	8.398	1.570	0.641	3.674	1.734	3.750	0.965	2.556
43	1.446	1.742	6.899	0.867	4.188	9.155	2.093	0.531	0.830	9.829	1.783	0.792	4.150	1.879	4.180	1.253	3.092
50	1.753	2.030	7.561	1.014	4.817	10.665	2.315	0.641	0.985	11.019	2.032	0.940	4.622	2.093	4.228	1.583	3.511
66	2.477	2.736	9.508	1.397	6.531	14.517	2.854	0.761	1.068	14.295	2.816	1.337	5.971	2.921	6.243	1.999	4.639
78	2.617	2.773	9.641	1.591	6.781	15.224	2.919	0.836	1.211	15.268	3.199	1.514	6.127	2.805	6.273	2.204	4.883
106	2.834	3.717	11.026	2.644	7.248	19.740	2.869	1.123	2.484	18.212	5.053	2.182	7.449	4.164	7.146	2.898	6.999
134	3.561	4.467	12.499	3.311	8.755	24.619	3.618	1.496	3.374	22.273	6.498	2.940	9.099	4.780	9.108	2.918	9.052
162	3.463	3.822	10.535	3.060	7.588	20.998	3.305	1.277	2.276	20.061	5.728	2.694	8.084	4.241	8.412	2.667	7.909
190	4.169	4.324	13.150	4.095	9.663	27.160	4.701	1.208	4.012	22.287	8.095	3.427	9.236	4.756	9.405	2.544	9.536
219	5.264	4.792	14.312	4.681	13.482	29.700	3.885	1.478	4.659	24.770	9.358	4.021	10.153	5.961	11.033	3.099	10.868
246	6.225	4.860	15.109	4.948	13.245	32.411	4.032	1.582	3.992	26.262	9.740	4.317	10.414	4.861	12.423	2.655	10.934

[a] Determined as phenylthiocarbamyl-derivatised free amino acids by RP-HPLC.

[b] Amino acid concentration levels in μmol g^{-1} Cheddar cheese.

FIGURE II.15: Amino acid concentrations in standard pH and reduced pH Cheddar cheese up to 246 days of maturation.

(A) Alanine.

(B) Arginine.

(C) Asparagine/Serine.

FIGURE II.15(A)

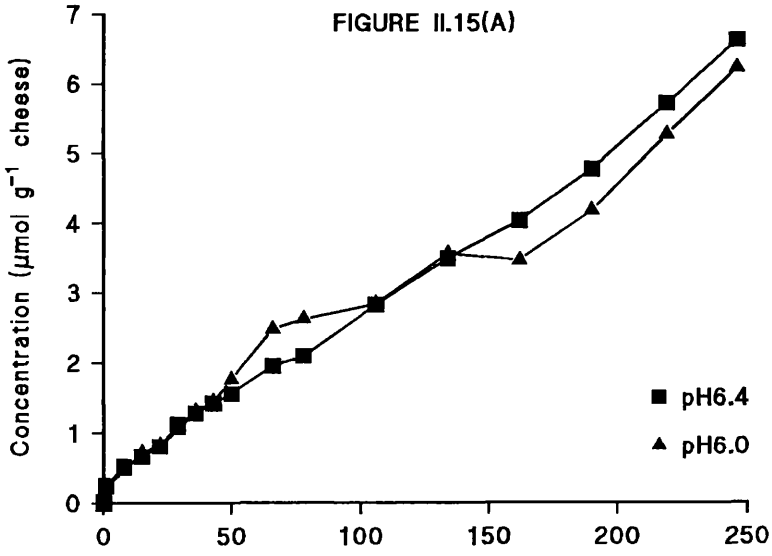

FIGURE II.15(B)

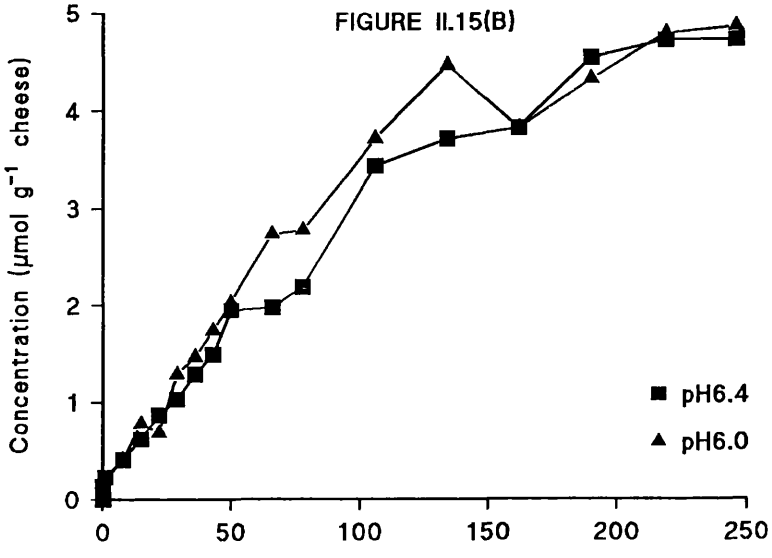

FIGURE II.15(C)

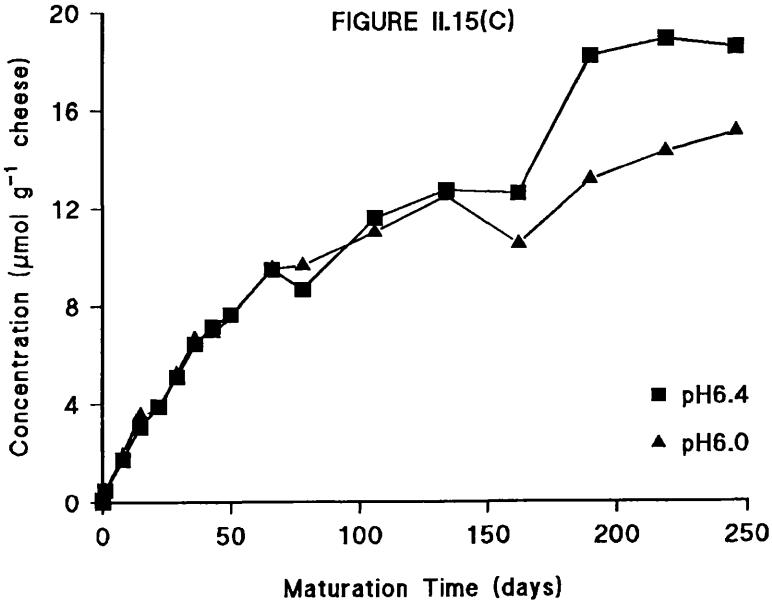

FIGURE II.16: Amino acid concentrations in standard pH and reduced pH Cheddar cheese up to 246 days of maturation.

(A) Aspartate.

(B) Glutamine.

(C) Glutamate.

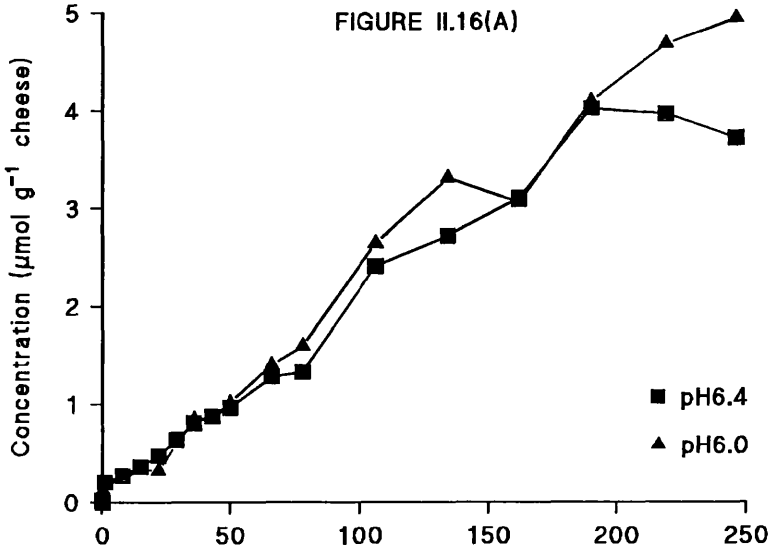
FIGURE II.16(A)
Concentration (μmol g⁻¹ cheese)
0
1
2
3
4
5
0
50
100
150
200
250
pH6.4
pH6.0

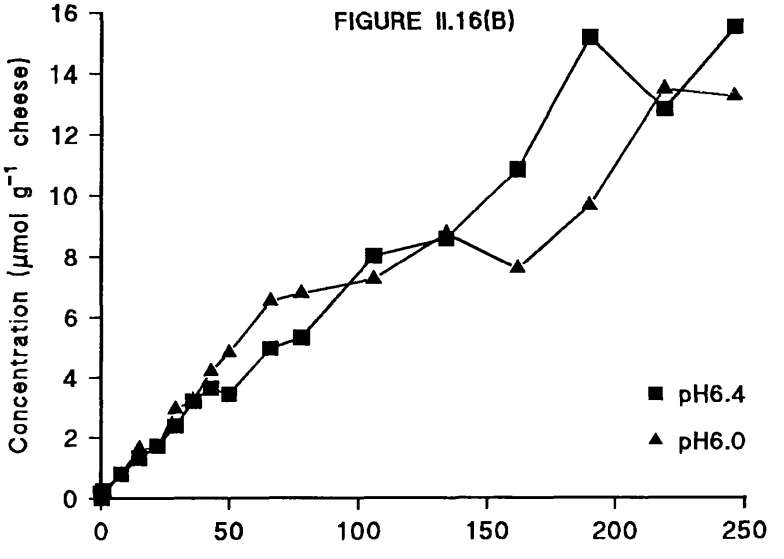
FIGURE II.16(B)
Concentration (μmol g⁻¹ cheese)
0
2
4
6
8
10
12
14
16
0
50
100
150
200
250
pH6.4
pH6.0

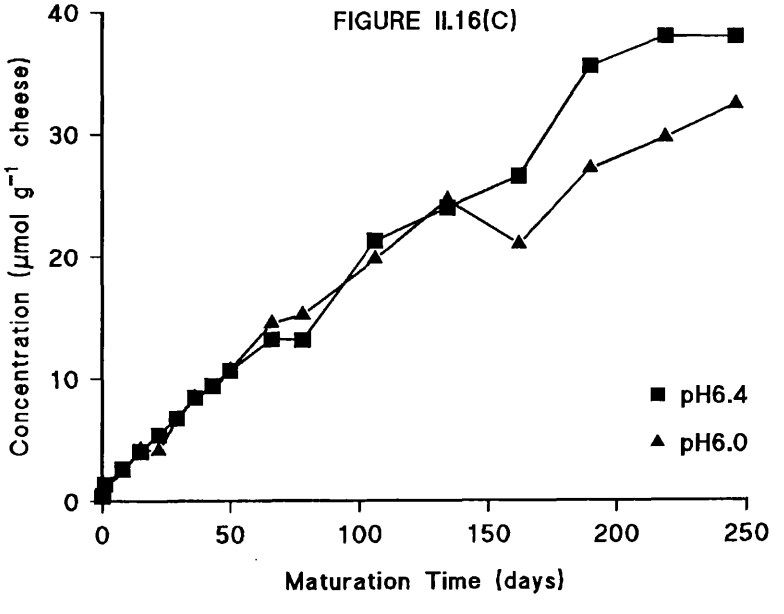
FIGURE II.16(C)
Concentration (μmol g⁻¹ cheese)
0
10
20
30
40
0
50
100
150
200
250
Maturation Time (days)
pH6.4
pH6.0

FIGURE II.17: Amino acid concentrations in standard pH and reduced pH Cheddar cheese up to 246 days of maturation.

(A) Glycine.

(B) Histidine.

(C) Isoleucine.

FIGURE II.17(A)

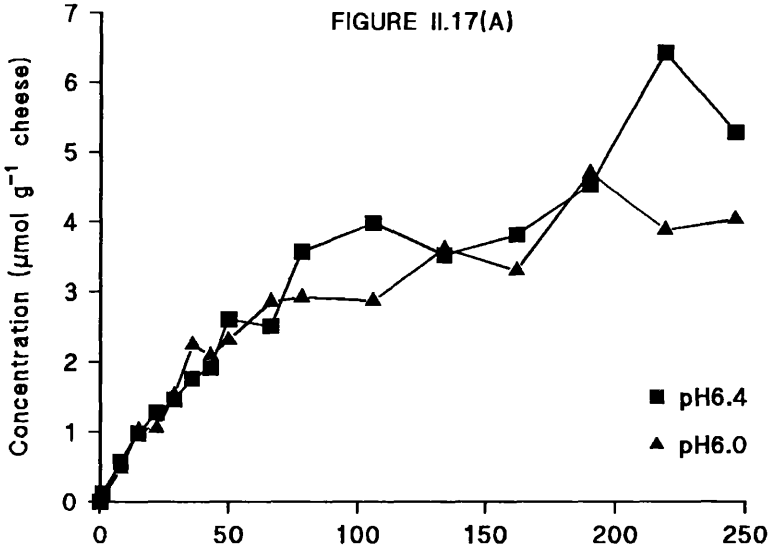

FIGURE II.17(B)

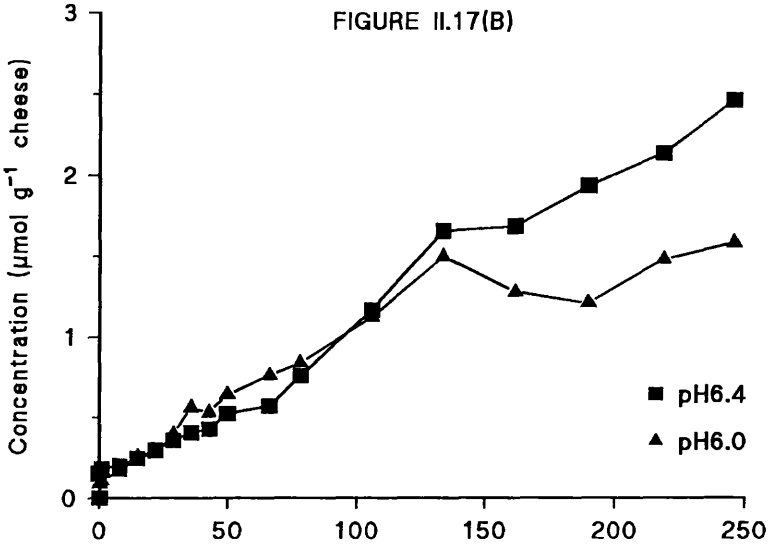

FIGURE II.17(C)

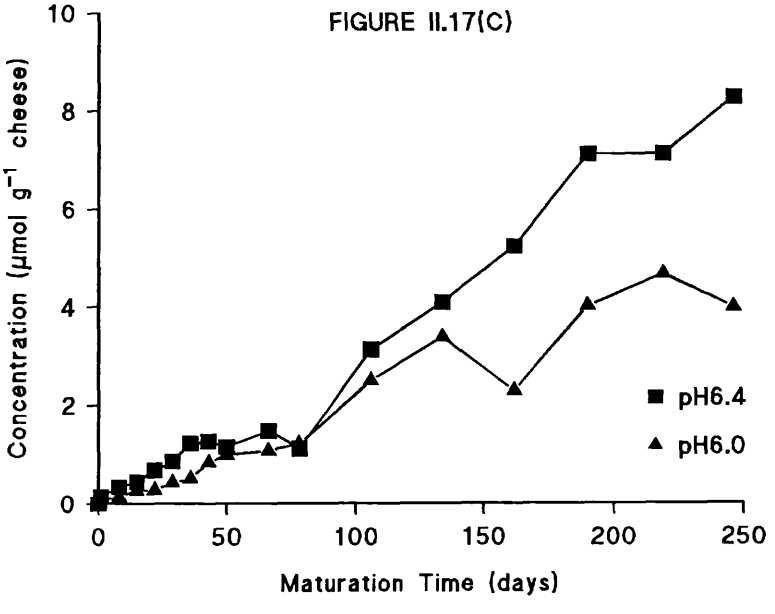

FIGURE II.18: Amino acid concentrations in standard pH and reduced pH Cheddar cheese up to 246 days of maturation.

(A) Leucine.

(B) Lysine.

(C) Methionine.

FIGURE II.18(A)

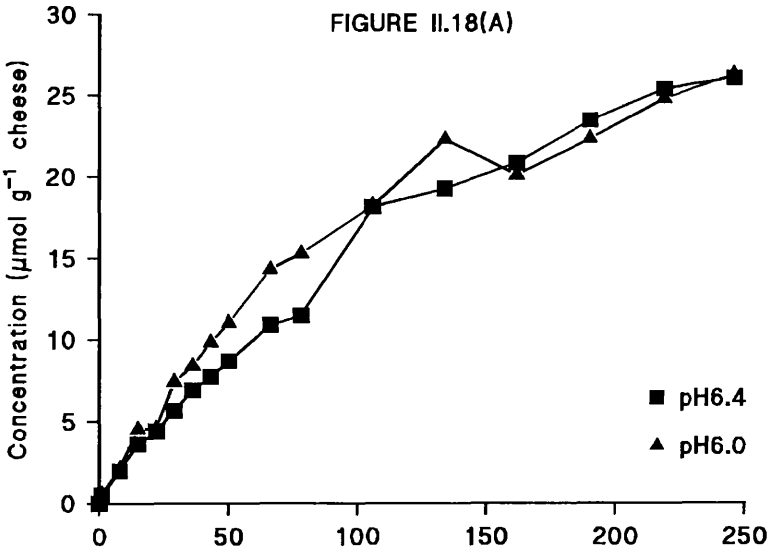

FIGURE II.18(B)

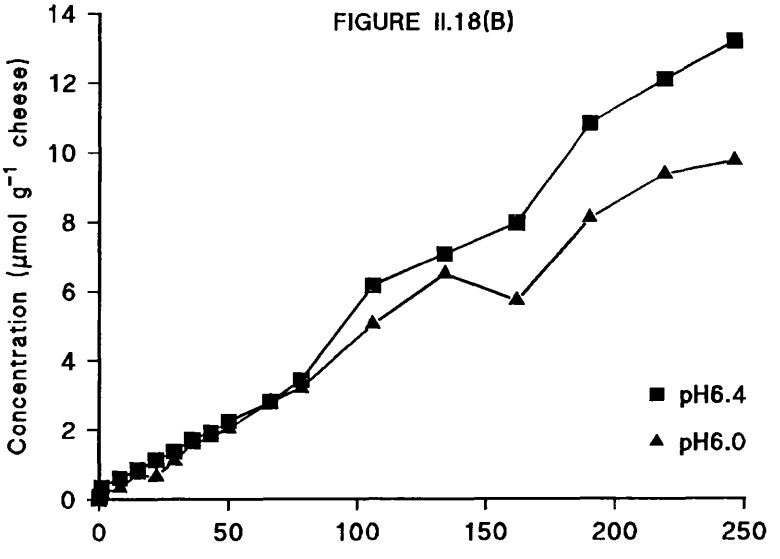

FIGURE II.18(C)

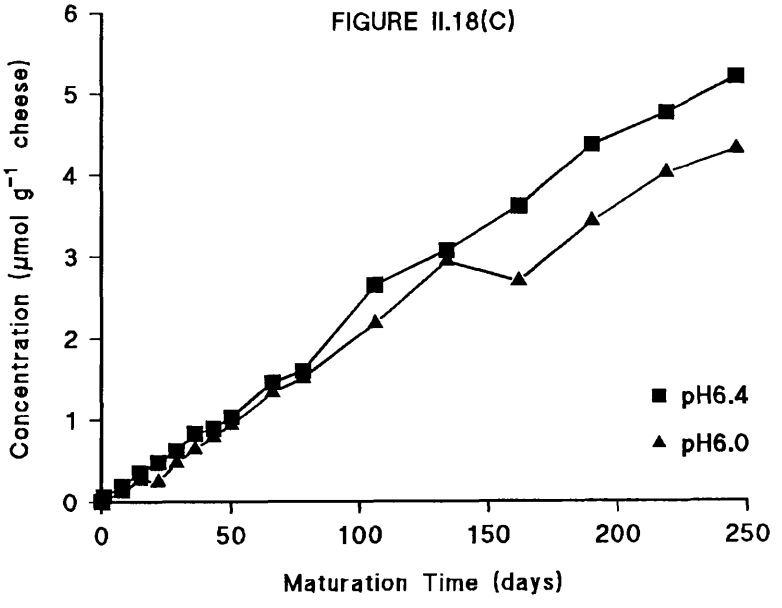

FIGURE II.19: Amino acid concentrations in standard pH and reduced pH Cheddar cheese up to 246 days of maturation.

(A) Phenylalanine.

(B) Proline.

(C) Threonine.

FIGURE II.19(A)

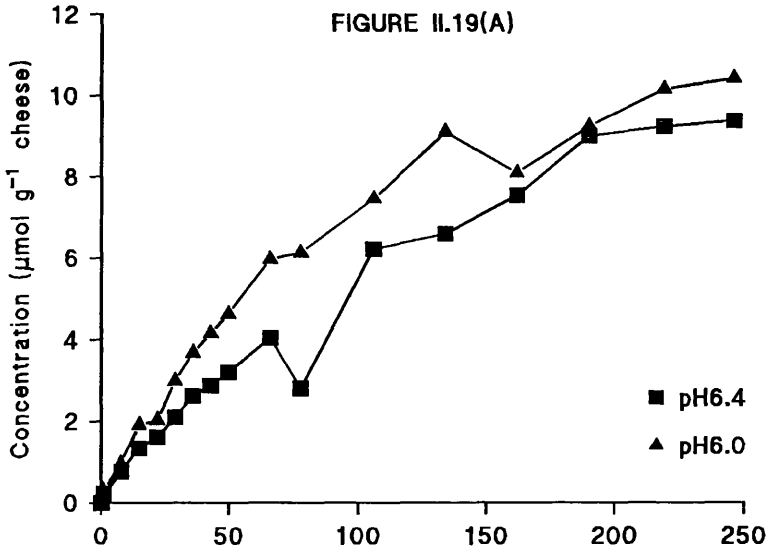

FIGURE II.19(B)

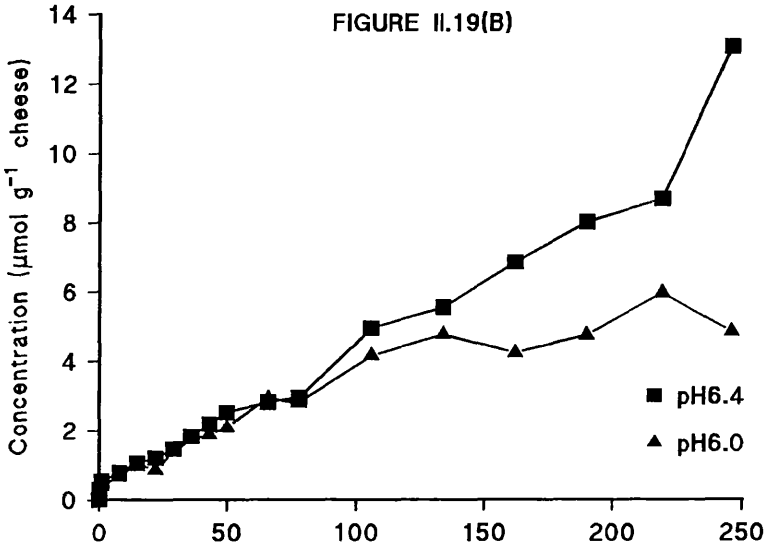

FIGURE II.19(C)

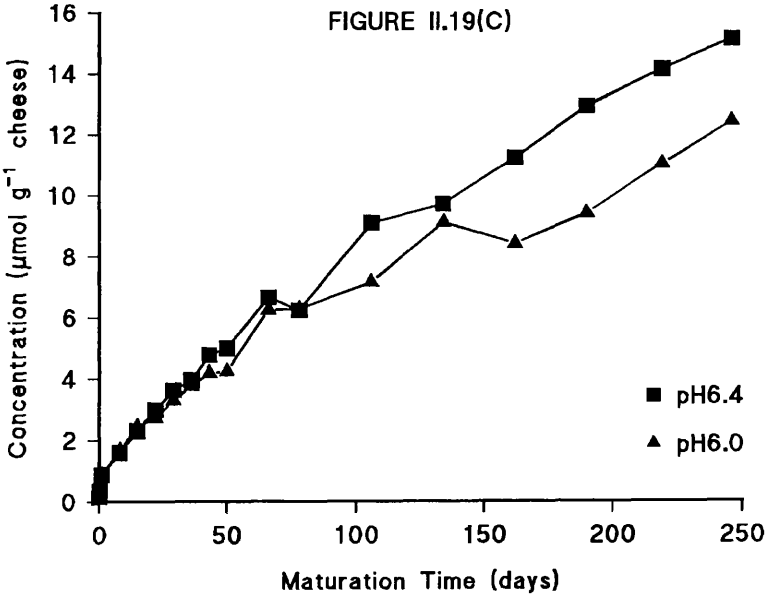

FIGURE II.20: Amino acid concentrations in standard pH and reduced pH Cheddar cheese up to 246 days of maturation.

(A) Tyrosine.

(B) Valine.

FIGURE II.20(A)

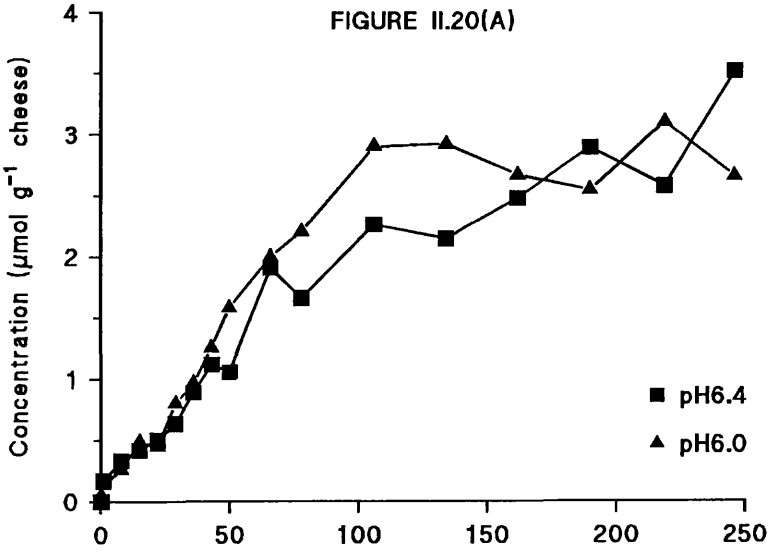

FIGURE II.20(B)

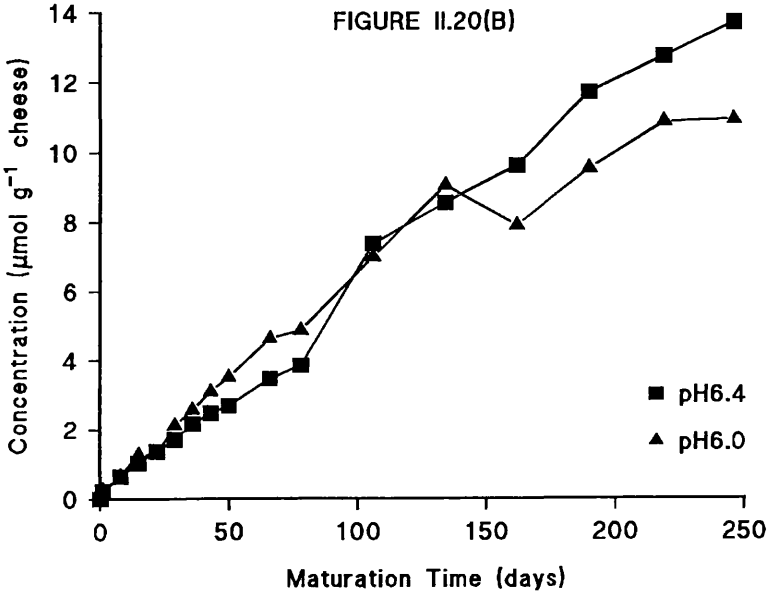

TABLE III.1: Percentage values of the water-soluble extract RP-HPLC chromatogram segment areas from standard pH and reduced pH milk Cheddar cheese at days 1, 22, 78, 190 and 246 of maturation.

Day	pH	Area 1	Area 2	Area 3	Area 4	Area 5	Area 6	Area 7	Area 8	Area 9	Area 10	Area 11	Area 12	Area 13	Area 14	Area 15	Area 16	Area 17
1	6.4	5.34	0.63	1.45	3.39	3.70	6.61	9.24	0.11	0.59	1.64	0.53	12.23	27.01	3.03	0.58	16.98	6.95
22	6.4	4.72	1.82	0.87	5.82	5.16	5.07	8.84	0.93	0.90	4.99	0.51	3.52	32.56	0.13	3.90	17.32	2.94
78	6.4	4.46	2.09	1.85	6.20	6.35	5.10	8.43	3.16	0.35	4.73	0.52	5.13	34.71	0.04	1.38	15.43	0.06
190	6.4	4.26	1.26	2.72	6.71	6.59	3.51	8.53	2.94	0.23	3.80	1.01	11.21	37.94	0.16	0.79	7.26	1.08
246	6.4	3.51	0.95	1.89	5.17	5.88	2.29	7.97	2.33	0.21	4.13	1.00	9.07	44.15	0.16	1.92	8.26	1.13
1	6.0	7.49	0.94	2.73	4.78	5.38	6.37	7.32	2.47	1.36	3.52	0.32	2.67	31.85	0.01	1.21	16.03	5.54
22	6.0	7.56	2.07	3.64	6.79	8.31	5.85	8.90	9.17	2.73	5.39	1.42	3.61	21.38	0.00	1.84	9.04	2.31
78	6.0	2.95	1.67	3.73	9.88	8.60	5.42	8.18	9.78	2.64	4.60	0.58	3.85	21.71	2.41	1.36	9.63	3.01
190	6.0	2.72	2.96	4.79	10.22	7.48	4.76	6.30	5.16	2.06	4.73	0.61	4.18	26.33	4.87	2.04	7.79	3.00
246	6.0	2.80	4.93	6.78	9.79	7.28	6.08	4.85	3.19	2.21	4.65	1.39	8.03	25.02	3.61	2.65	5.16	1.57

TABLE III.2: Amino acid concentrations in standard pH and reduced pH milk Cheddar cheese at days 1, 22, 78, 190 and 246 of maturation.

Day	pH	[a]Ala	Arg	Asn Ser	Asp	Gln	Glu	Gly	His	Ile	Leu	Lys	Met	Phe	Pro	Thr	Tyr	Val
1	6.4	[b]0.244	0.229	0.486	0.206	0.242	1.402	0.119	0.179	0.137	0.535	0.344	0.067	0.219	0.538	0.882	0.172	0.220
22	6.4	0.805	0.870	3.893	0.471	1.731	5.367	1.276	0.298	0.683	4.409	1.125	0.484	1.611	1.202	2.983	0.500	1.364
78	6.4	2.088	2.186	8.673	1.325	5.326	13.150	3.574	0.758	1.114	11.465	3.434	1.603	2.800	2.950	6.228	1.662	3.846
190	6.4	4.767	4.543	18.238	4.021	15.164	35.574	4.525	1.933	7.123	23.419	10.811	4.369	8.992	8.018	12.913	2.889	11.715
246	6.4	6.623	4.731	18.607	3.718	15.503	38.016	5.280	2.459	8.287	26.049	13.178	5.216	9.371	13.056	15.147	3.514	13.707
1	6.0	0.240	0.202	0.496	0.183	0.202	1.223	0.073	0.108	0.067	0.585	0.176	0.033	0.296	0.391	0.877	0.138	0.241
22	6.0	0.826	0.686	3.742	0.320	1.645	4.125	1.057	0.274	0.281	4.552	0.653	0.243	2.036	0.865	2.721	0.454	1.290
78	6.0	2.617	2.773	9.641	1.591	6.781	15.224	2.919	0.836	1.211	15.268	3.199	1.514	6.127	2.805	6.273	2.204	4.883
190	6.0	4.169	4.324	13.150	4.095	9.663	27.160	4.701	1.208	4.012	22.287	8.095	3.427	9.236	4.756	9.405	2.544	9.536
246	6.0	6.225	4.860	15.109	4.948	13.245	32.411	4.032	1.582	3.992	26.262	9.740	4.317	10.414	4.861	12.423	2.655	10.934

[a] Determined as phenylthiocarbamyl-derivatised free amino acids by RP-HPLC.

[b] Amino acid concentration levels in μmol g^{-1} Cheddar cheese.

FIGURE III.1: RP-HPLC chromatograms of water-soluble extracts from standard pH and reduced pH Cheddar cheese at day 0.2 of maturation.

(A) Water-soluble extract from standard pH Cheddar cheese, dissolved in water (final concentration 5mg mL^{-1}), and eluted with a gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

(a) Water-soluble extract from standard pH Cheddar cheese, dissolved in water (final concentration 10mg mL^{-1}), and eluted with a modified gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

(B) Water-soluble extract from reduced pH Cheddar cheese, dissolved in water (final concentration 5mg mL^{-1}), and eluted with a gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

(b) Water-soluble extract from reduced pH Cheddar cheese, dissolved in water (final concentration 10mg mL^{-1}), and eluted with a modified gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

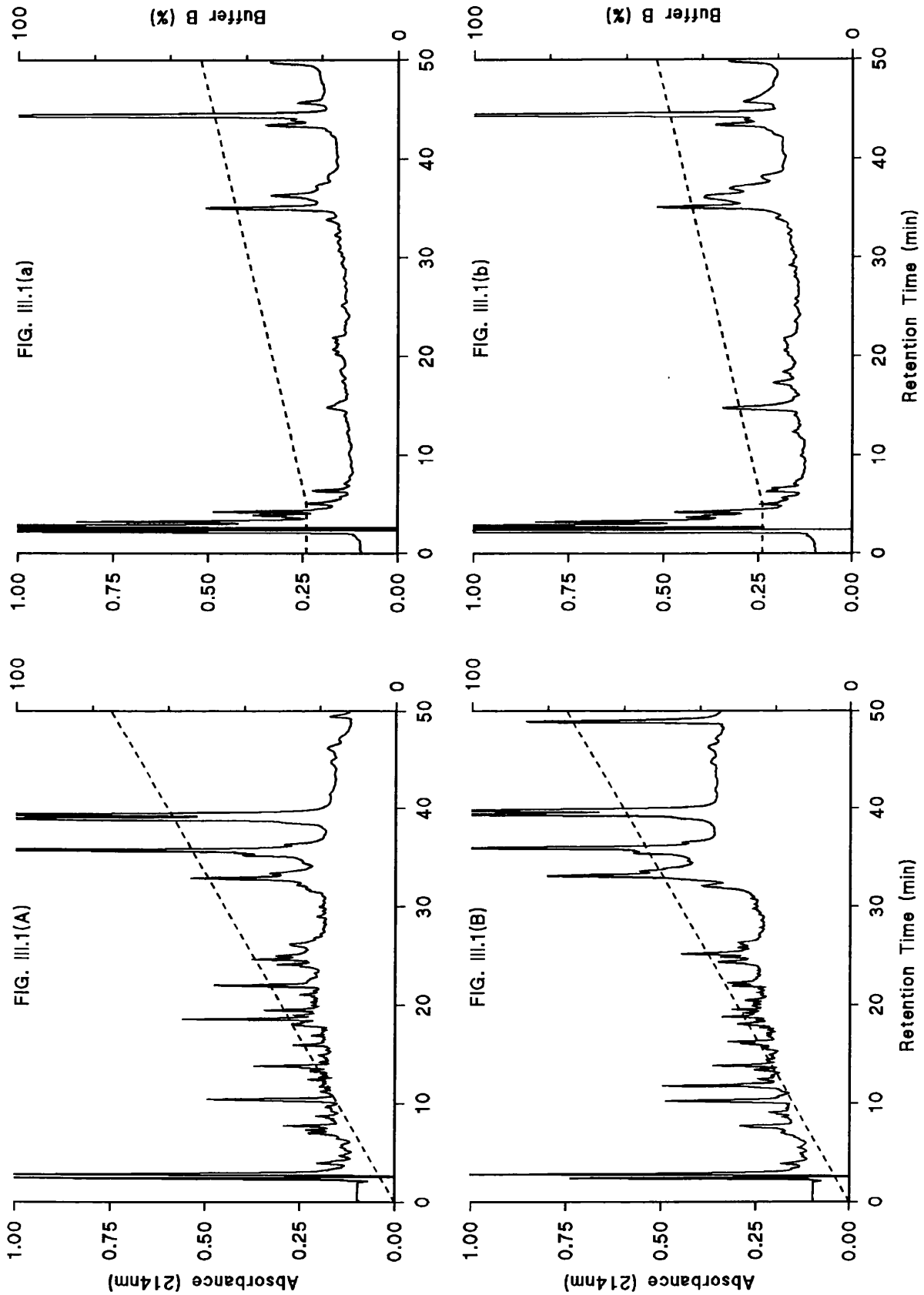
FIG. III.1(A)
FIG. III.1(B)
FIG. III.1(a)
FIG. III.1(b)
Absorbance (214nm)
Buffer B (%)
Retention Time (min)
1.00
0.75
0.50
0.25
0.00
100
0
50
40
30
20
10
0

FIGURE III.2: RP-HPLC chromatograms of water-soluble extracts from standard pH and reduced pH Cheddar cheese at day 1 of maturation.

(A) Water-soluble extract from standard pH Cheddar cheese, dissolved in water (final concentration 5mg mL^{-1}), and eluted with a gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

(a) Water-soluble extract from standard pH Cheddar cheese, dissolved in water (final concentration 10mg mL^{-1}), and eluted with a modified gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

(B) Water-soluble extract from reduced pH Cheddar cheese, dissolved in water (final concentration 5mg mL^{-1}), and eluted with a gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

(b) Water-soluble extract from reduced pH Cheddar cheese, dissolved in water (final concentration 10mg mL^{-1}), and eluted with a modified gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

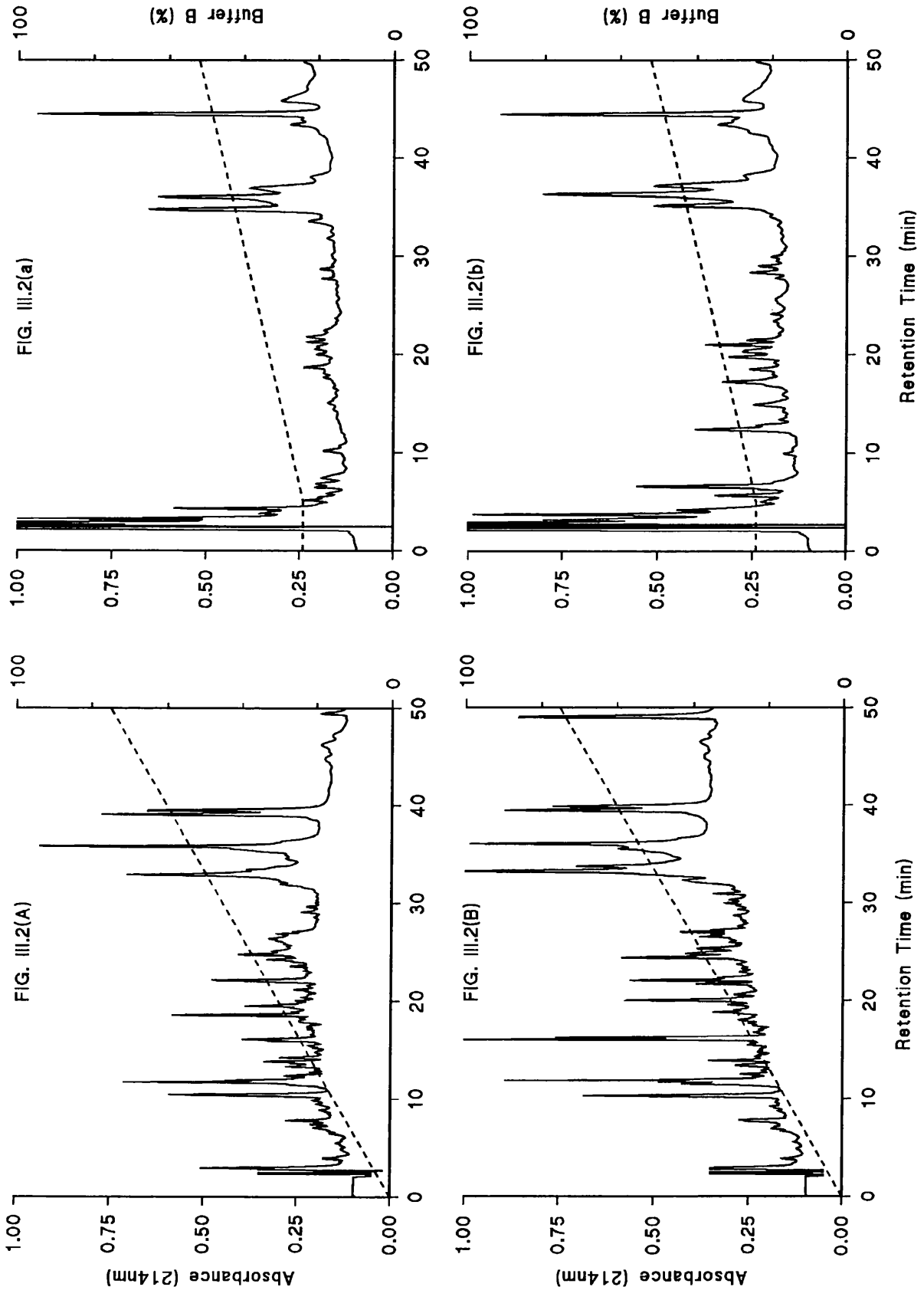
FIG. III.2(A)
FIG. III.2(B)
FIG. III.2(a)
FIG. III.2(b)
Absorbance (214nm)
Buffer B (%)
Retention Time (min)
1.00
0.75
0.50
0.25
0.00
100
0
10
20
30
40
50

FIGURE III.3: RP-HPLC chromatograms of water-soluble extracts from standard pH and reduced pH Cheddar cheese at day 8 of maturation.

(A) Water-soluble extract from standard pH Cheddar cheese, dissolved in water (final concentration 5mg mL^{-1}), and eluted with a gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

(a) Water-soluble extract from standard pH Cheddar cheese, dissolved in water (final concentration 10mg mL^{-1}), and eluted with a modified gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

(B) Water-soluble extract from reduced pH Cheddar cheese, dissolved in water (final concentration 5mg mL^{-1}), and eluted with a gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

(b) Water-soluble extract from reduced pH Cheddar cheese, dissolved in water (final concentration 10mg mL^{-1}), and eluted with a modified gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

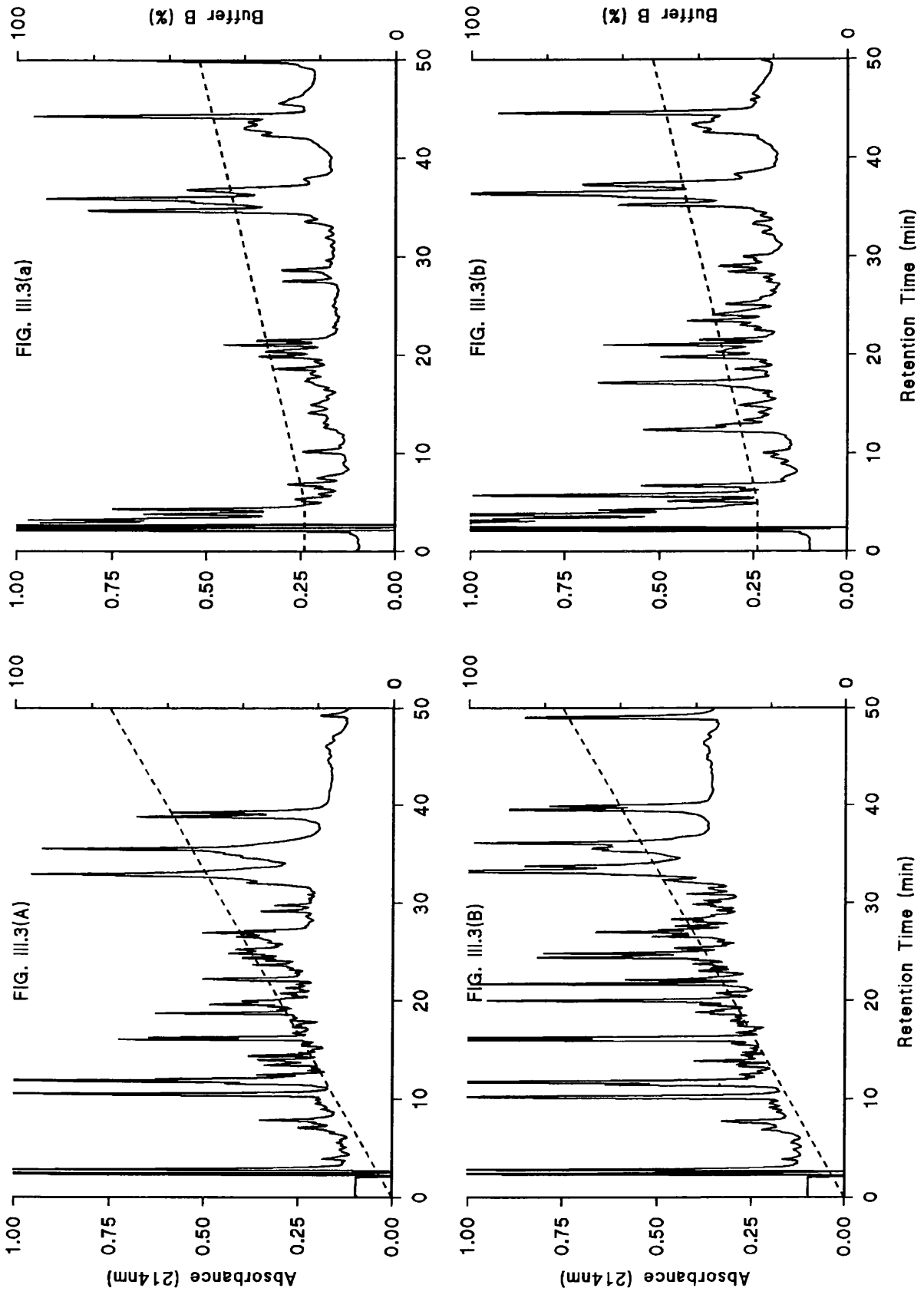
FIG. III.3(A)
FIG. III.3(a)
FIG. III.3(B)
FIG. III.3(b)
Absorbance (214nm)
Buffer B (%)
Retention Time (min)
1.00
0.75
0.50
0.25
0.00
100
0
10
20
30
40
50

FIGURE III.4: RP-HPLC chromatograms of water-soluble extracts from standard pH and reduced pH Cheddar cheese at day 15 of maturation.

(A) Water-soluble extract from standard pH Cheddar cheese, dissolved in water (final concentration 5mg mL^{-1}), and eluted with a gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

(a) Water-soluble extract from standard pH Cheddar cheese, dissolved in water (final concentration 10mg mL^{-1}), and eluted with a modified gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

(B) Water-soluble extract from reduced pH Cheddar cheese, dissolved in water (final concentration 5mg mL^{-1}), and eluted with a gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

(b) Water-soluble extract from reduced pH Cheddar cheese, dissolved in water (final concentration 10mg mL^{-1}), and eluted with a modified gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

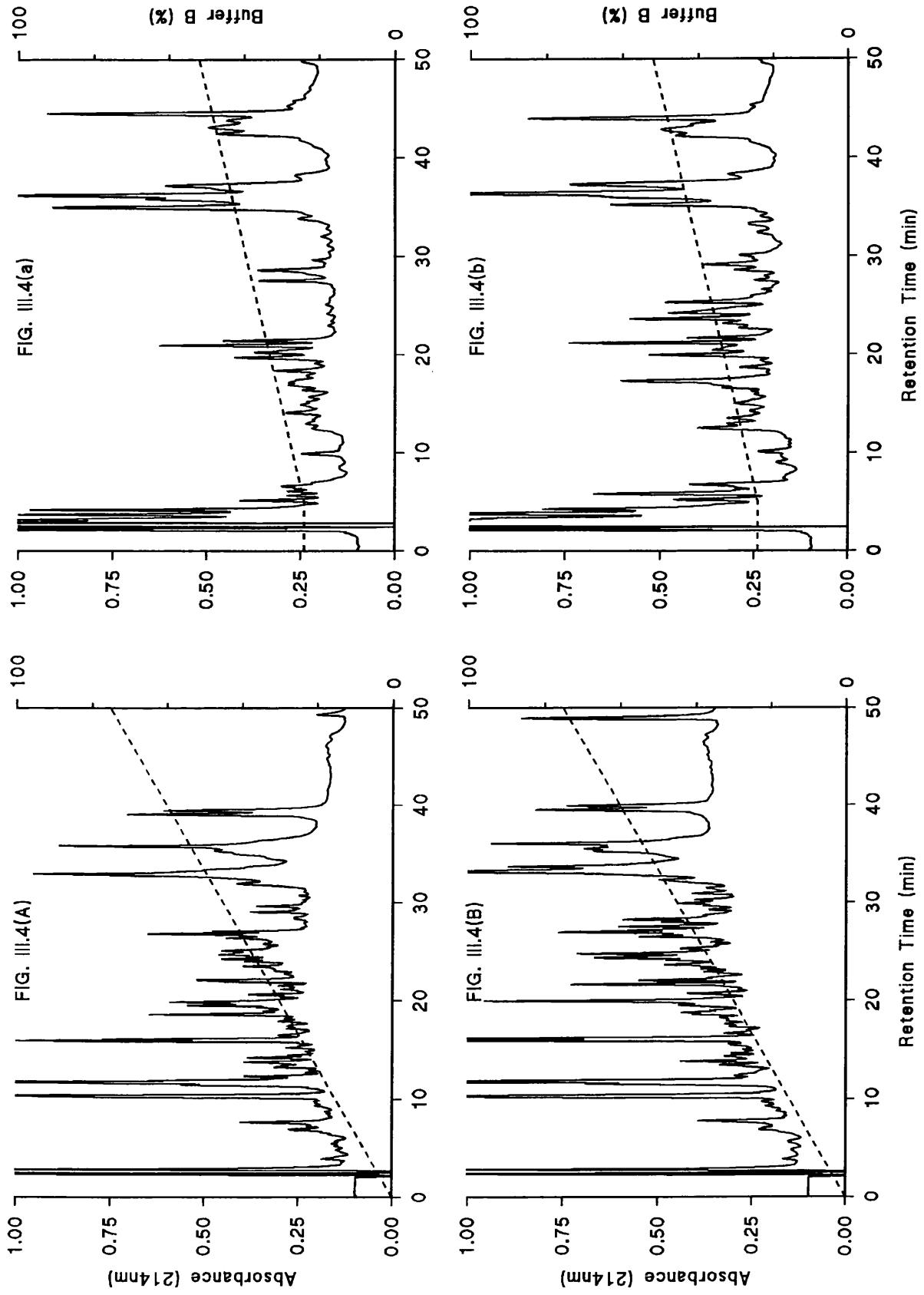
FIG. III.4(A)
FIG. III.4(B)
FIG. III.4(a)
FIG. III.4(b)
Absorbance (214nm)
Buffer B (%)
Retention Time (min)
1.00
0.75
0.50
0.25
0.00
100
0
10
20
30
40
50

FIGURE III.5: RP-HPLC chromatograms of water-soluble extracts from standard pH and reduced pH Cheddar cheese at day 22 of maturation.

(A) Water-soluble extract from standard pH Cheddar cheese, dissolved in water (final concentration 5mg mL^{-1}), and eluted with a gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

(a) Water-soluble extract from standard pH Cheddar cheese, dissolved in water (final concentration 10mg mL^{-1}), and eluted with a modified gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

(B) Water-soluble extract from reduced pH Cheddar cheese, dissolved in water (final concentration 5mg mL^{-1}), and eluted with a gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

(b) Water-soluble extract from reduced pH Cheddar cheese, dissolved in water (final concentration 10mg mL^{-1}), and eluted with a modified gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

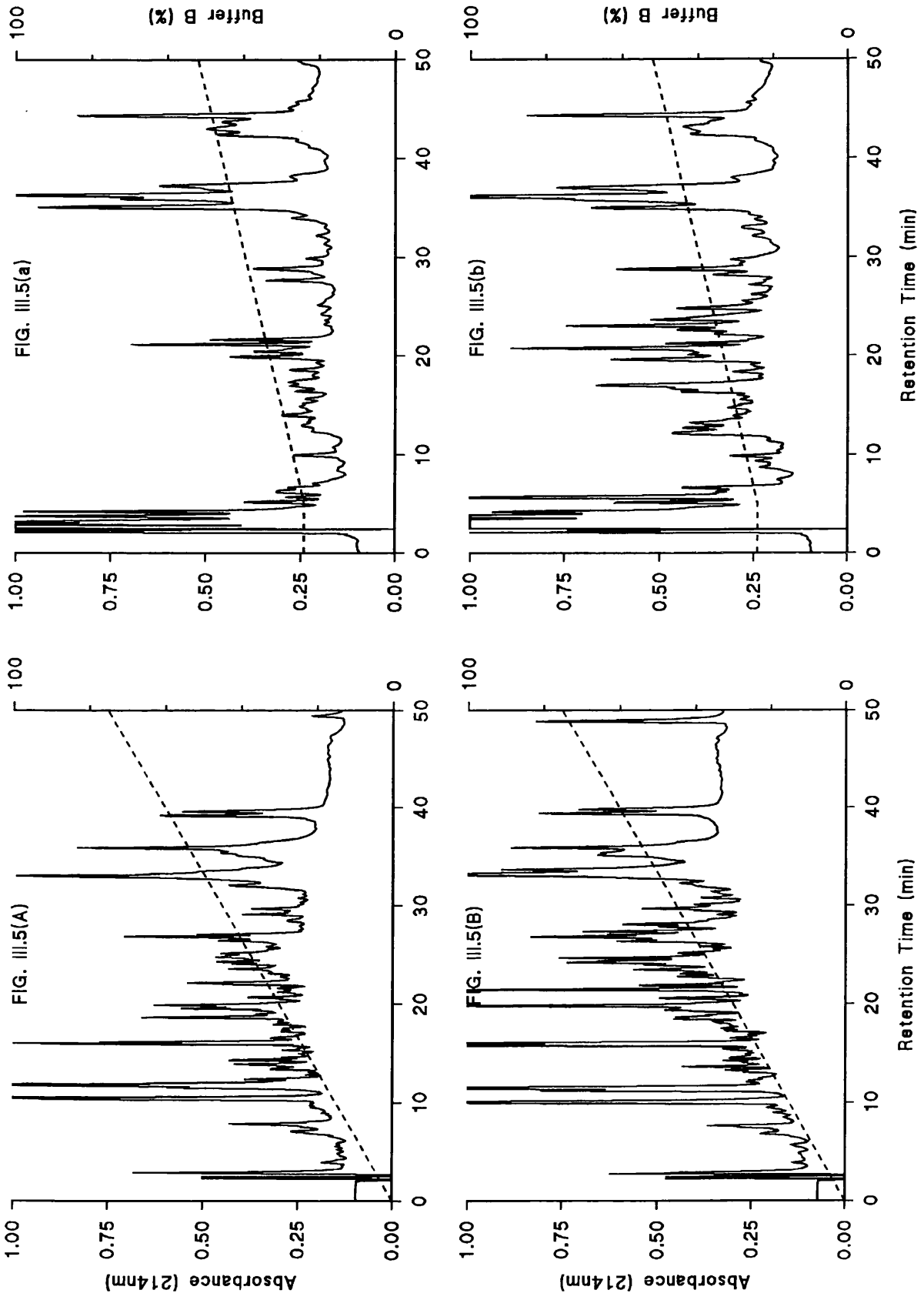
FIG. III.5(A)
FIG. III.5(B)
FIG. III.5(a)
FIG. III.5(b)
Absorbance (214nm)
Buffer B (%)
Retention Time (min)
1.00
0.75
0.50
0.25
0.00
100
0
10
20
30
40
50

FIGURE III.6: RP-HPLC chromatograms of water-soluble extracts from standard pH and reduced pH Cheddar cheese at day 36 of maturation.

(A) Water-soluble extract from standard pH Cheddar cheese, dissolved in water (final concentration 5mg mL^{-1}), and eluted with a gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

(a) Water-soluble extract from standard pH Cheddar cheese, dissolved in water (final concentration 10mg mL^{-1}), and eluted with a modified gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

(B) Water-soluble extract from reduced pH Cheddar cheese, dissolved in water (final concentration 5mg mL^{-1}), and eluted with a gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

(b) Water-soluble extract from reduced pH Cheddar cheese, dissolved in water (final concentration 10mg mL^{-1}), and eluted with a modified gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

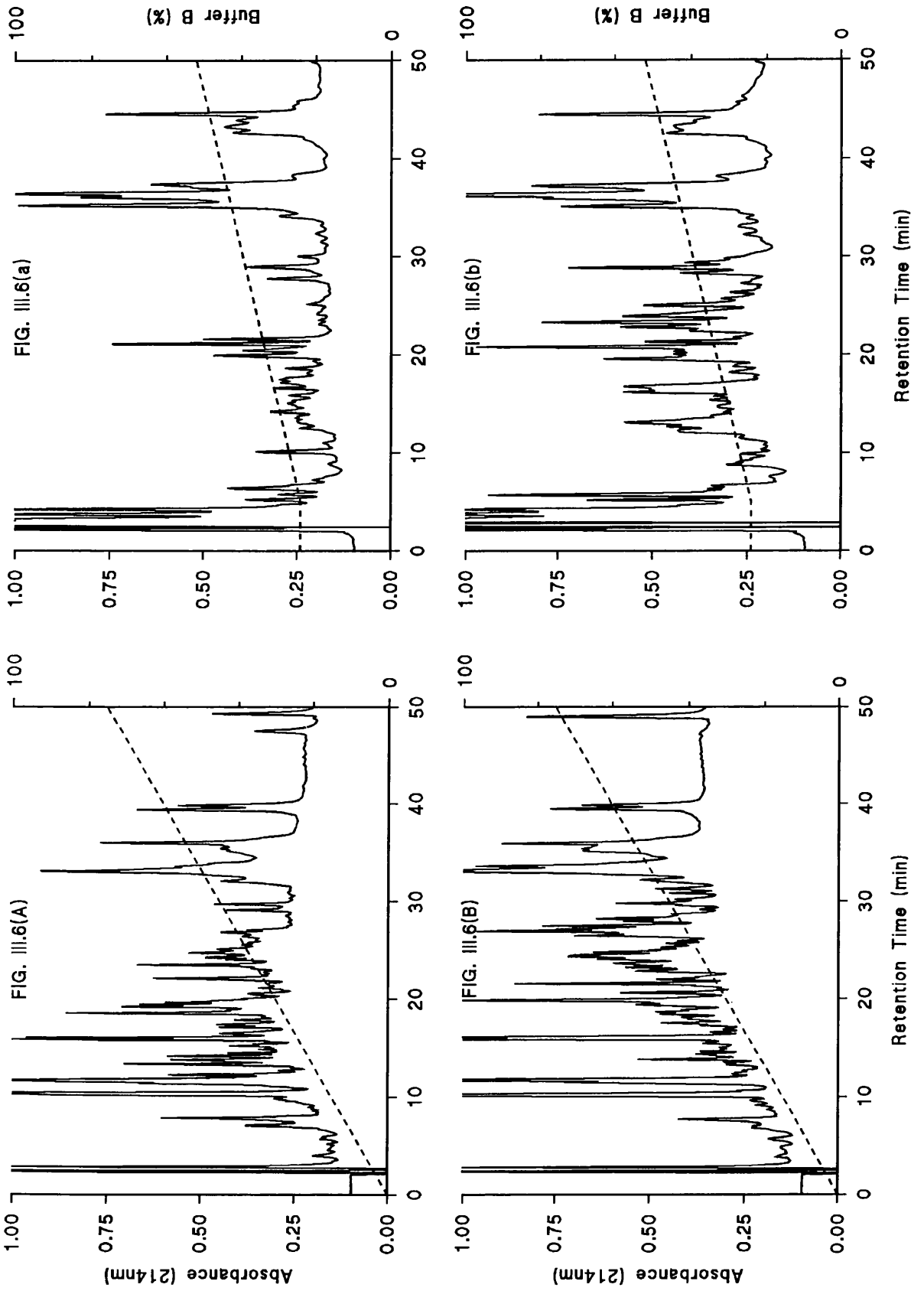
FIG. III.6(A)
FIG. III.6(B)
FIG. III.6(a)
FIG. III.6(b)
Absorbance (214nm)
Buffer B (%)
Retention Time (min)
1.00
0.75
0.50
0.25
0.00
100
0
10
20
30
40
50

FIGURE III.7: RP-HPLC chromatograms of water-soluble extracts from standard pH and reduced pH Cheddar cheese at day 50 of maturation.

(A) Water-soluble extract from standard pH Cheddar cheese, dissolved in water (final concentration 5mg mL^{-1}), and eluted with a gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

(a) Water-soluble extract from standard pH Cheddar cheese, dissolved in water (final concentration 10mg mL^{-1}), and eluted with a modified gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

(B) Water-soluble extract from reduced pH Cheddar cheese, dissolved in water (final concentration 5mg mL^{-1}), and eluted with a gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

(b) Water-soluble extract from reduced pH Cheddar cheese, dissolved in water (final concentration 10mg mL^{-1}), and eluted with a modified gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

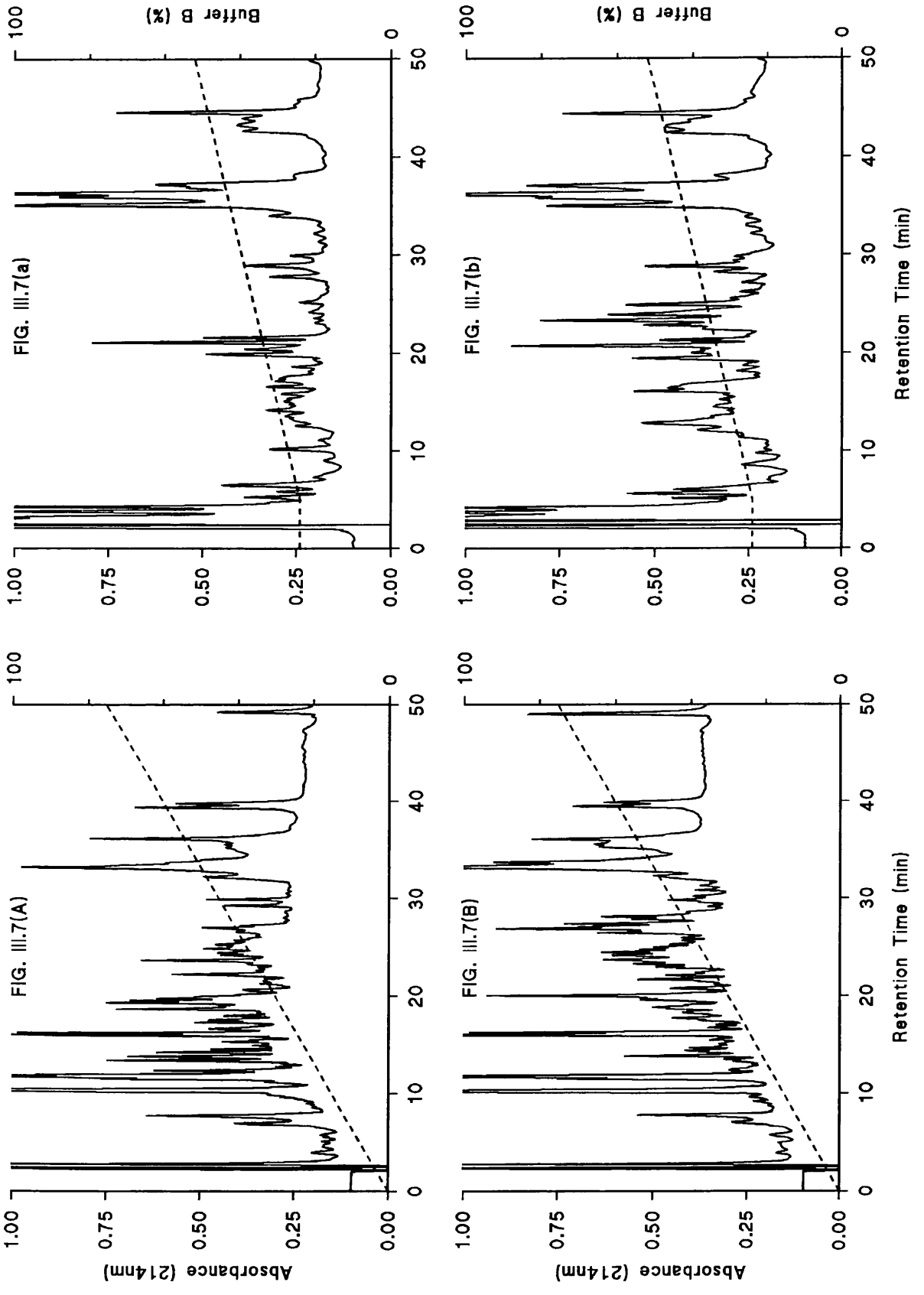
FIG. III.7(A)
FIG. III.7(a)
FIG. III.7(B)
FIG. III.7(b)
Absorbance (214nm)
Buffer B (%)
Retention Time (min)
1.00
0.75
0.50
0.25
0.00
100
0
10
20
30
40
50

FIGURE III.8: RP-HPLC chromatograms of water-soluble extracts from standard pH and reduced pH Cheddar cheese at day 66 of maturation.

(A) Water-soluble extract from standard pH Cheddar cheese, dissolved in water (final concentration 5mg mL^{-1}), and eluted with a gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

(a) Water-soluble extract from standard pH Cheddar cheese, dissolved in water (final concentration 10mg mL^{-1}), and eluted with a modified gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

(B) Water-soluble extract from reduced pH Cheddar cheese, dissolved in water (final concentration 5mg mL^{-1}), and eluted with a gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

(b) Water-soluble extract from reduced pH Cheddar cheese, dissolved in water (final concentration 10mg mL^{-1}), and eluted with a modified gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

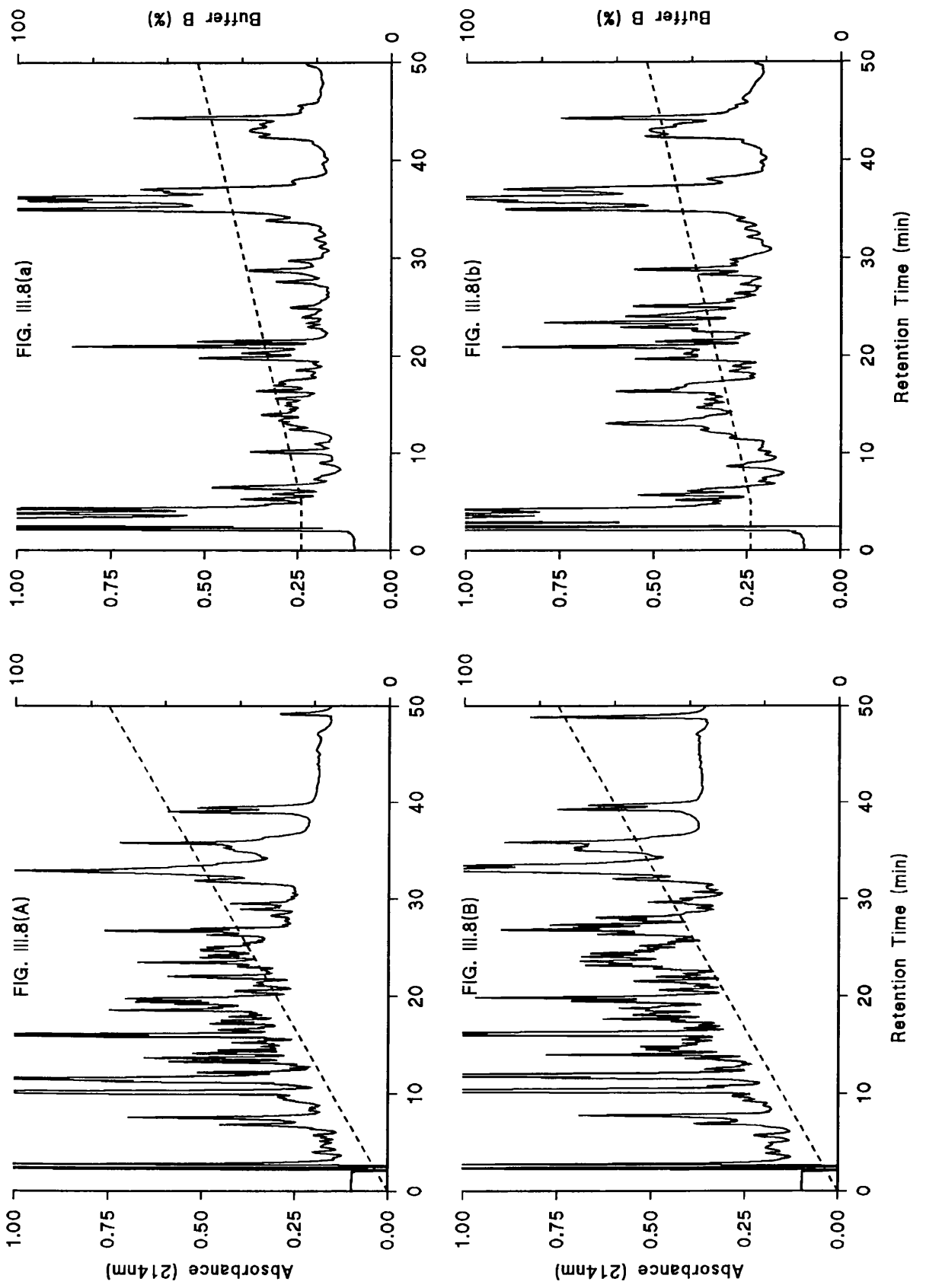
FIG. III.8(A)
FIG. III.8(B)
FIG. III.8(a)
FIG. III.8(b)
Absorbance (214nm)
Buffer B (%)
Retention Time (min)
1.00
0.75
0.50
0.25
0.00
100
0
10
20
30
40
50

FIGURE III.9: RP-HPLC chromatograms of water-soluble extracts from standard pH and reduced pH Cheddar cheese at day 78 of maturation.

(A) Water-soluble extract from standard pH Cheddar cheese, dissolved in water (final concentration 5mg mL^{-1}), and eluted with a gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

(a) Water-soluble extract from standard pH Cheddar cheese, dissolved in water (final concentration 10mg mL^{-1}), and eluted with a modified gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

(B) Water-soluble extract from reduced pH Cheddar cheese, dissolved in water (final concentration 5mg mL^{-1}), and eluted with a gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

(b) Water-soluble extract from reduced pH Cheddar cheese, dissolved in water (final concentration 10mg mL^{-1}), and eluted with a modified gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

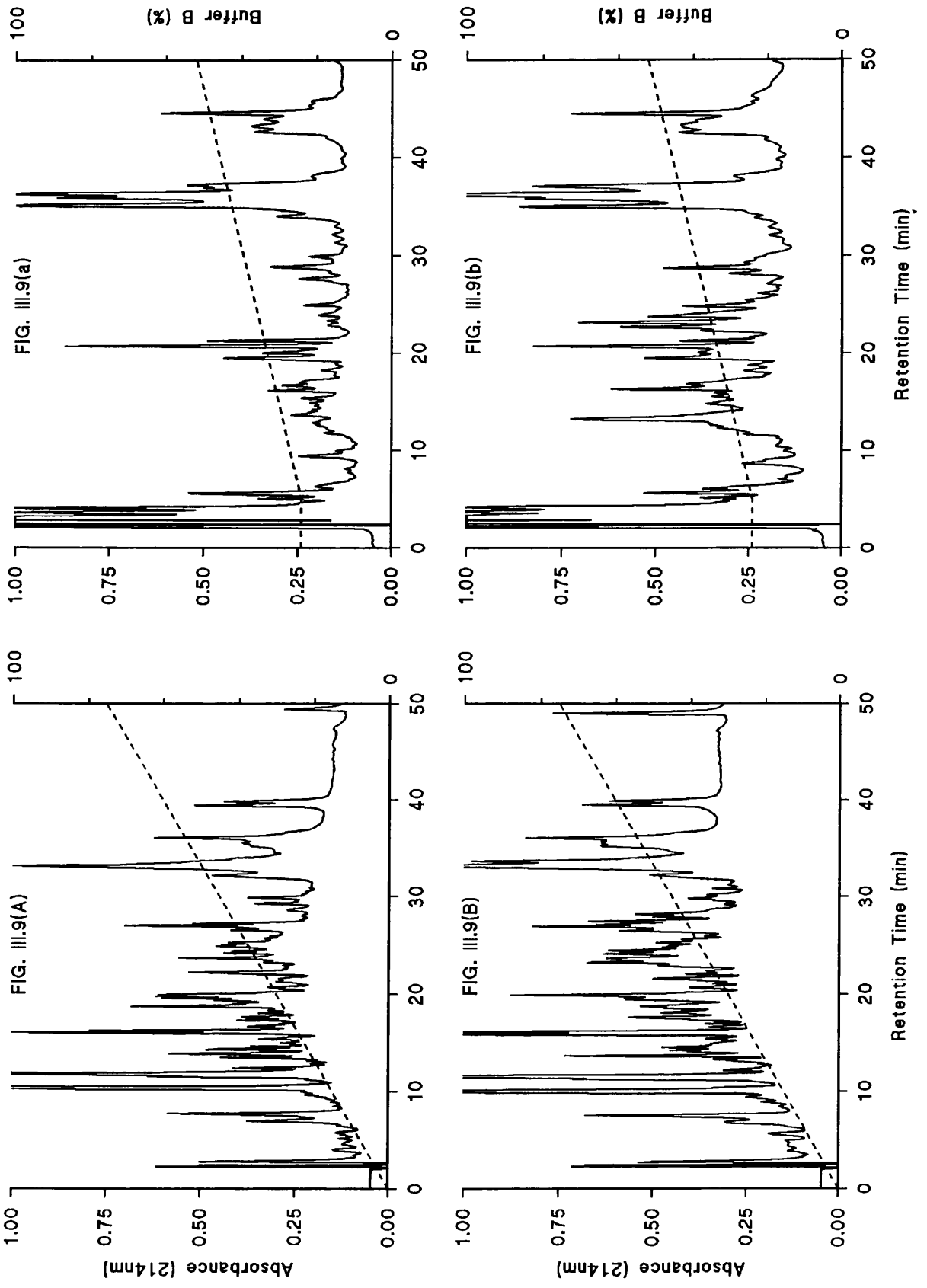
FIG. III.9(A)
FIG. III.9(B)
FIG. III.9(a)
FIG. III.9(b)
Absorbance (214nm)
Buffer B (%)
Retention Time (min)
1.00
0.75
0.50
0.25
0.00
100
0
10
20
30
40
50

FIGURE III.10: RP-HPLC chromatograms of water-soluble extracts from standard pH and reduced pH Cheddar cheese at day 106 of maturation.

(A) Water-soluble extract from standard pH Cheddar cheese, dissolved in water (final concentration 5mg mL^{-1}), and eluted with a gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

(a) Water-soluble extract from standard pH Cheddar cheese, dissolved in water (final concentration 10mg mL^{-1}), and eluted with a modified gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

(B) Water-soluble extract from reduced pH Cheddar cheese, dissolved in water (final concentration 5mg mL^{-1}), and eluted with a gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

(b) Water-soluble extract from reduced pH Cheddar cheese, dissolved in water (final concentration 10mg mL^{-1}), and eluted with a modified gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

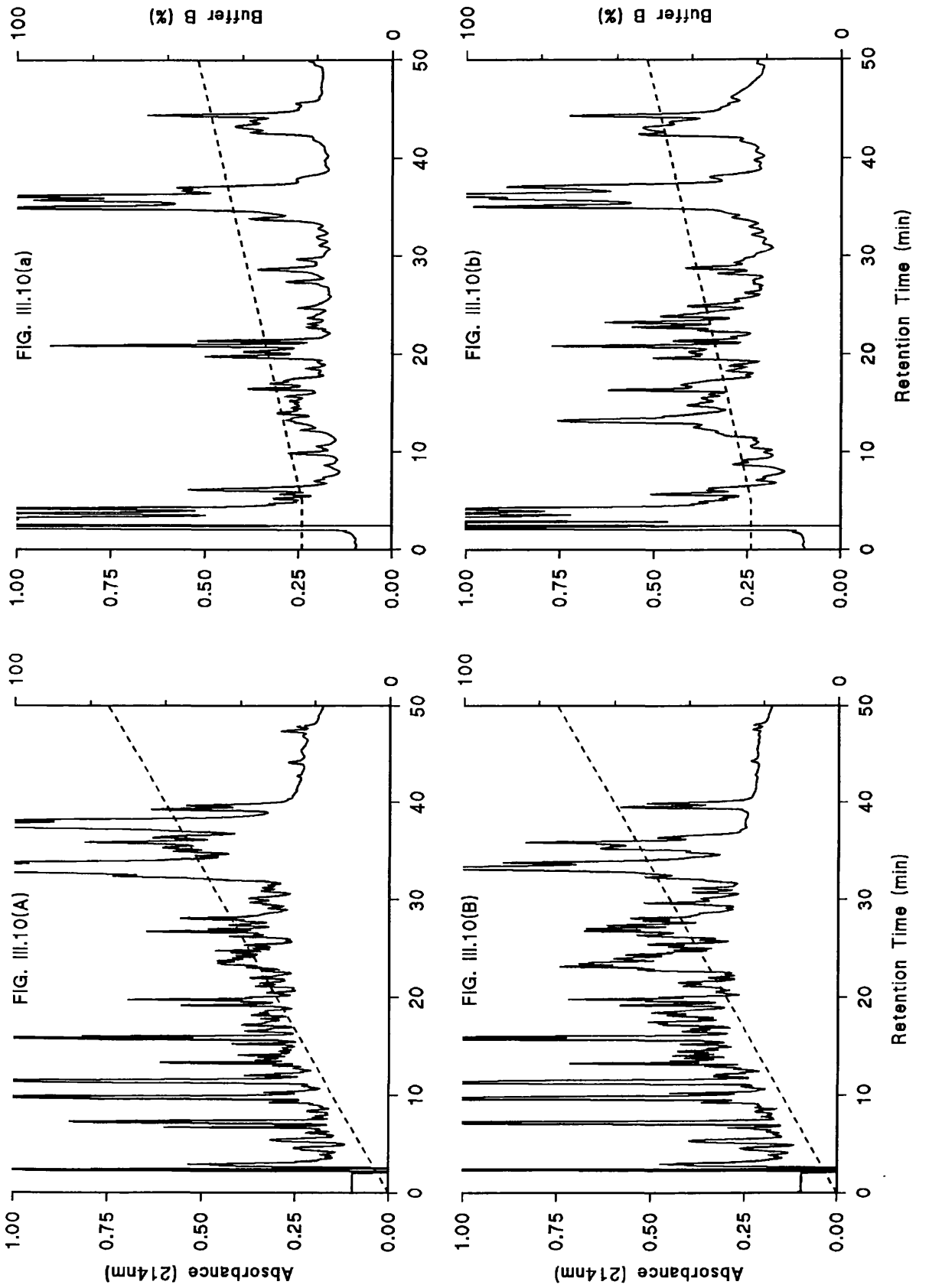
FIG. III.10(A)
FIG. III.10(B)
FIG. III.10(a)
FIG. III.10(b)
Absorbance (214nm)
Buffer B (%)
Retention Time (min)
1.00
0.75
0.50
0.25
0.00
100
0
10
20
30
40
50

FIGURE III.11: RP-HPLC chromatograms of water-soluble extracts from standard pH and reduced pH Cheddar cheese at day 134 of maturation.

(A) Water-soluble extract from standard pH Cheddar cheese, dissolved in water (final concentration 5mg mL^{-1}), and eluted with a gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

(a) Water-soluble extract from standard pH Cheddar cheese, dissolved in water (final concentration 10mg mL^{-1}), and eluted with a modified gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

(B) Water-soluble extract from reduced pH Cheddar cheese, dissolved in water (final concentration 5mg mL^{-1}), and eluted with a gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

(b) Water-soluble extract from reduced pH Cheddar cheese, dissolved in water (final concentration 10mg mL^{-1}), and eluted with a modified gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

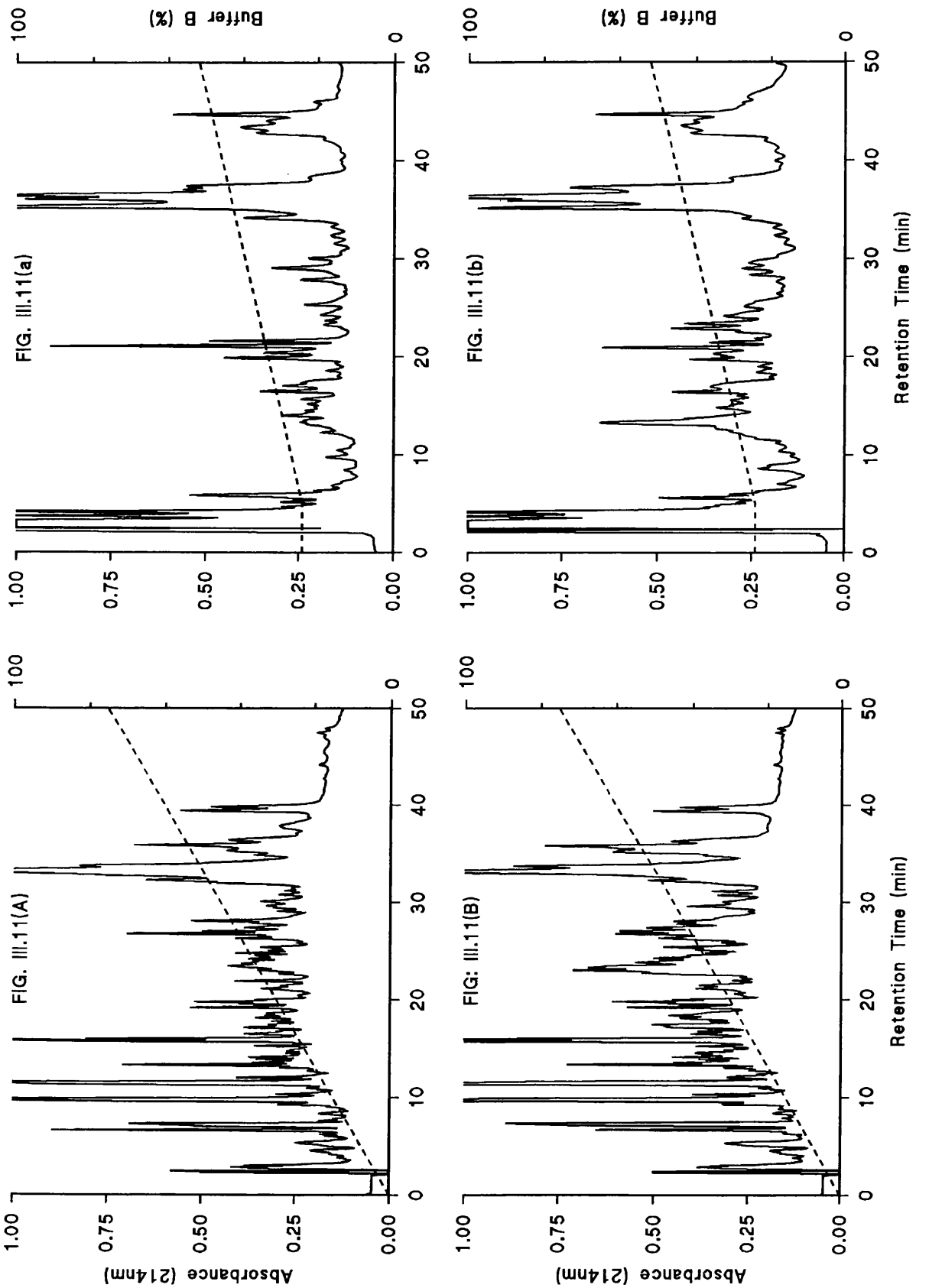
FIG. III.11(A)
FIG. III.11(a)
FIG: III.11(B)
FIG. III.11(b)
Absorbance (214nm)
Buffer B (%)
Retention Time (min)
0.00
0.25
0.50
0.75
1.00
0
10
20
30
40
50
100

FIGURE III.12: RP-HPLC chromatograms of water-soluble extracts from standard pH and reduced pH Cheddar cheese at day 162 of maturation.

(A) Water-soluble extract from standard pH Cheddar cheese, dissolved in water (final concentration 5mg mL^{-1}), and eluted with a gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

(a) Water-soluble extract from standard pH Cheddar cheese, dissolved in water (final concentration 10mg mL^{-1}), and eluted with a modified gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

(B) Water-soluble extract from reduced pH Cheddar cheese, dissolved in water (final concentration 5mg mL^{-1}), and eluted with a gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

(b) Water-soluble extract from reduced pH Cheddar cheese, dissolved in water (final concentration 10mg mL^{-1}), and eluted with a modified gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

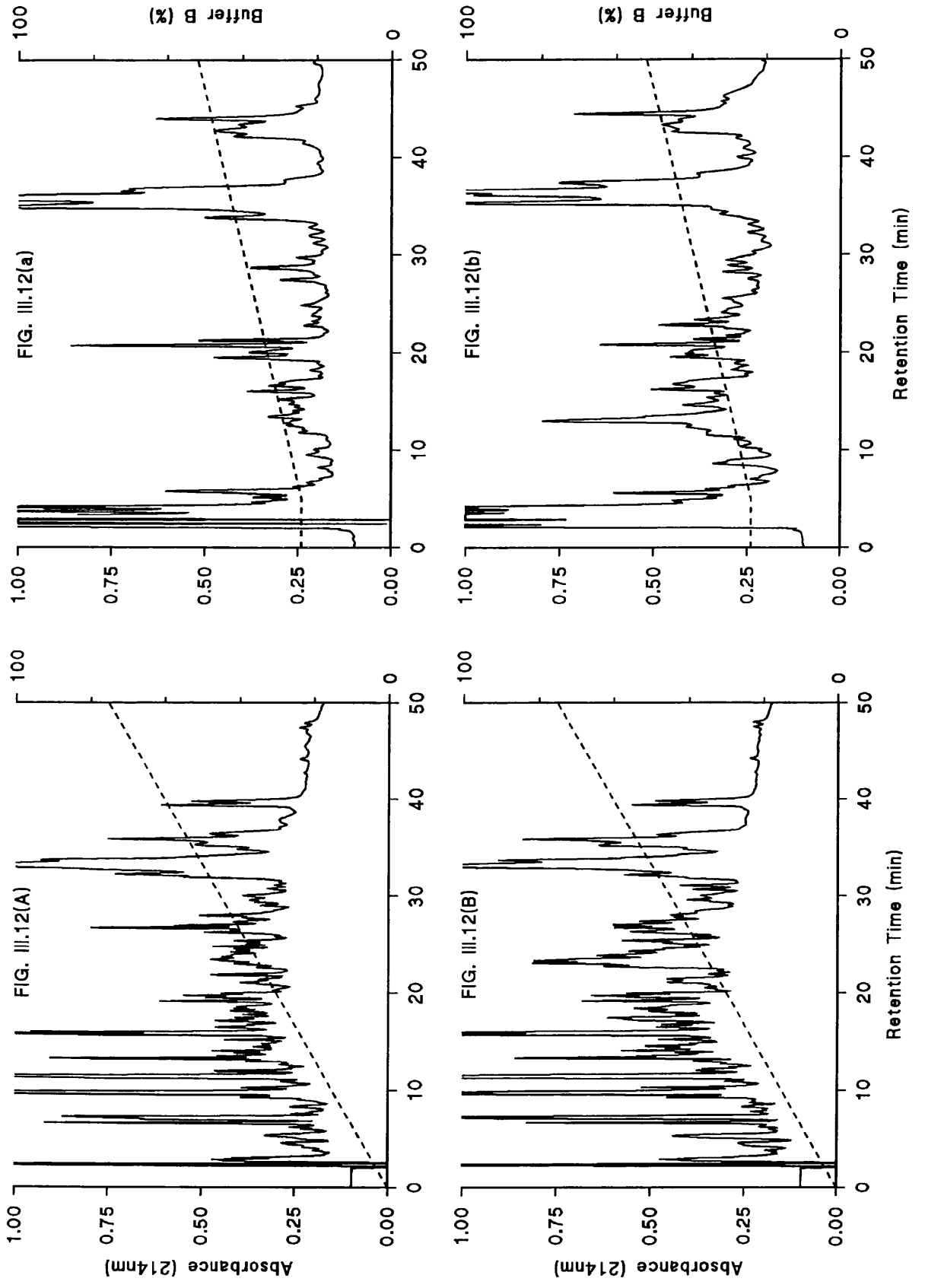
FIG. III.12(A)
FIG. III.12(B)
FIG. III.12(a)
FIG. III.12(b)
Absorbance (214nm)
Buffer B (%)
Retention Time (min)
1.00
0.75
0.50
0.25
0.00
100
0
10
20
30
40
50

FIGURE III.13: RP-HPLC chromatograms of water-soluble extracts from standard pH and reduced pH Cheddar cheese at day 190 of maturation.

(A) Water-soluble extract from standard pH Cheddar cheese, dissolved in water (final concentration 5mg mL^{-1}), and eluted with a gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

(a) Water-soluble extract from standard pH Cheddar cheese, dissolved in water (final concentration 10mg mL^{-1}), and eluted with a modified gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

(B) Water-soluble extract from reduced pH Cheddar cheese, dissolved in water (final concentration 5mg mL^{-1}), and eluted with a gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

(b) Water-soluble extract from reduced pH Cheddar cheese, dissolved in water (final concentration 10mg mL^{-1}), and eluted with a modified gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

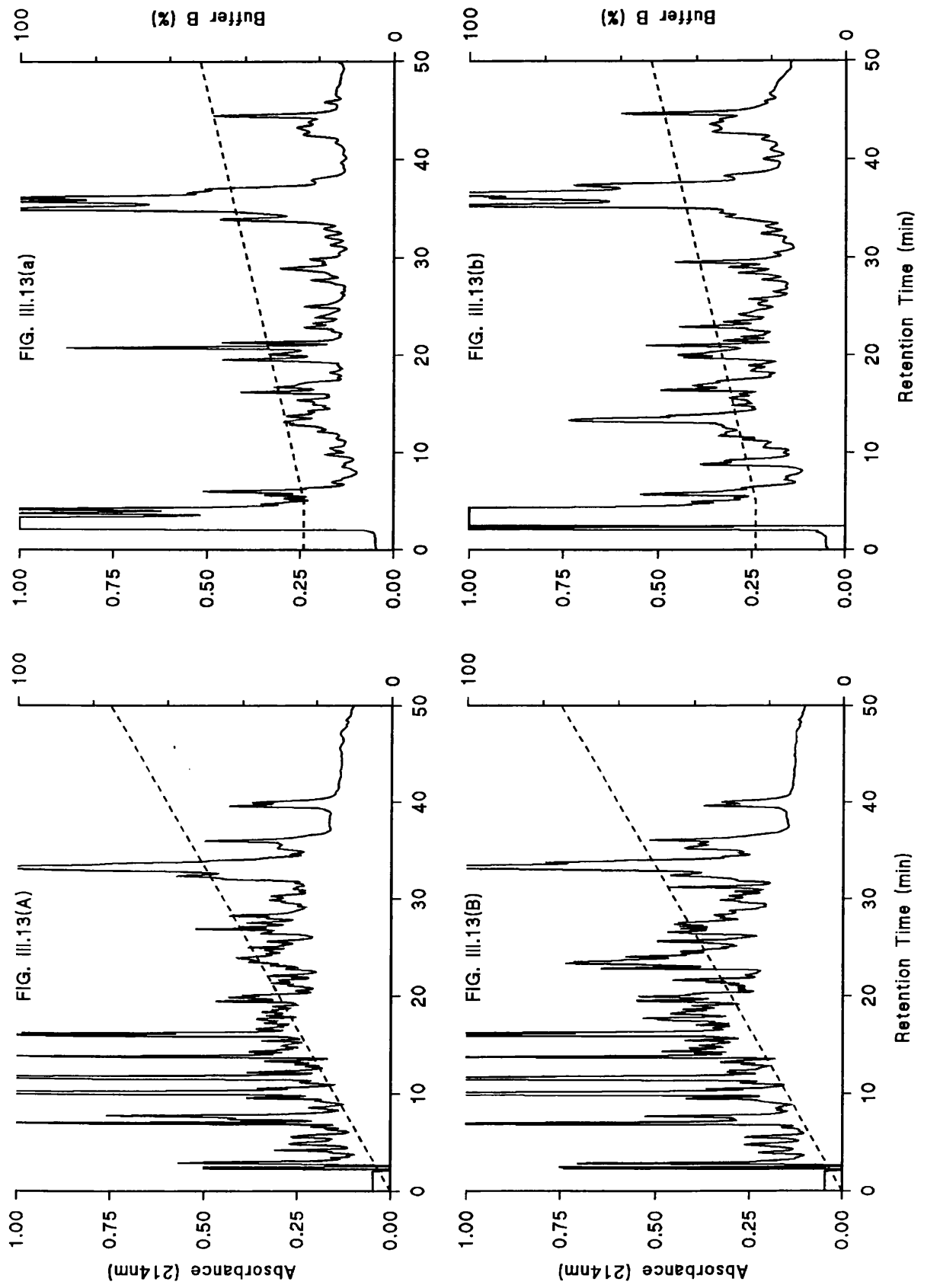
FIG. III.13(A)
FIG. III.13(B)
FIG. III.13(a)
FIG. III.13(b)
Absorbance (214nm)
Buffer B (%)
Retention Time (min)
1.00
0.75
0.50
0.25
0.00
100
0
10
20
30
40
50

FIGURE III.14: RP-HPLC chromatograms of water-soluble extracts from standard pH and reduced pH Cheddar cheese at day 219 of maturation.

(A) Water-soluble extract from standard pH Cheddar cheese, dissolved in water (final concentration 5mg mL^{-1}), and eluted with a gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

(a) Water-soluble extract from standard pH Cheddar cheese, dissolved in water (final concentration 10mg mL^{-1}), and eluted with a modified gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

(B) Water-soluble extract from reduced pH Cheddar cheese, dissolved in water (final concentration 5mg mL^{-1}), and eluted with a gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

(b) Water-soluble extract from reduced pH Cheddar cheese, dissolved in water (final concentration 10mg mL^{-1}), and eluted with a modified gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

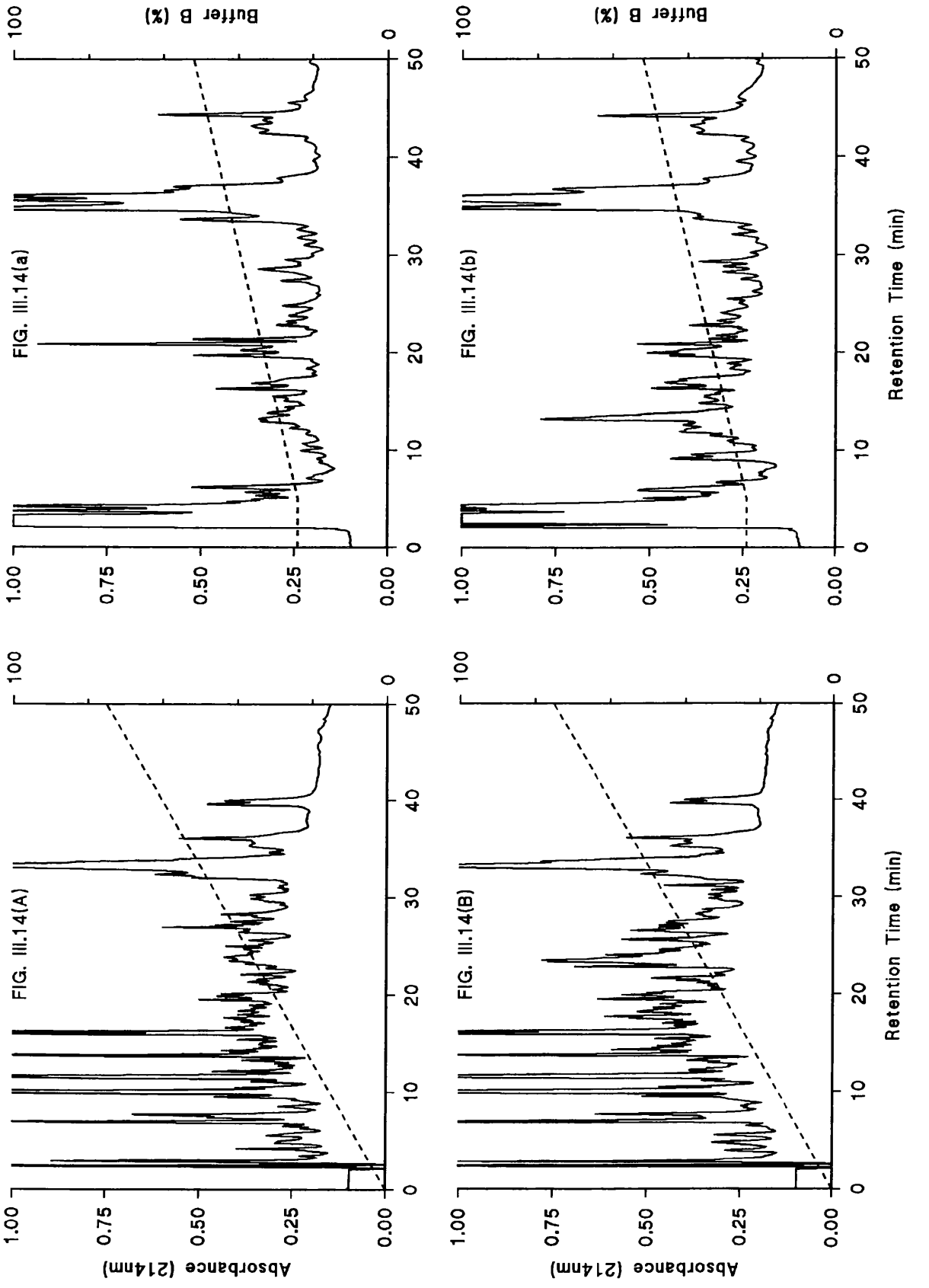
FIG. III.14(A)
FIG. III.14(B)
FIG. III.14(a)
FIG. III.14(b)
Absorbance (214nm)
Buffer B (%)
Retention Time (min)
1.00
0.75
0.50
0.25
0.00
100
0
10
20
30
40
50

FIGURE III.15: RP-HPLC chromatograms of water-soluble extracts from standard pH and reduced pH Cheddar cheese at day 246 of maturation.

(A) Water-soluble extract from standard pH Cheddar cheese, dissolved in water (final concentration 5mg mL^{-1}), and eluted with a gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

(a) Water-soluble extract from standard pH Cheddar cheese, dissolved in water (final concentration 10mg mL^{-1}), and eluted with a modified gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

(B) Water-soluble extract from reduced pH Cheddar cheese, dissolved in water (final concentration 5mg mL^{-1}), and eluted with a gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

(b) Water-soluble extract from reduced pH Cheddar cheese, dissolved in water (final concentration 10mg mL^{-1}), and eluted with a modified gradient of 0.1% TFA in 90% acetonitrile/water (v/v) (buffer B; --------) as described in the text.

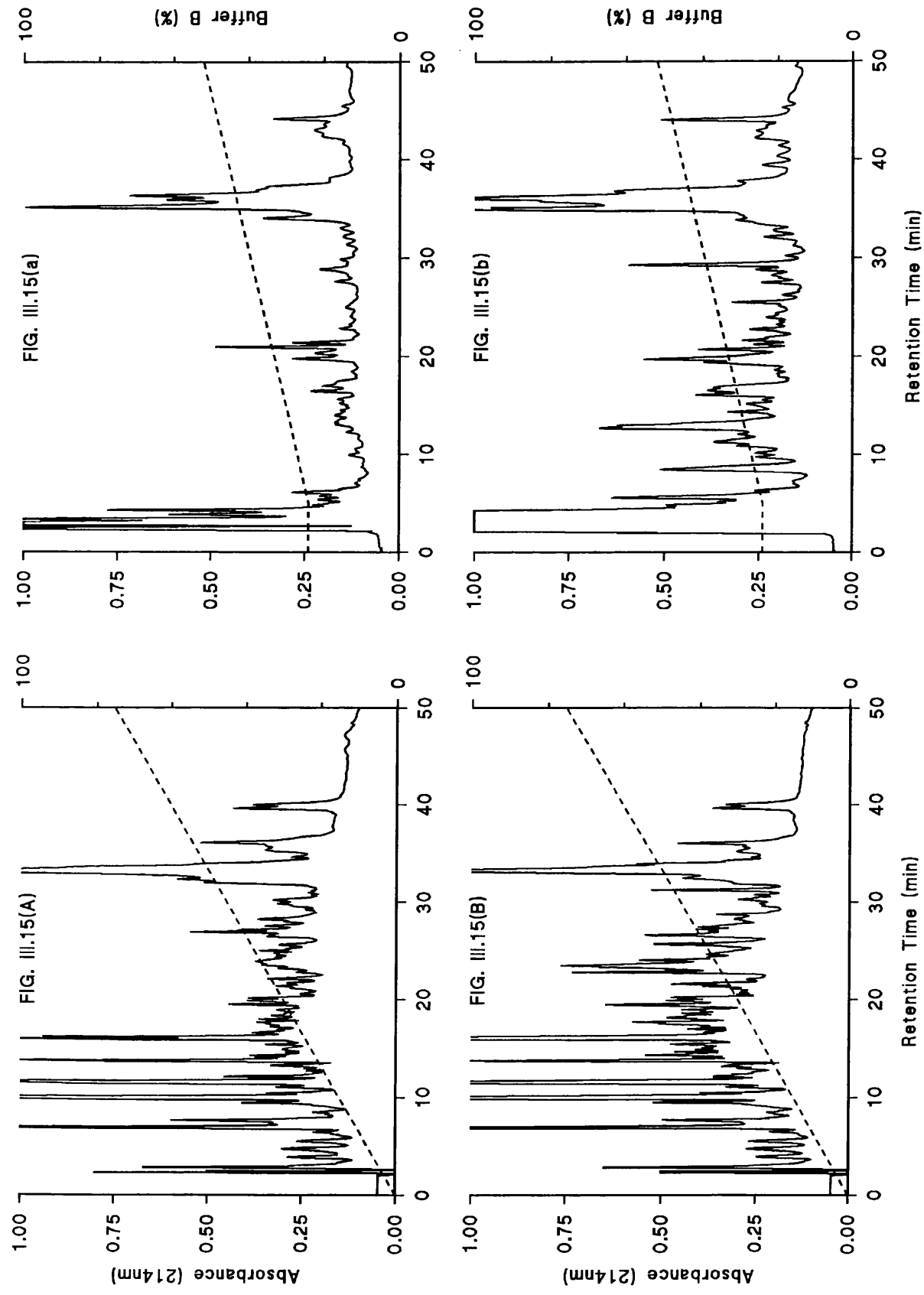
FIG. III.15(A)
FIG. III.15(B)
FIG. III.15(a)
FIG. III.15(b)
Absorbance (214nm)
Buffer B (%)
Retention Time (min)
0.00
0.25
0.50
0.75
1.00
0
10
20
30
40
50
100

FIGURE III.16: Changes in the modified gradient water-soluble extract RP-HPLC chromatogram segment areas in standard pH and reduced pH Cheddar cheese over 246 days of maturation.

(A) Segment area 1.

(B) Segment area 2.

(C) Segment area 3.

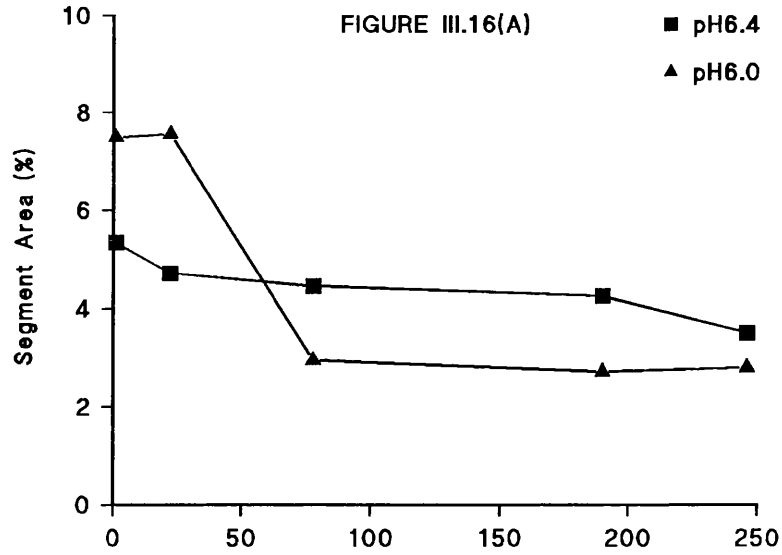
FIGURE III.16(A)
pH6.4
pH6.0
Segment Area (%)
0
2
4
6
8
10
0
50
100
150
200
250

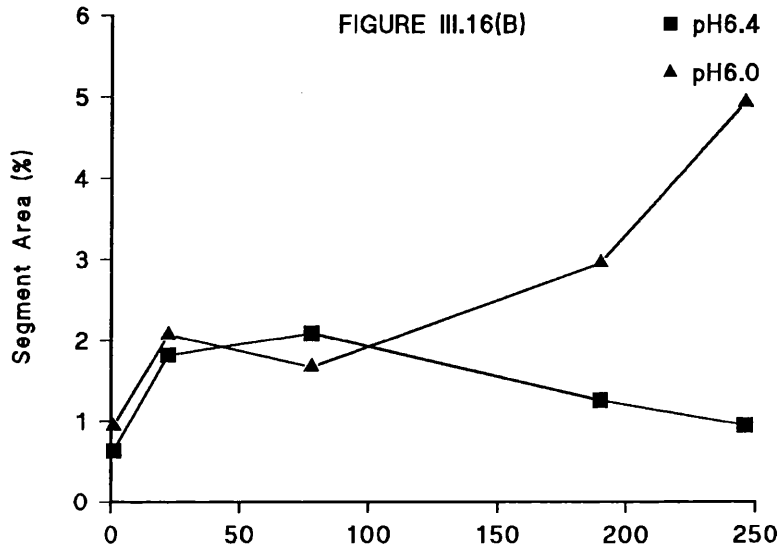
FIGURE III.16(B)
pH6.4
pH6.0
Segment Area (%)
0
1
2
3
4
5
6
0
50
100
150
200
250

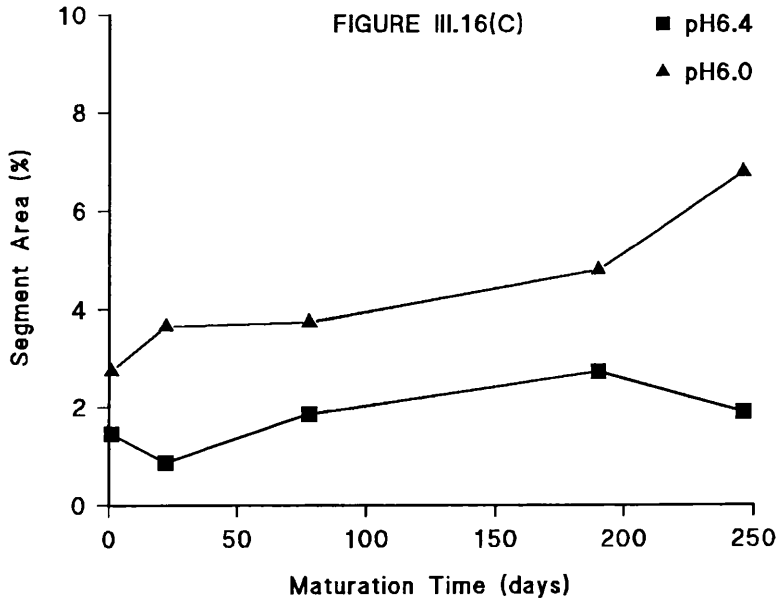
FIGURE III.16(C)
pH6.4
pH6.0
Segment Area (%)
0
2
4
6
8
10
0
50
100
150
200
250
Maturation Time (days)

FIGURE III.17: Changes in the modified gradient water-soluble extract RP-HPLC chromatogram segment areas in standard pH and reduced pH Cheddar cheese over 246 days of maturation.

(A) Segment area 5.

(B) Segment area 6.

(C) Segment area 7.

FIGURE III.17(A)

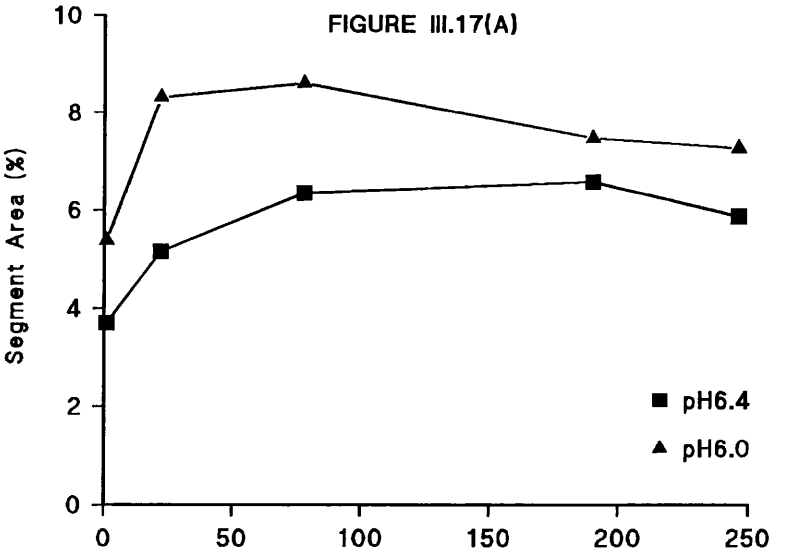

FIGURE III.17(B)

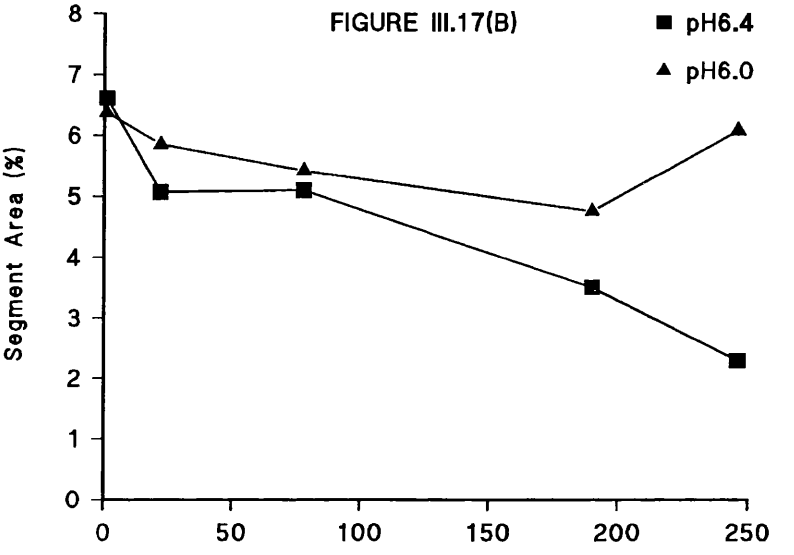

FIGURE III.17(C)

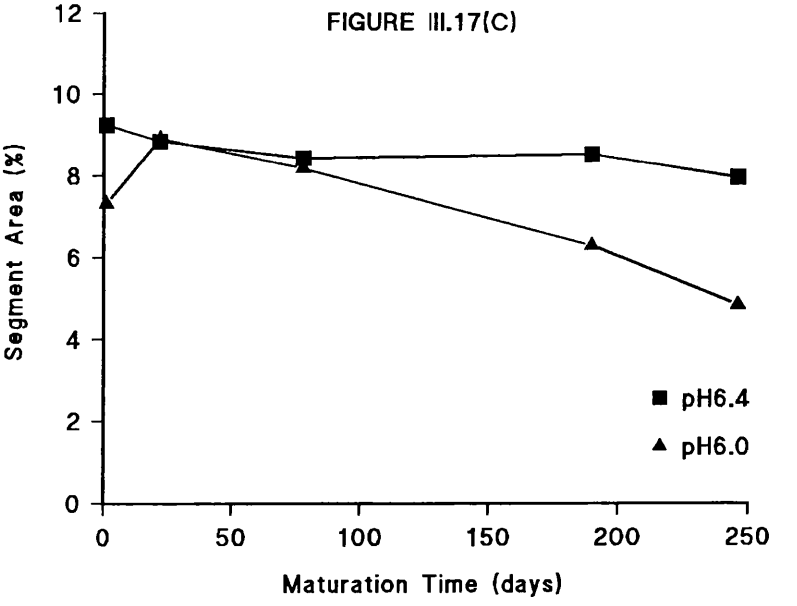

FIGURE III.18: Changes in the modified gradient water-soluble extract RP-HPLC chromatogram segment areas in standard pH and reduced pH Cheddar cheese over 246 days of maturation.

(A) Segment area 9.

(B) Segment area 10.

(C) Segment area 11.

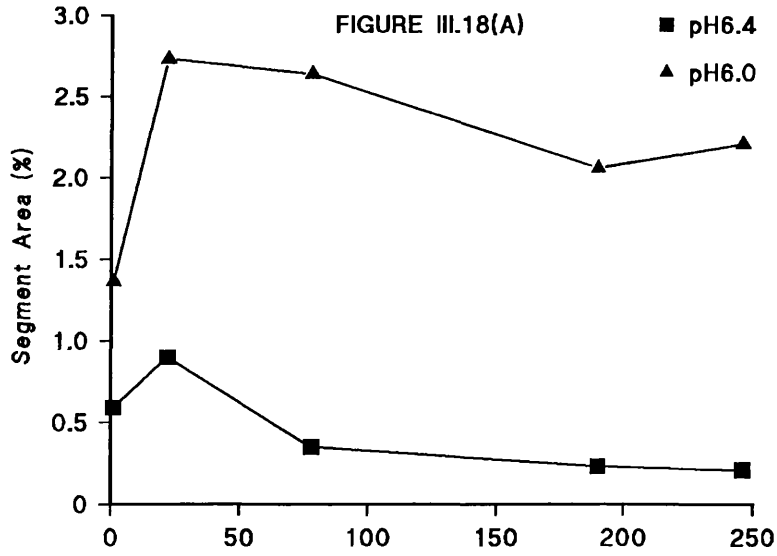
FIGURE III.18(A)
pH6.4
pH6.0
Segment Area (%)
3.0
2.5
2.0
1.5
1.0
0.5
0
0
50
100
150
200
250

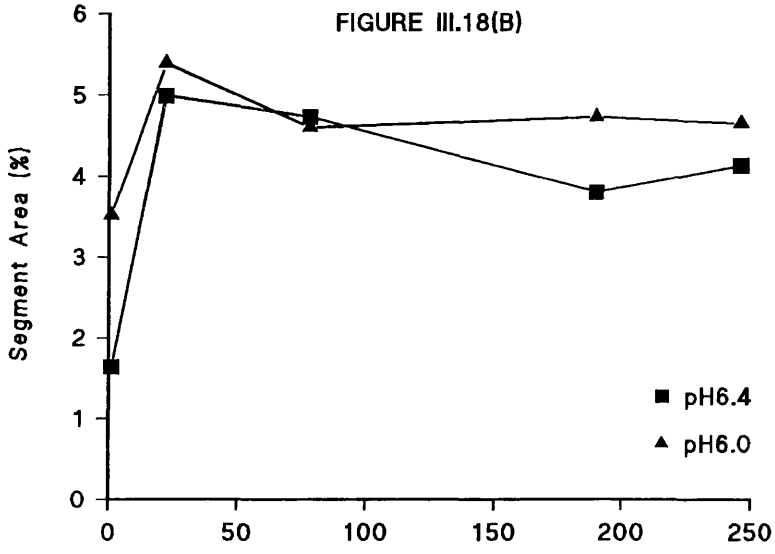
FIGURE III.18(B)
Segment Area (%)
6
5
4
3
2
1
0
pH6.4
pH6.0
0
50
100
150
200
250

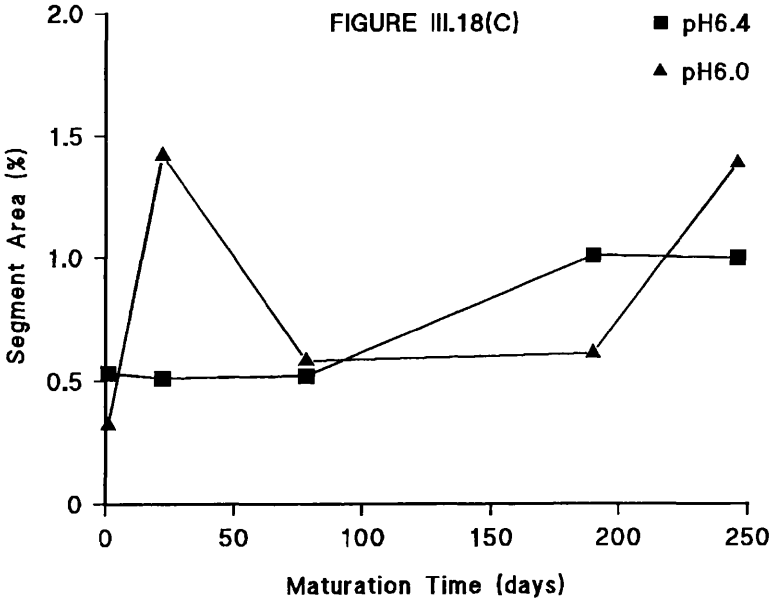
FIGURE III.18(C)
pH6.4
pH6.0
Segment Area (%)
2.0
1.5
1.0
0.5
0
0
50
100
150
200
250
Maturation Time (days)

FIGURE III.19: Changes in the modified gradient water-soluble extract RP-HPLC chromatogram segment areas in standard pH and reduced pH Cheddar cheese over 246 days of maturation.

(A) Segment area 14.

(B) Segment area 15.

(C) Segment area 17.

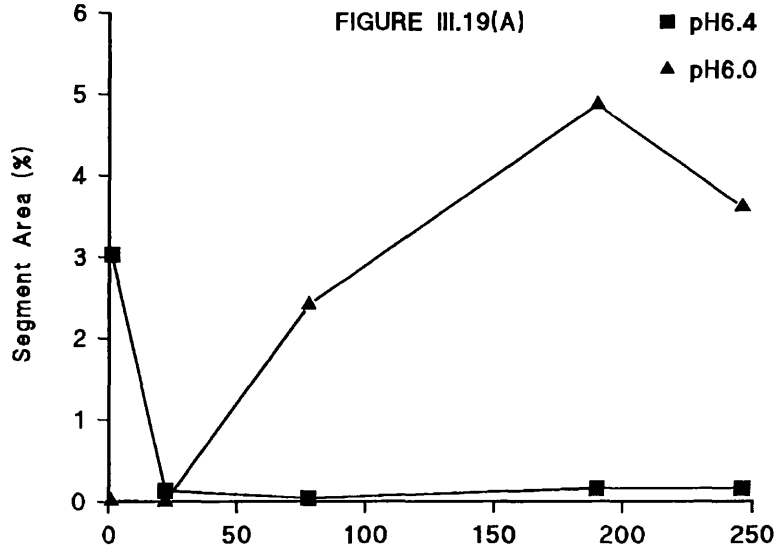
FIGURE III.19(A)
pH6.4
pH6.0
Segment Area (%)
0
1
2
3
4
5
6
0
50
100
150
200
250

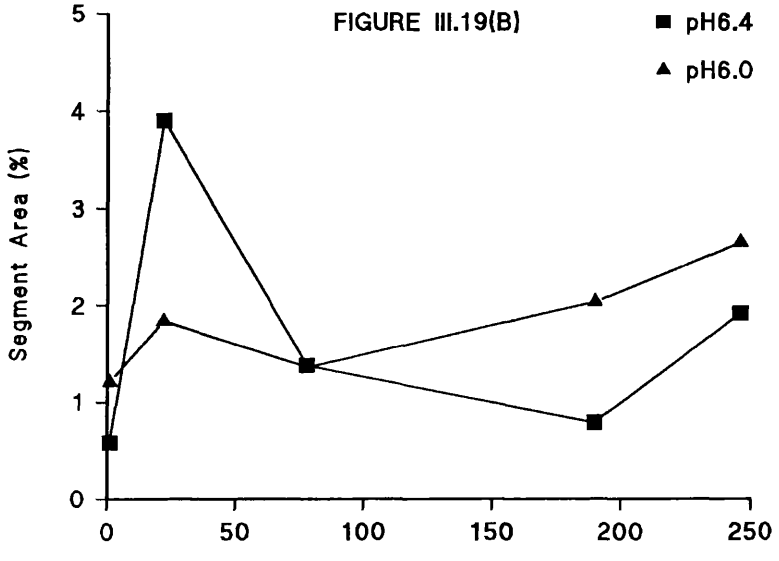
FIGURE III.19(B)
pH6.4
pH6.0
Segment Area (%)
0
1
2
3
4
5
0
50
100
150
200
250

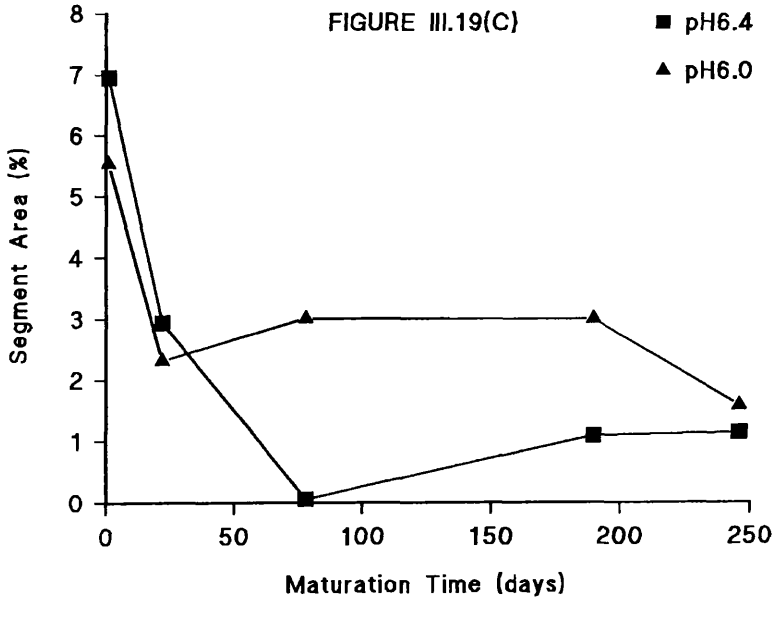
FIGURE III.19(C)
pH6.4
pH6.0
Segment Area (%)
0
1
2
3
4
5
6
7
8
0
50
100
150
200
250
Maturation Time (days)

TABLE IV.1: Changes in the levels of α_{s1}-casein, α_{s1}-I-casein and para-κ-casein in water-insoluble extracts from standard pH and reduced pH Cheddar cheese up to 246 days of maturation.

	α_{s1}-Casein		α_{s1}-I-Casein		Para-κ-casein	
Time (days)	pH6.4	pH6.0	pH6.4	pH6.0	pH6.4	pH6.0
0.0	[a]2.841	7.678	0.211	0.794	1.382	2.824
0.2	37.749	51.966	2.473	15.302	11.633	18.888
0.4	49.239	61.297	5.046	24.684	15.138	23.746
1.0	50.410	31.955	8.883	25.557	16.241	15.337
8.0	33.015	18.317	19.504	37.324	12.947	16.199
15.0	26.823	12.161	29.896	39.670	14.391	15.209
22.0	22.349	8.027	31.509	33.478	13.396	15.450
29.0	17.085	7.253	33.396	33.475	12.477	15.204
36.0	10.697	7.069	29.814	31.355	10.572	15.562
43.0	11.013	4.550	32.057	30.382	11.226	15.427
50.0	9.008	5.791	29.755	32.682	10.263	14.867
78.0	5.960	3.864	30.363	17.230	10.104	13.212
106.0	3.767	2.041	31.582	10.862	14.075	12.061
134.0	1.740	1.447	26.221	8.662	13.990	12.044
162.0	2.365	1.935	24.401	6.067	14.158	12.127
190.0	1.763	1.329	21.535	5.063	12.985	11.821
219.0	1.913	1.215	21.566	3.505	13.655	10.035
246.0	2.254	1.707	20.912	2.602	13.134	10.859

[a] Standardised for 1g "wet weight" Cheddar cheese from initial peak area percentage.

TABLE IV.2: Changes in the levels of β^{A1}-casein, β^{A2}-casein and β^{B}-casein in water-insoluble extracts from standard pH and reduced pH Cheddar cheese up to 246 days of maturation.

	β^{A1}-Casein		β^{A2}-Casein		β^{B}-Casein	
Time (days)	pH6.4	pH6.0	pH6.4	pH6.0	pH6.4	pH6.0
0.0	[a]5.564	8.789	2.679	4.159	0.903	1.575
0.2	48.753	67.371	23.254	30.008	8.356	10.928
0.4	63.406	81.659	30.297	37.262	11.066	14.460
1.0	68.733	54.492	32.090	25.944	11.471	9.675
8.0	54.176	53.977	26.561	25.177	8.972	9.932
15.0	55.663	57.315	28.624	29.623	10.026	10.527
22.0	52.484	47.799	26.923	24.630	8.754	8.769
29.0	47.184	47.243	25.231	24.299	7.958	8.685
36.0	37.899	45.941	20.495	24.405	5.820	8.781
43.0	39.023	45.290	21.213	23.011	6.209	8.450
50.0	35.301	53.901	19.165	25.833	5.724	10.465
78.0	34.203	41.476	19.257	26.575	5.521	6.561
106.0	33.271	30.435	17.972	17.566	5.209	4.726
134.0	27.728	28.350	15.741	17.127	4.107	4.250
162.0	29.456	27.444	16.136	17.540	4.340	4.240
190.0	25.678	27.184	14.885	19.793	3.661	4.253
219.0	25.641	23.066	17.041	17.865	3.552	3.887
246.0	22.299	22.813	14.832	17.641	3.158	3.488

[a] Standardised for 1g "wet weight" Cheddar cheese from initial peak area percentage.

TABLE IV.3: Levels of α_{s1}-casein, α_{s1}-I-casein, para-κ-casein, β^{A1}-casein, β^{A2}-casein and β^{B}-casein in water-insoluble extracts from standard pH and reduced pH Cheddar cheese at days 1, 22, 78, 190 and 246 of maturation.

Time (days)	pH	α_{s1}-Casein	α_{s1}-I-Casein	Para-κ-casein	β^{A1}-Casein	β^{A2}-Casein	β^{B}-Casein
1	6.4	[a]50.410	8.883	16.241	68.733	32.090	11.471
22	6.4	22.349	31.509	13.396	52.484	26.923	8.754
78	6.4	5.960	30.363	10.104	34.203	19.257	5.521
190	6.4	1.763	21.535	12.985	25.678	14.885	3.661
246	6.4	2.254	20.912	13.134	22.299	14.832	3.158
1	6.0	31.955	25.557	15.337	54.492	25.944	9.675
22	6.0	8.027	33.478	15.450	47.799	24.630	8.769
78	6.0	3.864	17.230	13.212	41.476	26.575	6.561
190	6.0	1.329	5.063	11.821	27.184	19.793	4.253
246	6.0	1.707	2.602	10.859	22.813	17.641	3.488

[a] Standardised for 1g "wet weight" Cheddar cheese from initial peak area percentage.