Objective Differentiation of Cheese Type and Maturity

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This thesis is dedicated to my parents Eric and Sylvia,
my husband Neil
and children Nicholas, Michael, Justin and Timothy

ABSTRACT

The main objectives of this study were to develop instrumental methods for determining cheese maturity and for differentiating variety irrespective of maturity. Traditionally this has been done by sensory (texture and taste) evaluation, but it is desirable to systematise the process, for both non-varietal cheese that is destined to become an ingredient in a complex food and for table cheese.

An initial study developed a size exclusion (high performance liquid) chromatography (SE-HPLC) method for the characterisation of proteolysis in cheese. The method provided predictable elution of peptide and amino acid standards on the basis of hydrodynamic volume (which equates well with molecular weight), although the presence of a net positive charge caused smaller peptides and amino acids to elute slightly earlier than anticipated. The repeatability of the elution time and peak intensity of a range of molecular weight standards was excellent, and their elution time was used to develop a standard curve for determining molecular weight.

The potential of the SE-HPLC method for characterising proteolysis in cheese was compared with commonly used methods by examining seven cheeses of different types. Non-casein nitrogen and non-protein nitrogen were useful for demonstrating differences in the overall amounts of protease and peptidase activity; alkaline urea-polyacrylamide gel electrophoresis (urea-PAGE) of the urea-soluble fraction (USF) of cheese was useful for comparing the amounts of chymosin and plasmin action; urea-PAGE of the water-soluble fraction (WSF) of cheese was useful for comparing the amounts of several large peptides (>~5000 Da) and reverse phase (RP)-HPLC of the WSF was useful for comparing patterns of peptides in different cheese types. SE-HPLC was useful for demonstrating differences in the pattern of molecular weight distribution of the peptides and amino acids present in the WSF of different cheese types, but was less useful for demonstrating the molecular weight distribution of the caseins (which eluted as a single peak), peptides and amino acids present in the USF. Good repeatability of the extraction of the WSF was demonstrated.

A larger study compared the SE-HPLC (WSF) method with alternative methods (urea-PAGE of the USF and WSF, and RP-HPLC of the WSF) for following the maturation of five cheese types (New Zealand-style Cheddar, Elsberg, Gouda, Mozzarella and Swiss) from pressed curd to beyond normal maturity. Principal component analysis (PCA) was used to reduce the data from a large number of peaks to a few principal components that accounted for most of the variation in the data set. Depending on the method of sample analysis, the correlation form of PCA accounted for between 72% and 83% of the variation in the data within the first three principal components, and generally provided better differentiation of cheese type than the covariance form of PCA which accounted for between 85 and 97% of the variation in the data and was better for determining maturity. Plots of the first three principal components showed that urea-PAGE of the USF and SE- and RP-HPLC of the WSF could be used to differentiate the cheese types throughout ripening; urea-PAGE of the USF, which measured primary proteolysis, provided useful maturity trends in the early stages of ripening of most cheese types and throughout the ripening of slowly maturing cheese while RP- and SE-HPLC of the WSF, which mainly measured secondary proteolysis, provided useful maturity trends throughout the ripening of each cheese type.

The final study used RP- and SE-HPLC of the WSF to examine the diversity of a range of first grade mild to mature commercial cheese of one type (Cheddar), and to examine the potential of the instrumental methods for establishing a cheese maturity index that could accurately predict a sensory maturity score (SMS; 1.00 – 9.00 scale) provided by a trained sensory panel. Each cheese sample (77 samples of Cheddar cheese manufactured at 8 New Zealand factories over 2 seasons and ripened at 5, 10 or 13°C for 3, 6, 12 or 15 months) was analysed using both methods, as well as traditional methods for determining maturity (non-protein nitrogen (NPN), water-soluble nitrogen (WSN)). The relationship between each set of instrumental results and the SMSs was modelled using multivariate statistics and equations were developed for predicting cheese sensory maturity. Multiple linear regression (MLR) analysis showed that the simplest measures of maturity provided a reasonable correlation with the SMS ($R = 0.88$, R^2 (adj) = 0.77 for NPN and total nitrogen (TN); and $R = 0.90$ and R^2 (adj) = 0.80 for WSN and TN). The covariance form of PCA accounted for the most variation in the data (PC1 to PC3 = 88% for RP-HPLC; and PC1 to PC3 = 93% for SE-HPLC) and provided trends that were more closely associated with sensory maturity. MLR analysis showed the close correlation between the first three principal components and the SMSs (RP-HPLC: $R = 0.94$, R^2 (adj) = 0.87 for PC1, PC2, PC3 and TN; and SE-HPLC: $R = 0.92$, R^2 (adj) = 0.84 for PC1, PC2, PC3 and TN). The PCA factor loading scores were used to select influential peaks and MLR was used to demonstrate their close correlation with the SMS (RP-HPLC: $R = 0.99$, R^2 (adj) = 0.98 for 32 peaks and TN (Model K); and SE-HPLC: $R = 0.96$, R^2 (adj) = 0.90 for 15 'peaks' and TN (Model M)). The regression equations for Models K and M were validated. RP-HPLC or SE-HPLC could be used to provide effective indices of maturity.

It was possible to conclude that: varietal differences could be captured using either the SE-HPLC method or RP-HPLC; differentiation of the source of cheese within a variety was not possible using SE- or RP-HPLC methods; a standardised SE-HPLC procedure or a RP-HPLC procedure could be developed for accurately predicting the sensory maturity Cheddar cheese; and that the SE-HPLC procedure provided the most practical instrumental means of estimating the sensory maturity of Cheddar cheese.

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4.2.3 Methods for the analysis of proteolysis in cheese, casein purity and casein hydrolysis

4.3 APPENDIX

1.0 INTRODUCTION

New Zealand produces approximately 20% of the cheese traded on the world market, with a net worth of approximately US $600 million. The current business goal of the NZ dairy industry is to further increase revenue from cheese over the next 5 years, with product development expected to play a significant role. An excellent understanding of the manufacturing processes and storage regimes responsible for cheese diversity and maturation is required to achieve this goal.

Cheese is comprised of a casein matrix in which fat, water, lactose, minerals, vitamins, bacteria and enzymes are entrapped. The basic principles for cheese-making are the same for most cheese types. Their manufacture involves the removal of water from milk with a consequent six to ten-fold (Fox, 1989) concentration of the protein, fat, minerals and vitamins.

The diversity in the texture and flavour of the many hundreds of cheese types produced around the world is due mainly to differences in milk type and cheese manufacture that affect cheese composition, and to differences in the storage temperature and length of time of storage of the cheese (Lawrence et al, 1983, 1993; Lawrence and Gilles, 1987; Lane et al, 1997).

Although some cheese types are consumed while fresh, many undergo a period of ripening or maturation. During maturation, the cheese changes from a firm, rubbery curd with little flavour, to a flavoursome cheese with textural and functional properties appropriate to the type. These changes occur as a result of several biochemical processes that include glycolysis, lipolysis, proteolysis as well as the secondary fermentations that take place when the products of these processes are catabolised.

Proteolysis is recognised as one of the most important and complex of these biochemical processes. It involves the hydrolysis of the caseins that form the cheese matrix to progressively smaller peptides and amino acids by proteinases including chymosin, plasmin and the starter cell-envelope proteinases and the peptidases of the starter and non-starter lactic acid bacteria (NSLAB). The quantity and rate of activity of these enzymes in cheese is influenced by even small changes in cheese composition or storage temperature.

The breakdown of the protein matrix by enzymes in the cheese, is responsible for changes in the textural and functional properties of the cheese (e.g. de Jong 1976, 1977, 1978; Creamer and Olson, 1982; Johnston et al, 1994; Watkinson et al, 1997; Lane et al, 1997) as well as many of the flavour components in cheese (e.g. McGugan et al, 1979; Aston and Creamer, 1986; Fox et al, 1993; Engels and Visser, 1994). The small peptides and amino acids may contribute directly to flavour or indirectly when they are catabolised to amines, acids, thiols and thioesters etc. (Fox et al, 1993, 1995a).

Most aspects of proteolysis in cheese have been extensively reviewed and published (e.g. Grappin et al, 1985; Rank et al, 1985; Law, 1987; Fox, 1987; Fox and Stepaniak, 1993; Fox et al, 1994, 1995a,b; Law and Mulholland, 1995; Fox and McSweeney, 1996; McSweeney, 1997; Kunji et al, 1996; Bastian and Brown, 1996; Law and Haandrikman, 1997).

A number of methods have been used to examine proteolysis in cheese or monitor proteolysis during cheese maturation and these have been the subject of various reviews (e.g. Fox et al, 1995b; McSweeney and Fox, 1997; Wallace and Fox, 1998). Traditional methods involve fractionation of the cheese and examination of the distribution of (Kjeldahl) nitrogen (N) among the fractions (e.g. total N (TN), non-casein N (NCN) and non-protein N (NPN)) or measurement of the concentration of free amino acids. Information on the hydrolysis of individual caseins and the formation of some of the largest peptides is provided by alkaline urea-polyacrylamide gel electrophoresis (urea-PAGE), and separation of the smaller peptides is usually provided by reverse phase-HPLC (RP-HPLC), following some form of fractionation.

The advent of mass spectrometry has meant that recent focus has been on the identification of peptides produced during cheese maturation, the assignment of peptides to particular enzymes responsible for their presence, and the elucidation of pathways for their generation (e.g. Gouldsworthy et al, 1996; Singh et al, 1994, 1995, 1997; Breen et al, 1995; Exterkate et al, 1997; Fernández et al, 1998; Mooney et al, 1998).

The development of powerful computer processors and statistical methods such as linear discriminant analysis (DA), principal component analysis (PCA) and multiple linear regression analysis (MLR), has provided the means to efficiently handle the often large volumes of data provided by the various methods used to analyse proteolysis in cheese. This has enabled a more global approach to analysing the data, that has been used to objectively differentiate cheese of different types (driven more recently by the desire to protect 'Appellation of Origin' or 'Protected Designation of Origin' cheese) (e.g. Smith and Nakai, 1990; Lopez-Fandiño et al, 1994; Dewettinck et al, 1997), or to broadly group cheese according to maturity (e.g. Pham and Nakai, 1984; Santa-Maria et al, 1986; O'Shea et al, 1996; Dewettinck et al, 1997; Garcia Ruiz et al, 1998). The complexity of proteolysis and cheese maturation, and in some cases the inadequacy of the methods and resultant data sets (e.g. O'Shea et al, 1996), has resulted in varying levels of success.

There is a clear need for simple methods of sample analysis that capture differences in proteolysis between cheese types and are sufficiently sensitive to capture differences related to maturation. Appropriate statistical analysis of the resultant data could provide information to assist in product development (for example, to group cheeses made according to similar processes), to group cheese according to its origin, to determine the stage of maturity, and perhaps to develop a cheese maturity index. This study explores the means to achieve these objectives.

2.0 LITERATURE REVIEW

2.1 INTRODUCTION

This review covers literature published before 1999. Relevant literature published since this date is covered in the discussion sections of the various chapters.

The focus of the following review is on proteolysis in cheese. The caseins, casein micelles, and the processes of cheese manufacture and curd formation are discussed briefly. The enzymes present in cheese, including the coagulant enzymes, the indigenous milk enzymes and those of the starter bacteria, starter adjuncts, and NSLAB are described. Current knowledge on the effect of manufacturing processes on the activity of the enzymes present and on cheese texture and flavour is reviewed. Methods for the analysis of proteolysis in cheese and the use of statistical analysis to interpret the data so that it can be used for the differentiation of cheese type and maturity are also discussed.

2.2 CASEINS AND MICELLES

Milk is the raw material used in the manufacture of cheese. It exists as a complex system in which fat, micellar casein, whey protein, lactose, salts and indigenous enzymes are dispersed in an aqueous phase. Milk composition is influenced by factors such as breed of cow, genetic polymorphism, seasonal and dietary variability and stage of lactation. Any differences in milk composition can be expected to influence the technological behaviour of milk in cheese manufacture and to influence cheese composition, which in turn may influence the textural and functional properties of the cheese. Manufacturing procedures can compensate for some variation in milk composition.

2.2.1 The casein fraction of milk

Bovine milk caseins (24-28 $g.L^{-1}$ milk) are synthesized in the mammocytes through the expression of a series of co-dominant alleles that are located in close proximity on chromosome-6 of cattle.

The four proteins that comprise the casein fraction of milk are: the κ-casein-1P group (169 amino acid residues) (Fig. 2.1) that has many different forms with different degrees of glycosylation; the α_{S1}-group of caseins (199 amino acid residues) which is comprised of α_{S1}-casein B-8P and α_{S1}-

casein B-9P (α_{S0}-casein) (Fig. 2.4); β-casein-5P (209 amino acid residues) (Fig. 2.5); and the α_{S2}-group of caseins (207 amino acid residues) which is composed of α_{S2}-casein A-10P, α_{S2}-casein A-11P, α_{S2}-casein A-12P and α_{S2}-casein A-13P (Fig. 2.6) (Grappin et al, 1985).

2.2.1.1 α_{S1}-Casein

α_{S1}-Casein (12-15 g.L^{-1} milk) is one of the main proteins in bovine milk and may be the principal structural casein in cow's milk cheese. It is calcium sensitive (insoluble in 5 mM $CaCl_2$ solution at pH 7.0) and forms strong self-association complexes as well as complexes with other caseins. The primary sequence of α_{S1}-casein B (Fig. 2.4) was established by Mercier et al (1971) and Grosclaude et al (1973). α_{s1}-Casein B is the main variant and a small proportion (approximately 10%) has an additional phosphorylation at position 41.

Two distinct hydrophobic regions are discernible in α_{s1}-casein. One at the N-terminus between Arg_1 and Ile_{44}, and the other at the C-terminus between Arg_{90} and Trp_{199} (Fig. 2.4). The region between Ile_{44} and Arg_{90} contains seven of the eight phosphoseryl residues (between $SerP_{46}$ and $SerP_{75}$) and it is widely believed that these residues are implicated in the cross-linking of the caseins in cheese by colloidal calcium phosphate.

2.2.1.2 β-Casein

β-Casein (9-11 g.L^{-1} milk) is the second most abundant casein in milk. The primary structure of β-casein A^2 (Fig. 2.5) was reported by Ribadeau-Dumas et al (1973) (Fig. 2.5). The most common genetic variants of β-casein are A^1, A^2, A^3 and B.

β-Casein is the most hydrophobic of the caseins, with the highly charged region at the N-terminus clearly separated from a large hydrophobic region. The unusually high number of prolyl residues (34-35) probably influences the number of β-turns present (Andrews et al, 1979; Graham et al, 1984) and is responsible for the open structure of this casein. β-Casein is a strongly amphipathic molecule that is monomeric at low temperatures and self-associates into detergent-like micellar aggregates of about 40 molecules at physiological temperatures. This self-association has been linked to the very hydrophobic C-terminus of the protein (Creamer and Berry, 1975).

2.2.1.3 α_{S2}-Casein

α_{s2}-Casein is present in milk at a concentration of 3-4 g.L^{-1}. Brignon et al (1977) reported the primary structure of α_{S2}-casein (Fig. 2.6). It consists of 207 residues, possibly existing as dimers linked at Cys_{36} or Cys_{40} via a disulphide bond. The four genetic variants of this protein are A, B, C and D, with the A variant predominant. The D variant differs from the A by the deletion of 9 residues around the phosphoseryl residue cluster $SerP_{56}$ - $SerP_{58}$ (Grosclaude et al, 1979).

Hydrophobic residues are distributed throughout the length of α_{S2}-casein (Fig. 2.6), although they are concentrated in three distinct regions that are separated by charged regions. α_{s2}-Casein is strongly dipolar, with a cluster of negative charges at the N-terminus and positive charges at the C-terminus, suggesting that electrostatic interactions may be an important factor in determining the structural characteristics.

2.2.1.4 κ-Casein

κ-Casein (3-4 g.L^{-1} milk) plays an important role in milk chemistry by providing colloidal stability to the casein micelles (Walstra, 1990; Creamer, 1991). Mercier et al (1973) determined the primary structure of κ-casein (Fig. 2.1). Two variants are common, with the B variant differing from the A variant by the substitution of Ile for Thr$_{136}$ for and Ala for Asp$_{148}$. The A variant has an allelic frequency of 0.826 in Friesian and 0.519 in Jersey cows, with the remainder comprised of the B variant (Winkelman and Wickham, 1996). The B variant is associated with higher expression levels of κ-casein. Walsh et al (1998) compared the effect of the κ-casein A and B variants, at similar casein concentration, on the moisture-adjusted cheese yield. They found that the yield was significantly improved when milk with the κ-casein B variant was used.

NH$_2$-Glu-Glu-Gln-Asn-Gln-Glu-Gln-Pro-Ile-Arg^{10}-Cys-Glu-Lys-Asp-Glu-Arg-Phe-Phe-Ser-Asp^{20}-Lys-Ile-Ala-Lys-Tyr-Ile-Pro-Ile-Gln-Tyr^{30}-Val-Leu-Ser-Arg-Tyr-Pro-Ser-Tyr-Gly-Leu^{40}-Asn-Tyr-Tyr-Gln-Gln-Lys-Pro-Val-Ala-Leu^{50}-Ile-Asn-Asn-Gln-Phe-Leu-Pro-Tyr-Pro-Tyr^{60}-Tyr-Ala-Lys-Pro-Ala-Ala-Val-Arg-Ser-Pro^{70}-Ala-Gln-Ile-Leu-Gln-Trp-Gln-Val-Leu-Ser^{80}-Asp-Thr-Val-Pro-Ala-Lys-Ser-Cys-Gln-Ala^{90}-Gln-Pro-Thr-Thr-Met-Ala-Arg-His-Pro-His^{100}-Pro-His-Leu-Ser-Phe●Met-Ala-Ile-Pro-Pro^{110}-Lys-Lys-Asn-Gln-Asp-Lys-Thr-Glu-Ile-Pro^{120}-Thr-Ile-Asn-Thr-Ile-Ala-Ser-Gly-Glu-Pro^{130}-Thr-Ser-Thr-Pro-Thr-Thr-Glu-Ala-Val-Glu^{140}-Ser-Thr-Val-Ala-Thr-Leu-Glu-Asp-SerP-Pro^{150}-Glu-Val-Ile-Glu-Ser-Pro-Pro-Glu-Ile-Asn^{160}-Thr-Val-Gln-Val-Thr-Ser-Thr-Ala-Val^{169}-COOH

Fig. 2.1 *Sequence of κ-casein A-1P (19035 Da).* In the B variant, Ile is substituted for Thr$_{136}$, and Ala is substituted for Asp$_{148}$. The phosphoseryl residue is indicated in red. Potential phosphorylation sites are underlined in red. The primary chymosin (●) cleavage site is indicated.

In contrast to α_{s1}-, α_{s2}- and β-casein, κ-casein has one, two or three phosphoseryl residues and is less sensitive to precipitation by calcium ions. κ-Casein has two distinct regions, one of which (the N-terminus) is very hydrophobic and is buried within the micelle. The C-terminus of the molecule is more hydrophilic and protrudes from the casein micelle to provide colloidal stability.

2.2.2 Casein micelles

The caseins, α_{s1}-, α_{s2}-, β- and κ-casein, achieve segregation of their hydrophobic regions from the water in milk by self-aggregation (Mora-Gutierrez et al, 1996), and exist in milk in the presence of calcium (30 mM) as colloidal casein complexes or micelles of ~200 nm in diameter. These micelles have a hydrophobic core, contain some exchangeable 'trapped water' (Mora-Gutierrez et al, 1996), and have polar groups at the surface that maintain micelle stability.

The micelles are made up of clusters of inhomogeneous sub-micelles that are incorporated into the colloidal micelle whilst retaining most of their structural and dynamic properties (Farrell et al, 1990). According to Walstra (1990), the sub-micelles are roughly spherical aggregates of several casein molecules held together by hydrophobic bonds and salt bridges. Regions on the surface of the sub-micelles are linked by micellar calcium phosphate (Ca^{2+} and HPO_4^{2-}) (Gatti and Pires; 1995; Holt, 1998) *via* ester phosphate groups to form micelles. Casein micelles are stabilised against aggregation due to steric repulsion of the hydrophilic filaments of κ-casein (and possibly by β-casein filaments at low temperature) that protrude from the micelle surface (Holt and Horne, 1996).

2.3 MILK AND CHEESE MANUFACTURE

During the manufacture of cheese, a large proportion of the water as well as most of the whey proteins, lactose and dispersed salts in milk are removed. The remaining components, including the fat, and some of the moisture, lactose, salts, indigenous enzymes, added coagulant enzyme and starter bacteria are retained in the casein network of the coagulum.

Several stages in the manufacture of cheese influence the chemical composition of the curd and either directly or indirectly influence cheese structure, texture and flavour; these are outlined in the following paragraphs.

In general, the manufacture of cheese involves:

i) Preparation of the cheese milk: In New Zealand, milk is chilled to 4°C on the farm and transported under refrigeration to the factory where it is stored in silos at approximately 10°C prior to processing. The milk is standardised to the required protein to fat ratio by separating a portion of the milk and blending the skim milk with the whole milk. Standardisation is necessary to achieve the desired fat in dry matter (FDM) in the cheese. Excess fat in the milk reduces moisture loss from the curd and increases the moisture in the non-fat substance (MNFS), resulting in a cheese that has a weak body. The standardised milk is then thermised

(65°C/15 s) and held at 4.7°C prior to pasteurisation at 72°C for 15 s to destroy pathogenic organisms. The milk is then cooled to 32°C and pumped into the cheese vat.

ii) Acidification of the milk: It is usual for starter microorganisms to be used to convert lactose to lactic acid. Alternatively, or perhaps in conjunction with the starter microorganisms, an acidifying agent such as lactic acid, citric acid, malic acid, acetic acid or gluconic acid-δ-lactone can be added directly to the milk to achieve the desired pH. The activity of the coagulant enzyme, the amount of enzyme retained in the curd, and as a consequence, the amount of proteolysis, are dependent on the amount of acid produced during the initial stages of cheese-making (Stadhouders and Hup, 1975; Holmes et al, 1977; Creamer et al, 1985; Larsson and Andrén, 1997). The caseins, at a higher pH, have a net negative charge and absorb moisture, resulting in a casein matrix with a higher moisture content (Creamer and Olson, 1982; Lawrence et al, 1987; Lucey, 1990). Acidification of the cheese-milk causes the negatively charged groups on the caseins to become protonated and, as a consequence, casein solvation decreases and there is gradual dissolution of colloidal calcium phosphate. During this process the renneted casein micelles gradually increase in volume to a maximum at pH 5.4 to 5.6, and then as the pH continues to fall, they decrease in volume as the attractive forces between the caseins diminish and they begin to dissociate, possibly in the form of sub-micelles (Creamer, 1985; Roefs et al, 1985; van Hooydonk et al, 1986). The dissolution of colloidal calcium phosphate and consequent dissociation of the caseins alters the susceptibility of the caseins to proteolysis during manufacture and storage, and influences the rheological properties (such as texture) of the cheese (Lawrence et al, 1987, 1993).

iii) Coagulation of the casein fraction to form a gel is usually achieved by the addition of an enzyme preparation known as rennet. Several types of rennet are available and it is probable that the selection will influence the properties of the final cheese. During coagulation, rennet cleaves the glycomacropeptide from κ-casein, resulting in destabilisation of the casein micelles and their eventual aggregation.

iv) Dehydration or syneresis of the coagulum, which is the loss of whey as the result of cutting, stirring, cooking, salting, or pressing the curd. Syneresis is also influenced by the rate and extent of acidification.

v) Stretching the curd (Mozzarella style cheeses), involves the application of heat (~62-72°C) to the curd while it is kneaded, either manually or mechanically, to alter the orientation of the proteins and align them along the same plane. The application of heat during stretching creates the distinct "plastic curd" type cheeses with characteristically good melt and stretch properties, such as Mozzarella cheese. The temperature and duration of stretching diminishes subsequent enzyme activity in the cheese.

vi) Addition of salt, can be either directly to the curd prior to stretching or moulding (dry-salting) or the stretched and moulded curd can be placed in a brine solution (brine-salting). Salt acts as a preservative by controlling microbial growth and enzyme activity. The presence of salt increases syneresis of the curd and the lower moisture content of the cheese affects the rate of enzyme activity. Salt is also involved in physical changes in cheese proteins which influence texture, protein solubility, conformation and susceptibility to hydrolysis by enzymes.

2.3.1 Milk coagulation and curd formation

The active enzyme in most rennet preparations cleaves κ-casein at the Phe_{105} - Met_{106} bond (Delfour et al, 1965). An exception is the *Cryphonectria parasitica* rennet (Surecurd) that cleaves at the Ser_{104}-Phe_{105} bond (Drohse and Foltman, 1989).

During the primary phase of rennet action, the hydrophilic carboxy-terminal peptide (glycomacropeptide) is released to the whey. Calcium then binds to the negatively charged phosphoseryl residues or carboxylic acid groups which results in an overall reduction of micelle charge and a decrease in repulsion (Lucey, 1990). When approximately 86-90% (Dalgleish, 1979) of the κ-casein has been hydrolysed, the modified micelles begin to aggregate, in the presence of > 6 mM calcium and at a temperature of >15°C (Berridge, 1942), due in part to van der Waals attractions and to hydrophobic interactions (Dalgleish, 1993). This is often referred to as the secondary phase of rennet action.

The rates of enzyme action and coagulation are dependent on casein concentration (with the aggregation rate proportional to the square of casein concentration), rennet concentration (Lomholt et al, 1998; Lomholt and Qvist, 1999), temperature, micelle size (Niki et al, 1994), as well as milk pH and calcium concentration (Green and Marshall, 1977; van Hooydonk et al, 1984; Hyldig, 1993 (reported by Lomholt and Qvist, 1999).

The coagulum is comprised of an open network of aggregated casein micelles (Fig.2.2) that contracts rapidly as moisture is expelled. After 24 hours the micelles are no longer evident.

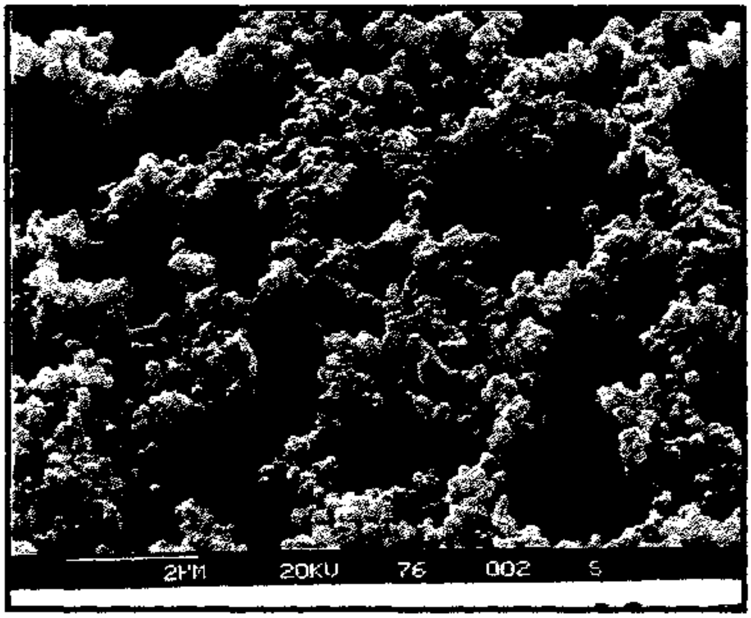


Fig. 2.2 *Scanning Electron Micrograph showing the 3-dimensional casein micelle network of the casein matrix during coagulum formation* (supplied by R Hirst of the Microstructure Unit, Food Science, Fonterra Research Centre). Thin sections of epoxy resin-embedded cheese curd that had been prepared for transmission electron microscopy (TEM) were chemically etched (using a method based on that of Brooker and Wells (1984)) and examined by SEM.

Tunick et al (1997) used EM imaging to examine changes in the sub-micelle structure of Mozzarella cheese during storage. They showed that the spacing patterns of regular structures with the dimensions of casein sub-micelles changed during storage at 4°C, with clusters formed at longer than average spacings. Results suggested that ultrastructural changes were related to proteolysis. They concluded that changes in sub-micelle size and distribution could explain some of the changes in physical properties, especially texture.

2.3.2 Factors that influence curd formation

Several factors influence curd formation, including stage of lactation, refrigeration, application of heat or homogenisation of cheese-milk. Some of these milk variations can be overcome using manufacturing procedures.

2.3.2.1 Stage of lactation

The rennet coagulation time and the gel strength deteriorate in late lactation milk (White and Davies, 1958; O'Keeffe et al, 1982; O'Keeffe, 1984), resulting in a coagulum with a lower gel strength and syneresis rate and a higher moisture content (O'Keeffe et al, 1982; O'Keeffe, 1984), poor body and texture, and poor melting and stretching properties (Fox and Stepaniak, 1993). The longer coagulation time has been attributed to the activity of plasmin in the milk, however, extensive proteolysis must occur before any significant effect on the coagulation properties is seen (Grufferty and Fox, 1988b).

2.3.2.2 Refrigeration of milk

Milk is normally stored at refrigeration temperatures to prevent spoilage. However, the storage of milk at low temperatures prior to the addition of rennet results in an increase in coagulation time (e.g. Qvist, 1979; Ali et al, 1980). This is believed to be due to the solubilisation of colloidal calcium phosphate and the diffusion of β-casein (Creamer, 1978a) and κ-casein from the micelles. The effect on coagulation time is not completely reversed by holding at the renneting temperature (~32°C). Although pasteurisation at 72°C/15 seconds only partially reverses this effect (e.g. van Hooydonk et al, 1984; Dalgleish, 1987), the coagulation properties can be restored if calcium chloride (0.1 $g.L^{-1}$) is added to the cold milk before pasteurisation (Qvist, 1979). It is well known that heating at 60°C for 30 minutes restores the original coagulation time and gel firmness (e.g. Ali et al, 1980).

2.3.2.3 Application of heat to milk

An increase in cheese yield can be obtained by the application of heat to milk (prior to the addition of rennet) at $pH < 6.6$ and at temperatures which denature β-lactoglobulin. The resultant increase in the rennet coagulation time (Morrissey, 1969) is believed to be caused by the formation of κ-casein-β-lactoglobulin (heat denatured) complexes on the outside of the casein micelles that probably make the Phe-Met bond inaccessible to rennet (Sawyer 1969; Singh et al, 1988, 1995).

Heating milk alters the distribution of calcium between the micelles and the serum phase of milk (Jenness and Patton, 1959). The effect on calcium distribution can be almost totally reversed by lowering the pH of the milk to about 5.5 (to partially dissociate the calcium phosphate) and then bringing it back up to pH 6.6 prior to the addition of rennet (Banks et al, 1987; Singh et al, 1988; Lucey et al, 1994). The effectiveness of pre-acidification suggests that heat induced precipitation of calcium and phosphate (Schmidt and Both, 1987) may be one factor affecting gel strength, curd syneresis and cheese functional properties. The addition of Ca^{2+} can also be used to reverse, at least partially, the effect of this heat treatment (Dalgleish, 1987; Lucey et al, 1994).

2.3.2.4 Homogenisation of milk

Homogenisation of milk prior to rennet addition does not appear to adversely affect the rate at which the coagulum is formed. Homogenisation breaks up the milkfat globules and the newly formed milkfat globules, stabilised by casein adsorbed at the surface (Wiese and Palmer, 1932; Buchheim et al, 1986), tend to behave as large casein micelles. Normally a coagulum consists of a casein matrix containing entrapped fat. These casein coated fat globules, acting as large casein micelles, form part of the casein matrix, and this results in changes in the properties of the coagulum (Shaker et al, 1990) and lowers fat losses during syneresis.

2.4 COAGULANT ENZYMES

Most varieties of cheese are coagulated enzymatically by rennet. The rennet can be of animal, microbial or plant origin, or a recombinant `nature identical' rennet. The rennet incorporated in the cheese curd hydrolyses the proteins to peptides and amino acids during the maturation of the cheese.

2.4.1 Animal rennet

Chymosin, an acid (aspartyl) proteinase, is extracted from the stomach linings of young milk-fed animals. As the animals mature, substances other than milk enter the diet, and chymosin is replaced by another acid proteinase known as pepsin (e.g. Andrén et al, 1982). In New Zealand, calves are slaughtered soon after birth and the active enzyme in the rennet extracts is about 95% chymosin. In other countries calves may be older, and the rennet extracts therefore contain different ratios of pepsin to chymosin.

Chymosin and pepsin from different species are traditionally used in the manufacture of cheese. Of the animal rennets, calf chymosin has the highest milk clotting to proteolytic activity ratio. The proteolytic activities of the animal rennets decrease in the following order: porcine pepsin > ovine pepsin > bovine pepsin > calf rennet (Fox, 1969, 1987; Green, 1972; O'Leary and Fox, 1972; Gouda et al, 1988).

An increased ratio of pepsin to chymosin results in a decrease in the rate of protein breakdown during maturation (Fox and Walley, 1971). It is believed that this 'reflects the lower pH and heat stability of bovine pepsin during cheese manufacture and the consequent lower level of residual rennet activity in the curd' (Guinee and Wilkinson, 1992). A rennet-free cheese can be produced by using ovine pepsin to form the coagulum and then increasing the pH to above 7 to denature pepsin (Lane et al, 1997).

2.4.2 Fermentation-derived (recombinant) rennet

There are several genetically engineered or 'nature identical' rennets available commercially. For each, the appropriate gene sequence (obtained from either calf abomasum cells or synthesised) is inserted into the DNA of a bacterial or fungal cell that is subsequently cloned and grown in fermentation vessels. The first commercially available genetically engineered rennet was Chy-max

(Pfizer and Co. Inc., USA), produced in an inactive form by *Escherichia coli* K12, and chemically extracted and activated to form chymosin (genetic variant A). This enzyme is no longer sold. A second genetically engineered rennet, known as Maxiren (Royal Gist Brocades NV, Delft, Netherlands), is secreted into the growth medium as prochymosin by the host organism *Kluyveromyces lactis* (a species of yeast). The prochymosin is activated to form a chymosin that contains a sugar molecule but is otherwise identical to natural chymosin. McSweeney et al (1993d, 1994a) showed that the action of Maxiren and calf chymosin on α_{s1}- and α_{s2}-casein produced identical peptides. A third genetically engineered rennet is known as Chymogen (Genencor International, San Francisco, CA, USA and Chr. Hansen Ltd (Chr. Hansen's Laboratorium, Danmark A/S, Denmark) and is produced by *Aspergillus niger* (a species of fungus). The organism secretes inactive prochymosin into the growth medium. The prochymosin is extracted and activated to form a chymosin (genetic variant B) that also contains a sugar molecule but is otherwise identical to native chymosin. Chymogen is also available in a form that contains added pepsin of animal origin and is known as Chymogen S (information supplied by Dr K. Turner, Dairy Meats NZ Ltd, Enzyme Division, New Zealand). Cheese incorporating recombinant rennet has been shown to be indistinguishable from cheese made with calf rennet (Green et al, 1985; Koch et al, 1986; Hicks et al, 1988; Prokopek et al, 1988). These products have not gained acceptance in New Zealand although they are used widely in some countries.

2.4.3 Microbial rennet

Several different microbial (fungal) enzymes are available commercially and can be used as rennet substitutes. These microbial rennets are sometimes known as vegetable rennets and can be used for making Kosher cheese.

To enable a rennet substitute to be used successfully in cheese it must possess the following characteristics: a high ratio of milk clotting-to-proteolytic activity; a milk clotting activity that is not very pH-dependent in the pH region of 6.0 - 7.0; a similar stability to calf rennet in the pH and temperature range used in the manufacture of cheese; low thermostability during whey processing; produce cheese that has the desired flavour, body and texture characteristics (Scott, 1973; Phelan, 1977).

A frequently used enzyme known by tradenames such as Rennilase (Novo Industrie, Bagsvaerd, Denmark), Fromase (Royal Gist Brocades), Hanilase or Marzyme (Miles Laboratories, Madison, Wisconsin, USA) is obtained from *Rhizomucor miehei*, a species of fungus (Foltmann, 1987). This enzyme has a ratio of milk clotting to proteolytic activity that is similar to chymosin and pepsin. However, the enzyme has a different specificity to chymosin or pepsin and produces a different array of peptides (Creamer, 1978b). The three forms of this enzyme are designated L (heat stable and more proteolytic than chymosin), TL (heat labile, more pH dependent and less proteolytic than the L form) and XL (more heat labile, more pH dependent and less proteolytic than the TL form).

An enzyme with similar activity to the *Rhizomucor meihei* enzyme is known as Emporase (Dairyland Food Laboratories, WI, USA), Meito (Meito Sangyo Co. Ltd, Japan) or Noury (Vitex, Paris, France) and is produced by *Rhizomucor pusillus* (Foltmann, 1987). In Cheddar cheese this enzyme has been shown to have a similar specificity to the *Rhizomucor miehei* proteinase. The specificity is different to that of either calf rennet or a 50:50 bovine pepsin: porcine pepsin blend (e.g. Phelan, 1985 as reported by Guinee and Wilkinson, 1992). An examination of Cheddar cheese (at 26 weeks) incorporating either of these microbial enzymes revealed more extensive degradation of β-casein than in cheese made with calf rennet (Phelan, 1985 as reported by Guinee and Wilkinson, 1992). Similar findings were reported by Creamer et al (1988b) who compared casein degradation in a pair of Cheddar cheeses made using either calf rennet or a microbial coagulant (Rennilase).

Surecurd (Pfizer) is a coagulant produced from *Cryphonectria parasitica* that may contain lipase as well as protease activity and is sometimes used in the manufacture of Cottage, Swiss and Mozzarella cheeses. It has been shown to be very proteolytic, hydrolysing α_{s1}-casein more rapidly than κ-casein in cheese-milk (Vanderpoorten and Weckx, 1972; Tam and Whitaker, 1972; Phelan, 1977), resulting in relatively high N losses to cheese whey (Emmons, 1990).

2.4.4 Plant rennet

A coagulant that has received recent attention is the extract of the artichoke thistle flower of *Cynara cardunculus* L, that is used in Portugal to make traditional ewe's milk cheese (Serra and Serpa) and is considered superior to rennet for this purpose (Louro Martins et al, 1996). The dried extract cleaves κ-casein at the Phe_{105} – Met_{106} bond and has similar milk clotting activity to liquid coagulants, but differences in the drying process are responsible for great variability between extracts (Louro Martins et al, 1996). The rennet contains proteinases with chymosin (cardocin A) and pepsin-like (cardocin B) specificities (Verissimo et al, 1995). Barbosa et al (1981) compared the extract with calf rennet powder for making Grana cheese, with kid rennet paste for making Provolone and with liquid calf rennet for making Bel Paese cheese. Proteolysis in the Grana and Provolone cheese was extensive despite the high cook temperature (53-55°C) and caused the cheese to lose shape during ripening. Bel Paese cheese was bitter in comparison with cheese made with calf rennet. Sousa and Malcata (1998) found the *Cynara cardunculus* extract less proteolytic than calf rennet in ovine raw milk cheeses.

2.5 INDIGENOUS MILK ENZYMES

Bovine milk contains ~60 indigenous milk proteinases (Fox and Stepaniak, 1993), with most present at very low concentrations and having little proteolytic activity (Fox and Morrissey, 1981). The most important of the proteinases are plasmin (e.g. Farkye and Fox, 1990, 1991, 1992; Farkye and Lankammer, 1992; Le Bars and Gripon, 1989a,b, 1993; Mulvihill and McCarthy, 1993; McSweeney et al, 1993c) and cathepsin D (McSweeney et al, 1995, Larsen et al 1996).

2.5.1 Plasmin

Plasmin (milk alkaline proteinase), a seryl proteinase, is the major indigenous proteolytic enzyme present in bovine milk. It has optimum activity at 37°C and pH 7.5-8.0 (Chen and Ledford, 1971; Kaminogawa et al, 1972). This enzyme is normally found in blood where it is responsible for solubilising fibrin clots.

Freshly secreted milk contains the complete plasminogen activation system (Heegaard et al, 1994), including plasminogen, plasmin, plasminogen activators (PAs), plasminogen activator inhibitors and plasmin inhibitors. The PAs are seryl proteinases that are very specific in their action, catalysing the hydrolysis of the Arg_{557} - Ile_{558} bond in bovine plasminogen (Schaller et al, 1985) to give active plasmin (Lu and Nielsen, 1993). In mammals, there are two types of PAs, tissue-type (tPA) and urokinase-type (uPA) that convert plasminogen to plasmin (Saksela, 1985; Bastian and Brown, 1996). Deharveng and Nielsen (1990) detected at least four PAs in bovine milk. Two of these were isolated and purified and were believed to be of the uPA type. The main plasmin inhibitor is thought to be α_2-antiplasmin (α_2-AP) (Wiman and Collen, 1978a,b; Christensen et al, 1995).

The ratio of plasminogen to plasmin in bovine milk is ~8:1 (Korycka-Dahl et al, 1983). Both plasminogen and plasmin are associated with the casein fraction of milk (de Rham and Andrews, 1982; Korycka-Dahl et al, 1983; Richardson, 1983a) and are incorporated in cheese curd. According to Richardson (1983b) the concentrations of plasmin and plasminogen are greatest in late lactation milk, presumably because of an increased permeability of the blood vessels in the mammary gland. Baldi et al (1996) found that the ratio of plasminogen to plasmin decreased and the amount of plasmin and plasminogen activators increased in late lactation milk, indicating increased conversion of plasminogen to plasmin. In milk, the plasmin concentration ranges between 0.14 and 0.73 $\mu g.mL^{-1}$ and plasminogen between 0.55 and 2.75 $\mu g.mL^{-1}$ (Richardson and Pearce, 1981).

Plasmin is denatured by heating and has a half-life of 45 s at 85°C and about 6.5 min at 72°C (Alichanidis et al, 1986). However, following pasteurization, there may be a small increase in plasmin activity in milk and this may be due the heat denaturation of a plasmin inhibitor (Noomen, 1975). Because plasmin, plasminogen and trypsin inhibitor have similar half-lives at 70°C (33.3 - 35.3 min), and there is an increase in the activation of plasminogen after pasteurization, Richardson (1983a) postulated that the increase in plasmin activity could be due to the destruction of an inhibitor of the plasminogen activator. This view remains current (Fig. 2.3).

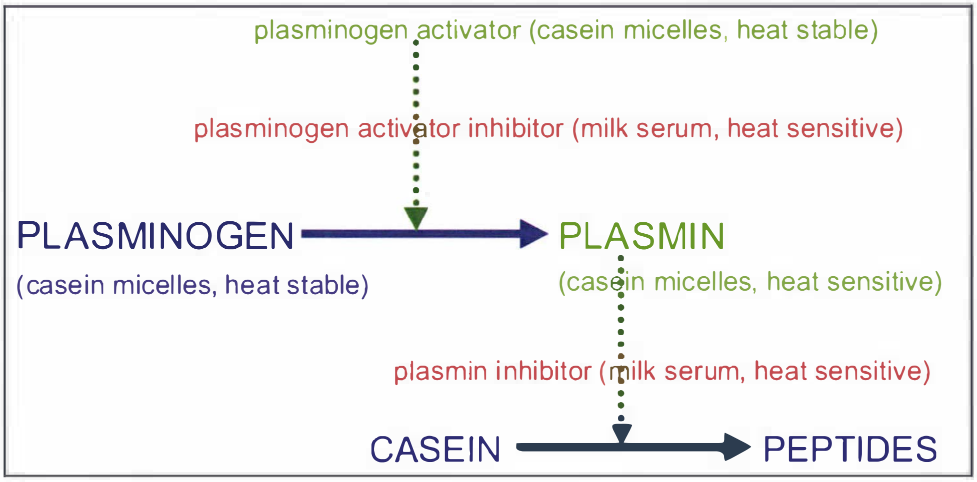


Fig. 2.3 *Activation of plasminogen to plasmin and the distribution of enzymes between the casein micelles and serum phase of milk.* Heat sensitivity and thermostability are included in brackets.

Plasmin has a specificity for Lys-X and Arg-X bonds (Weinstein and Doolittle, 1972) and in milk hydrolyses β-casein and α_{s2}-casein at approximately the same rate (Snoeren and van Riel, 1979; Richardson, 1983a), while α_{s1}-casein is hydrolysed more slowly (Chen and Ledford, 1971; Kaminogawa et al, 1972; Noomen, 1975; de Rham and Andrews, 1982) and κ-casein is more resistant to its action (Chen and Ledford, 1971).

2.5.1.1 Plasmin and the whey proteins

Both β-lactoglobulin and α-lactalbumin are resistant to plasmin. Heat denatured β-lactoglobulin has been shown to have an inhibitory effect on plasmin (Grufferty and Fox, 1986; Alichanidis et al, 1986). A possible mechanism for the inhibitory effect on plasmin activity may be interaction (via thiol-disulphide interchange) between the activated sulphydryl group of the denatured β-lactoglobulin and the disulphide bonds of plasminogen or plasmin (Alichanidis et al, 1986; Grufferty and Fox, 1988a; Benfeldt et al, 1997). Bastian et al (1993) showed that both native and denatured β-lactoglobulin inhibit the action of plasmin on casein especially when the reaction mixture was heated (60°C for 15 min). Politis et al (1993) demonstrated that β-lactoglobulin A, BSA and α-lactalbumin, at concentrations usually found in milk, had an inhibitory effect on plasmin activity whereas β-lactoglobulin B was a less potent inhibitor.

2.5.2 Cathepsin D

Cathepsin D is an indigenous acid (aspartyl) proteinase found in skim milk at a concentration of approximately 0.4 $\mu g.mL^{-1}$ (Larsen et al, 1996). The enzyme is mostly present as the inactive proenzyme, procathepsin D (Larsen et al, 1996). Its activity has been studied in solution (McSweeney et al, 1995; Larsen et al, 1996) and it has been demonstrated to have a similar specificity to chymosin. Cathepsin D is able to cleave GMP from κ-casein and cause milk to coagulate (Larsen et al, 1996).

2.6 PROTEOLYSIS IN CHEESE

During ripening, the caseins (α_{s1}-casein, β-casein, α_{s2}-casein, and para-κ-casein) that form the cheese matrix are gradually hydrolysed to smaller peptides and amino acids by proteinases, including the coagulant (chymosin, pepsin, microbial or fungal rennets, or plant extract from *Cynara cardunculus*), the indigenous milk enzymes including plasmin, Cathepsin D, and the proteinases and peptidases of the starter and non-starter lactic acid bacteria (NSLAB).

2.6.1 Rennet and proteolysis in cheese

Proteolysis of the caseins in cheese is often referred to as the tertiary phase of rennet action. The rennets are all acid proteinases and show specificity for peptide bonds to which hydrophobic residues supply the carboxyl group.

The amount of rennet retained in the curd varies with cheese type, and in Cheddar cheese it is usually about 6% of the initial amount added (Fox, 1989; Fox, Singh and McSweeney, 1994), while 15% has been reported for Gouda (Stadhouders and Hup, 1975). The rate and extent of rennet action depends on the initial rennet level, the cook temperature and the curd pH at draining (Stadhouders and Hup, 1975; Holmes et al, 1977; Creamer et al, 1985; Larsson and Andrén, 1997, Broome and Limsowtin, 1998), as well as the salt and moisture content of the curd, the final cheese pH (Watkinson et al, 1997), and the storage temperature of the cheese.

2.6.1.1 Chymosin action on α_{s1}-casein

During the first two months of Cheddar cheese maturation, ~80% of the α_{s1}-casein is hydrolysed (Johnston et al, 1994). The first α_{s1}-casein cleavage site is Phe_{23} - Phe_{24} and/or Phe_{24} -Val_{25} and results in two peptides, α_{s1}-casein f1-23 and α_{s1}-I-casein (f24/25-199) (Creamer and Richardson, 1974; McSweeney et al, 1993d) (Fig.2.4). Plasmin cleaves α_{s1}-casein at Arg_{22} - Phe_{23} (Le Bars and Gripon, 1993 (in solution)) and it is probable that the larger peptide formed has an electrophoretic mobility similar to that of α_{s1}-I-casein. The cleavage of α_{s1}-casein by chymosin is thought to be responsible for changes in cheese texture during the early stages of ripening (de Jong, 1976, 1977, 1978; Creamer and Olson, 1982; Johnston et al, 1994).

There is considerable variability in the rate of α_{s1}- and α_{s1}-I-casein hydrolysis in cheese, and some of this variability can be attributed to the effect of salt concentration. Thomas and Pearce (1981) examined proteolysis in commercially produced Cheddar cheese, after one month at 10°C, and found a linear relationship between salt concentration and the extent of proteolysis. At 4% salt-in-moisture (S/M), approximately 95% of the α_{s1}-casein was hydrolysed, but at 6% and 8% S/M, the amount of α_{s1}-casein that was hydrolysed was 70% and 40%, respectively. Kelly et al (1996) demonstrated that S/M influences the rate of formation of water-soluble nitrogen (WSN) and the susceptibility of some peptide bonds in Cheddar cheese. The Leu$_{101}$- Lys$_{102}$ bond in α_{s1}-casein was particularly sensitive to salt concentration.

Numerous peptides arising from α_{s1}-casein as the result of chymosin action have been identified in different cheese types by various authors (e.g. Singh et al, 1994, 1997; Breen et al, 1995; Gouldsworthy et al, 1996; Exterkate et al, 1997; Fernández et al, 1998; Mooney et al, 1998). The cleavage sites are shown in Fig. 2.4. Peptides produced by chymosin are often cleaved by plasmin, and the cell-envelope proteinases (CEP) and peptidases of the cheese microflora. These cleavage sites as well as some that have been identified but not attributed to a particular enzyme (but are often likely to be the result of aminopeptidase or carboxypeptidase activity) are included together for convenience.

NH$_2$-Arg-Pro-Lys🔵His-Pro-Ile-Lys🔵His🟢Gln🟢Gly^{10}⚫Leu-Pro-Gln🟢Glu🔴Val-Leu🟢Asn🟢Glu-Asn-Leu^{20}-
Leu🔴Arg🔵Phe🔴Phe🔴Val⚫Ala-Pro-Phe🔴Pro🔴Glu^{30}-Val🔴Phe🔴Gly🟢Lys🔵Glu🔴Lys🔵Val🟢Asn-Glu-Leu^{40}🔴
Ser-Lys-Asp-Ile-Gly-SerP-Glu-SerP-Thr-Glu^{50}-Asp-Gln-Ala-Met🔴Glu⚫Asp-Ile-Lys🔵Gln⚫Met^{60}🔴Glu-
Ala-Glu-SerP-Ile-SerP-SerP-SerP-Glu⚫Glu^{70}-Ile-Val-Pro-Asn🟢SerP-Val⚫Glu-Gln-Lys🔵His^{80}-Ile-Gln-
Lys-Glu🟢Asp-Val-Pro-Ser🟢Glu-Arg^{90}🔵Tyr⚫Leu⚫Gly-Tyr-Leu-Glu-Gln-Leu🔴Leu-Arg^{100}🔵Leu🔴Lys🔵
Lys🔵Tyr-Lys🔵Val-Pro-Gln-Leu🟣Glu^{110}-Ile-Val-Pro-Asn⚫SerP-Ala-Glu-Glu-Arg🔵Leu^{120}⚫His🟢Ser-Met-
Lys🔵Glu-Gly-Ile-His🔴Ala-Gln^{130}🟢Gln-Lys🔵Glu-Pro-Met-Ile-Gly-Val-Asn🟢Gln^{140}-Glu-Leu🔴Ala-Tyr-
Phe-Tyr-Pro-Glu🟢Leu🔴Phe^{150}🔴Arg🔵Gln-Phe🔴Tyr-Gln-Leu🔴Asp🟢Ala🔴Tyr🔴Pro^{160}-Ser🟢Gly-Ala-Trp-
Tyr🔴Tyr-Val-Pro-Leu🟢Gly^{170}-Thr-Gln-Tyr-Thr-Asp-Ala-Pro-Ser-Phe🔴Ser^{180}-Asp-Ile-Pro-Asn-Pro-
Ile⚫Gly⚫Ser-Glu-Asn^{190}🟢Ser🟢Glu-Lys🔵Thr-Thr-Met-Pro-Leu-Trp^{199}-COOH

Fig. 2.4 *Sequence of α_{S1}-casein B-8P (23614 Da) with identified cleavage sites marked.* Phosphoseryl (SerP) residues are highlighted in red. An additional phosphorylation site at position 41 (SerP) is present in the α_{S1}-casein B-9P. Cleavage sites indicative of chymosin (🔴), pepsin (🟣), plasmin (🔵), cell-envelope proteinase (🟢) or peptidase (⚫) action on α_{s1}-casein.

2.6.1.2 Chymosin action on β-casein

Chymosin action on β-casein is closely related to both pH and NaCl concentration (Fox and Walley, 1971; Mulvihill and Fox, 1978; Lawrence et al, 1987) and their effect on the conformation of the protein. The extent of salt induced aggregation of β-casein, and the consequent inaccessibility of the chymosin-sensitive bonds, increases as the salt in the moisture phase (S/M) increases and the water activity decreases (Creamer, 1976a). In solution and in cheese, β-casein (209 amino acid residues) has been shown to be hydrolysed by chymosin to β-I-casein (Arg_1 - Ala_{189}/Leu_{192}), β-II-casein (Arg_1 - Leu_{165}/Gln_{167}) and β-III-casein (Arg_1 - Leu_{139}) (Creamer et al, 1971; Pelissier et al, 1974; Creamer, 1976a; Visser and Slangen, 1977).

It is thought that the concentration of NaCl in most cheese varieties contributes to the inhibition of chymosin action on β-casein. However, even in the absence of NaCl the amount of β-casein degradation is small (Phelan et al, 1973). It is believed that only monomeric β-casein is susceptible to chymosin action (Creamer, 1976a) and that temperature-dependent hydrophobic association of β-casein may make potential cleavage sites inaccessible (Creamer, 1976a; Fox et al, 1993). One site in particular, Leu_{192} – Tyr_{193} is particularly sensitive to salt concentration (Kelly et al, 1996). Microbial rennets degrade α_{s1}- and β-casein in cheese at approximately the same rate (Creamer et al, 1988a,b; Phelan, 1985) and are less sensitive to NaCl (Phelan, 1985).

The cleavage sites identified by various authors (e.g. Singh et al, 1994, 1995, 1997; Breen et al, 1995; Gouldsworthy et al, 1996; Exterkate et al, 1997; Fernández et al, 1998; Mooney et al, 1998) as resulting from the action of chymosin on β-casein are summarised in Fig. 2.5. Plasmin and CEP cleavage sites as well as some cleavage sites likely to be the result of peptidase activity are included here for convenience.

NH$_2$-Arg-Glu-Leu-Glu-Glu-Leu🟢Asn🟢Val-Pro-Gly^{10}-Glu-Ile-Val-Glu-SerP⚫Leu🟢SerP-SerP⚫SerP⚫
Glu^{20}-Glu-Ser-Ile-Thr-Arg-Ile-Asn⚫Lys🔵Lys🔵Ile^{30}-Glu-Lys-Phe⚫Gln-SerP-Glu⚫Glu⚫Gln-Gln⚫Gln^{40}-
Thr-Glu-Asp🟢Glu⚫Leu🟢Gln🟢Asp-Lys-Ile-His^{50}-Pro-Phe🟢Ala-Gln🟢Thr-Gln🟢Ser🟢Leu🟢Val⚫Tyr^{60}-Pro-
Phe-Pro-Gly-Pro-Ile⚫Pro-Asn🟢Ser-Leu^{70}⚫Pro-Gln⚫Asn-Ile🟢Pro-Pro-Leu-Thr-Gln-Thr^{80}-Pro-Val-Val-
Val-Pro-Pro-Phe-Leu-Gln-Pro^{90}-Glu⚫Val⚫Met🟢Gly⚫Val⚫Ser⚫Lys🔵Val-Lys-Glu^{100}⚫Ala🟢Met-Ala-Pro-
Lys🔵His-Lys🔵Glu-Met-Pro^{110}-Phe-Pro-Lys🔵Tyr-Pro-Val-Glu-Pro-Phe-Thr^{120}-Glu-Ser-Gln-Ser-Leu-Thr-
Leu🔴Thr-Asp-Val^{130}-Glu-Asn-Leu-His-Leu-Pro-Leu-Pro-Leu🔴Leu^{140}-Gln-Ser-Trp-Met-His-Gln-Pro-His-
Gln-Pro^{150}-Leu🟢Pro-Pro-Thr-Val-Met-Phe-Pro-Pro-Gln^{160}🟢Ser-Val-Leu🔴Ser-Leu🔴Ser🟢Gln🔴Ser🟢Lys-
Val^{170}⚫Leu-Pro-Val-Pro-Gln🟢Lys🔵Ala-Val-Pro-Tyr^{180}-Pro-Gln🟢Arg🔵Asp-Met-Pro-Ile-Gln-Ala🔴Phe^{190}
🟢Leu🟢Leu🔴Tyr🟢Gln-Glu-Pro-Val-Leu-Gly-Pro^{200}-Val-Arg-Gly-Pro-Phe-Pro-Ile🟢Ile-Val^{209}-COOH

Fig. 2.5 *Sequence of β-casein A^2-5P (23982 Da) with identified cleavage sites marked.* His is substituted for Pro^{157} in the A^1 variant casein, Gln is substituted for His$_{106}$ in the A^3 variant casein, and His is substituted for Pro$_{67}$ and Arg for Ser$_{122}$ in the B variant casein. Phosphoseryl (SerP) residues are highlighted in red. Cleavage sites indicative of chymosin (🔴), plasmin (🔵), CEP (🟢) or peptidase (⚫) action on β-casein.

2.6.1.3 Chymosin action on α$_{s2}$-casein

The action of chymosin on bovine α$_{s2}$-casein in 100 mM sodium phosphate buffer, pH 6.5, at 30°C was investigated by McSweeney et al (1994a). Peptides soluble in sodium acetate buffer at pH 4.6 were isolated by RP-HPLC and identified from their amino acid sequence. The peptides identified were Tyr$_{89}$ - Tyr$_{95}$, Tyr$_{89}$ - Leu$_{99}$, Tyr$_{89}$ - ?, Leu$_{96}$ - ?, Tyr$_{98}$ - ?, Leu$_{99}$ - ?, Leu$_{164}$ - ?, Ala$_{175}$ - Tyr$_{179}$ and Ala$_{175}$ - ?. The primary cleavage site (in solution) was believed to be Phe$_{88}$ – Tyr$_{89}$. Although no large water-insoluble peptides have been identified in cheese (Mooney et al, 1998), several water-soluble peptides have been identified. The chymosin, plasmin, and CEP cleavage sites, as well as some cleavage sites likely to arise from peptidase action on peptides released by proteinases and reported by several authors (e.g. Singh et al, 1994, 1995, 1997; Breen et al, 1995; Fernández et al, 1998) are shown in Fig. 2.6.

NH$_2$-Lys-Asn-Thr-Met-Glu-His-Val-SerP-SerP-SerP^{10}-Glu-Glu-Ser-Ile-Ile-SerP-Gln-Glu-Thr-Tyr^{20}-
Lys🔵Gln-Glu-Lys🔵Asn-Met●Ala-Ile-Asn-Pro^{30}-Ser-Lys-Glu-Asn-Leu-Cys●Ser-Thr-Phe-Cys^{40}-Lys🔵Glu-
Val-Val-Arg-Asn-Ala-Asn-Glu-Glu^{50}-Glu-Tyr-Ser-Ile-Gly-SerP-SerP-SerP-Glu-Glu^{60}●SerP-Ala-Glu-
Val-Ala-Thr-Glu-Glu-Val-Lys^{70}🔵Ile●Thr-Val-Asp-Asp-Lys-His-Tyr-Gln🟢Lys^{80}-Ala-Leu-Asn-Glu-Ile-
Asn-Glu-Phe🟢Tyr-Gln^{90}-Lys-Phe-Pro-Gln-Tyr-Leu-Gln-Tyr-Leu-Tyr^{100}-Gln-Gly-Pro-Ile-Val-Leu-Asn-
Pro-Trp-Asp^{110}-Gln-Val-Lys-Arg🔵Asn🟢Ala-Val-Pro-Ile-Thr^{120}-Pro-Thr-Leu-Asn-Arg-Glu-Gln🟢Leu-
SerP-Thr^{130}-SerP-Glu-Glu-Asn-Ser-Lys-Lys-Thr-Val-Asp^{140}-Met-Glu-SerP-Thr-Glu-Val-Phe-Thr-Lys🔵
Lys^{150}🔵Thr-Lys-Leu-Thr-Glu-Glu-Glu-Lys-Asn-Arg^{160}-Leu-Asn-Phe-Leu-Lys-Lys🟢Ile-Ser-Gln-Arg^{170}-
Tyr-Gln-Lys-Phe🟢Ala●Leu-Pro-Gln🟢Tyr-Leu^{180}-Lys🔵Thr🟢Val-Tyr-Gln-His🟢Gln🟢Lys🔵Ala-Met^{190}●Lys-
Pro-Trp-Ile-Gln-Pro-Lys🔵Thr-Lys-Val^{200}-Ile-Pro-Tyr🟢Val●Arg-Tyr-Leu^{207}-COOH

Fig. 2.6 *Sequence of α_{S2}-casein A 11P (25229 Da) with identified cleavage sites marked.* Cleavage sites indicative of chymosin (🔴), plasmin (🔵), CEP (🟢) or peptidase (●) action on α_{s2}-casein.

The remaining milk proteins, para-κ-casein, β-lactoglobulin and α-lactalbumin (present at low levels in most cheese) are thought to be more resistant to rennet action.

2.6.2 Plasmin and proteolysis in cheese

Plasmin plays an important role in cheese ripening, and hydrolyses β-casein, α_{s2}-casein and α_{s1}-casein to a lesser extent. β-Casein is hydrolysed to the γ-caseins (β-casein f29-209 (γ_1-casein), β-casein f106-209 (γ_2-casein) and β-casein f108-209 (γ_3-casein)). Plasmin contributes to both the formation and degradation of water-soluble peptides in cheese (Farkye and Fox, 1991, 1992; Farkye and Landkammer, 1992). Figs. 2.4 - 2.6 show the cleavage sites for plasmin action on α_{s1}-, β- and α_{s2}-casein in cheese.

Plasmin, plasminogen and the plasminogen activator are associated with the casein micelles (de Rham and Andrews, 1982; Richardson, 1983a; Korycka-Dahl et al, 1983) and are incorporated into the cheese (Grufferty and Fox, 1988c), whereas, the plasmin inhibitors and the plasminogen activator inhibitors are found in the serum and are lost in the whey (Korycka-Dahl et al, 1983). The addition of 1 M NaCl to milk results in the release of plasmin from the casein micelles and it had been suggested that the method used to salt the cheese may affect the amount of plasmin incorporated in the cheese (Grufferty and Fox, 1988c). However, Farkye and Fox (1990) examined Cheddar and Emmental cheeses and found that the method of salting did not affect the retention and activity of plasmin. In the pH range 4.6 - 6.6, plasmin is associated with the casein micelles and it has been suggested that, within the normal pH range for draining the curd, plasmin retention in cheese is not affected by pH (Grufferty and Fox, 1988c).

The activity of plasmin in cheese is influenced by the heat treatment of either the cheese milk or the cheese curd and the final cheese pH. A range of cooking temperatures is used in the manufacture of the different varieties of cheese. Plasmin activity increases with increased cooking temperature within the range of 31-52°C in Cheddar cheese and 30-60°C in rennet curd (Farkye and Fox, 1990). In the same study it was shown that the amount of plasmin activity (7-amido-4-methyl coumarin (AMC) units/g cheese) in Emmental cheese (~52°C) was ~2.5 times greater than that in Cheddar cheese (~38°C) (Richardson and Pearce, 1981; Farkye and Fox, 1990) and that plasmin activity (AMC units / g cheese) in Cheshire cheese (~33°C) was less than in either Emmental or Cheddar cheese (Farkye and Fox, 1990). The values obtained for Gouda cheese were intermediate between Emmental and Cheddar cheese.

Farkye and Fox (1992) and Farkye and Landkammer (1992) showed that increasing the level of plasmin (approximately three-fold) in Cheddar cheese accelerated ripening, and although there was no significant difference in body, the cheese had consistently superior organoleptic quality (to the control cheese) with no trace of the bitterness that is often attributed to plasmin activity (Le Bars and Gripon, 1989b). An alternative approach has been to add urokinase to the cheese-milk. Bastian, Hansen and Brown (1991) used human urokinase to increase plasmin activity in ultrafiltered Havarti and Saint-Paulin cheeses and Bastian, Lo and David (1997) added urokinase to Swiss cheese to increase β-casein degradation and improve cheese quality.

In Cheddar cheese, with a pH of 4.95, proteolysis attributable to plasmin is slow. Thomas and Pearce (1981) in their experiment with Cheddar cheese with a NaCl gradient, found that 50% of the β-casein was hydrolysed when the S/M was 4%, but only 5% of the β-casein was hydrolysed when the S/M was 8%. The hydrolysis of β-casein was not attributed to either rennet or plasmin. Richardson and Pearce (1981) found a close relationship between the amount of β-casein hydrolysed and the amount of plasmin in Cheddar cheese. Noomen (1978) found that in simulated soft cheese the amount of plasmin activity was dependent on both pH and NaCl concentration. The degradation of β-casein was five to six times greater at pH 6.3 than at pH 5.4 after 28 days storage at 13°C, and although the addition of 2-4% S/M was found to increase plasmin activity, at higher concentrations of NaCl the activity of plasmin was reduced. At high salt concentration, providing the pH is sufficiently high (e.g. 6.3) plasmin is still active.

Reviews that include plasmin action in cheese have been written by Grappin et al (1985), Grufferty and Fox (1988a), Fox (1989), Fox (1991), Fox et al (1993), Fox and Stepaniak (1993), Visser (1993), Bastian and Brown (1996), and Grappin and Beuvier (1997).

2.6.3 Cathepsin D and proteolysis in cheese

Cathepsin D, an indigenous acid proteinase is present at low concentrations in milk and survives pasteurisation (Wium et al, 1998). It has a specificity similar to chymosin and may contribute to the formation of α_{s1}-I-casein (Kaminogawa et al. 1972, 1980; McSweeney et al, 1995; Lane et al, 1997). Other peptides produced by cathepsin D include β-I-, β-II- and β-III-casein, normally produced from β-casein by chymosin. According to McSweeney (1993), it is likely that cathepsin D will be active in the presence of normal levels of salt and over the pH range normally used in cheese. Wium et al (1998) reported cathepsin D-like activity in Feta cheese.

2.6.4 Microbial proteinases and peptidases

The proteinases and peptidases from starter species, starter adjuncts and non-starter lactic acid bacteria (NSLAB) play an important role in the hydrolysis of caseins and peptides in cheese (Exterkate et al, 1991, 1993, 1995; Law et al, 1993; Fox et al, 1994; Singh et al, 1994, 1995, 1997; McSweeney et al, 1993a; Lane and Fox, 1997; Fernández et al, 1998; and Lynch et al, 1999).

The cleavage sites on α_{s1}-, β- and α_{s2}-casein attributed to microbial proteinases and peptidases are shown in Figs. 2.4 – 2.6, respectively. Small peptides and amino acids produced by these enzymes may contribute directly to the flavour of cheese or they may be catabolised to form flavour components in cheese.

2.6.4.1 Starter Enzymes

The principal role of the lactic acid bacteria used as starters in cheese is to convert lactose to lactic acid. Two types of starter are used. Mesophilic starters (e.g. *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris* and *Leuconostoc* species), with an optimum temperature of ~30°C, are used in most common varieties of cheese (e.g. Cheddar, Gouda, Edam, Blue Vein and Camembert. Thermophilic starters (e.g. *Lactobacillus delbruekii* subsp. *bulgaricus*, *Lactobacillus helveticus* and *Streptococcus thermophilus)*, with an optimum growth temperature of ~45°C, are used in the manufacture of Swiss and Italian cheeses which are cooked at much higher temperatures (50-55°C).

Starter bacteria can be divided into two categories on the basis of their ability to coagulate milk at 22°C in 24 hours. Those strains able to coagulate milk in 24 hours are designated proteinase-positive (Prt^+) (Pearce et al, 1974). The remainder lack the plasmid that encodes the proteinase gene(s) and are designated proteinase-negative (Prt^-). Cheese made using Prt^- strains contains only small quantities of small peptides and amino acids because the growth rate of the starter is limited by the quantities of peptide and amino acid substrates produced by rennet and plasmin, whereas cheese made using Prt^+ starters contains greater quantities of pepides and amino acids and these contribute to the flavour intensity and acceptability of the cheese (Lane and Fox, 1997).

2.6.4.1.1 Proteinases

The starter proteinases are attached to the cell wall (cell-envelope proteinases) and are weakly proteolytic. The three types of proteinases have been given the designations P_I-, P_{II}- and P_{III}-type proteinases (Visser et al, 1986). The P_I-type proteinase acts preferentially on β-casein and produces peptides with similar electrophoretic mobilities to those produced by chymosin. This proteinase has also been shown to break down κ-casein and to a lesser extent α_{s1}-casein (Visser, 1993). The P_{II}-type proteinase is a heat labile form of the P_I-type proteinase. The P_{III}-type proteinase acts preferentially on β-casein (but with different specificity to the P_I-type enzyme) and produces peptides with a similar electrophoretic mobility to the γ-caseins produced by the action of plasmin on β-casein (Visser et al, 1986). The proteinase also hydrolyses κ- and α_{s1}-casein. Some strains of *L. lactis* have either the P_I-type proteinase or the P_{III}-type proteinase, but others have mixed P_I-type and P_{III}-type proteinases. Neither of the proteinase types can produce α_{s1}-I-casein (Visser, 1993) but both are able to hydrolyse α_{s1}-casein f1-23 (Exterkate et al, 1991).

2.6.4.1.2 Peptidases

The starter cells possess a range of intracellular peptidases that can be divided into endopeptidases and exopeptidases. Several endopeptidases ($PepO_1$, $PepO_2$, $PepF_1$, $PepF_2$) have been identified (Yan et al, 1987a,b; Tan et al, 1991), and none are able to hydrolyse intact casein. A range of exopeptidases has been identified. These peptidases include four aminopeptidases (PepN, PepA, PepC and PepP (a proline aminopeptidase), an iminopeptidase (PepI that release N-terminal proline), two prolyl dipeptidases (prolinase (PepR) and prolidase (PepQ)), an X-prolyl dipeptidyl aminopeptidase (PepX), a pyrrolidine carboxylyl peptidase (PCP), a tripeptidase (PepT) and several dipeptidases (a general peptidase (PepV) and a peptidase that hydrolyses di- and tri-peptides with Leu at the N-terminal (PepL) (Exterkate and Stadhouders, 1971; Mou et al, 1975; Exterkate, 1977; Schmidt et al, 1977; Law, 1979b; Kaminogawa et al, 1984; Kolstad and Law, 1985; Thomas and Pritchard, 1987; Kok, 1990; Booth et al, 1990; Meyer and Spahni, 1998. Caseins are rich in proline and bonds containing proline are resistant to most proteinases and peptidases. Those exopeptidases showing specificity for these bonds are therefore important for the degradation of peptides in cheese.

2.6.4.2 Enzymes of secondary microflora

There are several varieties of cheese which owe their characteristic flavour and texture to the presence of secondary microflora.

Propionibacteria (*Propionibacterium shermanii*), added with the starter culture in Emmental and Gruyère cheese, grow only after the cheese has been manufactured. They are only weakly proteolytic and their peptidases (PepN and PepX) contribute to proteolysis by releasing proline in particular (Langsrud et al, 1977, 1978).

Cheeses such as Danbo and Limburg are colonized at their surface by *Brevibacterium linens* and related coryneforms which contribute to their characteristic flavour. These organisms possess a range of proteinases (Friedman et al, 1953) and peptidases (Sorhaug 1981). The major proteinase is a serine proteinase which is active at the surface of smear-ripened cheeses. It hydrolyses both α_{s1}- and β-casein (Rattray et al, 1996, 1997). *B. linens* possesses both intracellular and extracellular peptidases which hydrolyse a range of dipeptide substrates (Law, 1987). The catabolism of amino acids by *B. linens* also contributes to the flavour of these cheeses.

Penicillium candidum or *Pe. camemberti* in Camembert and Brie cheeses and *Pe. roqueforti* in blue cheese possess highly proteolytic enzymes. These organisms secrete metallo- and aspartyl proteinases that hydrolyse α_{s1}- and β-casein (Gripon, 1993).

2.6.4.3 Enzymes of non-starter lactic acid bacteria

The non-starter lactic acid bacteria (NSLAB) in cheese are mostly lactobacilli although pediococci and micrococci are also present and can contribute, to a lesser extent, to cheese ripening (Fryer, 1969; Chapman and Sharpe, 1981). The predominant species are *Lactobacillus paracasei* subsp. *paracasei, Lactobacillus rhamnosus*, *Lactobacillus casei*, *Lactobacillus plantarum* and *Lactobacillus brevis*. The numbers of these adventitious bacteria in cheese are affected by the numbers in raw milk (Peterson and Marshall, 1990) and the extent of post-pasteurization contamination (Thomas, 1986). Typically their numbers are in the range of $10\text{-}10^4$/g in 10 day old Cheddar cheese, $10^6\text{-}10^7$/g in 20 day old cheese and plateau at 10^6 to 10^8/g between two and nine months of age (Naylor and Sharpe, 1958a,b; Martley and Crow, 1993; Fox et al, 1998). They are the dominant flora in ripening Cheddar cheese after three months and possess a range of enzymes that produce an array of peptide and amino acids that contribute to cheese flavour. There are numerous strains of NSLAB and while some enhance Cheddar flavour and have been selected as adjunct cultures, others are responsible for flavour defects including bitterness. The enzymes of the *L. casei* include extracellular and intracellular proteinases and peptidases with dipeptidase, tripeptidase, carboxypeptidase, aminopeptidase (leucine, valine and cysteine aminopeptidases) (Requena et al, 1991) and endopeptidase activities (El Soda et al, 1978).

L. plantarum possesses intracellular aminopeptidase and dipeptidase activities and lacks endopeptidase and carboxypeptidase activities (El Soda et al, 1983).

2.7 METHODS FOR THE ASSESSMENT OF PROTEOLYSIS IN CHEESE

Many methods are used to analyse the products of proteolysis in cheese and these have been the subject of several reviews (Bulletin of the International Dairy Federation No 261, 1991; Fox, McSweeney and Singh, 1995; McSweeney and Fox, 1997; Bulletin of the International Dairy Federation No 337, 1999). The simplest methods involve measuring the (Kjeldahl) nitrogen content of various cheese fractions, with non-casein nitrogen (NCN), non-protein nitrogen (NPN) and water-soluble nitrogen (WSN) being the most commonly used. Alkaline urea-polyacrylamide gel electrophoresis (urea-PAGE) and, more recently, reverse phase-high performance liquid chromatography (RP-HPLC) are the two methods most commonly employed to monitor the hydrolysis of casein and the formation of peptides by the enzymes in the cheese during ripening. There has been interest in the application of these methods for classification of cheese (e.g. Marcos et al, 1979; Haasnoot et al. 1989; Fox, 1993).

2.7.1 NCN, NPN and WSN

NCN provides a measure of the N soluble at pH 4.6 and is used to provide information on overall trends in proteolysis (e.g. Dahlberg and Kosikowski, 1947; Vakaleris and Price, 1959; Vakaleris et al, 1960; O'Keeffe et al, 1978; Creamer and Olson, 1982; Johnston et al, 1994). This fraction contains peptides of medium to low molecular weight, amino acids, proteose peptones, as well as whey proteins (Kuchroo and Fox, 1982a; Christensen et al, 1989).

NPN provides a measure of the extent of proteolysis in cheese. Measurement of NPN involves solubilisation of the cheese proteins and peptides, followed by TCA precipitation of the proteins and some peptides and determination of the amount of soluble N. This fraction contains peptides with less than 20 amino acids and free amino acids (Yvon et al, 1989). Different concentrations of TCA have been used varying from 2% (Kuchroo & Fox, 1982a), 2.5% (Reville & Fox, 1978), to 12% (Vakaleris and Price, 1959; Kuchroo and Fox, 1982a; Reville and Fox, 1978; Bican and Spahni, 1991; Bütikofer et al, 1993).

WSN provides an overall assessment of proteolysis in cheese. The water-soluble fraction (WSF) of Cheddar cheese contains peptides, amino acids and their degradation products, organic acids and their salts, and small quantities of whey proteins (McSweeney and Fox, 1997) and has typical Cheddar flavour (Aston and Creamer, 1986). The flavour of several cheese types was found to reside in the low molecular weight fraction (<500 Da) of the water-soluble fraction (WSF) (Engels and Visser, 1994). The WSN was found to be related to cheese maturity (Barlow et al, 1986; Farkye and Landkammer, 1992). Lopez-Fandiño et al (1994) found that the combination of pH, TN, WSN, trichloroacetic acid-soluble nitrogen (TCA-N), phosphotungstic acid-soluble nitrogen (PTA-N), urea-PAGE and SDS-PAGE was useful for distinguishing the most from the least mature cheese.

2.7.2 Alkaline urea-PAGE of the urea-soluble fraction of cheese

Urea-PAGE separates the proteins and peptides in cheese on the basis of both charge and size. The method used in New Zealand is a combination of the Davis (1964) and Ornstein (1964) system and the discontinuous system that is based on the methods of Peterson (1963), Thompson et al (1964) and Peacock et al (1965) and has a stacking and a resolving gel. Sample analysis is relatively fast and provides good resolution of the caseins and larger peptides. Variations of the method have been widely used to monitor the hydrolysis of the caseins and the formation of the largest of the breakdown products (α_{s1}-casein-I, derived from α_{s1}-casein by rennet action; the γ-caseins, derived from β-casein by plasmin action; and β-I, β-II- and β-III-casein, derived from β-casein by rennet action) during cheese maturation (e.g. Ledford et al, 1966; Edwards and Kosikowski, 1969; Creamer, 1970, 1976a; Phelan et al, 1973; Creamer and Richardson, 1974; Creamer and Olson, 1982; Shalabi and Fox, 1987; Wilkinson et al, 1992; McSweeney et al, 1994b; Johnston et al, 1994; Watkinson et al, 1997; Sousa and Malcata, 1998). These as well as many of the previously unidentified peptides in the water-insoluble fraction (WISF) of cheese were identified by McSweeney et al (1994b) and Mooney et al (1998).

Urea-PAGE has also been used to demonstrate differences in proteolysis between cheese types (e.g. Ledford et al, 1966; Marcos et al, 1979; Creamer, 1991; Fox, 1993). The ratio of γ- to β-casein has been proposed as a possible means of cheese classification by Fox (1993), as well as an indicator of the maturity of Gouda (Haasnoot et al, 1989) or Parmesan cheese (Mayer, 1997). Haasnoot et al (1989), recognising the importance of chymosin to primary proteolysis and cheese differentiation, suggested that the ratio of α_{s1}- to α_{s1}-I-casein was a better indicator of maturity in very young cheese.

2.7.3 Alkaline urea-PAGE of the water-soluble fraction of cheese

Urea-PAGE has been used to examine the peptides in the water-soluble fraction (WSF) of cheese (Law et al, 1993; McSweeney et al, 1993a; Singh et al, 1995), but these may be difficult to identify with reliability, particularly when cheese types are compared. Fox (1993) believed that analysis of the WSF by urea-PAGE was more discriminating than analysis of the whole cheese and could be used to distinguish cheese varieties, but has only applied subjectivity to the judgement.

2.7.4 Reverse phase-HPLC of the water-soluble fraction of cheese

RP-HPLC has been used by many researchers (e.g. Pham and Nakai, 1984; Aston et al, 1986; Cliffe and Law, 1991; Gonzalez de Llano et al, 1991) to separate the peptides in cheese or to monitor the appearance and disappearance of peptides during cheese maturation. More recently RP-HPLC has been coupled with mass spectrometry to identify the peptides in cheese (e.g. McSweeney et al, 1994b; Singh et al, 1994, 1995, 1997; Gouldsworthy et al, 1996; Ferranti et al, 1997; Alli et al, 1998), and this is usually done to elicit information on the pathway of proteolysis in the cheese.

Variations of the RP-HPLC method have been used to group cheese according to broad stages of maturity (e.g. Pham and Nakai, 1984; Furtula et al, 1994b; Bütikofer et al, 1998); and to classify cheese according to type. Smith and Nakai (1990) used a C8 RP-HPLC column to analyse the WSF of samples of Cheddar, Edam, Gouda, Swiss and Parmesan cheeses. Multivariate statistical analysis was used to demonstrate differences between the samples (discussed below). Fox (1993) reported distinctive RP-HPLC profiles for samples of Cheddar, Emmental, Gouda, Parmesan, Brie and Appenzeller cheese. Fox (1993), showed intravarietal differences between Cheddar cheeses of the same age, but made with different starter types, while Pannell and Olson (1991) showed differences between Cheddar cheeses of the same age and made at the same factory with the same starters. Gonzalez de Llano et al (1995) used RP-HPLC to demonstrate that the WSF of each of several types of artesanal cheese from Asturias was characteristic of the cheese type and changed throughout the maturation period of 60 d.

2.7.5 Size-exclusion chromatography

Size-exclusion chromatography (SEC) has been widely used, particularly in the analysis of whey protein hydrolysates, where the demonstration of proteolysis is important because it equates with enhanced nutritional value, loss of allergenicity or improved functionality. There are several types of column, ranging from low pressure Sephadex columns (Pharmacia, now Amersham Biosciences, Uppsala, Sweden) to the higher-pressure columns such as the TSK G-2000 SW (Toso-Haas, Japan) and Superdex-peptide (Pharmacia) columns used today. SEC separates proteins and peptides mainly on the basis of the hydrodynamic size of the molecule in the particular solvent used.

The main cheese-related application of SEC has been the fractionation of cheese extracts for further analysis (e.g. Singh et al, 1994; Breen et al, 1995; Fernández et al, 1998). Haasnoot et al (1989), in a search for methods suitable for the objective assessment of the maturity of Gouda cheese, examined SE-HPLC using a TSK G-3000SW and G-2000SW in series with a solvent comprised of

0.1 M sodium sulphate and 0.01 M sodium dihydrogenphosphate (pH 6.8) and reported that the method was unsuitable for examining the WSF of cheese.

Changes in peptide profiles during ripening were demonstrated by Wilkinson et al (1992) who used an FPLC Superose-12HR column (Pharmacia) to study the effects of commercial enzymes on proteolysis and ripening in Cheddar cheese. The Superose-12HR column was also found useful for characterising peptides in Cheddar cheese fractions by Breen et al (1995). Cliffe et al (1993), using a combination of SE-HPLC (with a Sephadex G-25 Superfine HPLC column and water as the eluent) and RP-HPLC, demonstrated that the higher molecular weight fractions obtained by SE-HPLC contained bitter peptides and corresponded to the later-eluting peaks on the RP-HPLC traces, whereas the lower molecular weight fractions corresponded to the early-eluting peaks.

2.8 OBJECTIVE ASSESSMENT OF ANALYTICAL RESULTS

The results obtained from the analysis of proteolysis can be complex and it is difficult to differentiate one cheese type from another or to determine the maturity of a range of cheeses. One way of doing this objectively is to analyse the data obtained from each analysis using multivariate statistics. Linear discriminant analysis (DA) and principal component analysis (PCA) are the two main types of analysis that have been used, either separately or in conjunction with one another.

2.8.1 Discriminant analysis

DA has been used to differentiate cheese and is particularly useful in binary situations, for distinguishing good from bad, young from old *etc*. In this type of analysis, it is usual for two to four categorical groups to be predicted by a number of independent variables that should be uncorrelated to achieve successful classification (Hair et al, 1998).

Pham and Nakai (1984) used stepwise DA of RP-HPLC (WSF) peak data (13 peaks from a C8 column) from 41 Cheddar cheese samples to categorise the cheese into mild, medium, old and extra-old maturity groups, but not every cheese in each category matched the maturity stated on the label.

Others used DA of data from different physico-chemical parameters to assign cheese to a small number of categories. Those who have used this approach to group cheese on the basis of maturity include: Santa-Maria et al (1986), who successfully classified Manchego cheese into fresh, medium ripe and aged categories using results from the analysis of various nitrogen fractions (TN, WSN, NPN, peptide N, amino acid N, Tyr and Trp, β-, α_{s1}- and α_{s1}-I-casein); and Alonso et al (1987), who distinguished fresh from frozen Cabrales cheese using results from a large number of physico-chemical characteristics (pH, fat, total solids, ash, NaCl), proteolysis (TN, WSN, NPN, free amino acids, casein N, γ-, β-, α_{s1}- and α_{s1}-I-casein) and lipolysis (4 indices of lipolysis as well as butyric and caproic acid).

DA of data from various physico-chemical parameters has also been used to determine cheese variety. For example, Millán et al (1996a), used results from chemical analyses (WSN, NPN, ammonia N, a_w moisture, fat, salt and pH), to classify 7 varieties of Spanish cheeses from the Canary Islands, with limited success and Millán et al (1996b), correctly classified 10 Spanish cheese types (8 samples of each type) using results from chemical analyses (NPN, ammonia N, pH, NaCl and moisture content) that were statistically selected from a larger set of variables that also included (TN, a_w and fat content).

2.8.2 Principal component analysis

Principal component (classification) analysis (PCA) is a powerful statistical tool that has been widely used to interpret large multivariate data sets. It provides the means to reduce the large number of interdependent variables (e.g. peaks or chemical results) represented in the original data set to a few (uncorrelated) independent variables or principal components that explain most of the variation in the data, and can be used to highlight the presence or absence of relationships between variables and cases. PCA is sometimes used to remove correlations between variables prior to using other forms of statistical analysis such as cluster analysis (CA), DA, or principal component similarity (PCS) for classification purposes, or multiple linear regression analysis (MLR) or partial least squares regression (PLS) to determine relationships.

PCA was first applied to cheese proteolysis by Amantea and Nakai (1984), who successfully differentiated first grade from downgraded cheese samples using cheese grader sensory assessment and RP-HPLC (WSF) peak data.

2.8.2.1 Differentiation of cheese maturity

PCA has been used to demonstrate differences in the maturation (RP-HPLC peptide profiles) of cheeses that have been made using different manufacturing processes. Examples include: Amantea et al (1995) who used PCA of RP-HPLC (WSF) peak area data to demonstrate the effects of accelerated ripening on the maturity of Cheddar cheese; Furtula et al (1994a) who used PCA of RP-HPLC peak area data followed by PCS to demonstrate differences in the ageing profiles of standard Cheddar cheese and cheese incorporating enzyme extracts of lactic bacteria; Furtula et al (1994b) who used PCA of RP-HPLC peak area data followed by PCS to broadly group Cheddar cheese into 3 m (mild), 6 m (medium), 9 m (sharp) and 12 m (extra-sharp) categories and to compare it with cheese that had been abused and rapidly aged under abnormal conditions; and Bütikofer et al (1998) who used PCA of the RP-HPLC peak height data to compare the maturation of Emmental cheese made with and without the addition of *Lactococcus casei* subsp. *casei* and sampled after 3, 6, 9 and 12 months. The first three principal components explained 89% of the variation in the data. The first principal component was associated with cheese age, although this was not straightforward. The fourth principal component accounted for 2% of the variation in the data, but more clearly differentiated the two treatments.

Classification of Cheddar cheese on the basis of maturity and quality was also attempted by O'Shea et al (1996). These authors used RP-HPLC to analyse the retentate (>10000 Da) and permeate (<10000 Da) of the WSF of 60 Cheddar cheese samples that varied in 'age', and quality as determined by cheese grader. PCA was used to analyse the peak area data from the retentate or permeate. Between 82-84% of the variability in the data was explained using 19 or 20 principal components, with PC1 accounting for 32.4 and 20.7% of the variation in the data from analysis of the retentate and permeate, respectively. DA of the principal component scores was used to group the cheese on the basis of 'age' (mild, mature or extra-mature) flavour (defective or non-defective) and texture (good body or weak body), as determined by the cheese grader. RP-HPLC discriminated defective from non-defective cheese, but provided poor differentiation of maturity, with 33% or 48% of samples correctly classified using the permeate and retentate, respectively. (The RP-HPLC traces depicted in the paper show inconsistent matching of the peaks, and it is possible that this contributed to the poor differentiation of maturity). These authors demonstrated that DA of the total free amino acid concentration was more effective than RP-HPLC for differentiating between mild, mature and extra-mature cheese, with an average of 70% of the cheeses correctly classified, although the value for mature cheese was only 20%.

Differences in free amino acid concentration also provided the basis for differentiation of four types of Mahón cheese at different stages of maturity and made from raw or pasteurised milk at different factories (Frau et al, 1997). PCA was used to analyse the data and group the cheese into fresh, half-ripened, ripened and old-ripened categories and to determine whether it was made from raw or pasteurised cheese milk, but there was considerable overlap of samples from each category and cheese source.

In a different approach, García Ruiz et al (1998) used multivariate statistical analysis, including MLR and PCR, to correlate instrumental results (water activity, pH, WSN/TN, PTA-N/TN and PAGE (α_{s1}-, β- and γ-casein) with the ripening time of Manchego cheese. The resultant regression equations were cross-validated by using them to assign 8 commercial samples of Manchego cheese to one of the three broad age-based categories (soft-ripened (2.5 - 3 m), semi-hard (3 - 5m), hard (> 5 m) used to define cheese maturity.

The diversity of medium, mature, extra-mature and vintage Cheddar cheese of factory or farmhouse origin and from several countries was studied by Muir et al (1997). PCA was used to analyse the sensory data (with flavour and texture characteristics considered separately) and PLS regression was used to relate the first three principal components to various physico-chemical results (pH, salt in moisture (S/M), fat in dry matter (FDM), moisture in non-fat solids (MNFS), TCA-N and sulfosalycilic acid-soluble nitrogen (SSA-N) and to model the relationship. PCA enabled raw milk cheeses to be differentiated from pasteurised milk cheeses, but the flavour attributes of the factory-made cheeses were similar. The best of the models was not considered to have strong predictive value.

The relationship between the volatile components and sensory attributes of Cheddar cheese during ripening was examined by Banks et al (1995). PCA was used to analyse data from measurements of the concentrations of volatile components (72% of the variance was explained by four principal components) and then to separately analyse the sensory data (84% of the variance was explained by four principal components) measured during the ripening of 4 commercial and 4 experimental Cheddar cheeses. PCR was used to demonstrate the close correlation between the principal component scores of the volatile components and three sensory attributes (flavour intensity, and acid and bitter flavours, and equations were constructed

PCA and PLS were used to evaluate the relationships between sensory textural data and instrumental data from the analysis of Appenzeller and Parmigiano Reggiano cheese (Noël et al, 1998). The instrumental data included pH, dry matter, NaCl, Ca, ash (Ca + NaCl), fat, TN, casein N (TN - pH 4.4 SN), larger peptide N (pH 4.4 SN), smaller peptide N (TCA-N), and amino acid N (PTA-N), β-casein, α_{s1}-casein, α_{s1}-I-casein, γ-casein and β-I-casein, RP-HPLC peak data plus each of the amino acids. The composition of free amino acids and the RP-HPLC peaks were the most promising tools for predicting sensory texture.

MLR and PCA with PCR were used to demonstrate the influence of particular compositional variables on the rheological properties (fracture stress, fracture strain and modulus of deformability) of Emmental cheese (Rohm et al, 1992). Both forms of analysis provided essentially the same result, but the authors expressed a preference for PCR because it eliminates correlations between independent variables.

2.8.2.2 Differentiation of cheese type

PCA has also been used to differentiate cheese varieties or cheese within a variety using data from various types of analyses. The most well known work of this type is that of Smith and Nakai (1990), who used a combination of PCA and DA to analyse RP-HPLC (required four C8 columns) peak area data from 106 random samples of Cheddar, Edam, Gouda, Swiss or Parmesan cheeses, in order to classify the cheese according to type. PCA reduced the data from 55 peaks to 17 principal components with Eigenvalues of greater than 1.0 (a common statistical cut-off point) that explained 74% (88 - 99% if data from individual columns was analysed separately) of the variation in the data. The proportion of variance explained by any one principal component was no greater than 13%. DA of the 17 principal components provided correct classification of 64% of the samples. This was improved to >90% if data from individual columns was analysed separately.

Four types of Belgian cheese sampled at different stages of maturity were differentiated using results from SDS-PAGE (Phast system) of the pH 4.6-insoluble protein fraction of the cheese (Dewettinck et al, 1997). PCA of the data gave correct classification of two types and less clear differentiation of the other two, while DA gave correct classification of the four types. There was no differentiation of maturity using either method of data analysis.

It appears that these modern multivariate statistical techniques provide the possibility of analysing a greater range of data so that cheese can be assessed more objectively than relying on grade scores, NPN/TN, the ratio of γ- to β-casein or the ratio of α_{s1}- to α_{s1}-I-casein.

3.0 OBJECTIVES

The focus of this study is on proteolysis in different cheeses during maturation, with the purposes of developing and testing methods for the objective differentiation of cheese types at all stages of maturity and the accurate estimation of cheese maturity within a variety.

At the outset, it seems likely that the chromatograms provided by RP-HPLC would be too complex, but that traditional methods for the determination of nitrogen in some cheese fraction (eg. NPN) would be too simplistic. However, a combination of the quantities of nitrogen measured in several different cheese fractions may be useful, and alkaline urea-PAGE may have some potential for differentiating cheese type. Using a combination of methods could be cumbersome and a single methodology would be advantageous. One possibility is a SE-HPLC method based on a procedure used to monitor the progressive hydrolysis of whey proteins. It seems likely that this method could be developed and used to separate the nitrogenous components of cheese into a number of measurable fractions (ie. chromatographic peak areas) and therefore has potential for differentiating both cheese type and maturity.

Therefore, the first objective of this study is to develop a SE-HPLC method for the analysis of cheese.

The second objective is to examine the potential of this SE-HPLC method for determining differences in proteolysis in a range of diverse cheese types.

A third objective is to compare the SE-HPLC results with those obtained using RP-HPLC (of the WSF) and urea-PAGE (of the USF and WSF) to analyse the same cheeses.

The fourth objective is to compare four potential methods (RP-HPLC, SE-HPLC and/or urea-PAGE of the USF and/or WSF) in a more in-depth study of the differentiation of several cheese types during 6 months maturation and to use appropriate statistical methods to analyse the data objectively.

The final objective is to examine one cheese variety in detail using the most appropriate methods to determine the natural variability of the cheese and the likely accuracy of estimating cheese sensory maturity by instrumental means.

4.0 MATERIALS AND METHODS

4.1 MATERIALS

4.1.1 Commercial cheese supplied as mature and "ready to eat" (Ch. 6)

Cheeses supplied by Mainland Products Ltd, New Zealand were Swiss at 4 months old, Elsberg (Jarlsberg-type) at 4 months old, Gouda at 2 months old, Feta at 2 months old and Blue at 2 months old. Those supplied by Kiwi Co-operative Dairies, New Zealand were Cheddar at 8.5 months old and Mozzarella at 2 weeks old.

The cheese were collected and stored at -85°C until they were analysed.

4.1.2 Commercial cheese and cheese manufactured at the NZDRI (Ch. 7)

The cheeses were: New Zealand-style Swiss, Elsberg (Jarlsberg-type), and Gouda cheese (20 - 40 kg of each type) supplied immediately following manufacture by Mainland Products Ltd, New Zealand; and Cheddar and Mozzarella (20 kg of each) manufactured at the New Zealand Dairy Research Institute (NZDRI).

4.1.3 Commercial Cheddar cheese (Ch. 8)

The cheeses were: New Zealand Cheddar cheese (77 samples) from 8 cheese factories that had been ripened at 5, 10 or 13°C for 3, 6, 12 or 15 months.

4.1.4 Polyacrylamide Gel Electrophoresis (urea-PAGE and SDS-PAGE)

The reagents were: Acrylamide/Bis mixture (37.5:1, 2.6% C), TEMED (tetramethylethylenediamine) and ammonium persulphate supplied by BioRad Laboratories, Hercules, California; Bromophenol Blue, 2-mercaptoethanol, Tris HCl (tris (hydroxymethyl)aminomethane), urea, EDTA, boric acid, sodium dodecyl sulphate, hydrochloric acid, glycerol, glacial acetic acid and isopropanol supplied by BDH Laboratory Supplies, Poole, England; Coomassie Brilliant Blue R250 and glycine supplied by Sigma Chemical Co, St Louis, MO, USA; and deionised and distilled water (Milli-Q).

The standards were: skim milk supplied by Tararua Milk Co., Palmerston North, New Zealand; and rennet casein supplied by Anchor Products, Hautapu, New Zealand.

4.1.5 Size-Exclusion and Reverse Phase High Performance Liquid Chromatography

The reagents were: acetonitrile ('Far UV' grade) supplied by BDH Laboratory Supplies, Poole, England and filtered through a 0.45 µm filter (Millipore, Millipore Corporation, Bedford, MA, USA) before use; trifluoroacetic acid ('Far UV' grade) and the molecular weight standards supplied by Sigma Chemical Co, St Louis, MO, USA; and deionised water that was filtered through a 0.45 µm filter (Millipore).

The molecular weight standards supplied by Sigma Chemical Company were:

Glyceraldehyde-3-phosphate dehydrogenase	MW	36000 Da
Carbonic anhydrase	MW	29000 Da
Soybean trypsin inhibitor	MW	20100 Da
Lysozyme	MW	14300 Da
Aprotinin	MW	6500 Da
Insulin	MW	5730 Da
Insulin B-chain	MW	3400 Da
Bacitracin	MW	1420 Da
Phenylalanine	MW	165 Da

The amino acid, and di- tri-, tetra- and pentapeptide standards supplied by Sigma Chemical Company were:

Lysine (Lys)	MW	182.7 Da (Lys HCl)	(Lys MW 146.2 Da)
Arginine (Arg)	MW	210.7 Da (Arg HCl)	(Arg MW 174.2 Da)
Histidine (His)	MW	209.6 Da (His HCl H_2O)	(His MW 155.2 Da)
Leucine (Leu)	MW	131.2 Da	
Isoleucine (Ile)	MW	131.2 Da	
Phenylalanine (Phe)	MW	165.2 Da	
Tryptophan (Trp)	MW	204.2 Da	
Valine (Val)	MW	117.1 Da	
Methionine (Met)	MW	149.2 Da	
Alanine (Ala)	MW	89.09 Da	
Proline (Pro)	MW	115.1 Da	
Glutamic acid (Glu)	MW	147.1 Da	
Aspartic acid (Asp)	MW	133.1 Da	
Tyrosine (Tyr)	MW	181.2 Da	
Cysteine (Cys)	MW	121.1 Da	
Glutamine (Gln)	MW	146.1 Da	
Threonine (Thr)	MW	119.1 Da	
Glycine (Gly)	MW	75.07 Da	
Serine (Ser)	MW	105.1 Da	
Asparagine (Asn)	MW	132.1 Da	

Lys-Lys	MW	347.3 Da
His-Phe	MW	302.3 Da
Asp-Lys	MW	261.3 Da
Tyr-Phe	MW	328.4 Da
Met-Phe	MW	296.4 Da
Gly-Phe	MW	222.2 Da
Ala-Met	MW	220.3 Da
Leu-Gly	MW	188.2 Da
Pro-Ala	MW	186.2 Da
Gly-Val	MW	174.2 Da
Gly-Ala	MW	146.2 Da
Gly-Asn	MW	189.2 Da
Gly-Gly	MW	132.1 Da
Leu-Gly-Phe	MW	335.5 Da
Gly-Pro-Ala	MW	243.3 Da
Gly-Ala-Ala	MW	217.2 Da
Val-Gly-Asp-Glu	MW	418.4 Da
Gly-Gly-Gly-Gly	MW	246.2 Da
Gly-Gly-Gly-Gly-Gly	MW	303.3 Da

4.1.6 Casein purification

The caseins were purified from mineral acid casein prepared in the laboratory from skim milk.

The reagents were: urea supplied by Petrochem, Balance Agri-Nutrients, New Zealand; and sodium acetate-3 hydrate, sodium chloride, sodium hydroxide and hydrochloric acid supplied by BDH Laboratory Supplies, New Zealand. The dialysis tubing (33 mm wide with a 10000 Da molecular weight cut-off) was supplied by Union Carbide Corporation, a subsidiary of the Dow Chemical Company, MI, USA.

4.1.7 Casein hydrolysis

The caseins were α_{s1}-, β- α_{s2}- and κ-casein purified by the author (Ch. 4, section 4.2.5).

The enzymes were: calf rennet (59 Rennin Units (RU)/mL) supplied by Dairy Meats NZ Ltd, Enzyme Division, New Zealand; and plasmin supplied by Boehringer Mannheim, Germany.

The reagents were sodium phosphate, disodium phosphate and sodium azide supplied by BDH Laboratory Supplies.

4.1.8 Cheese manufacture

The coagulant was calf rennet (59 RU/mL) supplied by Dairy Meats NZ Ltd, Enzyme Division, New Zealand.

4.2 METHODS

4.2.1 Cheese manufacture

Known details of the commercial cheese manufacture are included in Ch. 6, section 6.4.1, Table 6.1.

4.2.1.1 Cheddar cheese

Cheddar cheese was manufactured using mid-season milk (November) according to a standard protocol. Whole milk was standardised to a protein:fat ratio of 0.80, resulting in a final milk composition of 4.24% fat, 3.38% protein and 4.85% lactose. The standardised milk was thermised (65°C/15 s) and held overnight at 4.7°C prior to pasteurisation (72°C/15 s).

The vat was filled with milk and the temperature brought to 33°C prior to the addition of 2.5% starter (*Lactococcus lactis* subsp. *cremoris* strains). New Zealand standard strength (59 RU/mL, >95% pure chymosin) calf rennet was added (16.0 mL/100 L milk) to the milk in the vat. The vat contents were mixed mechanically and the milk was left to set at 33°C. After 40 min, the coagulum was cut and stirring was commenced while the temperature was raised to 38°C and was continued during cooking. At pH 6.22, the curds and whey were pumped out on to a conveying system, where the whey was drained from the curd which was then cheddared, milled, salted, hooped and presssed. The next day, the cheese was vacuum-sealed into 20 kg blocks. The cheese was ripened at 13°C.

4.2.1.2 Mozzarella cheese

Mozzarella cheese was manufactured using mid-season milk (November) according to a standard protocol. Whole milk was standardised to a protein:fat ratio of 1.46, resulting in a final milk composition of 2.37% fat, 3.47% protein and 5.01% lactose. The standardised milk was thermised (65°C/15 s) and held overnight at 3.9°C prior to pasteurisation (72°C/15 s).

The vat was filled with milk and the temperature brought to 33°C prior to the addition of 4.0% starter (*Lactococcus lactis* subsp. *cremoris* strains) and held at this temperature for 65 min (pH 6.3). New Zealand standard strength (59 RU/mL, >95% pure chymosin) calf rennet was added (4.0 mL/100 L milk) to the milk in the vat. The vat contents were mixed mechanically and the milk was left to set at 33°C. After 55 min, the coagulum was cut, and stirring was commenced while the temperature was raised to 36°C and was continued during cooking. The whey was drained at pH 5.90, the curd was milled, dry-salted (to gain early control of the growth of the microorganisms), stretched at 68°C, hooped and cooled. The next day, the cheese was vacuum-sealed into 20 kg blocks. The cheese was ripened at 13°C.

4.2.2 Chemical analyses of cheese

Samples of cheese were analysed for composition and pH according to standard procedures (New Zealand Ministry of Agriculture and Fisheries, 1979). Moisture, fat, total nitrogen (TN), water-soluble nitrogen (WSN), non-casein nitrogen (NCN), non-protein nitrogen (NPN), salt, calcium and ash contents were determined by the Analytical Services Group of the Food Science Section. The salt-in-moisture (S/M) and moisture in the non-fat substance (MNFS) were derived from these values.

4.2.2.1 Moisture content (NZDRI Method ACCA12)

The moisture content was determined by drying a known weight of grated cheese curd sample in an oven at 105°C for 16 h, cooling it in a desiccator for 1 h and weighing it. The content of moisture in the MNFS was derived from the fat and moisture contents.

4.2.2.2 Fat content (NZDRI Method ACCA03)

Grated cheese (9.0 g ± 0.001 g) was placed in a Babcock bottle and dissolved with 17.5 mL sulphuric acid, added in three or four portions. The test bottles were placed in a waterbath at 65°C for 5 min and then centrifuged at 165 g for 5 min. The content of fat-in-dry matter was derived from the values for fat and moisture.

4.2.2.3 Total nitrogen (NZDRI Method ACCA06.1)

Grated cheese (1 g ± 0.0001 g) was analysed using a Kjelfoss (Foss Electric, Hillerød, Denmark) nitrogen analyser. This is a semi-automated protein analyser following the Kjeldahl principle, using hydrogen peroxide to boost digestion. The equipment was calibrated with accurately prepared ammonium sulphate solutions.

4.2.2.4 Water-soluble nitrogen (Method of C Coker, supplied in 1995)

The method of Coker (1994) was adopted as a routine method for the analysis of WSN in cheese. Cheese (3 g ± 0.0001 g) sub-sampled from a larger sample that had been finely grated and well mixed was dispersed in 30.0 mL of water. The samples were heated to 40°C and held at that temperature for 30 min, prior to blending with an Ultra-Turrax T25 at approximately 24000 rev/min for 1 min. The warm samples were centrifuged at 10000 rev/min at 4°C for 10 min to separate the insoluble material from the water phase and to solidify and separate the fat. The aqueous subnatant was analysed for nitrogen using a Kjelfoss nitrogen analyser.

4.2.2.5 Non-casein nitrogen (NZDRI Method ACCA06)

This is the nitrogen soluble at pH 4.6, using a sodium acetate/acetic acid fractionation method that is a modification of the method of Dahlberg and Kosikowski (1947). Grated cheese (1 g ± 0.0001 g) was dispersed in 20 mL of distilled and deionised water, and solubilised by the addition of 1 mL of 1.5 M NaOH at 35°C. Acetic acid (10% w/v) was added (4 mL), followed by 1 mL of a 2 M sodium acetate solution and the volume was brought to 50.0 mL with distilled and de-ionised water to precipitate the protein at pH 4.6. The precipitated protein was removed using Whatman No. 40 filter paper and the nitrogen in the filtrate was measured using a Kjelfoss nitrogen analyser.

4.2.2.6 Non-protein nitrogen (NZDRI Method ACCA06)

This is the nitrogen soluble in a 12% TCA solution (Vakaleris and Price, 1959). Grated cheese (2 g ± 0.0001 g) was solubilised in 0.1 M NaOH. Diluted trichloroacetic acid (TCA; 15%) was then added to give a final concentration of 12% TCA. The precipitated protein was removed by filtration using Whatman No. 42 filter paper and the filtrate was analysed for nitrogen using the Kjelfoss instrument.

4.2.2.7 Salt (NZDRI Method ACCA28)

Grated cheese (2 g ± 0.001 g) was weighed into a titration sample cup and mixed with 40 mL of dilute (2%) HNO_3 at 65°C. After 30 min the sample container was attached to the measuring electrode of the autotitrator and titrated with silver nitrate. The salt in moisture (S/M) was derived from the salt and moisture contents.

4.2.2.8 Calcium (NZDRI Method ACME01)

Grated cheese (0.5 g ± 0.001 g) was mixed with 10 mL of 0.1 M HCl and diluted with the addition of 90 mL of water. Solid NaOH (0.5 g) was added and stirred until it dissolved. The solution was titrated with 0.02 M EDTA solution using Patton and Reeder's indicator.

4.2.2.9 Ash (NZDRI Method ACCA17)

Grated cheese (2 g ± 0.001 g) was weighed (in duplicate) into 50mm diameter silica crucibles and dried at 102°C overnight. The crucibles were held over a flame until the sample was charred and smoke emission had stopped. They were placed in a muffle oven set at 550°C for 5 h and then transferred to a desiccator to cool for a minimum of 1 h before being weighed. Ash was calculated as follows:

$$\text{Ash} = (M_1 - M_2)/(M_0 - M_2) \times 100$$

Where, M_1 was the mass of the crucible and ashed sample, M_2 was the mass of the crucible only, and M_0 was the mass of the crucible and initial sample. A factor of 100 was used to convert from g/g to g/100g (ie, %).

4.2.2.10 pH (NZDRI Method ACCA24)

A PHM 82 standard pH meter (Radiometer, Copenhagen, Denmark) with a N61 Schott Gerate (Mainz, Germany) electrode was standardised using a 0.025 M phosphate buffer at pH 6.88 and 0.05 potassium hydrogen pthalate buffer at pH 4.00. Grated curd or cheese at 25°C was tightly packed into a 10 mL glass beaker, the electrode was inserted and the pH determined.

4.2.2.11 Values derived from the composition

The MNFS, FDM and S/M contents were calculated as follows:

$$\%\,\text{MNFS} = (M/(100 - F)) \times 100$$

$$\%\text{FDM} = (F/(100-M)) \times 100$$

$$\%\,\text{S/M} = (S/M) \times 100$$

Where M was percentage moisture, F was percentage fat and S was percentage salt in the cheese curd.

4.2.3 Methods for the analysis of proteolysis in cheese, casein purity and casein hydrolysis

Each cheese sample was frozen at -85°C until all the samples for the particular experiment were collected. (Proteolysis is effectively stopped at temperatures below -26°C (Coker, 1994)). Proteolysis in the whole cheese or urea-soluble fraction (USF) was examined using alkaline urea-polyacrylamide gel electrophoresis (urea-PAGE) or Size Exclusion-High Performance Liquid Chromatography (SE-HPLC). Peptides in the water-soluble fraction (WSF) were examined by urea-PAGE, SE-HPLC or reverse phase-HPLC (RP-HPLC).

4.2.3.1 Urea-PAGE

Proteins in the cheese were separated by urea-PAGE using the Bio-Rad Mini-Protean II system with 10 slot gels of 0.75 mm thickness and a Bio-Rad model 1000/500 power supply. This method was also used to examine the purity of individual caseins and to examine the peptides produced when purified caseins were hydrolysed by either chymosin or plasmin.

The method is a combination of the Davis (1964) and Ornstein (1964) system with a stacking and a resolving gel, in a discontinuous buffer system at alkaline pH that is similar to that of Peterson (1963), Thompson et al (1964) and Peacock et al (1965). The chamber buffer was a Tris-EDTA-borate buffer.

4.2.3.1.1 Gel preparation

The resolving gel was prepared by adding 8.0 mL of a 30% (w/v) stock solution of acrylamide/bis mixture (37.5:1, 2.6% C) to 11.9 mL of resolving gel buffer (9.2 g Tris, 0.8 mL HCl, 54.0 g urea in 100 mL water, adjusted to pH 8.8 with HCl and brought to 200 mL with water). The gel solution was warmed to 20°C and degassed for 15 min by stirring on a magnetic stirrer while evacuating with a water pump. TEMED (10 μL) was then carefully mixed in to the degassed mixture. This was followed by 100 μL of ammonium persulphate solution (100 mg/1.0 mL water) and the solution was again carefully mixed before 3.3 mL was poured between two glass plates and overlaid with 400 μL of water. After the gel was set, the water was removed, the gel surface was dried using filter paper, and the stacking gel solution (1.3 mL of the acrylamide/bis stock solution in 8.64 mL of urea sample buffer that was degassed for 15 min at 20°C before 10 μL of TEMED was added and carefully mixed in, and then 50 μL of ammonium persulphate solution (100 mg/mL of water) was added and carefully mixed in) was applied to the top of the resolving gel and a 10 slot comb was inserted. After the gel was set the comb was removed and the gel slots washed with water to remove unpolymerised gel solution and dried with filter paper.

4.2.3.1.2 Preparation of samples – urea-soluble fraction of cheese

Cheese that had been previously been frozen to –85°C and thawed, was grated and thoroughly mixed. A portion (0.5 g ± 0.0005 g) was dispersed in 25.0 mL of alkaline urea sample buffer (0.928 g EDTA, 10.8 g Tris, 5.5 g boric acid, 360 g urea dissolved in 500 mL of water and adjusted to pH 8.4 with HCl. The solution was then made up to 1 L with water). The samples were heated to 40°C and held at that temperature for 1 h, to get the fat into the liquid phase, prior to blending with an Ultra-Turrax T25 (Janke and Kunkel, IKA-Labortechnik, Staufen, Germany) at approximately 24000 rev/min for 20 s. The warm samples were then centrifuged at 10000 rev/min at 4°C for 10 min in a Sorvall RC2 refrigerated centrifuge (Ivan Sorvall Inc., Norwalk, Connecticut, USA) to separate and solidify the fat. The aqueous subnatant (2 mL) was mixed with 10 μL/mL of both 2-mercaptoethanol and a 0.1% (w/v) solution of bromophenol blue and held for 18 h. Each slot in the gel slab was loaded with 5 μL of the mixture.

4.2.3.1.3 Preparation of samples – water-soluble fraction of cheese

Cheese that had been previously been frozen to –85°C and thawed, was grated and thoroughly mixed. A portion (3 g ± 0.0005 g) was dispersed in 30.0 mL of water and the WSF extracted using the protocol outlined in Ch. 4, section 4.2.2.4. The extract was frozen in 1.5 mL aliquots at -85°C.

The extracts were thawed, diluted 1:1 with alkaline urea sample buffer, mixed with 2-mercaptoethanol (10 μL/mL) and a 0.1% (w/v) solution of bromophenol blue (10 μL/mL) and held for 18 h. Each slot in the gel slab was loaded with 25 μL of the mixture.

4.2.3.1.4 Preparation of samples – purified caseins

Each purified casein solution was diluted 1:1 with alkaline urea sample buffer, and mixed with 2-mercaptoethanol and bromophenol blue (as above). A slot in the gel was loaded with 10 μL of the mixture.

4.2.3.1.5 Preparation of samples – casein hydrolysates

Each sample taken during the hydrolysis of the purified caseins (α_{s1}-, α_{s2}- and β-casein) was heated to 100°C for 2 min in a sealed container (to quench enzyme activity), cooled, diluted 1:1 with urea sample buffer and mixed with 2-mercaptoethanol and bromophenol blue (as above). A slot in the gel was loaded with 5 μL of the mixture.

4.2.3.1.6 Preparation of standards

A rennet casein standard was prepared by dissolving 12.0 mg of rennet casein in 6.0 mL of sample buffer. After stirring for 1 h the standard was diluted to give a final concentration of 1.0 mg/mL. A second standard was prepared by diluting 0.1 mL of skim milk with 3.9 mL of urea sample buffer. Each standard (2 mL) was mixed with 10 μL/mL of both 2-mercaptoethanol and a 0.1% (w/v) solution of bromophenol blue and held for 18 h. Each slot in the gel was loaded with 10 μL of the mixture.

4.2.3.1.7 Gel electrophoresis

A pair of gels was fitted into the electrode assembly and placed in the buffer chamber. A stock solution of chamber buffer (21.58 g Tris, 10.96 g boric acid, 1.85 g EDTA in 1800 mL water, adjusted to pH 8.4 and brought to 2 L with water) was diluted 1:4 with water and 400 mL was used to completely fill the inner buffer chamber and partially fill the outer buffer chamber. The cheese samples (2% (w/v) cheese) and the standards (5 μL and 10 μL respectively) were applied to the gel and electrophoresed until the dye front reached the bottom of the gel (approximately 1.7 h). The upper limits of the power supply were set at 210 V, 70 mA (an upper arbitrary value) and 6.5 W (the setting for two gels electrophoresed simultaneously).

4.2.3.1.8 Staining

The gels were stained with 50 mL Coomassie brilliant blue R solution (1.0 g Coomassie Blue R250, 500 mL isopropanol and 200 mL glacial acetic acid made up to 2 L with water) for 1 h and destained in 100 mL of Coomassie destain solution (200 mL isopropanol and 200 mL glacial acetic acid made up to 2 L with water) for 1 h. The destain was then replaced and destaining was continued for a further 19 h. The staining and destaining were done in sealed containers with continuous agitation.

The linearity between the amount of protein loaded on to the gel and the amount of dye bound was checked. α_{s1}-Casein and β-casein were purified from mineral acid casein, and then dissolved in alkaline urea buffer at a range of concentrations. The range of standard solutions was loaded on to each of several gels. The band absorbance (see next section) was plotted against the casein concentration. There was a linear relationship between band absorbance and casein concentration (see appendix, section 4.3, N White and C Coker, 1999).

4.2.3.1.9 Gel analysis and band quantitation

The gels were scanned using a Personal Densitometer (Molecular Dynamics, Sunnyvale, California, USA) and photographed. The gel images were analysed using ImageQuant (version 5.0) software. The gel image was viewed in logarithmic scale and an appropriate shape, usually a rectangle, was drawn around each band. The absorbance associated with each pixel within the rectangle was determined and the background absorbance was subtracted from it before the absorbance values of all of the pixels were summed. The background absorbance was determined manually by examining at least seven empty regions on the gel.

4.2.3.1.10 Photography

The gels were placed on a light box and back-lit with fluorescent lighting. The photographs were taken with a Pentax K-1000 single lens reflex camera fitted with a 50 mm macro (close-focusing) lens, and a combination of orange and green Hoya filters to restrict light at both high and low wavelengths and obtain the greatest contrast between the background and the bands. The film (Kodak 100 ASA T-Max) was selected because it is sensitive to wavelengths from below 400 to about 620 nm.

4.2.3.2 SDS-PAGE

Proteins and peptides in the κ-casein hydrolysate were separated by SDS-PAGE using the Bio-Rad Mini-Protean II system with 10 slot gels of 0.75 mm thickness and a Bio-Rad model 1000/500 power supply.

4.2.3.2.1 Gel preparation

The gel was prepared by combining 4.04 mL water, 5.00 mL of 1.5 M Tris-HCl buffer (18.5 g Tris base was dissolved in 60 mL water, the pH was adjusted to 8.8 with 1 M HCl, and the volume brought to 100 mL with water), 200 μL of a 10% SDS stock solution, and 10.60 mL of a 30% Acrylamide stock solution (30 g Bis/Acrylamide mixture (37.5:1 (2.6% C)) brought to 100 mL with water). The gel solution was warmed to 20°C and degassed for 15 min by stirring on a magnetic stirrer while evacuating with a water pump. TEMED (10 μL) was carefully mixed in. This was followed by 100 μL of ammonium persulphate solution (100 mg/1.0 mL water) and the solution was again carefully mixed before 3.3 mL was poured between two glass plates and overlaid with 400 μl of water. After the gel was set the water was removed, the gel surface was dried using filter paper, stacking gel solution (6.1 mL of water, 2.5 mL of 0.5 M Tris-HCl stock solution (6.0 g Tris base was dissolved in 60 mL of water, the pH was adjusted to 6.8 with 1 M HCl, and the volume was brought to 100 mL with water), 100 μL of the 10% SDS stock solution and 1.3 mL of the 30% Acrylamide/Bis stock solution was degassed at 20°C for 15 min before 10 μL of TEMED was added and carefully mixed in, and then 50 μL of ammonium persulphate solution (100 mg/mL of water) was added and carefully mixed in) was applied to the top of the resolving gel and a 10 slot comb was inserted. After the gel was set the comb was removed and the gel slots washed with water to remove unpolymerised gel solution and then dried with filter paper.

4.2.3.2.2 Preparation of samples – κ-casein hydrolysates

The samples, taken at regular intervals during the hydrolysis of the pure caseins, were heated at 100°C for 2 min in sealed containers, cooled and diluted 1:1 with sample buffer (50 mL distilled water, 12.5 mL of the 0.5 M Tris HCl stock solution (Ch. 4, section 4.2.3.2.1), 10.0 mL of glycerol and 20.0 mL of a 10% SDS solution). Each sample was treated with 2-mercaptoethanol (10 μL/mL) and a 0.1% solution of bromophenol blue (10 μL/mL), heated at 95°C for 4 min, cooled and loaded (5 μL) into a slot in the gel.

4.2.3.2.3 Preparation of standards

A rennet casein standard was prepared by dissolving 10.0 mg of rennet casein in 8.0 mL of sample buffer (Ch. 4, section 4.2.3.2.1). After stirring for 1 h the standard was diluted to give a final concentration of 1.0 mg/mL. A second standard was prepared by diluting 0.1 mL of skim milk with 3.9 mL of sample buffer. Each standard was treated with 2-mercaptoethanol (10 μL/mL) and a 0.1% solution of bromophenol blue (10 μL/mL), heated at 95°C for 4 min, cooled and loaded (10 μL) into a slot in the gel.

4.2.3.2.4 Gel electrophoresis

A pair of gels was fitted into the electrode assembly and placed in the buffer chamber. A stock solution of chamber buffer (9.0 g Tris base, 43.2 g glycine, 3.0 g SDS in 600 mL water, pH 8.3) was diluted 1:4 (60 mL of stock solution plus 240 mL of water) and 400 mL was used to completely fill the inner buffer chamber and partially fill the outer buffer chamber. The samples and the standards were applied to the gel and electrophoresed until the dye front was at the bottom of the gel (approximately 1 h). The upper limits of the power supply were set at 210 V, 70 mA and 6.5 W.

The procedures for staining and destaining the gels, measuring the band density and photographing the gels were the same as those used for urea-PAGE (Ch. 4, sections 4.2.3.1.8–10).

4.2.3.3 SE-HPLC

Urea-soluble proteins and peptides and water-soluble peptides and amino acids in the cheese, as well as the molecular weight standards, di-, tri-, tetra- and pentapeptides, and amino acid standards, were examined by SE-HPLC using a modification of the method of Swergold and Rubin (1983).

The HPLC was a Waters system (Waters Associates, Millipore Corp., Waters Chromatography Division, MA, USA) comprised of a Waters 600E System Controller and a Waters 700 Satellite Wisp autoinjector connected to a Hewlett-Packard 1040A multiwavelength detection system (Hewlett-Packard Company, Camas, Washington, USA) linked to a Hewlett-Packard ChemStation for data collection and analysis. For later experiments, the HPLC was replaced with a Hewlett-Packard 1100 series HPLC linked to a Hewlett-Packard ChemStation for data collection and analysis.

4.2.3.3.1 Preparation of samples – urea-soluble fraction of cheese

The USF (1.0 mL) prepared for urea-PAGE (0.5 g (± 0.0005 g) cheese in 25.0 mL urea sample buffer) (Ch. 4, section 4.2.3.1.2) was diluted 1:9 with the mobile phase to give a final cheese concentration of 0.2% and 50 μL was loaded on to the column.

4.2.3.3.2 Preparation of samples - water-soluble fraction of cheese

The WSF (200 μL) prepared for PAGE (3 g (± 0.0005 g) cheese dispersed in 30.0 mL of water) using the protocol outlined in Ch. 4, section 4.2.3.1.3 and frozen in 1.5 mL aliquots at -85°C, was thawed, diluted 1:49 (v/v) with the mobile phase to give a final cheese concentration of 0.2% and 50 μL was loaded on to the column.

4.2.3.3.3 Preparation of molecular weight standards

Standards in the effective molecular weight range of the column (100 Da - 36000 Da) (Ch. 4, section 4.1.5) were made to a concentration of 1 mg/10 mL mobile phase and 50 μL was loaded on to the column.

4.2.3.3.4 Preparation of amino acid and di-, tri- tetra- and pentapeptide standards

The non-aromatic amino acids and each of the di-, tri-, tetra-, and pentapeptides (Ch. 4, section 4.1.5) were made to a concentration of 10 mg/5 mL of water, diluted 1:1 with the mobile phase and 10μL was loaded on to the column. The aromatic amino acid solutions were prepared to a concentration of 5 mg/5 mL of water, diluted 1:1 with the mobile phase and 5 μL was loaded on to the column, to give a final loading that was 25% of the loading used for the non-aromatic amino acids.

4.2.3.3.5 Chromatography

The separation used a silica TSK G-2000 SWXL column (7.5 mm I.D. x 30 cm) (Tosoh Corporation, Tokyo, Japan) with a TSK-GEL SW guard column (7.5 mm I.D. x 7.5 cm). The mobile phase consisted of 36% acetonitrile, 0.1% trifluoroacetic acid (TFA) in water.

The column was equilibrated with the mobile phase at a flow rate of 0.4 mL/min. The molecular weight standards (50 μL), cheese samples (50 μL), non-aromatic amino acids (10 μL), aromatic amino acids (5 μL) or di-, tri-, tetra- or pentapeptides (10 μL) were injected on to the column at a flow rate of 0.4 mL/min and elution was complete after 45 min. The absorbance was monitored at 205, 210, 220, 280 and 295 nm.

4.2.3.4 RP-HPLC

Water-soluble peptides and amino acids in the cheese were separated by RP-HPLC using the HPLC system used for SE-HPLC (Ch.4, section 4.2.3.3). For later experiments (Ch. 6), the HPLC was replaced with a Hewlett-Packard 1100 series HPLC (Ch.4, section 4.2.3.3).

4.2.3.4.1 Sample preparation

The WSF prepared for PAGE (3 g (± 0.0005 g) cheese dispersed in 30.0 mL of water) using the protocol outlined in Ch. 4, section 4.2.2.4), was diluted 1:1 (v/v) with solvent A (5% acetonitrile and 0.1% TFA in water) prior to loading 100 μL on to the column. (This sample concentration was 50 times greater than that used for SE-HPLC.)

4.2.3.4.2 Chromatography

The separation used two Pharmacia PepRPC HR 5/5 FPLC columns in series (Amersham Pharmacia Biotech AB, Uppsala, Sweden).

The columns were equilibrated and the samples run at a constant flow rate of 0.70 mL/min. The gradient consisted of 100% solvent A for 5 min, a linear gradient from 0 to 100% solvent B (60% acetonitrile and 0.1% TFA in distilled and deionised water) over 75 min and 100% solvent A for 15 min. The absorbance was monitored at 205, 210, 220, 280 and 295 nm.

4.2.4 Purification of caseins

The caseins, α_{s1}-, α_{s2}-, β- and κ-casein, were purified using the method of Plowman and Burr (1992) using a Pharmacia BioPilot system in multipurpose configuration and BioPilot Manager software. The separation was achieved using cation exchange chromatography.

4.2.4.1 Sample preparation

A stock solution was prepared by dissolving wet mineral acid casein curd (125 g of casein containing approximately 50% water) in 1 L of Buffer A (6 M urea, 20 mM sodium acetate at pH 5.0 (1801.8 g of urea granules and 13.61 g of sodium acetate-trihydrate were dissolved in distilled water, brought to a volume of 4.9 L, and filtered under vacuum through Whatman GF/A filter paper followed by a Millipore 0.45 μm filter. The pH of the filtrate was adjusted to pH 5.0 using 2 M HCl, and the volume was brought to 5.0 L with water)). This solution was titrated to pH 7.0 with sodium hydroxide and frozen in 100 mL aliquots.

A 100 mL aliquot of the casein solution was diluted to 1100 mL with Buffer A and centrifuged at 5000 g for 10 min. The fat layer was removed and the fat-free supernatant was decanted. The pH of the casein solution was then adjusted to 5.0 before the disulphide bonds were reduced using 0.01% dithiothreitol.

4.2.4.2 Casein separation

The column was a Pharmacia BPP113/15 column with a bed height of 15.0 cm and a diameter of 11.3 cm, and the media was S-Sepharose Fast Flow (Pharmacia).

The column was equilibrated using Buffer A. The casein solution was loaded on to the column at a flow rate of 25 mL/ min over a 40 min period. The elution was as follows: 0 - 40 min, 100% Buffer A; 40 – 220 min, a linear gradient to 40% Buffer B (6 M urea, 20 mM sodium acetate trihydrate and 1 M sodium chloride at pH 5.0 (the preparation of Buffer B was the same as for

Buffer A, except that 292.2 g of sodium chloride was added)); 220 – 244 min, 100% Buffer B; 244 – 304 min, 100% Buffer A. The effluent was monitored at 214 nm and fractions corresponding to each of the peaks were collected.

The fractions were poured into dialysis tubing (33 mm wide with a 10000 Da molecular weight cut-off) and dialysed twice against 5 L of deionised water. The fractions were removed from the dialysis tubing and centrifuged at 5000 g for 10 min. The precipitate was resuspended in water and the pH adjusted to ~8.0 with 0.01M NaOH to solubilise the casein. The casein solution was poured into dialysis tubing and dialysed extensively against deionised water (5 changes of 5 L water). Several batches of casein were fractionated in this way.

The dialysed fractions were diluted 1:1 with urea-PAGE sample buffer and checked for purity using urea-PAGE (Ch. 4, section 4.2.3.1) or SDS-PAGE (Ch. 4, section 4.2.3.2). The remainder of each fraction was freeze-dried. If a particular fraction contained a mixture of caseins, it was pooled with the same fraction from other batches (if impure), dissolved in Buffer A and the fractionation procedure was repeated.

4.2.5 Hydrolysis of caseins

Purified protein, α_{s1}-, β-, α_{s2}- or κ-casein, was dissolved in 100 mM Na phosphate buffer containing 0.01% (w/v) NaN_3 at pH 8.0 for the plasmin experiments and pH 5.8 for the chymosin experiments to give a protein concentration of 10 mg/mL. Initial samples were removed and diluted 1:1 with alkaline urea sample buffer for urea-PAGE analysis (Ch. 4, section 4.2.3.1) or 1:1 with SDS sample buffer for SDS-PAGE analysis (Ch. 4, section 4.2.3.2) and 1:24 with SE-HPLC mobile phase (36% acetonitrile, 0.1% TFA in water) for SE-HPLC analysis (Ch. 4, section 4.2.3.3). The protein solution was brought to 37°C and either New Zealand standard strength rennet (59 RU/mL) or bovine plasmin (5 U/mL) was added to give a final concentration of 2.36 RU/mL or 0.2 U/mL, respectively. Further samples were taken at 1, 5, 10, 20, 40 (chymosin only) and 60 min and 2, 5 and 24 h and heated at 100°C for 2 min in sealed containers prior to diluting 1:1 with urea-PAGE sample buffer (for α_{s1}-casein, β-casein and α_{s2}-casein hydrolysates) or SDS-PAGE sample buffer (for κ-casein hydrolysates). Samples for SE-HPLC were diluted 1:24 with the mobile phase and this was sufficient to stop the reaction.

4.2.6 Multivariate Statistical Analyses

Multivariate statistical analyses allow several related variables to be considered simultaneously, with each variable being equally important at the start of the analysis (Manly, 1994). Two methods, principal component analysis (PCA) and multiple linear regression analysis (MLR) were used in the current study.

4.2.6.1 Principal component analysis

Principal component analysis (PCA) is a mathematical procedure used to determine the structure of a large multidimensional data set. The data set may contain a large number of variables, and if many of these variables are correlated, PCA can reduce it to a smaller number of uncorrelated principal components that more concisely account for most of the original information. The principal components are numbered in order of the amount of variation in the original data set that they explain, so that the first principal component accounts for the most variation, and each subsequent principal component accounts for as much of the remaining variation as possible. It is often possible to view the structure in the data by plotting two or three of the most important principal components (djmw, 1999; Statistica 6.0, StatSoft Inc., OK, USA, 2001). The best results are achieved when the original variables are highly correlated, and if they are uncorrelated PCA does nothing (Manly, 1994). The objectives of PCA are therefore "data reduction and interpretation" (Pripp et al, 2000b).

Two forms of PCA are typically used. The correlation form of PCA may be used in situations where the internal variance of a variable is large in comparison with the internal variance of other variables, and must be used when variables have different measurement units. For this form of PCA, the data set is standardised (scaled) before the analysis, so that each variable has a mean of zero and a standard deviation of one. The covariance form of PCA does not require standardisation (scaling) of the data prior to analysis, but uses data that has been centred around the mean for the particular variable, and is useful in situations where the relativity of the variance of the variables is important.

In more mathematical terms,

PCA takes p variables (peaks) $X_1, X_2, \ldots, X_p$ for n cases (samples) and finds linear combinations of these to produce indices $Z_1, Z_2, \ldots, Z_p$ that are uncorrelated and therefore measure different dimensions in the data. The indices are ordered so that the variance of $Z_1 \geq Z_2 \geq \ldots \geq Z_p$, with Z_i being the i th principal component.

Therefore, for the matrix

	Variable			
Case	X_1	X_2	...	X_p
1	x_{11}	x_{12}	...	x_{1p}
2	x_{21}	x_{22}	...	x_{2p}
.	...	...	...	...
.	...	...	...	...
.	...	...	...	...
n	x_{n1}	x_{n2}	...	x_{np}

OR

$$X = \begin{bmatrix} x_{11}, x_{12}, \ldots, x_{1p} \\ x_{21}, x_{22}, \ldots, x_{2p} \\ \ldots, \ldots, \ldots, \ldots \\ x_{n1}, x_{n2}, \ldots, x_{np} \end{bmatrix}$$

where x_{ij} is the value (loading coefficient) of the j th variable of the i th case, standardised or centred about its respective mean, the equation for the i th principal component is

$$Z_i = x_{i1}X_1 + x_{i2}X_2 + \ldots + x_{ip}X_p$$

4.2.6.2 Multiple linear regression

Multiple linear regression is used to determine the relationship between multiple independent or predictor variables and a dependent variable (Statistica v. 6.0).

A line in a two-dimensional space is defined by the equation

$$Y = a + b{*}X$$

where a is a constant (intercept), b is the slope (regression coefficient or B coefficient) and X is a variable (peak).

When there is more than one independent variable, the regression line cannot be visualised in two-dimensional space. In this instance, multiple regression analysis will estimate a linear equation:

$$Y = a + b_1{*}X_1 + b_2{*}X_2 + \ldots + b_p{*}X_p$$

where p represents the p th variable.

5.0 DEVELOPMENT OF SIZE EXCLUSION-HPLC FOR CHEESE ANALYSIS

ABSTRACT

The objective of the current study was to characterise the size-exclusion (SE)-HPLC method of Swergold and Rubin (1983), that had been modified by Motion (1992) for analysis of whey protein hydrolysates, and to develop it for the analysis of cheese.

A series of standards ranging in molecular weight from 165 to 36000 Da was used to demonstrate that within this range the method separated proteins on the basis of molecular weight, and that peak elution time and area were repeatable. A series of peptide and amino acid standards was used to demonstrate the effect on elution time of relatively large differences in the ratio of charge or hydrophobicity to size of very small peptides and amino acids. Hydrophobicity had only a minor effect, with the more hydrophobic di- and tripeptides and amino acids eluting slightly earlier than expected on the basis of molecular weight. Charge had a greater influence, with the more positively charged amino acids (Arg, His and Lys) and dipeptides that incorporated them eluting earlier than expected on the basis of their molecular weight. The effect of acetonitrile concentration (±1%) was examined and it was demonstrated that an invariant solvent composition is critical to obtain reproducible elution times for the proteins and peptides.

The SE-HPLC method was used to separate the proteins, peptides and amino acids in the USF and the peptides and amino acids in the WSF of Cheddar cheese into a series of peaks. Poorly resolved early-eluting peaks containing the caseins and largest peptides dominated the traces of the USF, and the later-eluting peaks containing small peptides and amino acids were small in comparison. In contrast, the early-eluting peaks on the trace of the WSF were small, indicating that most high molecular weight material was insoluble in water, and the later-eluting peaks were large. A comparison of the traces of the USF and WSF showed that the elution pattern of the small peptides and amino acids was similar on both traces.

The molecular weight distribution of the proteins and peptides on the SE-HPLC trace of the WSF of Cheddar cheese ranged from approximately 165 - 20000 Da and was calculated from a standard curve derived from the elution times of molecular weight standards analysed at the same time as the cheese extracts.

Finally, the repeatability of both the extraction of the WSF of cheese and SE-HPLC analysis of the extract was demonstrated. Fifteen extracts of one cheese were prepared and analysed by SE-HPLC. The relative standard deviation for the total peak area was 3.75%, while the average relative standard deviation for the area of the 14 peaks was 4.70%. Peak elution time was practically identical.

5.1 INTRODUCTION

A number of methods have been used to examine the caseins and their proteolysis products in cheese or to monitor changes to the proteins during cheese maturation. The simplest of these involves measuring the distribution of (Kjeldahl) nitrogen (N) among fractions, after some form of fractionation. The proportions of non-casein N (NCN) and non-protein N (NPN) in relation to total N (TN) or the concentration of free amino acids are commonly used for these purposes. Methods that provide more information are alkaline urea-polyacrylamide gel electrophoresis (urea-PAGE), which provides information on the loss of each casein and the formation of some of the largest peptides, and reverse phase-HPLC (RP-HPLC) which separates the peptides in the water-soluble fraction (WSF) and enables them to be viewed as peaks on a trace.

Proteolysis follows the general path:

Caseins ➔ large peptides ➔ small peptides ➔ amino acids

(➔ organic chemicals (via catabolic pathways)).

Therefore, the trend is towards peptides of ever decreasing size and amino acids. Hence, methods such as SDS-PAGE or size-exclusion chromatography (SEC) that separate on the basis of size may be useful for monitoring proteolysis and maturation. SEC has been widely used to fractionate different cheese extracts prior to some other form of analysis, such as sensory analysis, urea-PAGE, thin layer chromatography or more recently RP-HPLC. Commonly, Sephadex gels with different pore sizes were used in open or low-pressure systems. For example, Richardson and Creamer (1973) used Sephadex G-25 to fractionate bitter peptides associated with starter strains in Cheddar cheese prior to sensory analysis and urea-PAGE, Visser et al (1983) used Sephadex LH20 to fractionate bitter peptides associated with starter strains in Gouda cheese prior to sensory analysis and thin layer chromatography, and Singh et al (1994, 1995, 1997) used Sephadex G-25 to fractionate Cheddar cheese extracts prior to the isolation and identification of peptides using RP-HPLC and mass spectrometry.

New SEC matrices were developed for high performance chromatography systems, and some of these have been used to fractionate solubilised cheese. For example, a TSK G-3000 SW and G-2000 SW (Tosoh Corp) in series with a solvent comprised of 0.1 M sodium sulphate and 0.01 M sodium dihydrogenphosphate (pH 6.8) was found by Haasnoot et al (1989) to be unsuitable for the fractionation of peptides in Gouda cheese because there were only "a number of poorly separated components" present, but an FPLC Superose-12HR column (Pharmacia) was found to be useful for studying the effects of commercial enzymes on proteolysis in Cheddar cheese (Wilkinson et al, 1992) and for characterising peptides in Cheddar cheese fractions (Breen et al, 1995). A Sephadex G-25 Superfine HPLC column with water as the eluent was used by Cliffe et al (1993) to fractionate cheese peptides prior to RP-HPLC analysis. They demonstrated that the higher molecular weight fractions obtained by SE-HPLC contained bitter peptides and corresponded to the later-eluting peaks on the RP-HPLC traces, whereas the lower molecular weight fractions had a savoury flavour and corresponded to the early-eluting RP-HPLC peaks. These early-eluting peaks had previously been demonstrated to have characteristic Cheddar flavour by Aston and Creamer (1986). Salles et al (1995) used a TSK G-HW-40 S column with water as the eluent to fractionate Comté cheese and demonstrated that the low molecular weight fractions were associated with cheese flavour.

A SE-HPLC method that has not been applied to cheese to date, is that of Swergold and Rubin (1983). This method enables peptides to be grouped according to size, because of the characteristics of the column and the particular solvent system used. The original method employed a silica TSK G-3000 PW column and a low ionic strength acidic acetonitrile solvent system (36 or 45% acetonitrile in water; 0.1% TFA) that prevented peptide association. A modified version of the method, using a 60 cm TSK G-2000 SW column was used by Motion (1992) to monitor hydrolysis of whey proteins and to form a profile of molecular weight distribution. It seemed that cheese proteins and peptides might be considered a complex hydrolysate and that this method may have an application in monitoring the enzymatic progress.

In this chapter, the SE-HPLC method of Motion (1992) is explored and characterised using various protein, peptide and amino acid standards. The chapter includes an examination of the ability of the method to separate cheese proteins and the products of proteolysis and to provide a chromatogram that demonstrates their molecular weight distribution.

5.2 OBJECTIVES

The purpose of the current study is to develop a method for examining the molecular weight distribution of proteins, peptides and amino acids, or peptides and amino acids in cheese fractions. Such a method would be a useful tool for product development, for cheese differentiation or for establishing cheese maturity.

To achieve this objective it was necessary to:

a) determine the elution time of selected molecular weight standards,
b) determine the effect of size, charge and hydrophobicity on the elution time of amino acids standards as well as selected di-, tri-, tetra- and pentapeptides,
c) determine the repeatability of the elution time and area of the molecular weight standards,
d) establish a standard curve for determining the relationship between the elution time of known protein standards and their molecular weight,
e) determine the effect of small variations in acetonitrile concentration on elution time,
f) examine the absorbance of a selection of amino acids and peptides, and determine the most appropriate wavelength for routine use,
g) examine the USF and WSF of a single cheese type and determine whether useful separation of protein, peptide and amino acid peaks is achieved,
h) establish the molecular weight distribution of peptide and amino acid peaks in cheese, and
i) determine the repeatability of sample preparation and SE-HPLC analysis.

5.3 MATERIALS AND METHODS

5.3.1 Materials

The materials are described in Ch. 4, section 4.1.

5.3.2 Methods

Most methods are fully described in Ch. 4, section 4.2.

5.3.2.1 Preparation and analysis of individual molecular weight standards

Molecular weight standards covering the molecular weight range from 165 to 36000 Da (Ch. 4, section 4.1.5) were prepared according to the method described in Ch. 4, section 4.2.3.3.3. Each standard solution (50 μL) was examined separately using SE-HPLC, according to the method described in Ch. 4, section 4.2.3.3.

5.3.2.2 Preparation and analysis of amino acids and very small peptides

A series of amino acids and di-, tri-, tetra- and pentapeptides (Ch. 4, section 4.1.5) solutions were prepared as described in Ch. 4, section 4.2.3.3.4. Each amino acid or peptide solution was analysed separately by SE-HPLC, according to the method described in Ch. 4, section 4.2.3.3.

5.3.2.3 Preparation and analysis of standard sets A and B

The molecular weight standard solutions were divided into two sets:

Standard set A contained 1 mL of each of glyceraldehyde 3-phosphate dehydrogenase (36000 Da), soybean trypsin inhibitor (20100 Da), aprotinin (6500 Da) and insulin B-chain (3400 Da) solutions and 0.2 mL of the Phe (165 Da) solution that had been individually prepared at a concentration of 1 mg/10 mL.

Standard set B contained 1 mL of each of carbonic anhydrase (29000 Da), lysozyme (14300 Da), insulin (5730 Da) and bacitracin (1420 Da) solutions that had been individually prepared at a concentration of 1 mg/10 mL.

Each mixture (50 μL) was examined separately using SE-HPLC, according to the method described in Ch. 4, section 4.2.3.3.

5.3.2.4 Preparation and analysis of the USF and WSF of Cheddar cheese

The USF and WSF of a mature Cheddar cheese sample were prepared as described in Ch. 4, sections 4.2.3.3.1 and 4.2.3.3.2, respectively. Each cheese fraction (50 μL) was separately analysed by SE-HPLC, according to the method described in Ch. 4, section 4.2.3.3. The two sets of molecular weight standards (50 μL) were separately analysed in the same batch as the cheese samples and a molecular weight calibration curve ($\log_{10}$ molecular weight versus elution time) was formed.

5.3.2.5 Fractionation of the WSF of Cheddar cheese

The WSF of the mature Cheddar cheese was pre-filtered through a 0.8/0.2 μm filter and then centrifuged at 7500 g for 2 h at 25°C through the membrane of a Centricon ultrafiltration concentrator with a 3000 Da molecular weight cut-off. Both fractions (one containing peptides >3000 Da and the other peptides <3000 Da) were separately analysed using SE-HPLC, according to the method described in Ch. 4, section 4.2.3.3.

5.3.2.6 Repeatability of SE-HPLC sample preparation and analysis

A Cheddar cheese sample was grated and the WSF was prepared 15 times as described in Ch. 4, section 4.2.3.3.2. Each extract was analysed sequentially by SE-HPLC, according to the method described in Ch. 4, section 4.2.3.3. The chromatographic peaks were integrated and the peak area data transferred to a Microsoft Excel spreadsheet. The mean, standard deviation and relative standard deviation (r.s.d.) was determined for each peak. One sample was analysed 5 times by SE-HPLC to determine the repeatability of the sample analysis.

5.4 RESULTS

In the following sections a SE-HPLC method is characterised and developed for the analysis of cheese. The method employs a silica TSK G-2000 SWXL column with a TSK-GEL SW guard column and a mobile phase comprised of 36% acetonitrile and 0.1% trifluoroacetic acid (TFA) in water.

5.4.1 Elution of molecular weight standards

Nine molecular weight standards, with a wide spread of molecular weights (165 - 36000 Da), were prepared (1 mg/10 mL mobile phase) and analysed individually to build a composite picture of the relationship between molecular weight and elution time of proteins (Fig. 5.1).

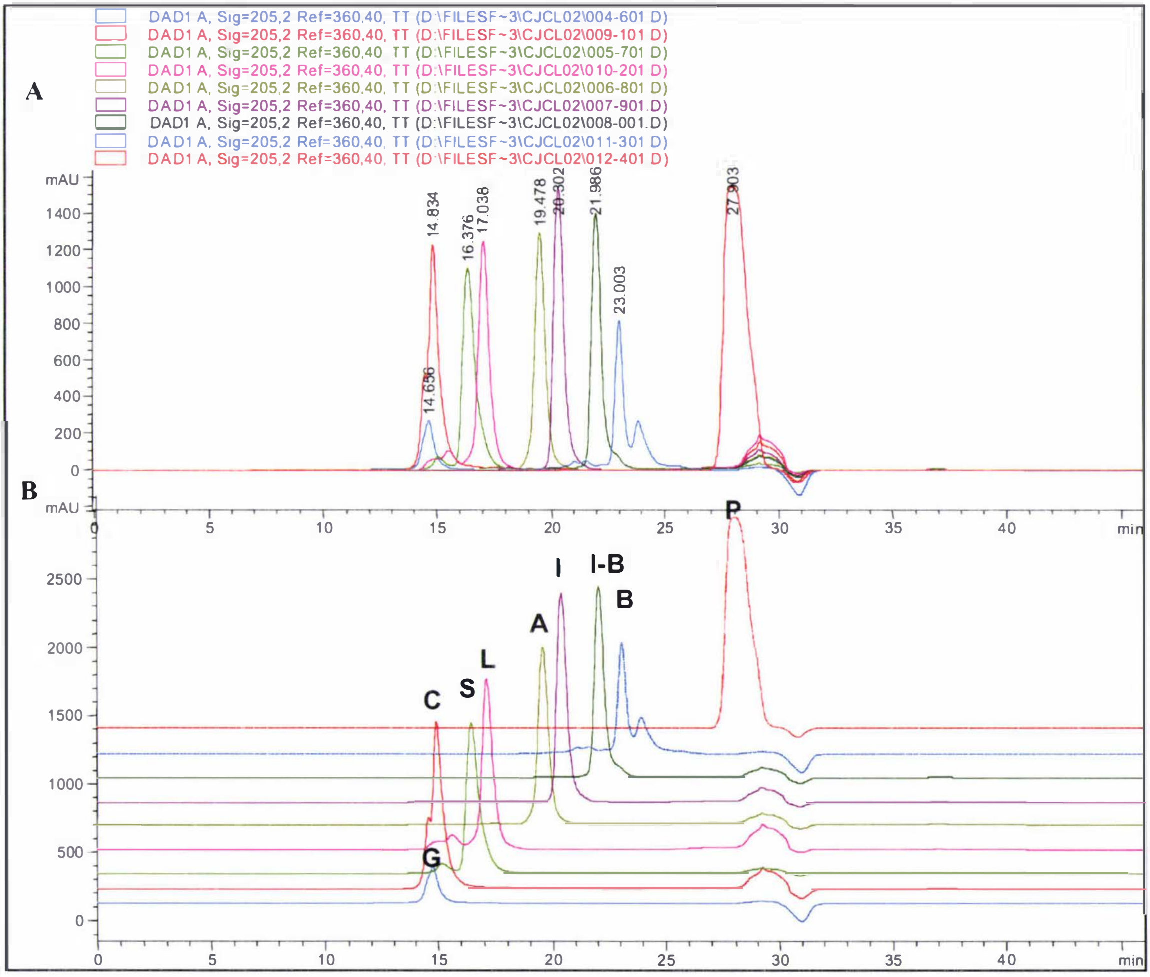


Fig. 5.1 *SE-HPLC - Analysis of individual molecular weight standards.* Glyceraldehyde 3-phosphate dehydrogenase (**G**) (36000 Da), carbonic anhydrase (**C**) (29000 Da), soybean trypsin inhibitor (**S**) (20100 Da), lysozyme (**L**) (14300 Da), aprotinin (**A**) (6500 Da), insulin (**I**) (5730 Da), insulin B-chain (**I-B**) (3400 Da), bacitracin (**B**) (1420 Da) and 9) phe (**P**) (165 Da) were prepared at a concentration of 1 mg/10 mL mobile phase and 50 μL was loaded on to the column. The mobile phase contained 36% acetonitrile and 0.1% TFA in water and the flow rate was 0.4 mL/min. The absorbance was measured at 205 nm. In Fig. 5.1 A, the traces for each standard have been overlaid, and in Fig. 5.1 B, they have been offset on the y-axis.

The offset (Fig. 5.1B) was done to account for some secondary peaks resulting from a lack of purity of some of the standards, particularly the soybean trypsin inhibitor, lysozyme and bacitracin, which each contained material that gave additional peaks. Several of the standards contained an impurity that gave rise to a peak that eluted around 30 min (Fig. 5.1A).

The standards were divided into 2 groups (standard sets A and B), each with a wide spread of non-overlapping peaks. The solutions within each set were combined (see Ch. 5, section 5.3.2.3).

5.4.2 Elution of amino acids and very small peptides

The effect of size, charge and hydrophobicity on the order of elution of amino acids and very small peptides that were expected to elute between 25 and 28 min was determined by analysing a series of amino acids and di-, tri-, tetra- and pentapeptides. The elution time for each of the amino acid and peptide standards was recorded (Table 5.1).

Table 5.1 *SE-HPLC – Elution times of selected di-, tri-, tetra- and pentapeptides and amino acids.* With the exception of the aromatic amino acids, the standards were made at a concentration of 10 mg/5 mL of water, diluted 1:1 with the mobile phase and 10 µL was loaded on to the column. The aromatic amino acid solutions were less concentrated (5 mg/5 mL of water) than the non-aromatic amino acid solutions and 5 µL was loaded on to the column. Refer to Fig. 5.1 caption for details of sample analysis.

	Amino acids	MW (Da)	Hydrophobicity (ethanol to water) ΔG° side chain* (J/mol)	Elution time (min)	Dipeptides	MW (Da)	Elution time (min)	Tri- tetra- and pentapeptides	MW (Da)	Elution time (min)
Basic	Lys HCl (Lys MW = 146.2)	182.7	6250	26.597						
	Arg HCl (Arg MW = 174.2)	210.7	3100	26.623	Lys-Lys	347.3	24.464			
	His HCl H_2O (His MW = 155.2)	209.6	2100	27.232	His-Phe	302.3	25.755			
Hydrophobic	Leu	131.2	10100	27.779	Asp-Lys	261.3	25.906			
	Ile	131.2	12400	27.802	Tyr-Phe	328.4	26.907			
	Phe	165.2	11100	27.848	Met-Phe	296.4	26.949			
	Trp	204.2	12550	27.880	Gly-Phe	222.2	27.348			
	Val	117.1	7050	28.023	Ala-Met	220.3	27.392			
	Met	149.2	5450	28.059	Leu-Gly	188.2	27.412	Leu-Gly-Phe	335.5	26.776
	Ala	89.09	3100	28.315	Pro-Ala	186.2	27.449	Val-Gly-Asp-Glu	418.4	27.142
	Pro	115.1	10850	28.571	Gly-Val	174.2	27.537	Gly-Pro-Ala	243.3	27.496
Acidic	Glu	147.1	2300	28.242	Gly-Ala	146.2	27.819	Gly-Ala-Ala	217.2	27.578
	Asp	133.1	2250	28.621						
Hydrophilic	Tyr	181.2	12000	27.815						
	Cys	121.1	4200	27.963						
	Gln	146.1	-400	28.542						
	Thr	119.1	1850	28.549						
	Gly	75.07	0	28.557	Gly-Gly	132.1	27.971	Gly-Gly-Gly-Gly-Gly	303.3	27.668
	Ser	105.1	170	28.663	Gly-Asn	189.2	28.037	Gly-Gly-Gly-Gly	246.2	27.761
	Asn	132.1	-40	28.663						

*Values for ΔG° reported by Cheftel et al (1985) and sourced from Bigelow (1967) and Ney (1972).

5.4.2.1 Effect of molecular weight on elution time

The results (Table 5.1) show that the general rule is that, with the exception of the basic amino acids and peptides, the higher molecular weight amino acids and peptides elute sooner. Examples of increasingly larger peptides were included in order to confirm this. The effect of increased chain length from Gly to Gly-Gly, Gly-Gly-Gly-Gly and Gly-Gly-Gly-Gly-Gly was a decrease in the elution time from 28.558 to 27.972 to 27.761 to 27.668 min in a non-linear fashion (Fig. 5.2). As Gly has no side-chain hydrophobicity, this is a molecular weight effect. The peak size reflects both the amount of Gly and the number of peptide bonds. Gly is depicted separately because it has a very low absorbance at 205 nm due to the absence of peptide bonds.

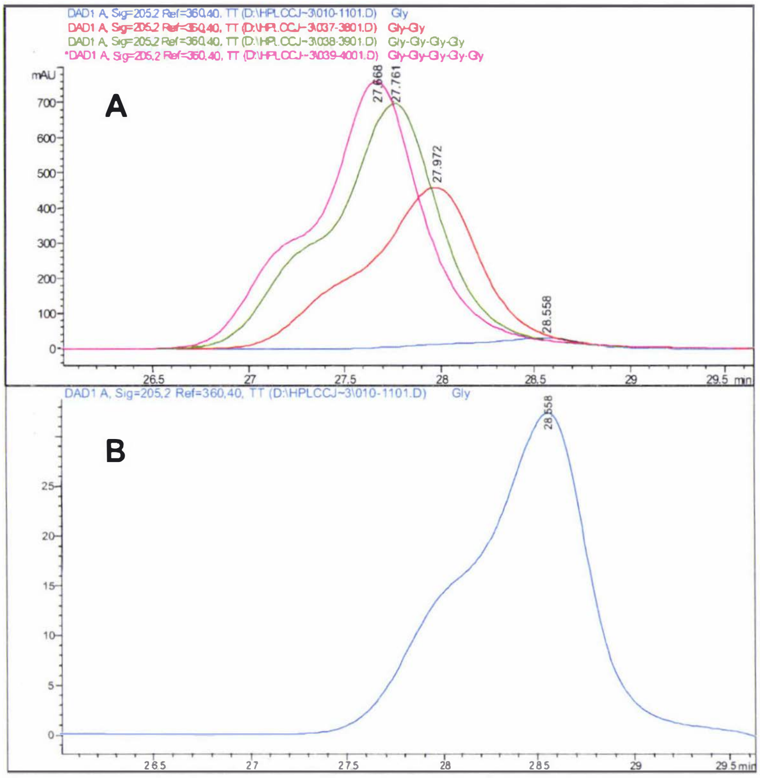


Fig. 5.2 *SE-HPLC – Effect of size on elution time.* The 205 nm traces for Gly and the Gly polymers Gly-Gly, Gly-Gly-Gly-Gly and Gly-Gly-Gly-Gly-Gly are depicted in B and A, respectively. These standards were prepared at a concentration of 10 mg/5 mL of water, diluted 1:1 with the mobile phase and 10μL was loaded on to the column. Refer to Fig. 5.1 caption for details of sample analysis.

An examination of the elution times of all of the dipeptides analysed (Table 5.1) showed that there were two groups. The first group contained no basic amino acids and elution time was closely related to the molecular weight, with the higher molecular weight dipeptides eluting earlier. The second group contained dipeptides with basic side-chains and within this group elution time was inversely related to the molecular weight.

5.4.2.2 Effect of hydrophobicity on elution time

The effect of increasing molecular weight and hydrophobicity on the elution time of Gly (MW 75.07, with no side-chain hydrophobicity) and Ala (MW 89.09; with a side-chain hydrophobicity of 3100 J/mol), to Gly-Ala (MW 146.2) and Gly-Ala-Ala (MW 217.2), was a decrease in elution time from 28.557 (Ala, 28.315) to 27.819 and 27.578 min, respectively (Table 5.2). The decrease in elution time was greater than occurred from Gly to Gly-Gly-Gly-Gly-Gly despite the lower molecular weight of Gly-Ala-Ala and this has been attributed to hydrophobicity.

Table 5.2 *SE-HPLC - Effect of size and hydrophobicity on the order of elution of di-, tri- and pentapeptides*

Amino acid or peptide	MW (Da)	Hydrophobicity (ethanol to water) ΔG^o side chain (J/mol)	Elution time (min)
Gly	75.07	0	28.557
Ala	89.09	3100	28.315
Gly-Ala	146.2		27.819
Gly-Ala-Ala	217.2		27.578
Gly-Gly-Gly-Gly-Gly	303.3		27.668

Similarly the effect of increasing molecular weight and hydrophobicity on the elution time of Gly (MW 75.07; with no side-chain hydrophobicity) and Leu (MW 131.2; with a side-chain hydrophobicity of 10100 J/mol) to Leu-Gly (MW 188.2) was a decrease in elution time from 28.557 (Leu 27.779) to 27.412 (Leu-Gly) (Table 5.3). The decrease in elution time was greater than would be expected from molecular weight alone (based on Gly to Gly-Gly-Gly-Gly-Gly) and it is likely to be a result of the combination of hydrophobicity and molecular weight.

Table 5.3 *SE-HPLC - Effect of hydrophobicity and size on the order of elution of di- and pentapeptides*

Amino acid or peptide	MW (Da)	Hydrophobicity (ethanol to water) ΔG^o side chain (J/mol)	Elution time (min)
Gly	75.07	0	28.557
Leu	131.2	10100	27.779
Leu-Gly	188.2		27.412
Gly-Gly-Gly-Gly-Gly	303.3		27.668

5.4.2.3 Effect of net charge on elution time

Where there was a positively charged species, the elution time was shorter than would be expected from the molecular weight. The amino acids Arg, His and Lys eluted at 26.623, 27.232 and 26.597 min, respectively, well ahead of the other amino acids (Tables 5.1 and 5.4). The dipeptides containing basic amino acids also eluted earlier than expected if molecular weight alone was responsible for their elution time (Table 5.4). In summary, the presence of basic R-groups markedly influenced the elution time, which is in agreement with the findings of Irvine and Shaw (1986).

Table 5.4 *SE-HPLC - Effect of net charge and size on the order of elution of amino acids, di- and pentapeptides.*

Amino acid or peptide	MW (Da)	Net charge at pH 2	Hydrophobicity (ethanol to water) ΔG^0 side chain (J/mol)	Elution time (min)
Asp	133.1	1	2250	28.621
Gly	75.07	1	0	28.557
Gly-Gly	132.1	1		27.971
Gly-Gly-Gly-Gly-Gly	303.3	2		27.668
His (His MW = 155.2)	209.6	2	2100	27.232
Arg HCl (Arg MW = 174.2)	210.7	2	3100	26.623
Lys HCl (Lys MW = 146.2)	182.6	2	6250	26.597
Asp-Lys	261.3	2		25.906
Lys-Lys	347.3	3		24.464

There are at least two possible explanations for the effect of positive charge on the order of elution of the amino acids and very small peptides. Although the silica matrix of the column is blocked to prevent interactions with the solute molecules, blocking is incomplete and at low pH the silanol groups on the column surface may take up a positive charge ($\equiv Si-OH_2^+$) (Irvine, 1987) and repel the positively charged amino acids. This would prevent entry of the basic amino acids and small peptides into the smaller pores of the matrix and they would elute earlier than expected. In an alternative explanation, the eluent incorporates TFA, a fluorinated acid that can form ion pairs with the basic groups (Lys, Arg, His) on the protein or peptide to prevent them reacting with any acidic silanol groups that are not blocked on the silica matrix of the column. This ion-pairing between the TFA anions (113 Da) and the positive charges on the amino acids also increases the apparent molecular weight of the small peptides and amino acids, and may be responsible for their order of elution. Although the number of positive charges affects the order of elution of the very small peptides and amino acids it is less likely to affect the larger peptides and proteins due the more uniform distribution of charge and the much greater ratio of molecular weight to net positive charge. It is probable that some combination of the two mechanisms is responsible for the order of elution of the peptides.

5.4.3 Repeatability of peak elution time and area

Two sets of molecular weight standards, standard sets A and B (Ch. 5, section 5.3.2.3), were analysed six times over a period of 36 h to examine the repeatability of the peak elution time and peak area. The results are presented in Figs. 5.3 and 5.4, respectively. In Figs. 5.3A and 5.4A, each of the six traces is laid directly over the others and in Figs. 5.3B and 5.4B, the same six traces are offset from one another. The values for peak elution time and peak area can be found in the calibration table (see appendix, section 5.8.1). Repeatability of elution time and peak area was excellent, with the peaks overlaying each other almost exactly.

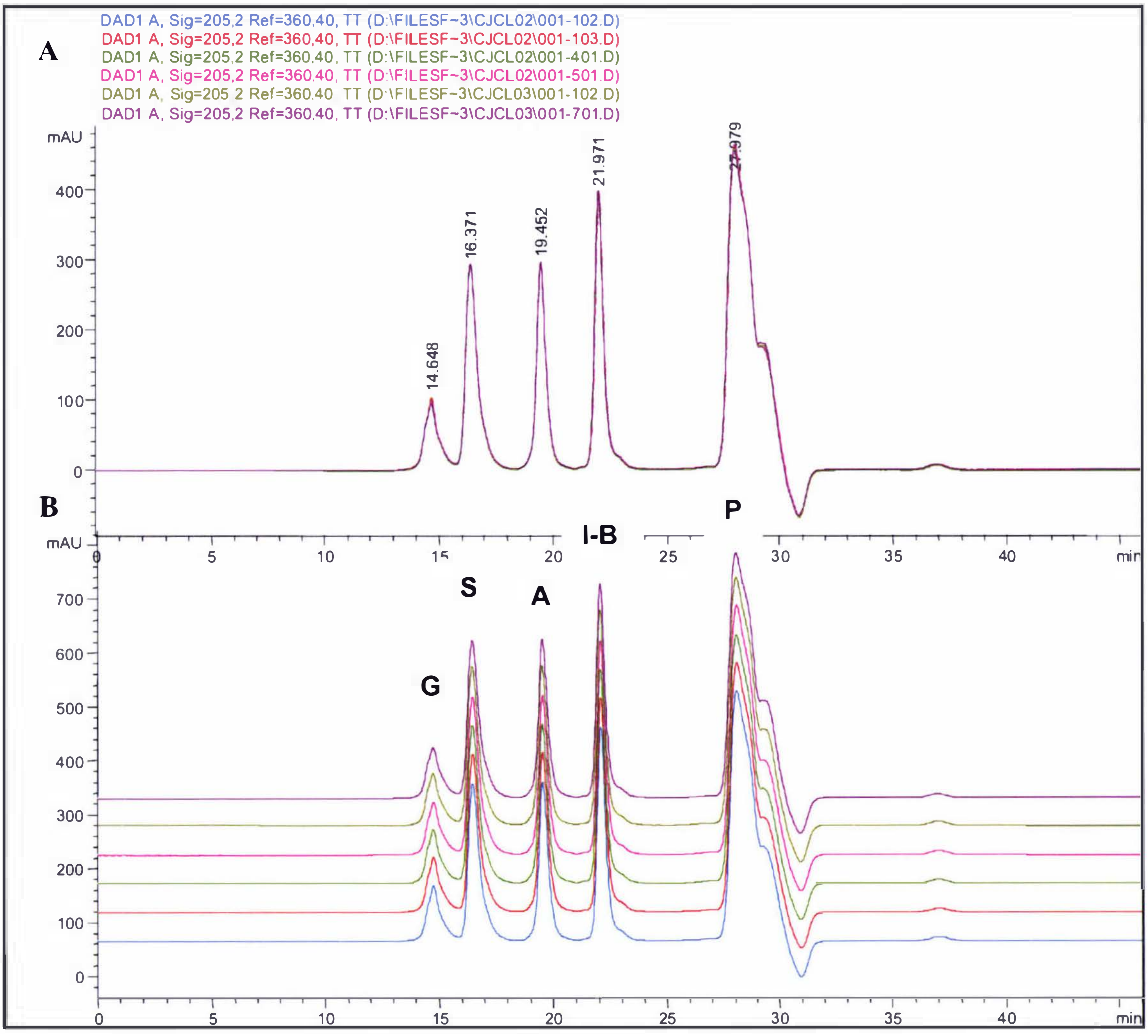


Fig. 5.3 *SE-HPLC – Repeatability of analysis of standard set A.* Standard set A contained 1 mL of each of glyceraldehyde 3-phosphate dehydrogenase (**G**) (36000 Da), soybean trypsin inhibitor (**S**) (20100 Da), aprotinin (**A**) (6500 Da) and insulin B-chain (**I-B**) (3400 Da) solutions and 0.2 mL of the phe (**P**) (165 Da) solution that had been individually prepared at a concentration of 1 mg/10 mL mobile phase. 50 μL of the mixture was loaded on to the column. Refer to Fig. 5.1 caption for details of sample analysis. The 205 nm traces are depicted.

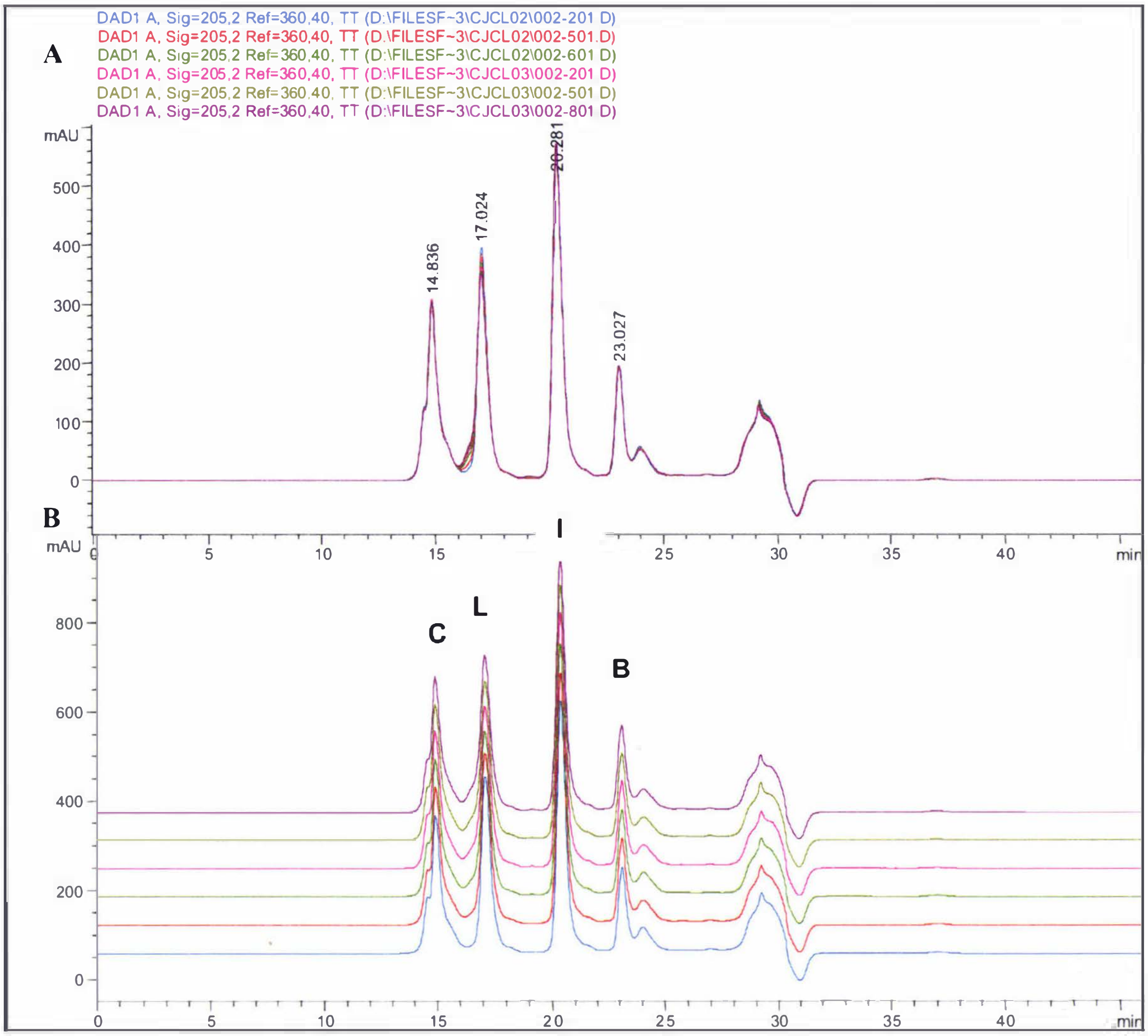


Fig. 5.4 *SE-HPLC - Repeatability of analysis of standard set B.* Standard set B contained 1 mL of each of carbonic anhydrase (**C**) (29000 Da), lysozyme (**L**) (14300 Da), insulin (**I**) (5730 Da) and bacitracin (**B**) (1420 Da) solutions that had been individually prepared at a concentration of 1 mg/10 mL mobile phase. 50 µL of the mixture was loaded on to the column. Refer to Fig. 5.1 caption for details of sample analysis. The 205 nm traces are depicted.

5.4.4 Calculation of molecular weight standard curve

A standard curve (Fig. 5.5) was calculated using the elution times of molecular weight standards (Figs. 5.3 and 5.4) shown in the calibration table (Ch. 5, section 5.8.1). Molecular weights for particular elution time ranges were calculated from the slope of the standard curve and can be found in the lower segment of the calibration table (see appendix, section 5.8.1).

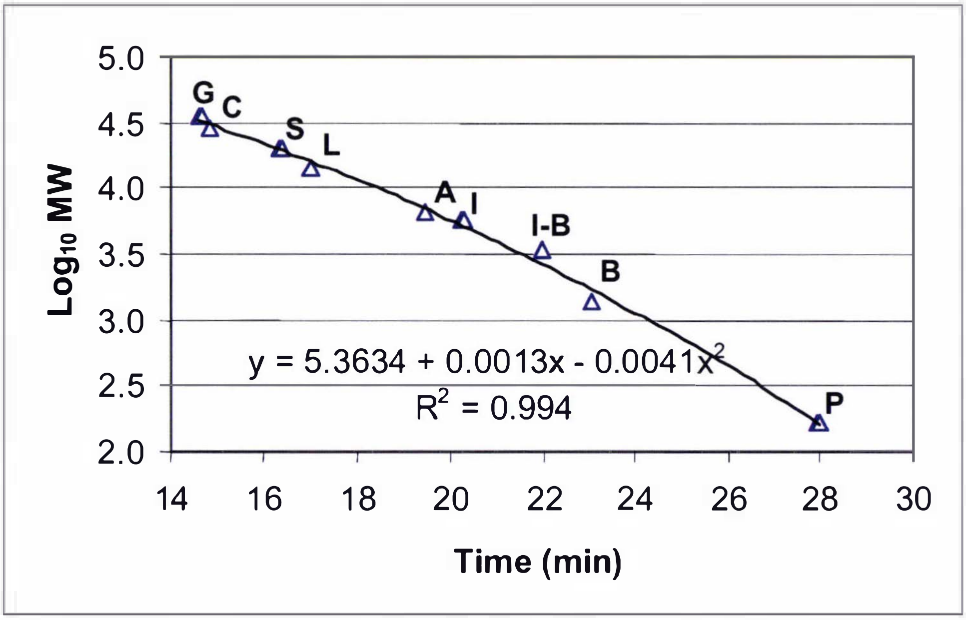


Fig. 5.5 *SE-HPLC - 2^nd^ order calibration curve derived from elution times of molecular weight standards.* Log_{10} molecular weight versus retention time (min) of glyceraldehyde-3-phosphate dehydrogenase (**G**) (36000 Da), carbonic anhydrase (**C**) (29000 Da), soybean trypsin inhibitor (**S**) (20100 Da), lysozyme (**L**) (14300 Da), aprotinin (**A**) (6500 Da), insulin (**I**) (5730 Da), insulin B-chain (**I-B**) (3400 Da), bacitracin (**B**) (1420 Da) and phe (**P**) (165 Da). The results from six replicates were plotted.

The results (Figs. 5.3, 5.4 and 5.5; appendix, section 5.8.1 (data)) indicate that the elution time for any protein was repeatable, with an average standard deviation of 0.414 s (the average of the standard deviations for the six elution times of each of the nine protein standards). Protein standards with a molecular weight of less than 29000 Da were well separated according to their respective molecular weights, but standards with molecular weights of 29000 and 36000 Da were poorly separated. However, because casein monomers and their products have molecular weights of less than 27000 Da, the method should be suitable for monitoring proteolysis in cheese.

5.4.5 Effect of acetonitrile concentration on protein elution time

The method described by Swergold and Rubin (1983) specified 36% (v/v) or 45% (v/v) acetonitrile in the mobile phase. It is usual when preparing the eluent solution to filter the water and acetonitrile separately under vacuum, and then to combine them before adding the TFA. If filtration was done after the solvents were mixed, some evaporation of the more volatile acetonitrile would probably occur, thus altering the acetonitrile concentration in the eluting solvent. The effect of acetonitrile concentration (±1%) was examined in order to test the sensitivity of the analysis results to solvent composition. Standard sets A and B were each analysed twice using an eluent containing 35, 36 or 37% acetonitrile. Fig. 5.6 depicts one trace from standard sets A and B at each acetonitrile concentration.

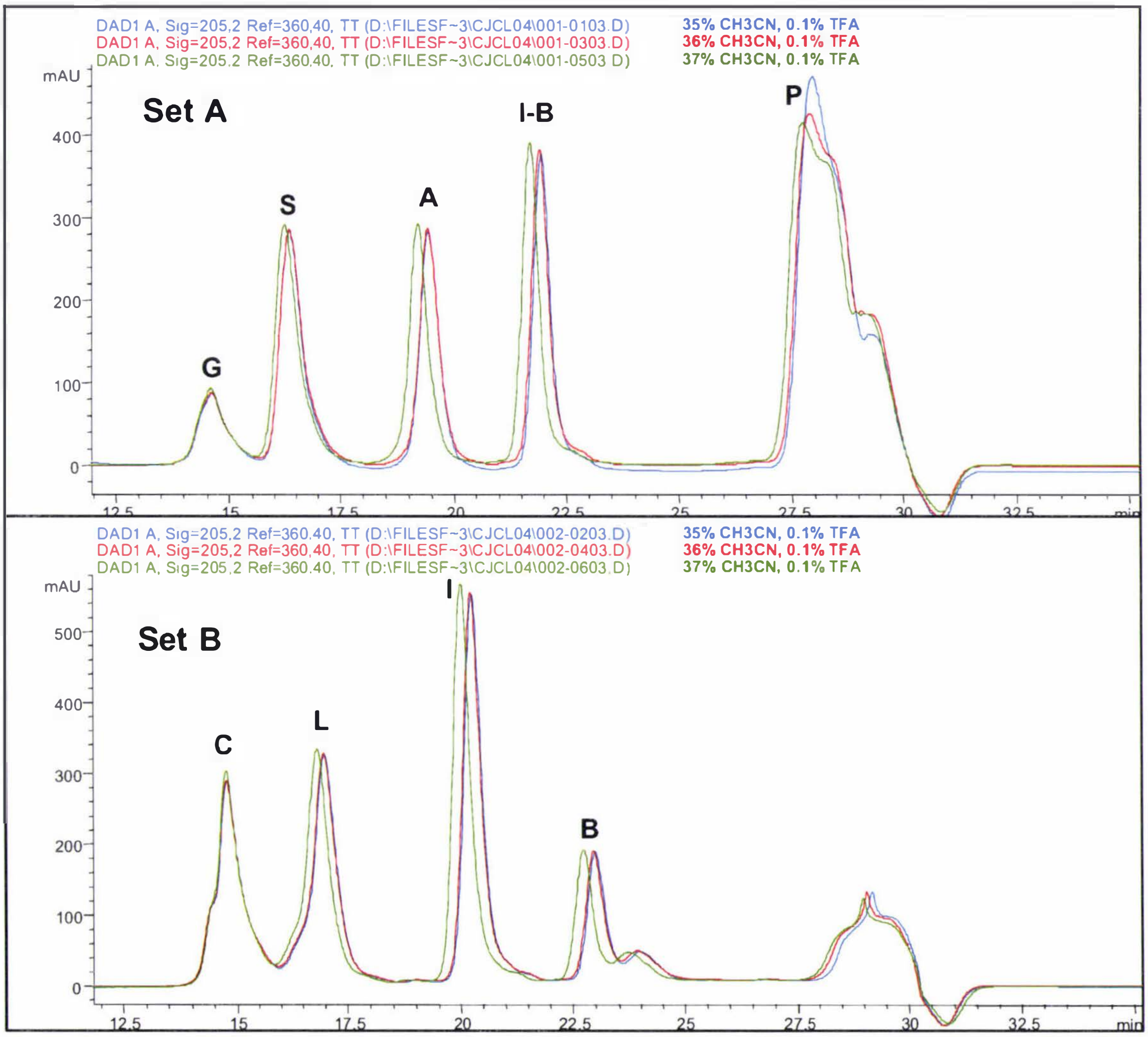


Fig. 5.6 *SE-HPLC – Effect of acetonitrile concentration on elution time of standard sets A and B.* Refer to captions of Figs. 5.3 and 5.4 for details of preparation of standards and meaning of symbols. The mobile phase contained *35%, 36% or 37%* acetonitrile and 0.1% TFA in water and the flow rate was 0.4 mL/min. The 205 nm traces are depicted.

Reducing the acetonitrile concentration of the mobile phase by 1% resulted in a very slight increase in elution time of the proteins that eluted after more than 20 minutes (Fig. 5.6) that would be unlikely to have any effect on the results. Increasing the acetonitrile concentration of the mobile phase by 1% had a more marked effect, with the proteins eluting after more than 20 min decreasing in elution time by up to 0.24 min. Protein elution times of duplicate samples are shown in the appendix (section 5.8.2).

Shifts in elution time achieved by using different concentrations of acetonitrile in the eluent could be used to advantage if it was necessary to spread out a particular region of the trace. For example, the region of the trace between 23 and 28 min in 36% acetonitrile could be spread out by using 37% acetonitrile to bunch the earlier peaks. Conversely, the region from 20 min could be spread out by using 35% acetonitrile to achieve an increasingly later elution time. These strategies could be useful if it was necessary to collect peaks within these regions for further analysis.

It is intended that this SE-HPLC method be used to analyse cheese fractions and to determine the approximate molecular weight of peaks within the fraction. As each set of analyses would be accompanied by standards, and a standard curve would be generated for each set, it is anticipated that there should be no effect on the calculation of peak molecular weight if the concentration of acetonitrile is a little different for some reason. Fig. 5.7 shows the standard curves generated for each acetonitrile concentration (35, 36 and 37%).

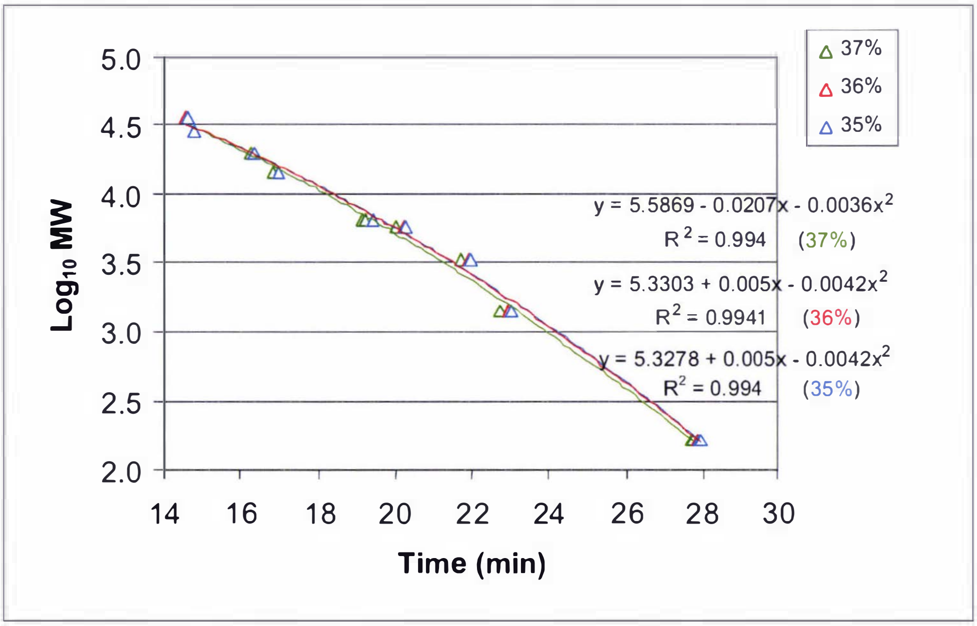


Fig. 5.7 *SE-HPLC - 2^{nd} order calibration curves of molecular weight standards eluted with 35, 36 or 37% acetonitrile in the mobile phase.* Two values are plotted for each protein standard at each acetonitrile concentration.

The slope of each of the three curves (Fig. 5.7) was used to calculate the molecular weight of the components of a peak that would elute at 20 min. The values obtained were 5595, 5627 and 5406. The values for 35 and 36% acetonitrile were in reasonable agreement with each other, indicating that the method is robust enough to withstand a small evaporative loss of acetonitrile that may occur during a long run. (In our laboratories, the temperature is constant at 22°C, the acetonitrile flasks are reasonably well sealed, and evaporation is not a problem even during a run that may take 48 h. As well, the standards are usually analysed first, after every 8th sample and last.)

It is anticipated that if the method proves useful for cheese analysis, peak selection would be automated so that the method can handle large numbers of samples. If peak selection is based on retention time, the concentration of acetonitrile should be precise. However, if the selection of peaks is based on molecular weight regions as calculated from the standard curve, concentrations between 35 and 36% would be appropriate.

5.4.6 Measurement of absorbance of peptides and amino acids

In order to obtain a semi-quantitative measure of the quantities of proteins, peptides and amino acids in cheese, their relative absorbance needs to be known. Before moving into the cheese system it was necessary to consider the absorbance spectra of various amino acids and peptides that occur in the WSF of cheese.

5.4.6.1 Amino acid absorption

To examine the effect of wavelength, each of the amino acids was separately analysed by SE-HPLC, the absorption spectra were measured, and the results compared (Fig. 5.8). The same two sets of traces are shown, the first drawn using absolute values which show the relativity of the spectra of the amino acids and the second using normalised values which show the spectra of the less absorbing amino acids more clearly.

The results (Fig. 5.8) indicated that measurement of the absorbance at 205 nm was appropriate for the display and measurement of results. This wavelength was sufficiently above the background noise for a flat baseline to be established and was close to the maximum absorbance of the non-aromatic amino acids. Many amino acids had very little absorbance at wavelengths above 205 nm and it was essential that the absorbance be determined at around 205 nm for their presence to be recorded. The aromatic amino acids, together with histidine, methionine and cysteine provided a disproportionate contribution to the absorbance, but at 205 nm their relative contribution was lower than it would have been at 214 nm.

In the past, a zinc lamp with a 214 nm emission was used as the light source for protein, peptide and amino acid detection. The 214 nm emission from a zinc lamp is stable and the absorbance of most proteins is similar at this wavelength. However, this is not true for smaller peptides, where the contribution to the absorbance is from a small range of amino acids. With the diode array detector used for the current work it was unnecessary to use 214 nm and preferable to use the lowest wavelength possible to measure the absorbance of small peptides and amino acids.

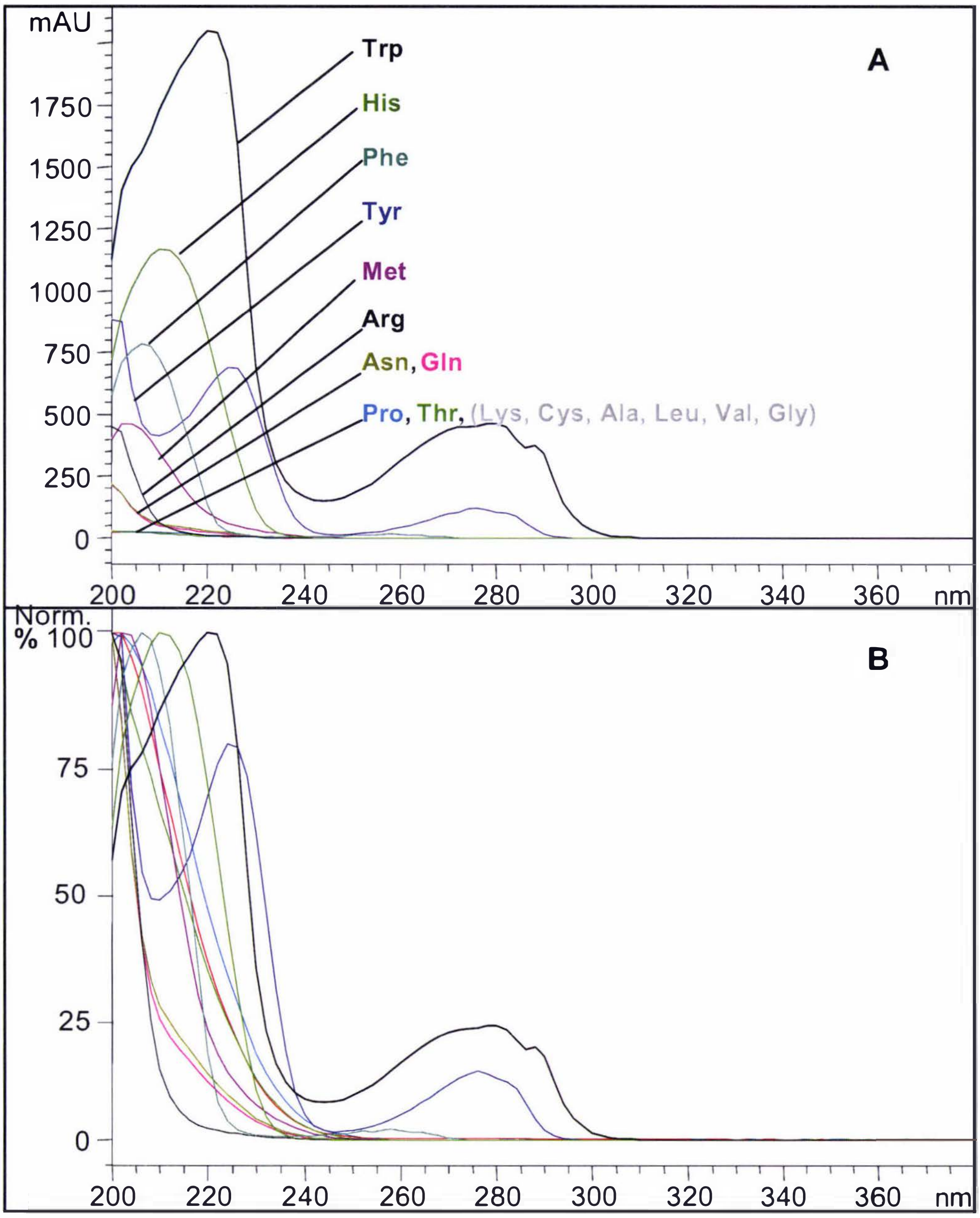


Fig. 5.8 *Absorption spectra of representative amino acids.* With the exception of the aromatic amino acids (Tyr, Trp and Phe), the standards were prepared at a concentration of 10 mg/5 mL of water, diluted 1:1 with the mobile phase and 10 μL was loaded on to the column. The aromatic amino acids were made to half the concentration of the non-aromatic amino acids and 5 μL was loaded on to the column, to a give a final loading that was 25% that of the non-aromatic amino acids. Refer to Fig. 5.1 caption for details of sample analysis.

5.4.6.2 Peptide bond absorption

Proteins absorb light in the far ultraviolet region of the spectrum, due to the presence of the peptide (amide) bonds that link individual amino acids together in a protein. The absorption band extends from 180 nm to 240 nm, and the absorbance is proportional to the number of peptide bonds, so that, on a molar basis, larger peptides absorb more strongly than small peptides. For example, dipeptides have a lower absorbance than tetrapeptides, because they have one peptide bond per molecule as opposed to three in the tetrapeptide (Fig. 5.9).

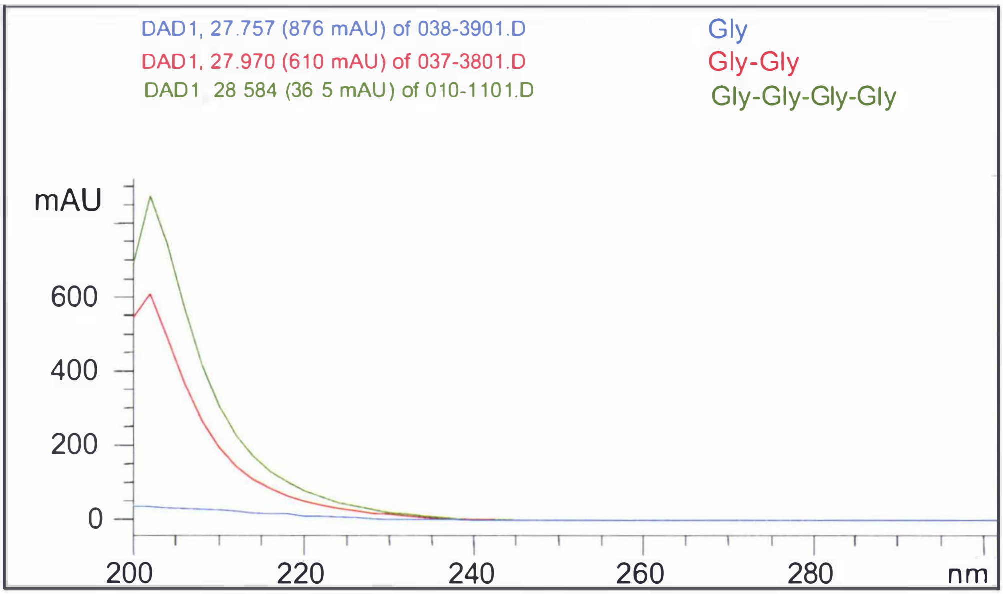


Fig. 5.9 *Absorption spectra of Gly, Gly-Gly and Gly-Gly-Gly-Gly.* Each solution was prepared at a concentration of 10 mg/5 mL of water, diluted 1:1 with the mobile phase and 10 μL was loaded on to the column. Refer to Fig. 5.1 caption for details of sample analysis.

The absorption spectra (Fig. 5.9) show that the wavelength of 205 nm is close to the maximum absorbance for peptide bonds.

As protein and peptide hydrolysis proceeds, there is a decrease in absorbance as the peptides are hydrolysed and the peptide bonds disappear. The relative contribution of the amino acids to the absorbance then becomes progressively greater. However, some amino acids have a greater absorbance than others and small peptides containing these amino acids (particularly aromatic amino acids) would have a disproportionately large absorbance relative to their concentration. Consequently, the observed absorbance of small peptides may not be directly proportional to the concentration of the peptide bonds nor the amount of material present. Therefore, the profiles should be used with caution when calculating the peak area or height distribution according to molecular weight.

It should be noted that, at wavelengths from 180 to 200 nm, the background absorbance of the solvent, oxygen in the air and the light intensity available result in a very noisy baseline that makes measurements at these wavelengths impractical.

5.4.7 SE-HPLC of the USF and WSF of Cheddar cheese

The SE-HPLC method was used to compare typical chromatograms of the WSF and USF of samples of mature Cheddar cheese. Samples of the WSF and USF of the cheese, prepared at the same concentration (0.2% cheese) (see Ch. 4, section 4.2.3.3) were loaded on to the column (50 μL) and analysed by SE-HPLC (Fig. 5.10). The scales on the two graphs (Fig. 5.10A and 5.10B) differ by a factor of ~11. Each graph shows the absorbance at 205, 210, 220, 280 and 295 nm.

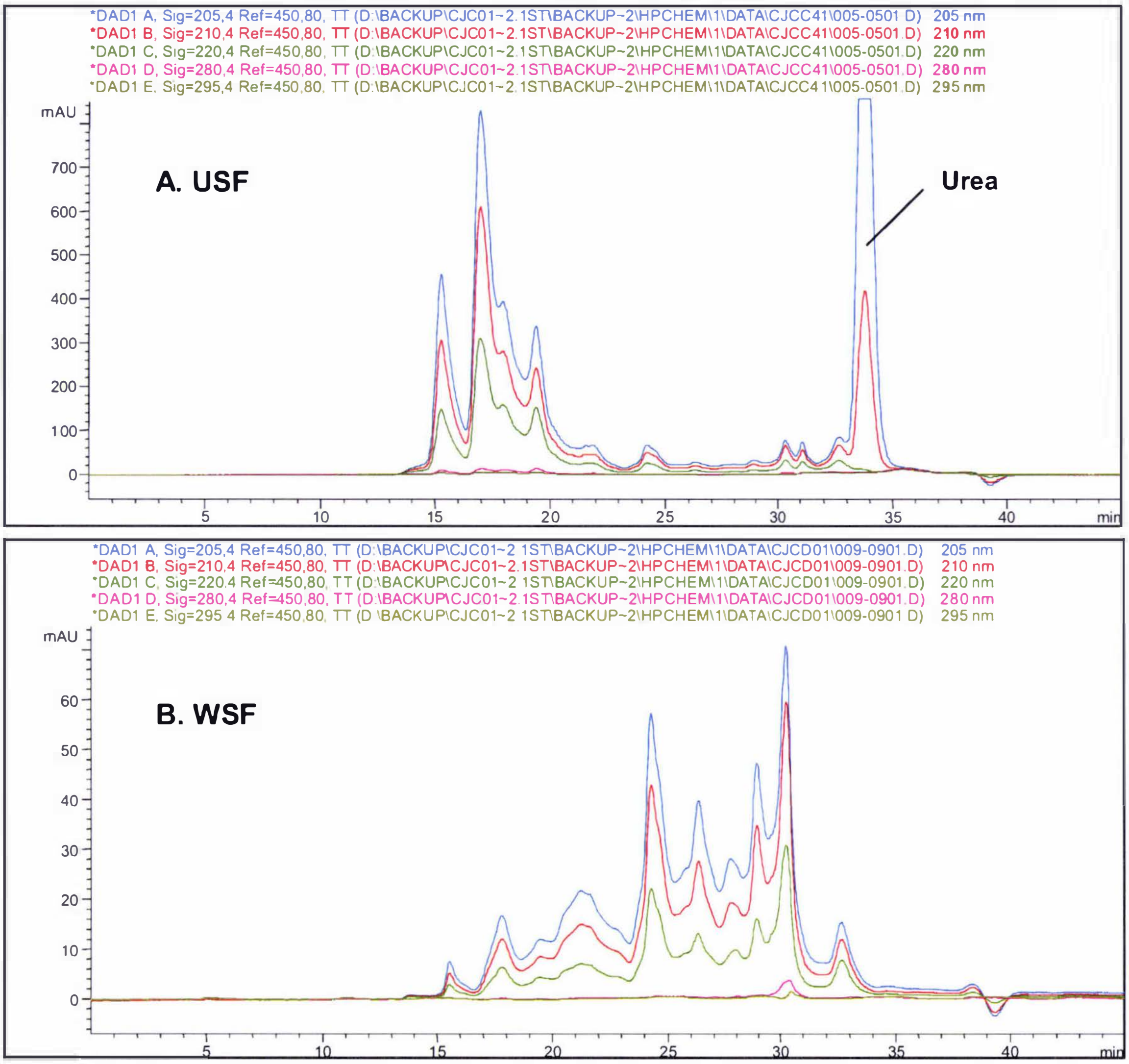


Fig. 5.10A and B *SE-HPLC - Analysis of the USF (A) and WSF (B) of mature Cheddar cheese* at 205, 210, 220, 280 and 295 nm. The cheese samples were prepared at the same concentration (0.2% cheese) and 50 μL of either the A) USF or B) WSF was loaded on to the column. The mobile phase contained 36% acetonitrile and 0.1% TFA in water and the flow rate was 0.4 mL/min.

At 205 nm the absorbance of all of the protein, peptide and amino acid peaks was greater than at any other wavelength (Fig. 5.10). At this wavelength, the absorbance of the peptide bonds (Fig. 5.9) and many of the amino acids (Fig. 5.8) is higher than at any of the other wavelengths and the contribution of the aromatic amino acids is reduced (Fig. 5.8). This wavelength is therefore considered the most appropriate for the analysis of cheese.

The 205 nm traces of the USF and WSF of mature Cheddar cheese were overlaid in the same scale (Fig. 5.11A), and with the signals in 'full' scale (Fig. 5.11B). Scaling was automated, with the largest peak on each chromatogram set to 100%, and the remaining peaks scaled accordingly.

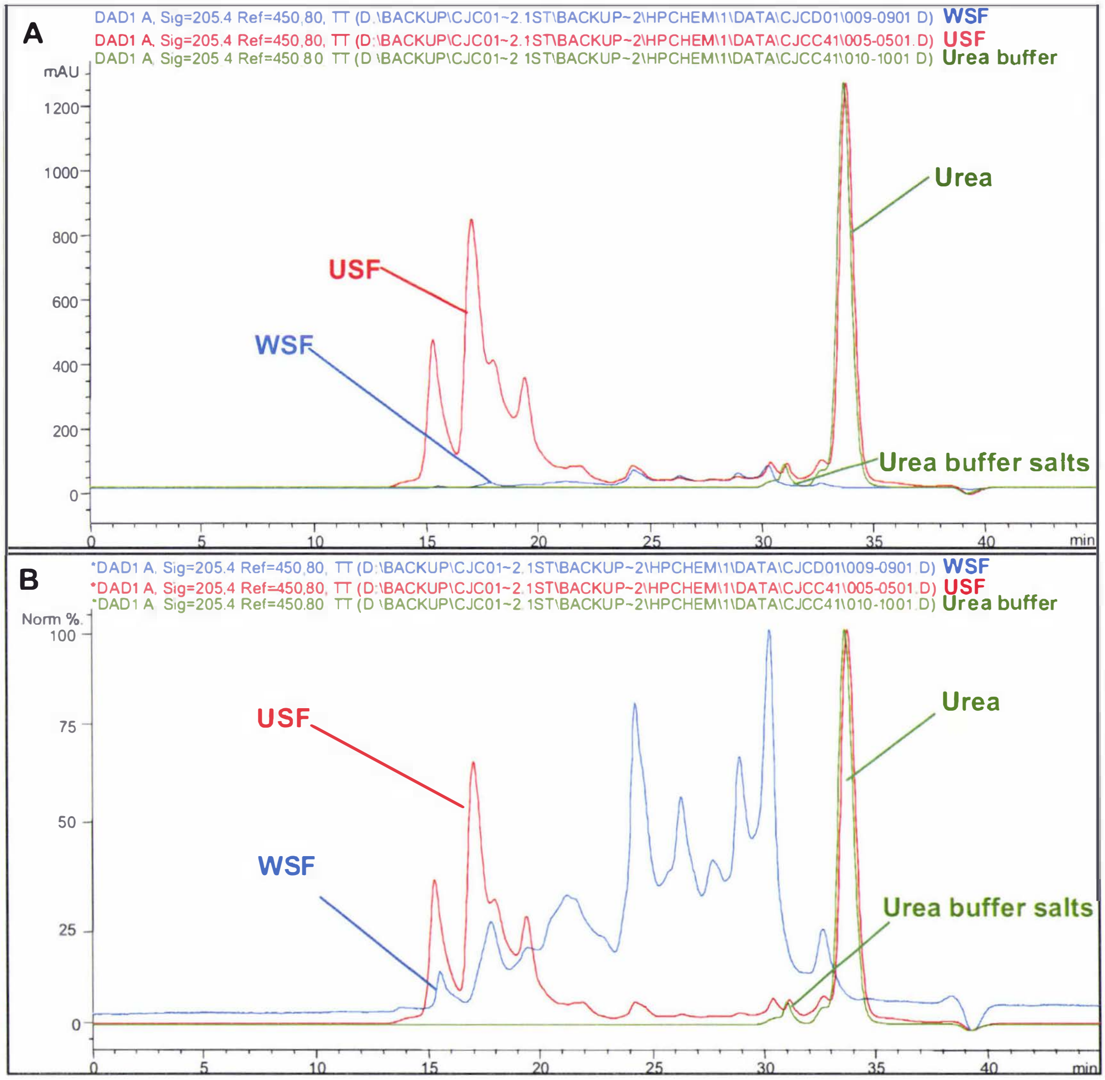


Fig. 5.11A and B *SE-HPLC- A comparison of the USF and WSF of mature Cheddar cheese at 205 nm.* The traces of the USF and WSF (Fig. 5.10) are displayed in the same scale (A) and in 'full' scale (B) (the urea peak in the USF was largest and set to 100%. The peak at ~30 min in the WSF was used to scale the chromatogram to 100%). Refer to Fig. 5.10 caption for details of sample preparation and analysis.

The USF contains all of the proteins, peptides and amino acids present in the cheese. An examination of the chromatograms (Fig. 5.11) showed that the proteins and large peptides that eluted between 15 and 23 min were dominant. The smaller later-eluting peptides were less evident because they were present at lower concentrations and because they have fewer peptide bonds. The large urea peak dominated the low molecular weight end of the chromatogram. The urea buffer contributed to the absorbance of peaks in the USF that eluted after 30 min.

The WSF (Fig. 5.11) contained large water-soluble peptides that eluted between 15 and 23 min, and smaller water-soluble peptides and amino acids that eluted after 23 min. The peptides and amino acids in the later-eluting peaks were predominant.

It was evident that the early-eluting peaks of the WSF (15 – 23 min) were minor components of the early-eluting peaks in the USF and that with the exception of these, most peptides in the USF were present in the WSF at about the same concentration.

5.4.8 Molecular weight distribution of cheese peptides

The WSF of a mature Cheddar cheese was prepared according to the method described in Ch. 4, section 4.2.3.3.3 and analysed by SE-HPLC. Two sets of molecular weight standards were prepared according to the method described in section 5.3.2.3, and separately analysed in the same set of 'runs' as the cheese sample. The molecular weight distribution of peptides and amino acids in the cheese (Fig. 5.12) was calculated from a standard curve (Fig. 5.13) and calibration table (see appendix, section 5.8.3) derived from the elution times of molecular weight standards in sets A and B.

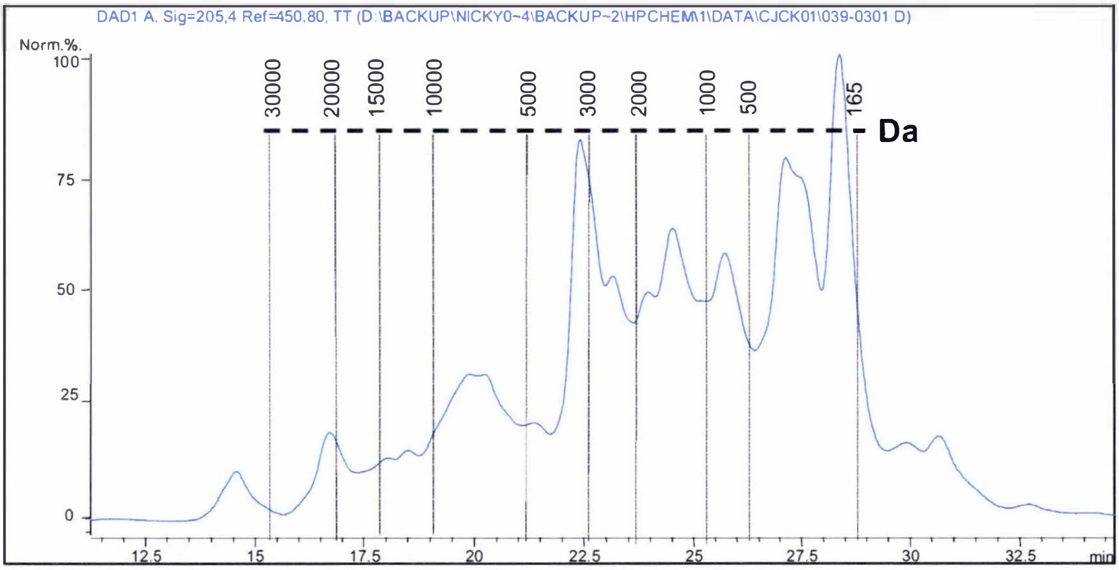


Fig. 5.12 *SE-HPLC – The molecular weight distribution of the WSF of mature Cheddar cheese.* Refer to Fig. 5.10 caption for details of sample preparation and analysis. The 205 nm trace is depicted. The molecular weight distribution scale (Da) was calculated from the protein standard calibration curve (Fig. 5.13).

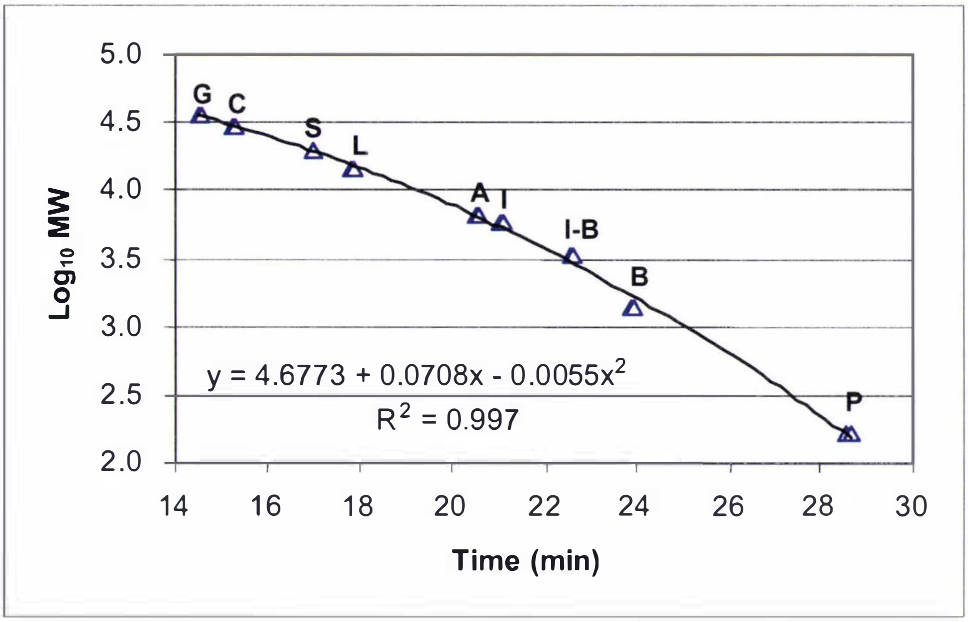


Fig. 5.13 *SE-HPLC - 2nd order calibration curve derived from elution times of molecular weight standards.* Log_{10} molecular weight versus elution time (min). There are three data points for each molecular weight standard. Equation used for estimates of molecular weight distribution (Fig. 5.12). Refer to Fig. 5.5 caption for meaning of symbols.

Differences between the equations depicted in Figs 5.5 and 5.8 were caused by shifts in retention time that occurred when a different HPLC and column were used and highlight the importance of including standards in the same set of 'runs' as the samples.

5.4.9 Repeatability of SE-HPLC sample preparation and analysis

The repeatability of the SE-HPLC method was determined by analysing the WSF of a Cheddar cheese sample 5 times. The WSF was prepared and analysed by SE-HPLC according to the methods described in Ch. 4, sections 4.2.3.3. The five integrated (205 nm) traces were overlaid so that the traces, drop-lines and bars were visible. The traces were almost identical to one another (Fig. 5.14) and it can be concluded that the analysis was very repeatable.

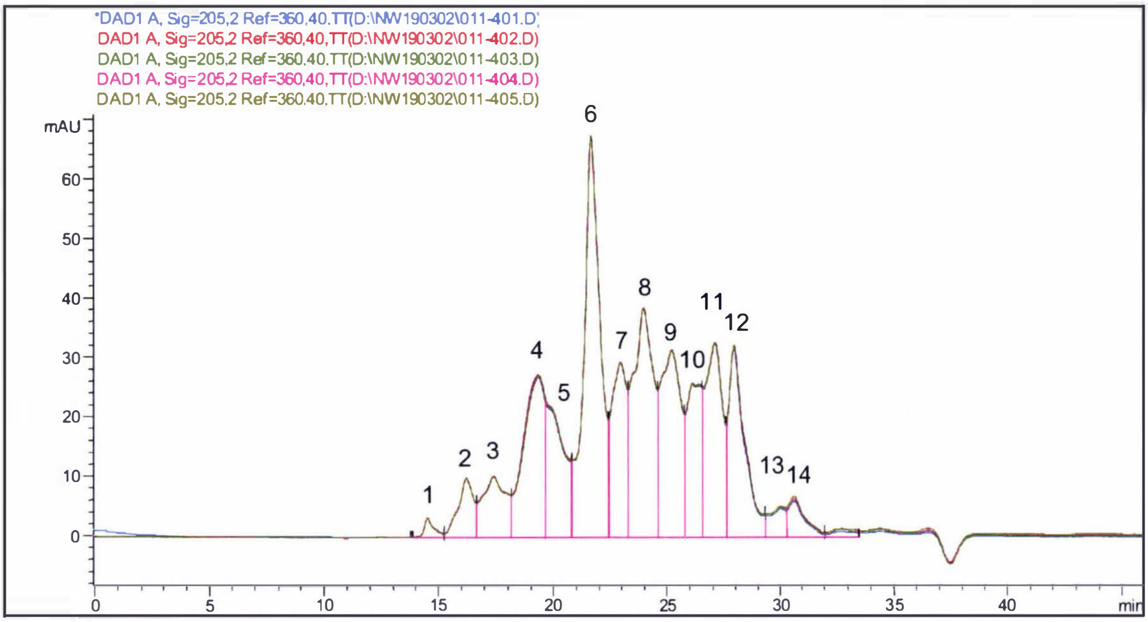


Fig. 5.14 *SE-HPLC - Repeatability of analysis of the WSF of cheese.* The WSF of a Cheddar cheese was analysed five times and the traces (205 nm) were overlaid. Refer to Fig. 5.10 for details of sample preparation and analysis.

The repeatability of the sample preparation was examined by sequentially analysing the WSF of a Cheddar cheese sample that had been prepared 15 times as described in Ch. 4, section 4.2.3.3.2. Each of the 15 extracts was analysed by SE-HPLC, the area of each peak (205 nm) was determined, and the mean, standard deviation and relative standard deviation were calculated for each peak (Table 5.5). The total peak area was reasonably constant for the 15 samples, with the relative standard deviation of 3.75% indicating that the method of fractionation was very repeatable.

Table 5.5 *SE-HPLC - Repeatability of sample preparation and analysis of the WSF of cheese.* The WSF of a Cheddar cheese was prepared fifteen times and analysed by SE-HPLC. The peak areas were determined and the mean, standard deviation and relative standard deviations were calculated. Refer to Fig. 5.10 for details of sample preparation and analysis.

Sample	P 1	P 2	P 3	P 4	P 5	P 6	P 7	P 8	P 9	P 10	P 11	P 12	P 13	P 14	Total Peak Area
1	95	460	739	1796	1124	3337	1354	2318	1976	1275	1418	1655	248	391	18166
2	100	520	757	1802	1097	3367	1343	2299	2003	1304	1390	1554	259	307	18101
3	97	490	723	1731	1163	3352	1351	2317	2017	1322	1399	1587	254	404	18208
4	76	440	661	1485	1082	3032	1226	2108	1820	1040	1455	1444	228	287	16383
5	85	455	646	1587	1130	3164	1284	2207	1898	1266	1328	1488	236	309	17085
6	96	465	732	1755	1183	3367	1358	2323	2003	1321	1400	1575	258	333	18169
7	93	445	697	1696	1155	3292	1331	2277	1986	1101	1558	1549	255	326	17760
8	99	585	771	1677	1257	3325	1350	2312	1939	1323	1385	1537	224	312	18095
9	94	537	758	1602	1174	3287	1324	2301	1910	1243	1432	1526	230	316	17732
10	78	502	690	1601	1133	3088	1246	2162	1808	1177	1351	1450	240	265	16791
11	95	563	762	1689	1298	3341	1356	2352	1973	1215	1538	1572	275	325	18355
12	80	545	760	1720	1256	3375	1356	2351	1965	1290	1441	1585	259	327	18310
13	96	509	755	1561	1313	3327	1351	2340	1961	1293	1443	1585	251	346	18130
14	73	628	797	1717	1368	3406	1384	2398	1989	1321	1483	1620	268	353	18806
15	99	610	805	1774	1328	3428	1392	2403	1996	1334	1476	1614	284	340	18884
Statistics															
Mean	90 31	516.96	736.85	1679 53	1204 15	3299.33	1333.66	2297 88	1949 62	1254.98	1433.13	1555 97	251 23	329 39	17933
Std Dev	8.91	58 23	44.50	90.10	88 28	111.21	45.21	78.80	62.33	84.59	61.02	57.94	16.84	34.37	672
Relative Std Dev	9.87	11 26	6.04	5.36	7 33	3.37	3.39	3.43	3.20	6.74	4.26	3.72	6 70	10 43	3.75
Average Relative Std Dev															4.70

The relative standard deviation for most peaks ranged from 3.20 to 7.33%. However, the very small peaks at beginning and end of each trace had a larger relative standard deviation of 9.87 to 11.26%. This was possibly caused by the difficulty of selecting precisely the same point on each trace to begin and end the peak integration. The effect of this would be amplified for small peaks. As well, the first peaks contained high molecular weight peptides and there may have been some problems with their solubility.

5.5 DISCUSSION

A method capable of measuring the molecular weight distribution of the proteins, peptides and amino acids in cheese could be used to monitor the effect of changes in cheese manufacturing processes on proteolysis, to differentiate cheese or to establish cheese maturity and would be a valuable asset to the New Zealand cheese industry.

The following discussion focuses on the characterisation and development of the SE-HPLC method and an examination of its potential for use in cheese analysis.

The SE-HPLC method can be used to separate proteins and peptides according to molecular weight. Using proteins of various molecular weights as standards, the method was proven highly repeatable in terms of both peak retention time and area (Figs. 5.3, 5.4 and 5.14). The separation was based predominantly on molecular weight, but it was demonstrated that the number of positively charged side-chain groups on amino acids and small peptides significantly decreased the elution time (Table 5.4). Larger peptides have a much greater ratio of size to charge and in most cases are likely to be affected similarly by positively charged side-chains. Similarly, the hydrophobic side-chain groups on amino acids and small peptides affected their elution time (Tables 5.2 and 5.3), and caused them to elute a little earlier than anticipated on the basis of their molecular weight. Fujinari and Manes (1997) also found that the use of an organic solvent (20% 2-propanol and 20% methanol in water and TFA (0.1%)) with a calibrated TSK G-2000 SW column resulted in estimated molecular weights for each peak that were in agreement with the actual molecular weights of the peptides contained within them.

Although the results were very repeatable for a series of analyses within a single HPLC 'run', and repeatability was very good between 'runs', it is possible to get minor shifts in retention time with column ageing, and when a different column is used the effect on elution time can be more marked (Fig. 5.5 and 5.13). Therefore it is essential that sets of molecular weight standards (standard sets A and B) be analysed, at least in duplicate, to obtain a calibration data set that can be used to determine the molecular weight distribution of the peaks within any samples analysed.

A high concentration of acetonitrile (36 or 45%) was recommended by Swergold and Rubin (1983) for reducing hydrophobic and ionic interactions between the solute and column matrix and promoting size exclusion behaviour that resulted in reproducible elution times. The importance of a precise concentration of 36% acetonitrile for reproduciblity of retention time was examined (Fig. 5.6). The effect of having a 1% decrease in acetonitrile concentration was minimal, with only a slight increase in the elution times of the later-eluting peaks. This is particularly important because acetonitrile is a volatile solvent and in some situations, such as vacuum assisted filtration or degassing, a small amount of evaporation may occur. The effect of having a 1% increase in acetonitrile concentration was marked, with a decrease in elution time of up to 0.24 min for peptides eluting between 18 and 28 min. This means that care must be taken with the preparation of the eluting solvents to prevent shifts in elution time. The sensitivity of elution time to concentration was determined for 35 - 37% acetonitrile and may not apply to other concentrations.

The SE-HPLC method had not been used to investigate cheese and it was necessary to identify suitable cheese fractions and select a suitable concentration for analysis using SE-HPLC. The first fraction (equivalent to the total nitrogen) was prepared by solubilising the cheese in an alkaline urea sample buffer, removing the fat and diluting the soluble fraction with the SE-HPLC mobile phase. SE-HPLC of this fraction (0.2% cheese) separated the proteins, peptides, amino acids and other small urea-soluble components in cheese into a series of peaks (Fig. 5.10A). The early-eluting peaks had a high absorbance and contained proteins and large peptides that dominated the trace. In contrast, the absorbance of the later-eluting peaks that contained small peptides and amino acids was low and the peaks were small. The second fraction (equivalent to the WSN) was prepared from the water-soluble components in the cheese and diluted with the SE-HPLC mobile phase. SE-HPLC separated the components of this fraction (0.2% cheese) into a number of peaks containing water-soluble peptides and amino acids (Fig. 5.10B). A possible advantage of analysing a fraction that did not contain casein and urea was that large water-soluble peptides were visible. These peptides co-eluted with the caseins and large water-insoluble peptides in the USF.

It was possible to calculate the molecular weight distribution of proteins, peptides and amino acids on the traces from the elution times of molecular weight standards analysed at the same time as the samples and used to generate a standard curve and calibration tables.

There is always concern that preparation of cheese extracts could be variable (Polychroniadou et al, 1999). The WSF is likely to exhibit greater variability than the USF in which all proteinaceous material is solubilised. In the present study, a large representative cheese sample was finely grated, mixed carefully, and always prepared to the same concentration (3 g ± 0.0005 g/30.0 mL water). The combination of this extraction protocol and SE-HPLC of the extract (WSF) resulted in repeatable traces (Fig. 5.14 and Table 5.5).

These preliminary investigations showed that SE-HPLC provided a useful pattern of the molecular weight distribution of the proteins and peptides in cheese. The method therefore has potential for examining differences in proteolysis in different cheese types and for monitoring changes in the molecular weight distribution of cheese proteins and peptides during ripening.

5.6 CONCLUSIONS

A SE-HPLC method was characterised and established for the analysis of cheese. The method was robust and provided highly repeatable results in the form of traces of the molecular weight distribution of the proteins and peptides in the urea-soluble and water-soluble fractions of cheese.

5.7 RECOMMENDATIONS

It is recommended that preliminary studies be undertaken to determine whether the SE-HPLC molecular weight distribution profiles of several cheese types are sufficiently different for the cheese to be differentiated. In this instance, it is further recommended that the results obtained using the SE-HPLC method be compared with results from urea-PAGE of the USF and WSF, and RP-HPLC of the WSF of the same cheese. The relationship between the methods should be determined.

6.0 CHARACTERISATION OF PROTEOLYSIS IN SEVEN VARIETAL CHEESES USING SE-HPLC AND TRADITIONAL ANALYTICAL METHODS – A PRELIMINARY STUDY

ABSTRACT

The potential of SE-HPLC for demonstrating differences in the molecular weight distribution of peptides in different cheese types (New Zealand-style Swiss, Elsberg, Cheddar, Mozzarella, Gouda, Feta and Blue cheese selected as 'mature and ready for market' by the manufacturer) was examined and compared with established methods.

NCN and NPN provided information on the amount and extent of proteolysis in each cheese type, and clearly, at the point in maturation that the cheese was sampled, the Mozzarella had undergone only a small amount of proteolysis while the Blue cheese had undergone extensive proteolysis. This enabled these cheeses to be distinguished from the others, which were similar.

Urea-PAGE of the whole cheese (USF) captured approximately 15 peaks that included caseins (β-, α_{s1}- and α_{s2}-casein) and large peptides (α_{s1}-I-, γ_1-, γ_2-, γ_3-, β-I- and β-II-casein) formed when the caseins were hydrolysed. Plasmin activity was characterised by the hydrolysis of β-casein to γ-caseins and was more extensive in the Swiss > Elsberg > Gouda > Mozzarella ≈ Cheddar (> Blue) > Feta. Chymosin activity was characterised by the hydolysis of α_{s1}-casein to α_{s1}-I-casein and was more extensive in the Cheddar (≈ Blue) > Elsberg > Gouda ≈ Feta > Swiss > Mozzarella. The results could be related to the known details of manufacture, storage temperature, storage time and composition.

Urea-PAGE of the WSF of each cheese showed approximately 16 peptides. These corresponded with large late-eluting peaks on the RP-HPLC trace and large early-eluting peaks of >5000 Da on the SE-HPLC trace. The absence of smaller peptides (on the gels) was likely to be due to both the quantity and the size of the peptides in relation to the pore size of the gel. The peptide patterns were different for most cheese types.

SE-HPLC was not as useful as urea-PAGE for examining the USF of the cheese because the caseins and large peptides were contained within a small number of early-eluting peaks and the later-eluting peaks were poorly defined.

Each SE-HPLC (WSF) chromatogram had about 16 distinct peaks, with only the first 5 captured by urea-PAGE. Elution time of most peptides was determined by size, with peptides of similar size grouped under a single peak. Each of the seven cheese types seemed to possess an identifiable

chromatogram that showed the molecular weight distribution of the peptides and could be likened to a fingerprint that represented the sum of the action of the various enzymes present.

RP-HPLC of the WSF of each cheese separated more than 60 peaks in most cheese types, with many common to several cheese types. The exception was Mozzarella cheese, which had few early-eluting low molecular weight peptide peaks because of the effect of the high stretch temperature on the quantity of chymosin, plasmin and especially the microbial enzymes. The Blue cheese was also different from the other cheese types, because of the high concentrations of small peptides and amino acids present. These were attributed to the very proteolytic enzymes of *Penicillium roqueforti* that supplemented chymosin, plasmin and microbial enzyme activity, and resulted in high concentrations of small peptides and amino acids. The complexity of the traces of the remaining cheese types meant that although the traces were different, it was difficult to subjectively distinguish one cheese type from another, particularly when the traces represented a point in maturation that was essentially a "snapshot" in time.

This preliminary study showed that urea-PAGE of the USF or WSF, RP-HPLC of the WSF, and SE-HPLC of the WSF provided a pattern of proteolysis that was different for each cheese type. It therefore seemed likely that each of these methods might be useful for distinguishing cheese types from one another.

6.1 INTRODUCTION

There are a large number of hard and semi-hard cheese varieties produced throughout the world. These cheeses are usually matured for a period that may extend to several months, or even years. During this time biochemical processes such as glycolysis, lipolysis and proteolysis transform the cheese from a flavourless rubbery curd to a cheese with flavour and texture attributes that are characteristic of the type. Proteolysis, the enzymic hydrolysis of the caseins in cheese, is perhaps the most important and complex of these processes and is responsible for many of the texture and flavour characteristics of cheese. The enzymes responsible arise from the cheese milk (e.g. plasmin and cathepsin D), the coagulant (calf, microbial or fungal rennets), the starter bacteria, the non-starter lactic acid bacteria (NSLAB) – either added or adventitious (e.g. *Lactobacillus rhamnosus* or *Lactobacillus paracasei* subsp. *paracasei* strains), and from moulds (e.g. *Penicillium camemberti* and *Penicillium roqueforti*) and other species added to provide flavour. The quantities and activities of these enzymes are dependent on the cheese-making process, the cheese composition and the cheese storage conditions (temperature and time). These processes dictate the way in which the caseins are hydrolysed and the particular peptides and amino acids that are formed.

An understanding of how small deviations in the manufacturing protocol influence proteolysis is important for being able to control cheese maturation (texture and flavour development) and for the design of new products. As a first step towards this understanding, it is important to have methods available for monitoring changes, and more particularly for monitoring changes to the caseins, caused by the enzymes in cheese. Methods capable of capturing the effect of cheese manufacturing processes on proteolysis should also be useful for differentiating cheese type (Ch. 7).

In this preliminary study, the size exclusion-high performance liquid chromatography (SE-HPLC) method developed for the analysis of cheese in the previous chapter (Ch. 5) is compared with traditional methods for examining proteolysis in cheese. In Ch. 5, this method was used to demonstrate the molecular weight distribution of the proteins, peptides and amino acids in the urea-soluble fraction (USF) and the peptides and amino acids in the water-soluble fraction (WSF) of Cheddar cheese. This type of separation should be useful for capturing differences in proteolysis caused by the different processes used in the manufacture of a range of cheese types.

The simplest of the traditional methods used in this study involve the determination of nitrogen in the pH 4.6-soluble (non-casein nitrogen (NCN)) and 12% TCA-soluble (non-protein nitrogen (NPN)) fractions of cheese using the Kjeldahl nitrogen assay. This approach has been used to provide information on overall trends in proteolysis and the extent of ripening in cheese (e.g. Vakaleris and Price, 1959; Law et al, 1993; Bütikofer et al, 1993; Johnston et al, 1994). The second method, alkaline urea-polyacrylamide gel electrophoresis (urea-PAGE) resolves caseins and larger peptides in the USF of cheese and has been widely used to study proteolysis in cheese (e.g. Melachouris and Tuckey, 1966; Creamer and Olson, 1982; McSweeney et al, 1993, 1994; Johnston et al, 1994; Watkinson et al, 1996; Singh et al, 1995 and 1997; Mooney et al, 1998). Urea-PAGE can also be used to separate peptides in the WSF of cheese and has been used to demonstrate differences in proteolysis between cheese types (e.g. Ledford et al, 1966; Marcos et al, 1979; Fox, 1993). A third method, reverse phase-HPLC (RP-HPLC), is mainly used to separate cheese peptides prior to identification (e.g. McSweeney et al, 1993, 1994; Singh et al, 1994, 1995, 1997; Gouldsworthy et al, 1996; Ferranti et al, 1997; Alli et al, 1998). This method has also been used to demonstrate differences between peptide profiles caused by different manufacturing processes (e.g. Fox, 1993; Bütikofer et al, 1998), to demonstrate broad stages of maturity (e.g. Pham and Nakai, 1984; Furtula et al, 1994b; Bütikofer et al, 1998), and to demonstrate differences between cheese types (e.g. Smith and Nakai, 1990; Fox, 1993; Gonzalez de Llano et al, 1995).

In this study, proteolysis in seven cheese types (New Zealand-style Swiss, Elsberg, Cheddar, Mozzarella, Gouda, Feta and Blue cheese selected as 'mature and ready for market' by the manufacturer) is examined using SE-HPLC and traditional methods to determine the relative usefulness of the SE-HPLC method and its potential for differentiating cheese on the basis of proteolysis. The fractions from SE-HPLC analysis of one cheese type are analysed by RP-HPLC and urea-PAGE to demonstrate the relationship between the methods.

6.2 OBJECTIVES

The major objectives of this study are to determine the potential of the SE-HPLC method developed for the analysis of cheese in chapter 5, for establishing differences in proteolysis between cheese types, and to compare the SE-HPLC method with traditional methods that have been used in the laboratory as tools to study cheese biochemistry.

To achieve these objectives it was necessary to:

a) obtain several cheese types at optimum ripeness ('mature and ready for market') from New Zealand cheese manufacturers,
b) subject the cheese to appropriate chemical analyses,
c) analyse the cheese using the new SE-HPLC method as well as traditional biochemical methods, including NPN, NCN, urea-PAGE and RP-HPLC,
d) compare the results from SE-HPLC analysis with the results of the traditional analyses,
e) determine the relationship between SE-HPLC, urea-PAGE and RP-HPLC,
f) examine the potential of the SE-HPLC method for differentiating cheese types, and
g) recommend methods for studies of cheese differentiation and maturation.

6.3 MATERIALS AND METHODS

6.3.1 Materials

All materials are described in Ch. 4, section 4.1.

6.3.2 Methods

Most methods are fully described in Ch. 4, section 4.2.

6.3.2.1 Cheese manufacture

Not all the details related to the manufacture of each cheese type were known. However, the details of cheese manufacture considered most important in determining the extent of proteolysis in a cheese are included in Table 6.1.

Table 6.1 *Cheese manufacture and storage*

Cheese type	Starter and other bacteria	Coagulant ml/100 L milk	Drain pH	Cook T (°C)	Curd treatment	Storage time and T (°C)
Swiss	*Streptococcus thermophilus* *Lactobacillus bulgaricus* *Propionibacterium shermanii*	calf chymosin 17	6.5	50°C	pressed brine-salted	12°C/14 d 20°C/21 d 4-6°C/3 m
Elsberg	Mesophilic starter (Flora Danica) *P. shermanii*	calf chymosin 17	6.4	37°C	washed pressed brine-salted	12°C/14 d 20°C/10 d 4-6°C/3 m
Cheddar	*Lactococcus lactis* subsp. *cremoris*	calf chymosin 16	6.1	38°C	Cheddared pressed dry-salted	13°C/8.5 m
Mozzarella	*Lc. lactis* subsp. *cremoris*	calf chymosin 4	5.9	36°C	dry-salted stretched at 68°C	13°C/14 d
Gouda	Mesophilic starter (Flora Danica)	calf chymosin 17	6.4	38°C	washed pressed brine-salted	6°C/2 m
Feta	Mesophilic starter (Flora Danica) *Lactobacillus helveticus*	calf chymosin 12	low	35°C	stored in whey brine	9°C/2 m
Blue	Mesophilic starter (Flora Danica) *Penicillium roqueforti*	calf chymosin 5	low	34-37°C	brine-salted	8°C/1 m 4-6°C/1 m

6.3.2.2 Determination of the chemical composition of cheese

The chemical composition (calcium, salt, pH, moisture, fat, TN, NPN and NCN) of each cheese was determined as described in Ch. 4, section 4.2.2.

6.3.2.3 Preparation and analysis of the USF and WSF of cheese

The USF and WSF of each cheese type were prepared and analysed using SE-HPLC, urea-PAGE and RP-HPLC (WSF only) according to the methods described in Ch. 4, sections 4.2.3.3, 4.2.3.1and 4.2.3.4, respectively.

6.3.2.4 Preparation and analysis of standard sets A and B

Two sets of molecular weight standard solutions were prepared according to the method described in Ch.5, section 5.3.2.3. Each solution (50 μL) was examined separately using SE-HPLC, according to the method described in Ch. 4, section 4.2.3.3.

6.3.2.5 The relationship between SE-HPLC, urea-PAGE and RP-HPLC

A large sample (200 μL) of the WSF of Mozzarella cheese was loaded on to the SE-HPLC column and analysed. The absorbance was monitored at 220 nm and the peak fractions were collected, concentrated by evaporation of the solvent, diluted 1:1 with either urea-PAGE buffer or RP-HPLC solvent A (as appropriate) and analysed by urea-PAGE and RP-HPLC. A diagram showing the relationship between the three modes of separation was constructed.

6.3.2.6 Purification of caseins (see appendix, section 6.8)

The caseins, α_{s1}-casein, β-casein, α_{s2}-casein and κ-casein, were purified as described in Ch. 4, section 4.2.4.

6.3.2.7 Hydrolysis of the caseins (see appendix, section 6.8)

Each purified casein was hydrolysed by chymosin or plasmin, and samples were collected as described in Ch. 4, section 4.2.5. The samples were analysed by SE-HPLC and urea-PAGE or SDS-PAGE so that the bands could be related to the SE-HPLC peaks. The results are included in the appendix (section 6.8).

6.4 RESULTS

Cheese was manufactured commercially and analysed at what was considered by the manufacturer to be its "optimum" maturity.

6.4.1 Chemical composition of the cheese

Cheese composition was determined to enable information about proteolysis determined by SE-HPLC, urea-PAGE and RP-HPLC to be discussed in the context of both composition and manufacturing parameters (Table 6.1). The pH, TN, NCN, NPN, fat, moisture, salt, calcium, S/M and MNFS contents of each of the cheeses are shown in Table 6.2.

Table 6.2 *Cheese chemical composition*

Composition	Swiss	Elsberg	Cheddar	Mozzarella	Gouda	Feta	Blue
TN (% w/w)	4.37	4.40	3.98	4.53	4.09	2.95	3.68
Fat (% w/w)	27.1	27.0	35.4	22.0	28.7	23.5	35.8
Moisture (% w/w)	38.2	40.0	33.1	45.6	41.6	54.0	38.5
Salt (% w/w)	0.98	1.87	1.73	1.50	1.87	3.56	2.55
Ca (mM/kg)	243	236	194	188	206	124	151
pH	5.58	5.51	5.10	5.72	5.75	4.83	5.66
S/M (%)	2.57	4.67	5.23	3.29	4.49	6.59	6.62
MNFS (%)	52.40	54.79	51.24	58.46	58.35	70.59	59.97
Nitrogen distribution							
TN (% w/w)	4.37	4.40	3.98	4.53	4.09	2.95	3.68
NCN (% w/w)	0.61	0.85	0.80	0.38	0.73	0.38	1.69
NPN (% w/w)	0.42	0.47	0.62	0.09	0.32	0.21	1.33
NCN/TN (%)	14.0	19.2	20.1	8.3	17.8	12.9	45.9
NPN/TN (%)	9.6	10.6	15.6	1.9	7.9	7.1	36.2
NPN/NCN	0.68	0.55	0.78	0.23	0.44	0.55	0.79

6.4.1.1 Nitrogen distribution - NCN and NPN

As a cheese matures, the quantity of peptides increases, and these can be extracted from the cheese and the quantity of N in the extract measured to provide an estimate of proteolysis. Three fractions are commonly used; TN captures all the nitrogen, NCN captures peptides and amino acids soluble at pH 4.6 (as well as whey protein) and NPN captures smaller peptides and amino acids soluble in 12% TCA. NPN is a subset of NCN.

The greatest amount of proteolysis (as estimated by NCN/TN (%)) (Table 6.2) was evident in the Blue cheese (45.90% of TN) and this was followed by Cheddar (20.1% of TN) > Elsberg (19.2% of

TN) > Gouda (17.8% of TN) > Swiss (14.0% of TN) > Feta (12.9% of TN) > Mozzarella cheese (8.3% of TN).

NPN is generally regarded as an indicator of the peptidolytic activity of the microflora (including the moulds) of the cheese. The amount of NPN (as estimated by NPN/TN (%)) (Table 6.2) was greatest in the Blue cheese (36.2% of TN) > Cheddar (15.6% of TN) > Elsberg (10.6% of TN) > Swiss (9.6% of TN) > Gouda (7.9% of TN) > Feta (7.1% of TN) > Mozzarella cheese (1.90% of TN).

6.4.2 SE-HPLC of the USF of seven cheese types

The USF of each of the cheese types was prepared and analysed by SE-HPLC and the resultant traces were overlaid (Fig. 6.1). Two sets of molecular weight standards, standard sets A and B were prepared and separately analysed in duplicate in the same set of 'runs' as the cheese samples. The elution times were used to generate a calibration curve ($\log_{10}$ molecular weight versus elution time). The equation for the calibration curve (Fig. 6.2) was used to calculate the retention times at set molecular weight values, and the resultant calibration table (see appendix, section 6.8.1) was then used to generate the molecular weight values on the traces.

To aid the interpretation of the results (Fig. 6.1) each of the caseins (α_{s1}-, α_{s2}-, β- and κ-casein) was purified and separately hydrolysed by both chymosin and plasmin. Samples were taken at regular intervals throughout the hydrolysis and each was analysed by SE-HPLC and either urea- or SDS-PAGE. The SE-HPLC traces (205 nm) were overlaid and are included with the PAGE results in the appendix (section 6.8.2 - 6.8.9).

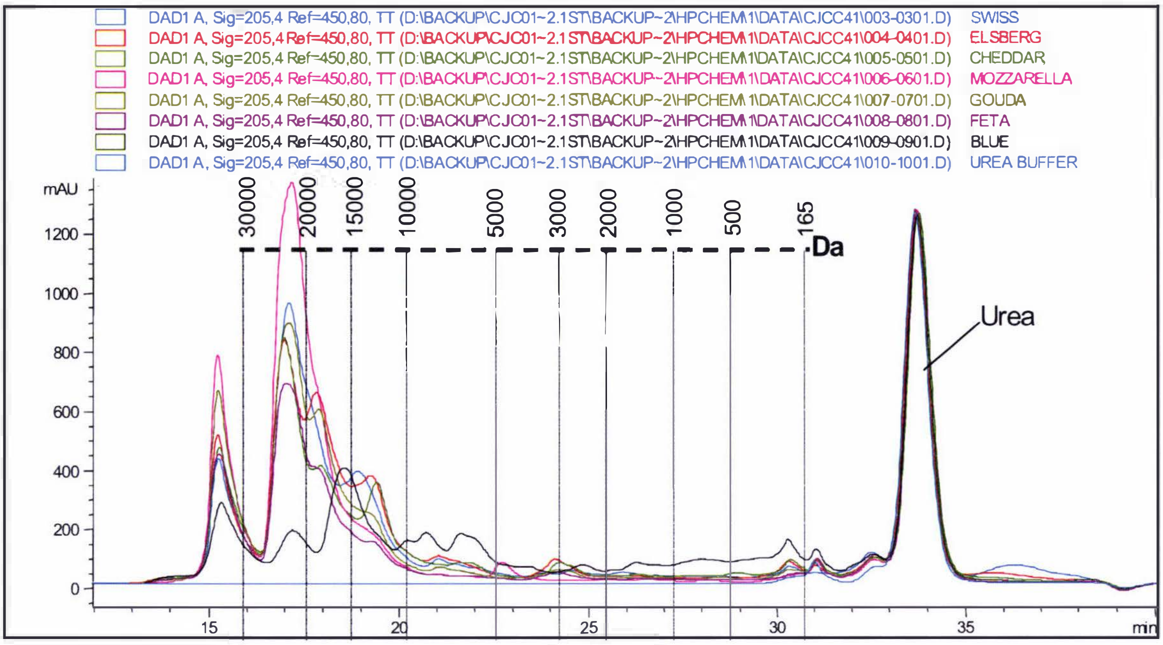


Fig. 6.1 *SE-HPLC - Traces of the USF of seven cheese types at optimum ripeness.* The USF (0.5 g ± 0.0005 g cheese in 25 mL of urea sample buffer) of Swiss ------, Elsberg ------, Cheddar ------, Mozzarella ------, Gouda ------, Feta ------ and Blue ------ cheese was defatted, diluted 1:9 (v/v) with the mobile phase (36% acetonitrile, 0.1% TFA) and 50 µL was loaded on to the column. The flow rate was 0.4 mL/min. The absorbance was measured at 205, 210, 220, 280 and 295 nm but only 205 nm was plotted. The molecular weight distribution scale (Da) was derived from the protein standard calibration curve (Fig. 6.2).

The ratio of high molecular weight proteins and peptides (14.5 – 22.5 min) to low molecular weight peptides and amino acids (22.5 – 32 min) in the USF (Fig. 6.1) was very large for all cheeses except Blue which had undergone extensive proteolysis.

The peak at 15.2 min has an apparent molecular weight of 32800 Da and was present in traces for each of the cheese types. It contains high molecular weight material that is larger than any casein monomers.

The peak at ~16.2 min (~24000 Da) is present in varying amounts in each cheese type and corresponds to β-casein (elution time 16.1 min (see appendix, section 6.8.4)) at the leading edge, followed by α_{s1}-casein (elution time 16.6 min (see appendix, section 6.8.2)) as the peak broadens. The size of this peak is greatest in the Mozzarella and Swiss cheeses, which is consistent with the smaller amounts of rennet activity in these cheese types due to the high stretch or cook temperatures.

Peaks from 17.78 to 19.40 min correspond to the large polypeptides formed by the action of chymosin and plasmin on β-, α_{s1}- and α_{s2}-casein and include β-I, β-II- and β-III-casein as well as γ_1-, γ_2- and γ_3-casein and α_{s1}-I-casein (see appendix, section 6.8.2 - 6.8.5).

In summary, SE-HPLC of the USF provided limited information about the cheese and, with the exception of the Blue cheese was not useful for differentiating cheese type. Most peaks were in the higher molecular weight range, where the column performance was at its worst, and were of similar molecular weight. In sharp contrast to the high concentration of high molecular weight peptides, the concentration of lower molecular weight peptides was low. The method was of more use for the analysis of the Blue cheese and casein hydrolysates (see appendix, section 6.8.2 – 6.8.9), and it is likely that it would have a place in the analysis of highly proteolysed cheese-based products such as enzyme modified cheese (EMC).

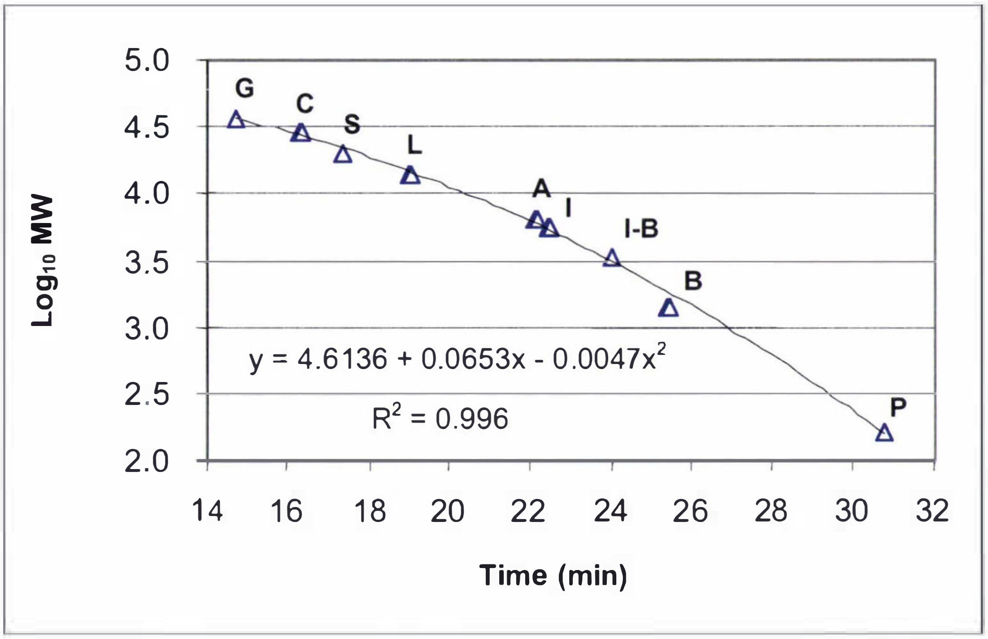


Fig. 6.2 *SE-HPLC - 2^{nd} order calibration curve for estimates of molecular weight distribution of the USF).* Log_{10} molecular weight versus retention time (min) of standards in sets A and B. Standard set A contained 1 mL of each of glyceraldehyde 3-phosphate dehydrogenase (**G**) (36000 Da), soybean trypsin inhibitor (**S**) (20100 Da), aprotinin (**A**) (6500 Da) and insulin B-chain (**I-B**) (3400 Da) solutions and 0.2 mL of the phe (**P**) (165 Da) solution, that had been individually prepared at a concentration of 1 mg/10 mL mobile phase. 50 μL of the mixture was loaded on to the column and analysed. Standard set B contained 1 mL of each of carbonic anhydrase (**C**) (29000 Da), lysozyme (**L**) (14300 Da), insulin (**I**) (5730 Da) and bacitracin (**B**) (1420 Da) solutions that had been individually prepared at a concentration of 1 mg/10 mL mobile phase. 50 μL of the mixture was loaded on to the column and analysed. Refer to Fig. 6.1 for details of sample analysis.

6.4.3 SE-HPLC of the WSF of seven cheese types

The WSFs of the seven cheese types were prepared and analysed by SE-HPLC and the resultant traces were overlaid (Fig. 6.3). (These traces were used to create 'thumbnail' diagrams to illustrate comparisons between cheese types discussed in the following sections.) Standard sets A and B were analysed in duplicate together with the cheese samples, and the elution times (see appendix, section

6.8.10) were used to generate a calibration curve ($\log_{10}$ molecular weight versus elution time) (Fig. 6.4). The equation for the calibration curve was used to calculate the retention times displayed on the traces at selected molecular weight values and to calculate the approximate molecular weights of particular peaks discussed in the text.

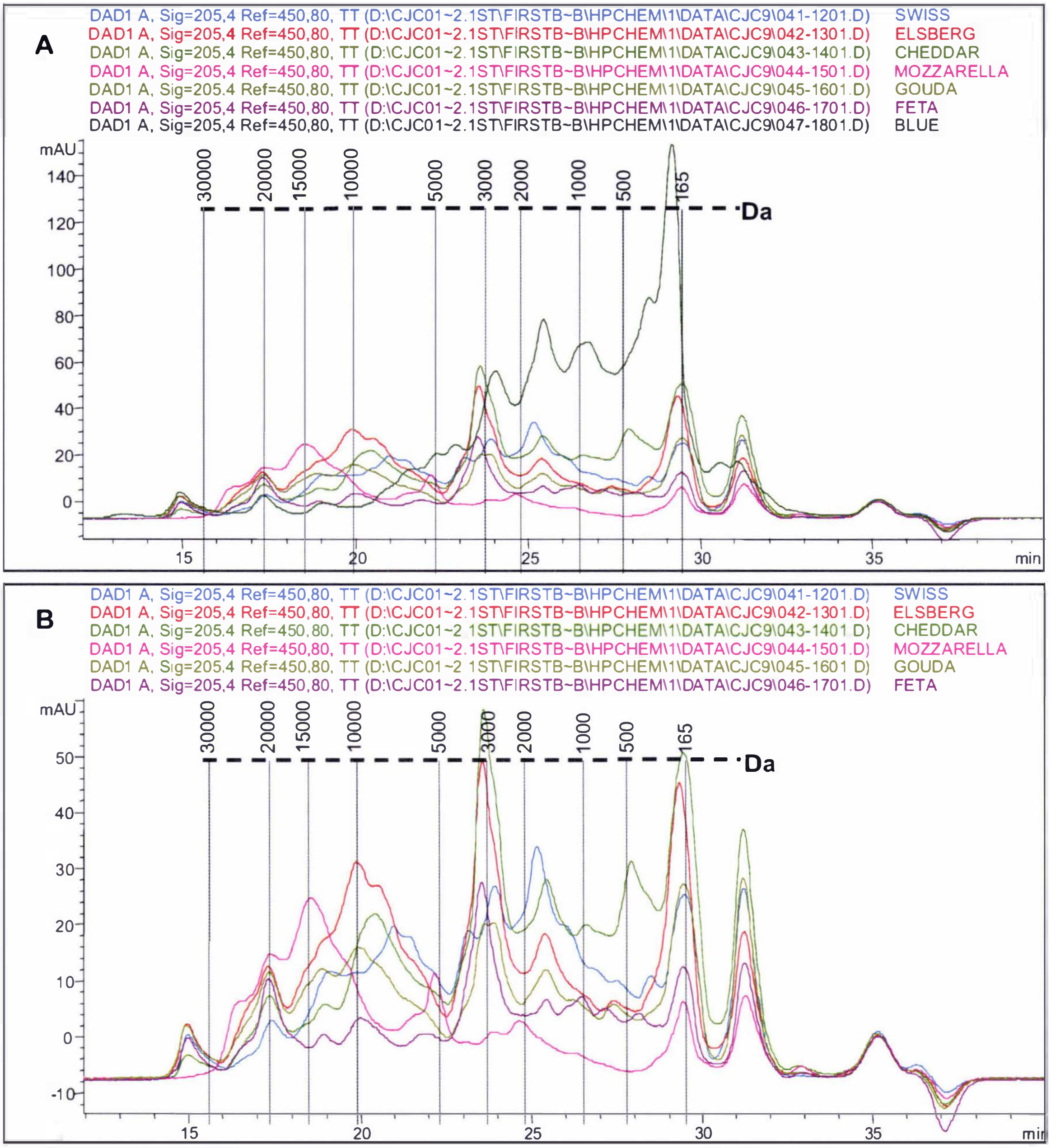


Fig. 6.3 *SE-HPLC – Traces of the WSF of A) seven cheese types and B) six cheese types at optimum ripeness.* The WSF (3 g ± 0.0005 g cheese in 30.0 mL water) of Swiss ----, Elsberg ----, Cheddar ----, Mozzarella ——, Gouda ----, Feta ---- and Blue —— cheese was defatted and diluted (200 μL with 9.8 mL of the mobile phase (36% acetonitrile, 0.1% TFA)) prior to loading 50 μL on to the column. See Fig. 6.1 for details of sample analysis and peak detection. The molecular weight distribution scale (Da) was derived from the protein standard calibration curve (Fig. 6.4).

SE-HPLC of the WSF (Fig. 6.3) grouped the peptides and amino acids in the WSF of each cheese on the basis of their molecular weight. The chromatograms were different for each cheese type. In all cases, the quantity of high molecular weight material (>~5000 Da) that eluted in the 14.5 – 22.5 min region was small compared with the quantity in the same region on the traces of the USF of the same cheeses (Fig. 6.1). The peaks that eluted after 22.5 min were more clearly defined than the same peaks on the chromatograms of the USF.

The peak that eluted at 14.9 min was small in comparison with the corresponding peak on the traces of the USF. The molecular weight of this peak was approximately 33000 Da (the accuracy is low in this molecular weight region), which is close to the molecular weight of the β-lactoglobulin (β-Lg) dimer. β-Lg is water-soluble and small quantities are present in most cheese types.

The peak shoulder that eluted at about 16.4 min was present in the WSF of each cheese type, and had an apparent molecular weight of approximately 26000 Da. Its elution time corresponded with the elution times of α_{s1}- and β-casein (16.6 and 16.1 min, respectively) (see appendix, section 6.8.2 and 6.8.4), which suggests that the peak may have contained low levels of casein.

The large water-soluble polypeptides (>~5000 Da) that eluted between 17.5 and 22.5 min were present at different concentrations in each cheese type and the quantity of each is likely to be dependent on the amount of primary proteolysis by the main proteolytic enzymes, chymosin and plasmin.

The peaks that eluted after 22.5 min contained peptides with a molecular weight of <~5000 Da and amino acids. They were present in the greatest quantities in the Blue and Cheddar cheese, and in the smallest quantities in the Mozzarella cheese, suggesting that they arose from secondary proteolysis by the enzymes of the cheese microflora.

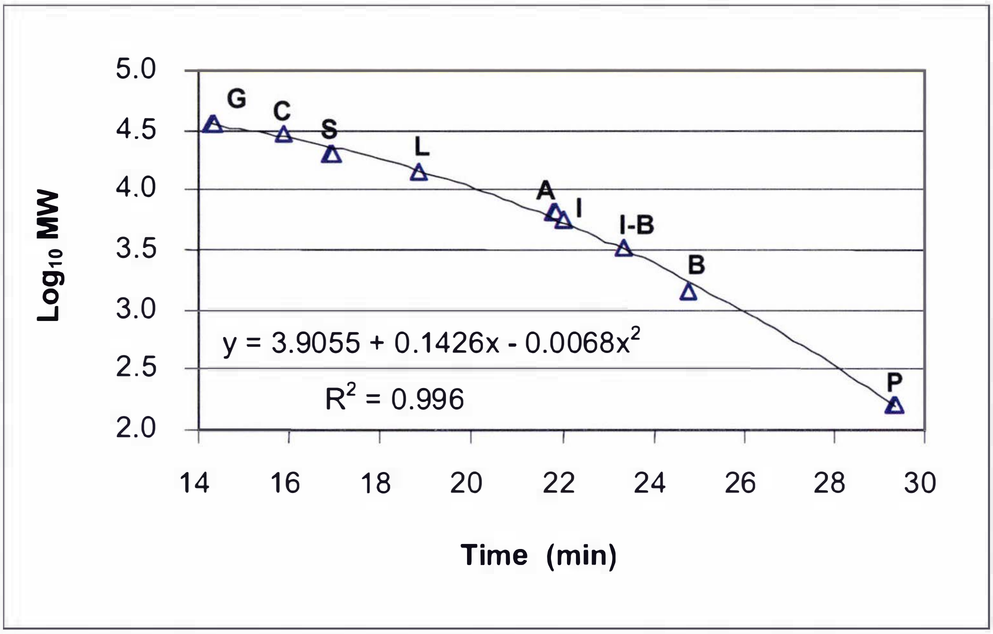
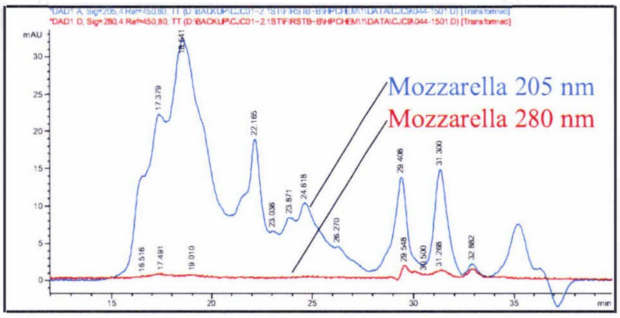


Fig. 6.4 *SE-HPLC - 2nd order calibration curve for the estimates of the molecular weights of peaks in Fig. 6.3.* Log_{10} molecular weight versus retention time (min). Refer to Fig. 6.2 for details of preparation and analysis of standards, and meaning of symbols.

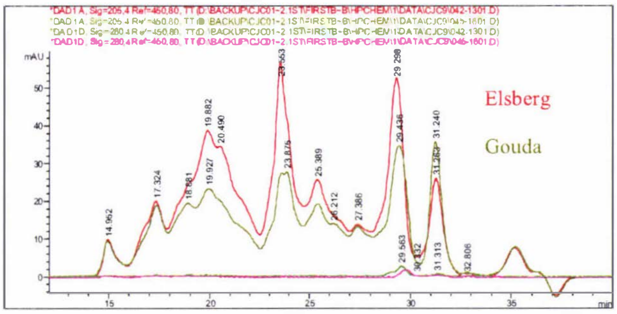
6.4.3.1 Mozzarella cheese



The distinguishing features of the chromatogram of the WSF of a sample of Mozzarella cheese ripened at 13°C for 14 d (Fig. 6.3B) were the absence of the peak at 14.9 min, the relatively large amount of material in the region from 16.4 – 22.5 min that corresponds to the caseins and higher molecular weight water-soluble peptides (26000 – 5000Da), and the small amount of material that eluted after 22.5 min that corresponds to the lower molecular weight water-soluble peptides and amino acids. The amino acid peak at 29.4 min and the peak that eluted at 31.3 min were smaller than the corresponding peaks on the traces of the other cheeses and absorbed light at 280 nm. The absence of the peak at 14.9 min that was tentatively identified as β-Lg, may mean that it was incorrectly identified or that at the high stretch temperature (68°C) β-Lg formed insoluble complexes via disulphide linkages between β-Lg molecules or α_{s2}-casein and para-κ-casein. It is likely that peptides in the first two peaks that eluted after 16 min contained aromatic amino acids, perhaps from the C-terminus of para-κ-casein, the C-terminus of the α_{s1}-casein f1-23 peptide, or the N- or C- terminus of α_{s1}-I-casein formed by chymosin or cathepsin D. The small amount of proteolysis observed is consistent with the cheese manufacture. Mozzarella cheese is stretched at high temperatures, and the sensitivity of chymosin to high temperature

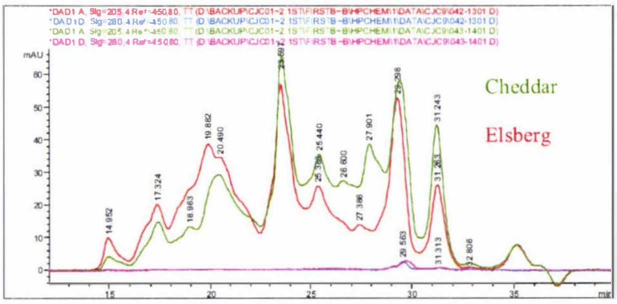
(Matheson et al, 1981) suggests that only a small amount of chymosin activity would be expected and it is likely that very few microorganisms would survive. As well, the high pH (5.72) is unfavourable for chymosin activity (Watkinson et al, 2001), although it is closer to the optimum for plasmin activity (pH 7.5 – 8.0 (Chen and Ledford, 1971; Kaminogawa et al., 1972)). Consequently, there are large quantities of casein (Fig. 6.1) and large polypeptides (Fig. 6.3) and very few smaller peptides and amino acids present.

6.4.3.2 Elsberg and Gouda cheese



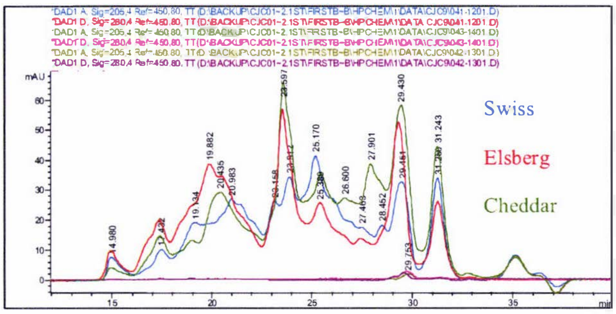
The chromatograms of the WSFs of Elsberg and Gouda cheese were similar (Fig. 6.3B), although most of the Elsberg peaks were larger than the corresponding Gouda peaks, indicating that there was more proteolysis in the Elsberg cheese. The peak at 17.3 min has an apparent molecular weight of 21700 Da and may contain small amounts of casein or perhaps α_{s1}-I-casein (20868 Da) and γ_1-casein (20520 Da), β-I-casein (21786 Da) and β-II-casein (19341/19125 Da). The cluster of large peaks at 18.3, 18.9, 19.9 and 20.5 min contains large polypeptides with molecular weights of approximately 17300, 14800, 11200 and 9300 Da. These molecular weights approximately correspond with β-III-casein (16147 Da), γ_2-casein (11822 Da) and γ_3-casein (11557 Da), but all are hydrophobic and not usually water-soluble (McSweeney et al, 1994). The peaks that eluted between 22.5 and 29.4 min contained small water-soluble peptides of <5000 Da and amino acids. The peak at 31.25 min may contain compounds derived from amino acids. The greater amount of proteolysis in the Elsberg cheese, probably influenced by the lower pH (pH 5.51), higher storage temperature and longer storage time (Tables 6.1 and 6.2), enabled the Elsberg and Gouda to be clearly distinguished from one another.

6.4.3.3 Cheddar cheese



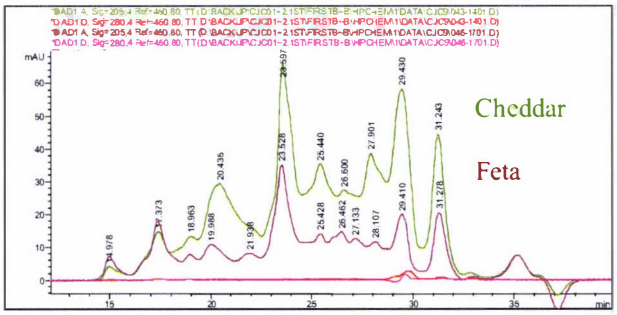
Proteolysis in the Cheddar cheese, which had been matured for longer than the other cheese types, was more extensive than in the Elsberg or Gouda cheese. The chromatogram of the WSF of Cheddar (Fig. 6.3B) had a similar distribution of peaks to the Gouda and Elsberg chromatograms. The distinguishing features of the Cheddar cheese chromatogram were the comparatively small amount of higher molecular weight material that eluted between 14.5 and 22.5 min (polypeptides of >5000 Da) and the relatively large amount of material that eluted after 22.5 min (small peptides of <5000 Da and amino acids). This molecular weight distribution of the peptides was probably indicative of a relatively large amount of chymosin activity at pH 5.1 that provided the substrates for a large amount of microbial enzyme activity. The peak that eluted at 31.25 min was larger than the corresponding peak in any of the other cheese types. This peak is believed to contain compounds such as pyroglutamic acid and catabolic end-products of proteolysis (Motion, 1992) that arise from starter or NSLAB activity (Williams, Noble and Banks, 2001).

6.4.3.4 Swiss cheese



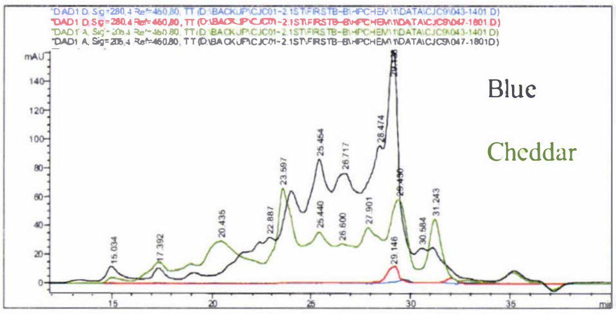
The chromatogram of the WSF of Swiss cheese (Fig. 6.3B) shows that the quantity of high molecular weight material (>5000 Da) in the peaks that eluted before 22.5 min was equivalent to the quantity in the Cheddar cheese, but less than in the Elsberg cheese. However, the quantity of lower molecular weight material (<1000 Da) in the peaks that eluted after 26.1 min was less than in the Cheddar, and, with the exception of the amino acid peak at 29.4 min, was more than in the Elsberg cheese. Although the Swiss and Cheddar cheese contained approximately the same quantities of large polypeptides, the smaller quantities of lower molecular weight material in the Swiss cheese suggest that the large polypeptides were accumulating in the cheese. As well, the quantities of each of the large polypeptides peaks that eluted between 18.0 and 22.5 min (18600 – 5000 Da) were different in the Swiss and Cheddar cheese. The high cheese pH (5.58) and high cook temperature (50°C) used during the manufacture of the Swiss cheese probably caused these differences. The high cook temperature would affect the survival of chymosin (Matheson et al, 1981) and result in less chymosin activity and possibly more plasmin activity (Richardson and Pearce, 1981) than in the Cheddar cheese, and the high pH would slow chymosin activity and enhance plasmin activity (Coker, unpublished). The small quantities of low molecular weight peptides and amino acids present in the Swiss cheese may indicate the limitation of the enzymes of the thermophilic starter and possibly low levels of NSLAB enzyme activity in this cheese type.

6.4.3.5 Feta cheese



The chromatogram of the WSF of Feta cheese (Fig. 6.3B) shows that it contained only small quantities of water-soluble peptides and amino acids. The peptides in the cheese were spread over a wide molecular weight range and most peaks present on the Feta chromatogram were present on the Cheddar chromatogram. This indicated that the enzymes active in the Cheddar cheese were also active in the Feta cheese. The smaller quantity of water-soluble peptides present in the Feta cheese was probably a consequence not only of the lower TN (2.95% w/w), lower level of rennet (12 mL/100 L milk), lower storage temperature (9°C) and shorter storage time (2 m), but also of the effect of the high salt concentration (3.56% w/w; S/M 6.59%) (Table 6.2) on rennet and plasmin activity, and on microbial growth and consequent enzyme activity. As well, the Feta cheese had a very high moisture content (54%) (which may enhance enzyme activity) and was stored in whey. This may have enabled small peptides and amino acids to leach from the cheese into the whey.

6.4.3.6 Blue cheese



The chromatogram of the WSF of Blue cheese (Fig. 6.3A) shows that the peaks that eluted in the first 22.5 min were very small, indicating that there were only small quantities of high molecular weight polypeptides of >5000 Da present in the cheese. Most of the water-soluble peptides in the Blue cheese eluted after 22.5 min (small peptides of <5000 Da and amino acids), and these peaks were large in comparison with those in the WSF of Cheddar, the most proteolysed of the other cheese types (Fig. 6.3A). The amino acid peaks that eluted between about 28.5 and 30.0 min were very large and had a high absorbance at 280 nm, indicating the presence of large quantities of the highly absorbing aromatic amino acids, particularly tyrosine (Tyr) and tryptophan (Trp). The small amount of high molecular weight peptide material in the USF (Fig. 6.1) and the large amount of low molecular weight material in the WSF are consistent with extensive proteolysis in the Blue cheese. This was due not only to chymosin, plasmin and the enzymes of the starter bacteria, but also to the highly proteolytic mould enzymes present in the cheese.

To summarise, the main findings were that the peptides and amino acids in the WSF of each of 7 cheese types could be divided into peaks on the basis of molecular weight, and that each cheese type appeared to have a distinctive profile of molecular weight distribution of the peptides and amino acids that could be related to the enzyme activity associated with that particular cheese type.

6.4.4 Gel electrophoresis of the USF and WSF of seven cheese types

Gel electrophoresis was used to compare the cheese types (Fig. 6.5). The amount of intact α_{s1}- and β-casein in each cheese type was determined (qualitatively), and from this, the relative contribution to proteolysis of the various enzymes in each cheese was assessed. This technique was also used to investigate the peptides in the WSF of each of the cheese types (Fig. 6.6). The USF of Cheddar, Elsberg and Blue cheese has been included on the gel as a reference for the bands in the WSF. The WSF was taken from 12.5 times more cheese than the USF to enable the bands to be seen.

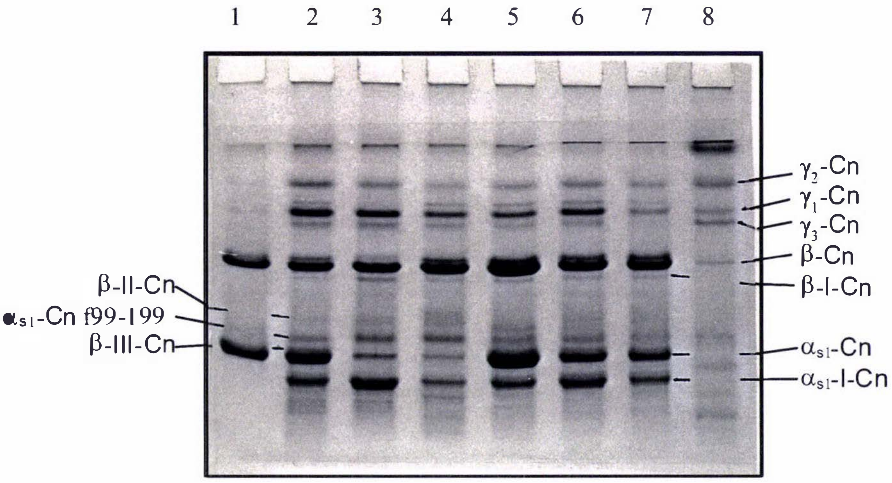


Fig. 6.5 *Urea-PAGE - Proteolysis in each of seven cheese types at optimum ripeness.* Lane 1, rennet casein standard (1 mg/mL, 10 μL volume loaded); lanes 2 to 8, Swiss, Elsberg, Cheddar, Mozzarella, Gouda, Feta and Blue cheese USF (0.5 ± 0.0005 g cheese in 25.0 mL of urea sample buffer (2% cheese), 5 μL sample volume loaded). The standard and samples were treated with 2-mercaptoethanol (10 μL/mL) and bromophenol blue (10 μL/mL), and held for 18 h before they were loaded on to the gel.

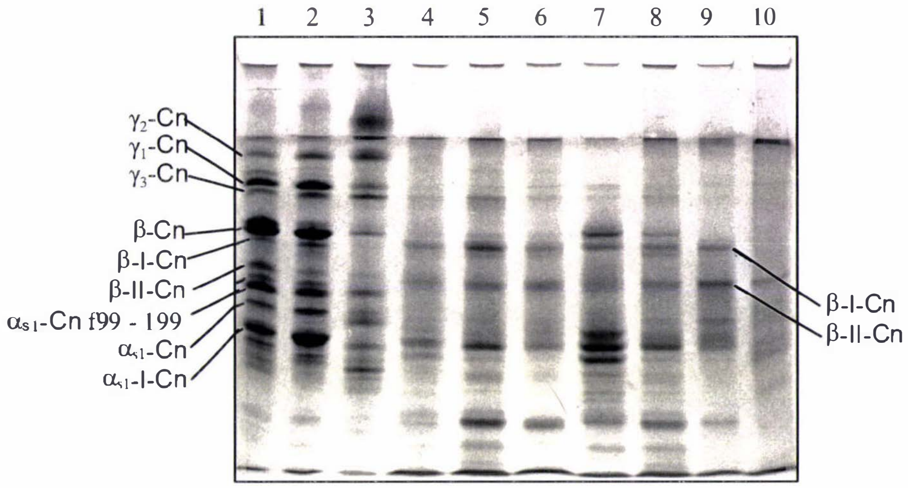


Fig. 6.6 *Urea-PAGE - Peptides in the WSF of each of seven cheese types at optimum ripeness.* Lanes 1 to 3, Cheddar, Elsberg and Blue cheese USF ((see Fig. 6.5 for details of sample preparation), 5 μL sample volume loaded); lanes 4 to 10, Swiss, Elsberg, Cheddar, Mozzarella, Gouda, Feta and Blue WSF (3 g ± 0.0005 g in 30.0 mL water (5% cheese), 25 μL sample volume loaded). The samples were treated with 2-mercaptoethanol (10 μL/mL) and bromophenol blue (10 μL/mL), and held for 18 h before they were loaded on to the gel.

Analysis of the USF using urea-PAGE provided information on primary proteolysis by the major proteolytic enzymes, chymosin and plasmin. Chymosin action results in the gradual loss of α_{s1}-casein and the formation of α_{s1}-I-casein during maturation (Creamer and Richardson, 1974), and in some types of cheese, it also hydrolyses β-casein to form β-I, β-II- and β-III-casein. Plasmin action results in the loss of α_{s2}-casein and β-casein, with the γ-caseins formed as a result of its action on β-casein. Secondary proteolysis was probably responsible for the loss of some of the products of primary proteolysis (e.g. α_{s1}-I-casein) but the products of secondary proteolysis were mostly not large enough to be retained in the gel. Table 6.3 summarises the findings of urea-PAGE of the USF and WSF and compares enzyme action in each of the seven cheese types. The significance of these findings is deferred to the discussion.

Table 6.3 A *summary of enzyme action in each of seven types of cheese at optimum ripeness*

Cheese Type	Chmosin action	Plasmin action	Microbial enzyme action
Swiss	++	+++++	+++
Elsberg	++++	++++	+++
Cheddar	+++++	++	++++
Mozzarella	+	++	+
Gouda	+++	+++	++
Feta	+++	+	++
Blue	+++++	+	++++++++++ (mainly mould activity)

6.4.5 RP-HPLC of the WSF of seven cheese types

RP-HPLC was used to examine the WSF of each cheese type (Fig. 6.7). These traces were used to create 'thumbnail' diagrams to illustrate comparisons between cheese types in the following sections.

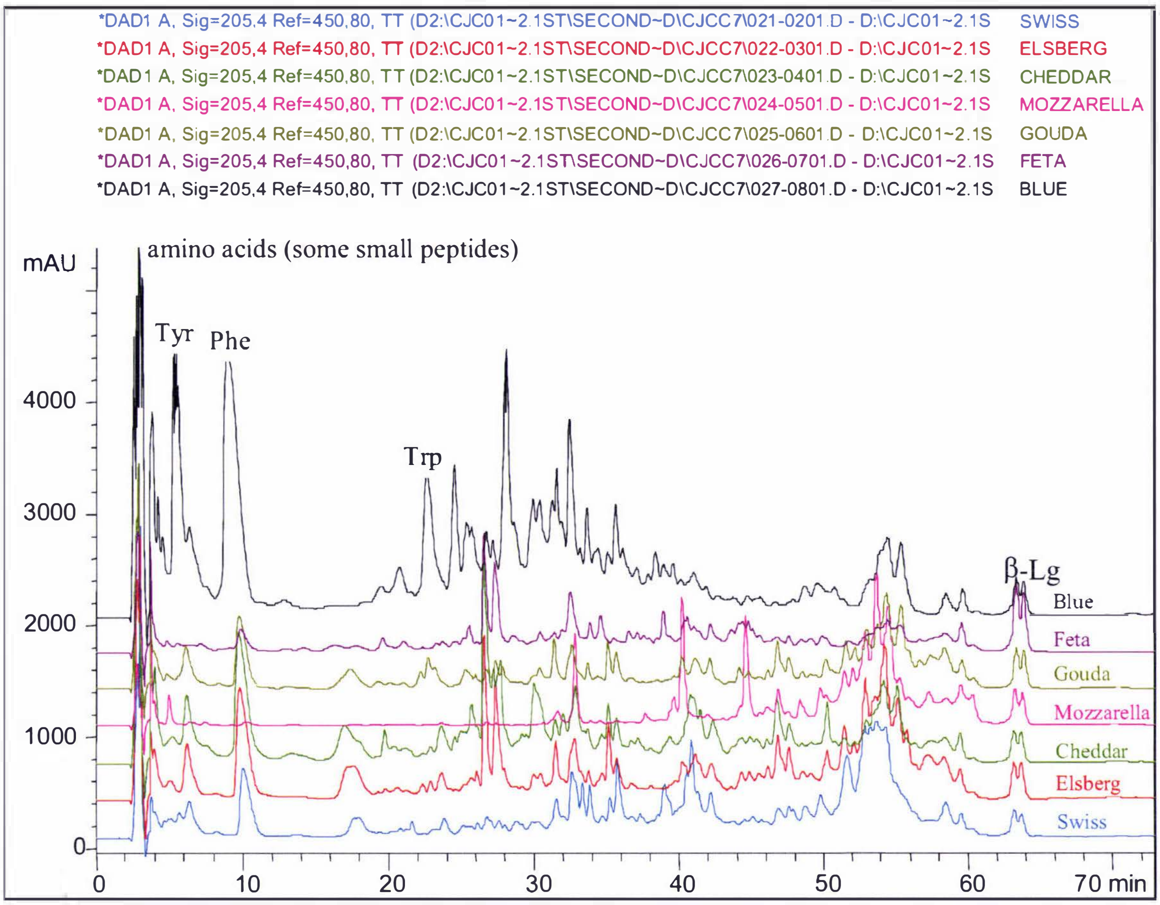
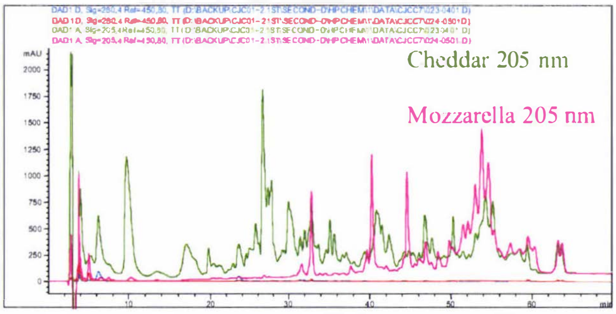


Fig. 6.7 *RP-HPLC – Traces of the WSF of seven cheese types at optimum ripeness.* The WSF (3 g ± 0.0005 g cheese in 30.0 mL water) of Swiss ------, Elsberg ——--, Cheddar -----, Mozzarella ——-, Gouda ------, Feta ——- and Blue ——- cheese was defatted and diluted 1:1 (v/v) with solvent A and filtered (0.45 μm filter) prior to loading 200 μL on to the column. Gradient: 100% solvent A for 5 min; 0 to 100% solvent B over 75 min; 100% solvent A for 15 min. Flow rate, 0.7 mL/min. The absorbance was measured at 205, 210, 220, 280 and 295 nm. Only the 205 nm trace is shown.

The RP-HPLC traces were complex with a great many peak because of the very large number of water-soluble peptides present in each cheese. Although the traces had many peaks in common there appeared to be significant differences between the traces.

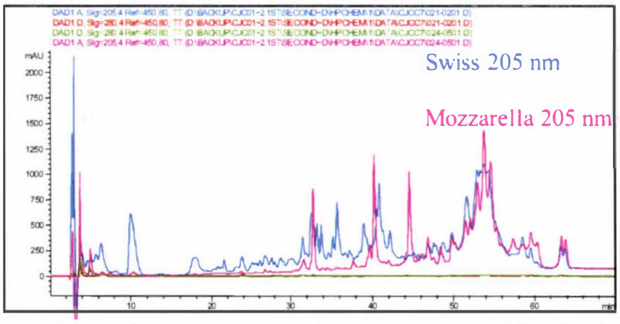
Peaks at 63.3 and 63.8 min (Fig. 6.7) corresponded to β-Lg (Coker, 1994) and were present in all of the cheese types. There was more β-Lg in the Feta cheese and this was due to the high moisture content (54.0 %; Table 6.2) and the use of brined whey for storage of this cheese type. Peaks between 58 and 61 min (Fig. 6.7) correspond to the caseins and the first of the degradation products. A small amount of casein and very high molecular weight peptide material was water-soluble, but most was insoluble in solvent A, used to dilute the samples prior to loading on to the column. This material was removed from the samples by filtration before they were loaded on to the column.

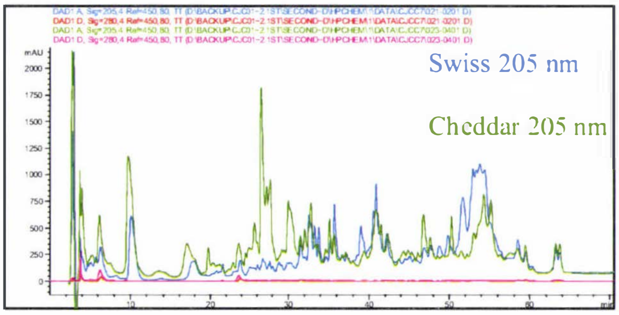
6.4.5.1 Mozzarella cheese



The chromatogram of the Mozzarella cheese WSF (Fig. 6.7) was very different to the chromatograms of the other cheese types. Although the quantity of β-Lg and the size of the peaks that eluted between 57 and 61 min were similar to the other cheese types, there were large quantities of later-eluting peptides, almost no early-eluting peptides and only a small quantity of amino acids present in the cheese. The peaks that eluted between 51 and 57 min were larger than in all cheese types except Swiss and seem likely to correspond with the early-eluting high molecular weight peaks on the SE-HPLC chromatogram (Fig. 6.3). The peaks that eluted between 31 and 51 min are likely to correspond with peptides of >5000 Da (Fig. 6.3) and were present in greater quantities than in the other cheese types. The lack of lack of early-eluting peptides of <5000 Da in the Mozzarella cheese, which had been stretched at high temperature (68°C), suggests that these peptides in the other cheese types arose from enzymes that were inactivated by the heat treatment that Mozzarella was subjected to. It is unlikely that much of the chymosin or any of the mesophilc starter could survive the high stretch temperature. It would therefore appear that the peptides that eluted after 31 min arose from plasmin activity and a small amount of chymosin, or perhaps cathepsin D, activity and accumulated in the cheese.

6.4.5.2 Swiss cheese



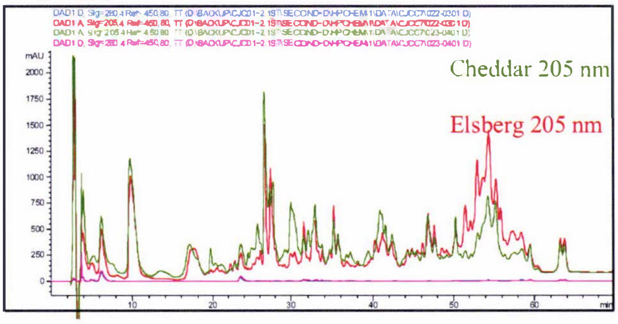


The amount of material that eluted after 50 min on the Swiss cheese WSF chromatogram (Fig. 6.7) was similar to the amount in the Mozzarella cheese. However, with the exception of the peptides that eluted at 32.8, 40.2 and 44.6 min, most of the peptides that eluted before 51 min and the amino acid peaks were present in greater quantities in the Swiss cheese, indicating a greater amount of enzyme activity in this cheese type. However, most of the larger peaks eluted between 31 and 50 min, which suggests that they were intermediates in the proteolytic process. A comparison with the Cheddar cheese (Fig.

6.7) showed that the early-eluting peaks were small in comparison with those on the Cheddar chromatogram. The Swiss cheese had a high pH (5.58), was made using thermophilic starters and was cooked at 50°C. It is therefore likely that there would be less chymosin activity in this cheese type than in the Cheddar cheese. The small quantities of low molecular weight peptides that eluted before 31 min suggest that the microflora of the Swiss cheese (thermophilic starter, propionibacteria and NSLABs) do not possess the peptidases that were active in Cheddar cheese (mesophilic starter and NSLABs), or that the peptidase activity was affected by the cheese environment. The first peak that eluted at about 3 min is likely to contain several of the amino acids (Ile, Ser, Leu, Asn, Lys, Ala, Cys, Ser, Thr, Gly, Arg, Pro (Coker, 1994)) and was relatively large, despite the apparently small amount of peptidase activity. The most highly absorbing of the amino acids that may elute in this peak, Arg and Asn, are not normally present in measurable quantities in Swiss cheese (Steffen et al, 1993). The propionibacteria in Swiss cheese possess peptidases with leucine aminopeptidase and proline iminopeptidase activities and are capable of producing large amounts of leucine and proline (Fox et al, 1993). Proline gives the cheese a sweet nutty flavour. Although these amino acids have only a small absorbance at 205 nm (see Ch. 5, Fig. 5.8), they are likely to be present in large enough quantities to account for much of the high absorbance of this peak.

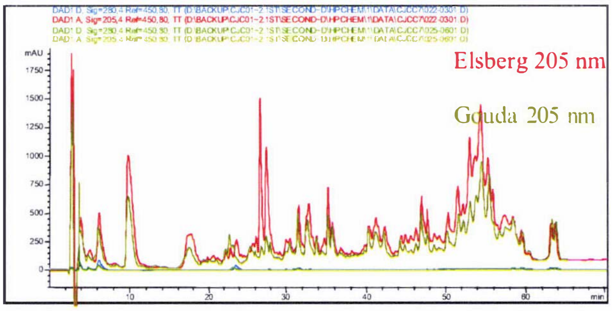
6.4.5.3 Cheddar cheese

The chromatograms (Fig. 6.7) showed that, with the exception of the Blue cheese, there was more proteolysis in the Cheddar cheese than in the other cheese types. The later-eluting peaks from 51.5 to 61 min were smaller and most of the earlier-eluting peaks as well as the amino acid peaks were larger than the peaks in the other cheese types. The Tyr and Phe peaks were larger in the Cheddar than in the other cheese types, with the exception of the Blue cheese. These free amino acids were probably released by the combined action of chymosin and the aminopeptidase activity of the cheese starter. The environment of the Cheddar cheese, and in particular the low pH of 5.10, favoured chymosin and microbial enzyme activity, but as well, the cheese had been ripened for 8 months at 13°C, which was longer than the ripening period of the other cheese types.



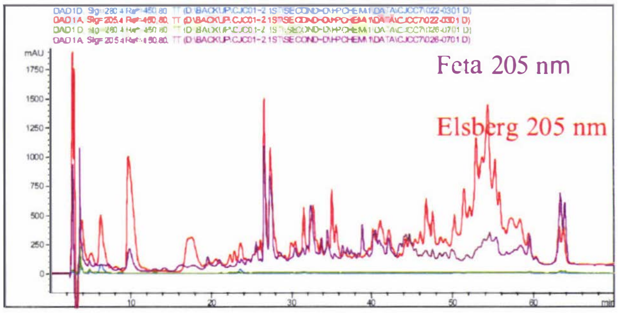
6.4.5.4 Elsberg and Gouda cheese

Most peaks present on the chromatogram of the WSF of Elsberg cheese (Fig. 6.7) were also present on the Cheddar and Gouda cheese chromatograms. Each of these cheese types



was made using approximately the same quantity of chymosin and mesophilic starters, however the Gouda and Elsberg are washed-curd cheeses and the removal of some whey and its replacement with water reduces the amount of chymosin and the number of microorganisms incorporated in the curd. With the exception of the peaks that eluted between 22 and 24 min, each Elsberg peak was larger than the corresponding Gouda peak. The pHs of the Elsberg and Gouda cheeses were 5.51 and 5.75, respectively. Both pHs are less favourable for chymosin activity than the Cheddar pH of 5.10. The differences between the Elsberg and Gouda chromatograms were caused by the lower pH, higher initial storage temperature and longer storage time at 6°C of the Elsberg cheese, which resulted in more enzyme activity and greater quantities of water-soluble peptides. The peaks that eluted between 26 and 28 min were present in large quantities in the Elsberg and Cheddar, but not in the Gouda or Swiss cheese. The large amino acid peak that eluted at about 3 min is likely to contain large quantities of proline and leucine produced by the peptidases of the propionibacteria. This peak was smaller than in the Swiss cheese and this is likely to reflect the washing step as well as the shorter time at higher temperature (Table 6.1).

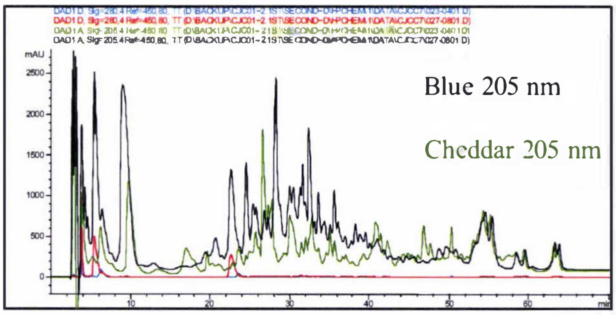
6.4.5.5 Feta cheese



Most peaks present on the Feta cheese WSF chromatogram (Fig. 6.7) were also present on the chromatograms of the Elsberg, Gouda and Cheddar cheeses, but many were much smaller than for the other cheese types. β-Lg eluted in two peaks between 63 and 65 min, and because of the high moisture content (54.0% w/w) was present in greater quantities in the Feta than any of the other cheese types. The peaks that eluted between 46 and 61 min contained large water-soluble polypeptides (Fig. 6.3) and were very small in comparison with the same peaks on the traces of the other cheese types. Of the peaks that eluted between 25 and 46 min, those that eluted between 26 and 28 min and 32 and 34 min were the same size as the corresponding peaks on the Elsberg trace, but the remainder were only a fraction of the size of the Elsberg peaks. The peaks of the amino acids and small peptides that eluted between 2 and 25 min were very small in comparison with those of most cheese types. The small amount of water-soluble material was a consequence of the low TN (2.95% w/w), and probably low rennet retention, as well as the effect of the high salt concentration (6.59% w/w) on enzyme activity and starter growth.

6.4.5.6 Blue cheese

The distribution of peaks on the Blue cheese WSF chromatogram (Fig. 6.7) indicated extensive proteolysis in the cheese. The quantity of material that eluted between 52 and 61 min was small and similar to the amount on the Cheddar cheese chromatogram. Peaks that eluted between 40 and 52



min were small in comparison with the Cheddar peaks in this region. However, the amino acid peaks and most peptide peaks that eluted between 2 and 40 min were very large in comparison with the peaks in this region on the chromatograms of the other cheese types. This result indicated that proteolysis was extensive and that most of the proteins in the cheese had been hydrolysed so that only small peptides and amino acids remained. The combination of chymosin, plasmin, and the enzymes of the cheese microflora including the very proteolytic enzymes of *P.roqueforti* caused this amount of proteolysis. It is interesting to note that β-Lg seems to be resistant to the enzymes of the mould species and is present at about the same concentration as in most other cheese types.

In summary, the RP-HPLC chromatograms were different for each cheese type. The differences could be related to cheese composition, the quantity of the various enzymes present, the storage time and storage temperature, and the effect of these on proteolysis.

6.4.6 The relationship between SE-HPLC, urea-PAGE and RP-HPLC

The WSF of the Mozzarella cheese was loaded on to the SE-HPLC column at high concentration and fractions were collected, concentrated, diluted 1:1 in appropriate buffer or solvent and analysed by urea-PAGE and RP-HPLC. The SE-HPLC fractions that could be seen on the gels were re-run on a single gel. A composite picture of the relationship between SE-HPLC, RP-HPLC and urea-PAGE was constructed from the results (Fig. 6.8).

Mozzarella cheese was selected because urea-PAGE of the WSF of each cheese (Fig. 6.6) showed that most, if not all the peptides visible on the gel were well represented in Mozzarella cheese. The quantities of these peptides were often greatest in Mozzarella, probably because the reduced microbial enzyme activity allowed them to accumulate rather than be broken down. It was therefore decided to use the WSF of an aged Mozzarella cheese (at 182 d) to compare the methods.

6.4.6.1 Urea-PAGE of the SE-HPLC fractions

Only the largest peptides corresponding to peaks 1 to 5 and perhaps peak 9 on the SE-HPLC trace, were visible using urea-PAGE. It was assumed that the remainder of the peaks were either too small or too highly charged to be trapped in the gel, or were not present in sufficient amounts to be visible after staining with Coomassie blue, or they were positively charged and did not migrate into the gel. The samples were analysed again so that all the visible peaks were present on a single gel (Fig. 6.8).

The faint mark that may have been a peptide in fraction 9 was not visible on the second gel and was assumed to be an artefact on the first gel. Each of the first five SE-HPLC peaks contained several peptides of similar size and these could be separated on the basis of charge using urea-PAGE.

6.4.6.2 RP-HPLC of the SE-HPLC fractions

The fractions collected from the SE-HPLC column were also analysed by RP-HPLC. To minimise the chance of misidentification of peaks caused by problems that were being experienced intermittently with the HPLC pump, the sample containing the WSF of the Mozzarella cheese was run after each fraction. In this way it was possible to accurately identify the location of the peaks (Fig. 6.8). Most peaks on the SE-HPLC trace could be related to peaks on the RP-HPLC trace. The early-eluting peaks on the SE-HPLC trace (Fig. 6.3) corresponded with the late-eluting peaks on the RP-HPLC trace (Fig. 6.7), and the late-eluting peaks on the SE-HPLC trace (Fig. 6.3) corresponded with the early-eluting peaks on the RP-HPLC trace (Fig. 6.7). However, there were anomalies because separation of the peptides by RP-HPLC was predominantly on the basis of hydrophobicity whereas separation of the SE-HPLC fractions was on the basis of size. In agreement with the findings using urea-PAGE, it was observed that there was often more than one peptide in each of the SE-HPLC peaks. The high molecular weight peaks observed at the end of the RP-HPLC trace (after 50 min) corresponded with the bands observed by urea-PAGE.

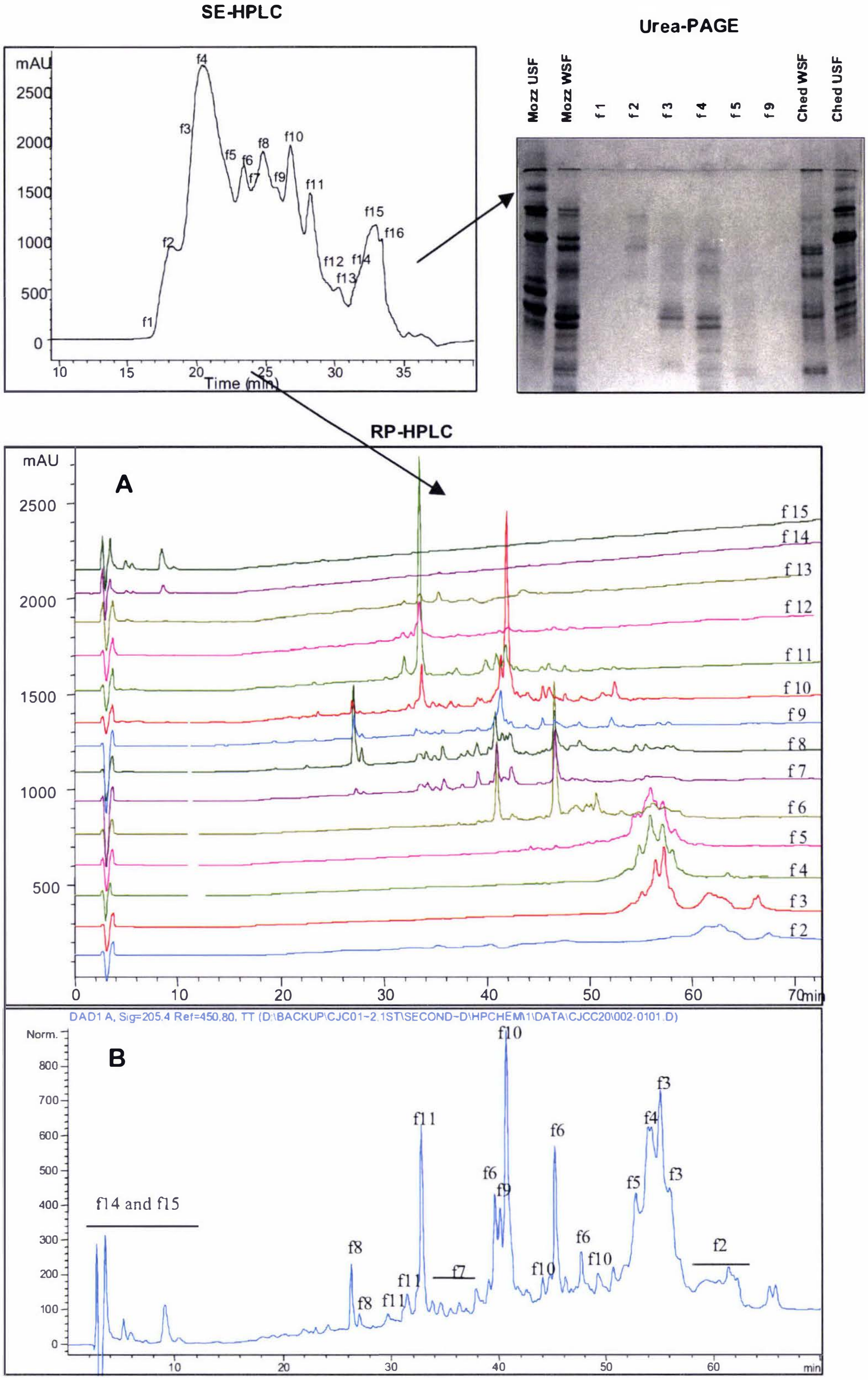


Fig. 6.8 *The relationship between SE-HPLC, urea-PAGE and RP-HPLC.* The WSF (200 µL) of aged Mozzarella cheese (3 g ± 0.0005 g cheese in 30 mL water) was loaded on to the SE-HPLC column and the elution was monitored at 220 nm. Refer to Fig. 6.3 for details of SE-HPLC. Fifteen fractions were collected, concentrated by evaporation of the solvent, diluted 1:1 with either urea-PAGE buffer or RP-HPLC solvent A (as appropriate) and analysed by urea-PAGE and RP-HPLC (refer to Figs. 6.6 and 6.7 for details of urea-PAGE and RP-HPLC). The RP-HPLC fractions (A) were used to assign SE-HPLC fraction numbers to each of the peaks of the "parent" Mozzarella cheese (B).

6.5 DISCUSSION

The main objective of the work in this chapter was to undertake preliminary experiments to determine the potential of SE-HPLC and traditional methods for characterising proteolysis in several cheese types that were ready for consumption, and for differentiating cheese types, so that methods could be selected for in-depth studies of cheese differentiation. In the process, information was gathered on the effect of cheese manufacture, composition and storage temperature on proteolysis in each of the cheese types.

The following discussion examines each of the methods used to analyse proteolysis in the seven cheese types, and compares the potential of the SE-HPLC method with NCN, NPN, urea-PAGE and RP-HPLC for use in cheese analysis.

6.5.1 Nitrogen distribution - NCN and NPN in seven cheese types

NCN and NPN are commonly used as indicators of proteolysis in cheese. According to Fox (1989), NCN and WSN provide similar results. However, the extraction of NCN is independent of cheese composition variables such as Ca or salt concentration that may influence the extraction of WSN. (There is no effect of pH on the extraction of WSN if the cheese pH < 6.0 (Bütikofer, 1992)).

Although it is often useful to determine and compare trends in the rate of increase in NCN or NPN during maturation, this type of analysis does not provide information as to the nature of the components of the NCN and NPN, and by itself, NCN or NPN content cannot be used to identify a cheese type and its degree of maturation. When comparing unrelated cheese samples it is only possible to discuss proteolysis in terms of the proportion of NCN (NCN/TN%) present, and to indicate the proportion of NPN present in the NCN (NPN/NCN%) and TN (NPN/TN%), so that the relative contributions of the proteolytic and peptidolytic enzymes can be assessed. Different storage conditions (temperature and time) were used for most cheese types, making it difficult to usefully compare enzyme activity in the cheese. However, the results of this study (Table 6.2 and Fig. 6.9) show that the Blue cheese had undergone more than twice the amount of proteolysis as any other cheese type, with about 46% of the TN hydrolysed to peptides contained in the NCN, with 79% of these being small enough to be present in the NPN. Because the cheese had been ripened at comparatively low temperatures (Table 6.1) for only 2 months it was possible to say that the results indicated more rapid and extensive proteolytic and peptidolytic enzyme activity in this cheese type than in any other. This is characteristic of this type of mould ripened cheese where the mould has very potent peptidolytic enzymes. In Cheddar cheese (pH 5.10) about 20.1% of the TN was NCN, but a high proportion (78%) of this was NPN, indicating extensive peptidolytic activity, probably by the enzymes of the starter and NSLAB. Because the cheese was ripened at a high temperature (13°C) for a long period (8.5 m) it was possible to speculate that the enzyme activity was low in comparison to that in Elsberg or Gouda cheese. Of the remaining cheeses, proteolytic activity (NCN) was greatest in Elsberg (19.2% TN) > Gouda (17.8% TN) Swiss (14.0% TN) > Feta (12.9% TN) > Mozzarella (8.3% TN) (Table 6.2). Peptidolytic activity (NPN) was greatest in Elsberg (10.6% TN) > Swiss (9.6% TN) > Gouda (7.9% TN) > Feta (7.1% TN) > Mozzarella (1.9% TN) (Table 6.2).

The small amount of NPN in the Mozzarella cheese is characteristic of the variety and indicative of the low level of starter addition, the growth-limiting effect of dry-salting and the loss of lactose during stretching, and the effect of stretch temperature (68°C) on the survival of chymosin, the mesophilic starter and NSLAB. The other cheese types each had significantly more microbial enzyme activity which resulted in the formation of the small peptides detected in the NPN. In cheeses with a full complement of proteolytic and peptidolytic enzymes, the ratio of enzyme activities is mainly dependent on the chemistry of the cheese, but can be manipulated to some extent by storage temperature.

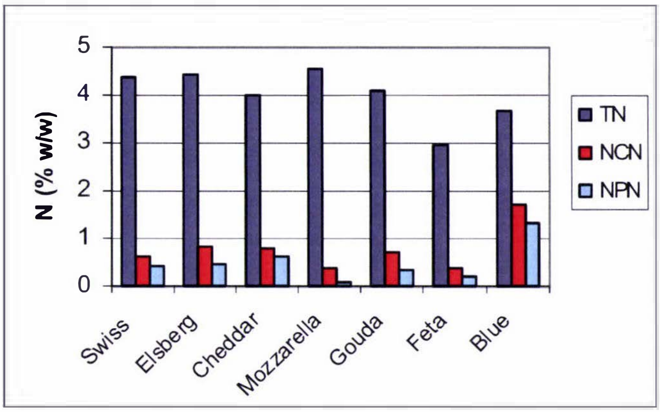


Fig 6.9 *Distribution of cheese nitrogen.* TN, NCN and NPN (% w/w) measurements for Swiss, Elsberg, Cheddar, Mozzarella, Gouda, Feta and Blue cheese.

In summary, when used in conjunction with NCN, the measurement of NPN provided insight into the proteolytic and peptidolytic activity in cheese. This combination of methods enabled the Blue and the Mozzarella cheese to be differentiated from each other and from the remainder of the cheeses. However, there may be considerable overlap in the amounts of NCN and NPN in different cheese types as they mature and it would not be possible to differentiate the remaining cheese types on the basis of the results of a single sample (see next chapter).

Although valuable tools, NCN and NPN provided no information as to which caseins were hydrolysed or the nature of the peptides produced.

6.5.2 SE-HPLC of the USF of seven cheese types

The SE-HPLC method can be used to demonstrate differences in the molecular weight distribution of the proteins, peptides and amino acids in the USF of a range of cheese types (Fig. 6.1). However, the differences between most cheese types were not large, suggesting that SE-HPLC of the USF may not be particularly useful for differentiating cheese type. The early-eluting higher molecular weight peptide peaks (>5000 Da) and the urea peak were so large in relation to the later-eluting lower molecular weight peptide peaks (<5000 Da) that some of the detail of the small peaks was lost, possibly as the result of tailing of the large peaks.

Although it is unlikely that SE-HPLC of the USF would be useful for differentiating cheese types, the wide molecular weight distribution of peaks observed in the Blue cheese (Fig. 6.1) suggested the possibility that the method could find a use for monitoring the hydrolysis of proteins and peptides in highly proteolytic cheese-based systems such as enzyme-modified cheese (EMC), used as a flavour ingredient in processed cheese (e.g. Wilkinson et al, 1992).

6.5.3 SE-HPLC of the WSF of seven cheese types

The peptides and amino acids in the WSF of cheese can be segregated into a series of peaks using SE-HPLC, and their molecular weight distribution can be calculated from a calibration curve prepared from the elution times of standards of known molecular weight (Ch. 5).

In the present study, seven cheeses that should demonstrate most differences in cheese proteolysis were selected so that the SE-HPLC method could be tested. The results showed that SE-HPLC produced a simple profile of molecular weight distribution of the peptides and amino acids in the WSF of each cheese type (Fig. 6.3). Whereas NCN could be divided into two fractions (NPN and non-NPN) based approximately on molecular weight distribution, WSN (approximately equivalent to NCN) could be divided into about sixteen fractions or peaks using SE-HPLC. The molecular weight distribution profile therefore provided more information on proteolysis (than NCN and NPN) and was distinctive for each of the seven cheese types. These profiles represent secondary proteolysis in the cheese and it was useful to consider them alongside the urea-PAGE (USF) results (see following section), which provided information on primary proteolysis. The profiles could be discussed in terms of the effect of manufacture, composition and storage temperature on the proteolytic and peptidolytic enzymes (rennet, (Cathepsin D), plasmin and microbial enzymes) responsible for the peptides and amino acids present in the SE-HPLC peaks of the WSF of ripened cheese (Ch.6, sections 6.4.3.1 – 6.4.3.6).

The molecular weight distribution of the Mozzarella cheese showed the limit of the action of the proteolytic enzymes, plasmin and chymosin, on the proteins in the cheese. This cheese was ready for sale and the molecular weight distribution (Fig. 6.3) fits with the amount of proteolysis required to achieve good functionality (stretch and melt) (C Coker and K Johnston, 1996, unpublished). That is, there was a large proportion of early-eluting high molecular weight peptides and only low concentrations of later-eluting lower molecular weight peptides. In most cheese types, the cheese microflora hydrolyse relatively large peptides in the cheese to form small peptides and amino acids (Thomas and Pritchard, 1987). However, the high stretch temperature (68°C) combined with dry-salting ensured that the survival of the cheese microflora was minimal in the Mozzarella cheese, as demonstrated by the lack of small peptides on the SE-HPLC trace. In contrast, it was clear that both proteolytic and peptidolytic enzymes had been active in the Blue cheese, resulting in a lack of higher molecular weight material and an abundance of small peptides and amino acids. The other cheese types, Swiss, Elsberg, Gouda, Cheddar and Feta cheese, each had a distinctive pattern of molecular weight distribution that was discussed in terms of the effect of cheese manufacture, composition and storage temperature on the types and amounts of enzyme activity in the cheese (Ch. 6, section 6.4.3).

The distinctive molecular weight distribution of peptides in each of the cheese types suggests that cheese type should be distinguishable regardless of age. To confirm this would require a study of selected cheese types at several stages during maturation.

6.5.4 Urea-PAGE of the USF of seven cheese types

Urea-PAGE is commonly used to analyse the USF of cheese (Creamer, 1991; Johnston et al, 1994), but the main constraint is that only the caseins and largest peptides are visible (Fig. 6.5). It is a more discriminating technique than NCN measurement and provides more information about the early stages of proteolysis than SE-HPLC of the USF.

Urea-PAGE of the USF of cheese (Fig. 6.5) provided information on the amount and type of casein hydrolysis. It was possible to determine which of the caseins had been hydrolysed in the various cheese types and by examination of the peptide products, predictions could be made with confidence as to which of the proteolytic enzymes were responsible.

Examination of the urea-PAGE patterns (Fig. 6.5) revealed differing concentrations of β-, α_{s1}-, α_{s1}-I-, γ_1-, γ_2-, γ_3-, β-I- and β-II-casein. Some of these differences could be attributed to differences in protein concentration in the cheese (Table 6.2, TN values), while others could be related to proteolysis in the cheese. At this stage, it is probably important not to over-interpret the results because the cheeses were all at different stages of maturation. However, the results fit the current knowledge of relative enzyme activities in these cheese types.

Hydrolysis of β-casein with the formation of γ-caseins (γ_1- (β-casein f29–209), γ_2- (β-casein f106-209) and γ_3- (β-casein f108-209) casein) is indicative of plasmin and perhaps thermophilic starter enzyme activity and this was most notable in the Swiss, Elsberg, and Gouda cheese. This is in agreement with the higher levels of plasmin activity reported for these cheese types (Richardson and Pearce, 1981; Farkye and Fox, 1990). The pH of each of these cheeses was greater than pH 5.5 (Table 6.2) and it is known that a higher pH results in greater plasmin activity (Noomen, 1975). The greatest plasmin action was observed in the Swiss cheese. The cook temperature of the Swiss cheese was 50°C and this is believed to inactivate the inhibitor of the plasminogen activator, resulting in a greater conversion of plasminogen to active plasmin (Richardson, 1983b; Deharveng and Neilson, 1990).

The formation of β-I- (β-casein f1-189/192), β-II- (β-casein f1-165/167) and β-III- (β-casein f1-139) casein is indicative of chymosin action on β-casein and was more pronounced in the Elsberg cheese. In this cheese type, it would seem that the β-casein was more accessible to both plasmin and chymosin. The cheese has a similar composition to Gouda, with a relatively high S/M (4.67%) (Table 6.2). Like Gouda cheese, Elsberg is a washed-curd cheese and would retain a similar amount of chymosin. However, the storage regime, which involved a period at high temperature (12°C for 14 d; 20°C for ~ 10 d), followed by long-term storage at low temperature (5°C) would facilitate greater chymosin activity than occurred in the Gouda cheese.

Hydrolysis of α_{s1}-casein with the concomitant formation of α_{s1}-I-casein (α_{s1}-casein f24-199) is indicative of chymosin (Creamer and Richardson, 1974) or perhaps cathepsin D activity (McSweeney et al, 1995). Cathepsin D, an indigenous milk protease, appears to survive pasteurisation (Hayes et al, 2001), is present in small quantities in cheese (Hurley et al, 2000), hydrolyses α_{s1}- and β-casein, and has a similar specificity to chymosin, producing α_{s1}-I-, β-I- and β-II-casein (McSweeney et al, 1995; Larsen et al, 1996). In some cheese types, most notably mature Cheddar and Blue cheese, little α_{s1}-casein or α_{s1}-I-casein remained and this suggested a high level of chymosin and perhaps microbial enzyme activity (or in Blue cheese, activity of the mould enzymes as well). Particularly noticeable in the Elsberg and Cheddar cheeses was the presence of a peptide with a lower electrophoretic mobility than the parent α_{s1}-casein (Fig. 6.5). A band in this position was identified by Mooney (1998) as α_{s1}-casein f99-199, a product of chymosin action.

The small band in the position α_{s1}-casein in the Cheddar cheese probably contained minor peptides that co-eluted with the casein. It has been demonstrated that four peptides account for ~ 5% of the α_{s1}-casein (calculated on the basis of the 1 day value) that appears to remain in aged Cheddar cheese (Coker, 1994).

Although the urea-PAGE profiles showed the caseins and products of primary proteolysis, and provided sufficient information to determine the enzymes responsible, there was insufficient information to differentiate the cheese types beyond moderately broad classifications. Apart from the loss of the products of primary proteolysis, there was no information about secondary proteolysis (proteolysis of the peptides produced by primary proteolytic enzymes) by microbial enzymes.

6.5.5 Urea-PAGE of the WSF of seven cheese types

One of the limitations of urea-PAGE is that only the most abundant water-soluble peptides that are effectively precipitated in the gel and stained by the particular staining system become visible (Fig. 6.6). Consequently, many peptides, and almost all that are < 5000 Da, are undetected and key information that is likely to be useful for characterising the cheese is unavailable.

An examination of the WSF (Fig. 6.6), at 12.5 times the concentration of the USF (to compensate for the low Coomassie stain adsorption and the small amount of material present), revealed the presence of many peptide bands in each cheese type. Although many water-soluble peptides have been identified in Cheddar cheese (e.g. McSweeney et al, 1994, Singh et al, 1995, 1997), there has been little focus on identifying them in other cheese types. In some cheese types the complement of bands and their intensity was similar (e.g. Elsberg and Gouda, or Cheddar and Feta). The only truly distinctive profiles were those of the Mozzarella and Blue cheese. Therefore, the differences between cheese types may be insufficient to differentiate them.

Urea-PAGE of the USF or WSF has not been discounted as a possible method for differentiating cheese types. It is possible that differences between cheese types would be highlighted if a ripening profile was established for each cheese and the results for band area were subjected to statistical analysis.

6.5.6 RP-HPLC of the WSF of seven cheese types

RP-HPLC separates peptides and amino acids on the basis of hydrophobicity, so that generally, the smaller and less hydrophobic peptides elute earlier and the larger and more hydrophobic peptides elute later. In young cheese or cheese that has undergone limited proteolysis, most peptides elute later. During the early stages of maturation there is an initial increase in the amount of later-eluting peptide material (in the region between 50 and 60 min (Fig. 6.7)), but as maturation progresses, these peptides are hydrolysed to smaller peptides that elute earlier. This general trend is observed in cheese types with a moderate or high level of enzyme activity such as Cheddar (Aston and Creamer, 1986; Coker, 1994).

The RP-HPLC chromatograms (Fig. 6.7) were different for each cheese type, and most contained a large number of peaks. The differences in the size and ratios of the peaks were created by differences in the manufacturing processes and storage protocol of each cheese type and their effect on the type and amount of proteolytic enzyme activity in the cheese (see Ch. 6, section 6.4.5).

In general terms, the RP-HPLC and SE-HPLC results provided similar information on secondary proteolysis, but RP-HPLC provided greater detail. RP-HPLC complemented NCN, NPN (Table 6.2), urea-PAGE (Fig. 6.5) and SE-HPLC (Figs. 6.1 and 6.3). For example, the low levels of NCN (8.3% TN) and NPN (1.9% TN) in Mozzarella cheese, corresponded with the high levels of intact α_{s1}- and β-casein observed on the urea-PAGE gels, the large amount of higher molecular weight material on the SE-HPLC trace and the small amount of material on the RP-HPLC trace that mainly contained later-eluting peptides (between 50 and 61 min). The lack of early-eluting peptides therefore appears characteristic of the Mozzarella cheese and provided a chromatogram with an easily recognisable pattern. The combined interpretation of the Mozzarella results would be that there was a small amount of chymosin (or Cathepsin D) action on α_{s1}-casein and a similarly small amount of plasmin action on β-casein that resulted in the release of α_{s1}-I- and γ-caseins as well as large water-soluble peptides from the parent casein, but further hydrolysis appears limited, probably as a result of the effect of the manufacturing process on microbial enzyme activity, although the short storage time (13°C/14 d) would be a contributing factor.

Cheddar cheese provided a different example. In this cheese type, NCN was comparatively high (20.1% TN) and NPN represented about 78% of this (15.6% TN) (Table 6.2), which indicates substantial proteolytic and peptidolytic activity. Urea-PAGE analysis showed that chymosin was the main contributor to the high NCN, and its activity had resulted in the hydrolysis of most of the α_{s1}- and α_{s1}-I-casein to peptides captured in the NCN fraction of this cheese. The contribution from plasmin was small in comparison, although it had hydrolysed a proportion of the β-casein with the consequent formation of the γ-caseins. Unlike Mozzarella cheese, the RP-HPLC chromatogram showed only a small proportion of later-eluting peptides and large quantities of early-eluting peptides and amino acids that accounted for the high NPN. This indicated substantial hydrolysis of the large peptides to small peptides and amino acids by the enzymes of the cheese microflora active in this cheese type.

The Elsberg, Gouda and Swiss cheeses each had a similar ratio of NPN/NCN, and most RP-HPLC peaks were common to each cheese, albeit at different ratios. The RP-HPLC chromatogram showed large quantities of amino acids in the first peak of the Swiss and Elsberg traces. This is likely to be as a consequence of the activity of the propionibacteria added during the manufacture of these cheeses. Urea-PAGE of the USF showed different amounts of chymosin and plasmin action in each cheese that could be attributed to the effect of differences in the cheese manufacturing process and storage protocol on the activity of the enzymes present. For example, the manufacturing protocol used for the Gouda and Elsberg cheese was similar (Table 6.1), with both cheeses made using the same mesophilic starters, the same amount of chymosin, cooked at a similar temperature and drained at the same pH. However, the storage protocol was different for each cheese type and the high storage temperature used for the Elsberg cheese during the first 24 days of ripening promoted greater enzyme activity that was largely responsible for differences in the RP-HPLC profiles. The storage protocol for the Swiss and Elsberg was similar, but different types of starter culture and the high cook temperature of the Swiss cheese promoted differences in proteolytic and peptidolytic enzyme activity.

The Blue cheese had a characteristic RP-HPLC pattern (Fig. 6.7) with large amino acid peaks and a large quantity of small peptides that eluted early in the trace (before 40 min). In this cheese, the high NCN (45.9% TN) and small amount of material remaining on the urea-PAGE gels indicated substantial proteolytic activity, while the high NPN (36.2% TN) and the large amount of early-eluting peptides and amino acids on the RP-HPLC trace (Fig. 6.7) indicated substantial peptidolytic activity. Extensive proteolysis is a typical feature of Blue cheese.

In summary, it was possible to distinguish some cheese types using RP-HPLC, but subjective differentiation of others was difficult. The traces may be sufficiently different to enable cheese type to be distinguishable regardless of age, but the complexity of the traces means that this would be a difficult task.

6.6 CONCLUSIONS

The results of this preliminary study were consistent with the concept that the manufacturing processes used to create different cheese types influence the type and amount of enzyme activity in the cheese, and the consequent progression from caseins to large peptides and then to small peptides and amino acids. This progression, or rather, the point in this progression when the samples were taken, cannot be adequately captured by any single method of analysis. However, it can be captured using a combination of methods, and the results can be used to determine the relative contribution of the different enzymes present.

Gross proteolysis could be estimated by measurement of NCN and the extent of this proteolysis could be determined by measurement of NPN. However, differences in the amounts of NCN and NPN or the ratio of NPN/NCN were insufficient for cheese differentiation.

Urea-PAGE of the whole cheese (USF) provided information on the extent of casein hydrolysis and by examining the peptide bands, it was possible to determine the relative contributions of chymosin and plasmin. The limiting factors with this method were that only the initial proteolytic steps could be quantified, most peptides in the cheese were not visible at the concentration used to measure the caseins and the differences between cheese types were not sufficiently great for the method to be used alone to differentiate cheese.

Urea-PAGE of the WSF revealed peptide profiles that were similar in some cheese types. The only distinctive profiles were those of the Mozzarella and Blue cheese. The limitations of this method were that only the largest and most abundant water-soluble peptides were visible and the differences between cheeses were not sufficient for cheese differentiation.

RP-HPLC of the WSF provided good separation of the peptides in the cheese but gave very complex patterns that were difficult to interpret when the cheese types were compared. A comparison of the concentrations of later-eluting larger peptides, intermediate peptides and early-eluting smaller peptides and amino acids showed that each cheese type had a different distribution of peptides. This and the obvious differences in peak areas of the large number of peaks suggest that this technique has the potential to differentiate cheese.

SE-HPLC gave a simple, useful molecular weight distribution of the components of the WSF. The pattern of molecular distribution was different for each cheese type, provided insight on the extent of proteolysis in each cheese, and may be useful for cheese differentiation.

In summary, urea-PAGE of the USF, and SE-HPLC and RP-HPLC of the WSF provided useful information on the type and extent of proteolysis in the cheese and may provide useful differentiation of cheese type. It was more difficult to draw firm conclusions on the usefulness of urea-PAGE of the WSF because some cheeses had a similar peptide complement and concentration at the point of examination. SE-HPLC of the USF was less useful for the analysis of most cheese types but it may be a useful method for monitoring proteolysis in cheese systems in which there is extensive proteolysis, such as in Blue cheese or EMC.

Of the methods examined, SE-HPLC of the WSF has the most potential for differentiating cheese because it provides simple separation of the components of the WSF to give a different pattern of molecular weight distribution for each cheese type that reflects the activity of the enzymes present. SE-HPLC could also be used in conjunction with urea-PAGE of the USF to monitor cheese ripening or to monitor the effects of changes in manufacturing protocol on proteolysis in cheese.

6.7 RECOMMENDATIONS

It is recommended that ripening profiles be established for selected cheese types, using urea-PAGE of the USF and urea-PAGE, SE-HPLC and RP-HPLC of the WSF. The band or peak areas should be determined and the results examined objectively using statistical analysis. In this way, it may be possible to select a single method that can differentiate cheese types at all stages of maturity.

7.0 DIFFERENTIATION OF CHEESE TYPE DURING MATURATION - A COMPARISON OF UREA-PAGE, SIZE EXCLUSION AND REVERSE PHASE-HPLC METHODS USING PRINCIPAL COMPONENT ANALYSIS

ABSTRACT

The enzymes present in each of five types of semi-hard to hard cheese (Cheddar, Gouda, Elsberg, Mozzarella and Swiss) hydrolysed the caseins to peptides of various sizes and amino acids during ripening. This transition was followed using a range of techniques that included urea-PAGE of the USF and WSF of cheese and RP-HPLC or SE-HPLC of the WSF of cheese.

It was possible to determine from the urea-PAGE results, that the rate of chymosin activity, characterised by the hydrolysis of α_{s1}- to α_{s1}-I-casein, was greatest in the Elsberg > Cheddar > Gouda ≈ Swiss > Mozzarella; and the rate of plasmin activity, characterised by the hydrolysis of β-casein to γ-caseins, was greatest in the Elsberg ≈ Swiss > Gouda ≈ Mozzarella > Cheddar: The ratios of α_{s1}-I- to α_{s1}-casein and γ- to β-casein were useful for monitoring proteolysis during the early stages of maturation of each cheese type or throughout the maturation of slowly ripening cheese types. Examination of the large number of peptide peaks on the RP-HPLC traces revealed trends associated with ripening and differences between cheese types. The SE-HPLC traces were simple in comparison, and it was clear that the molecular weight distribution of the peptides changed during maturation and was different for each cheese type.

Multivariate statistical techniques provided the possibility of differentiating cheese types and examining maturity using the complete data set from each type of analysis. Each data set was analysed using both the correlation and covariance forms of PCA. PCA of the urea-PAGE (USF) data was useful for differentiating cheese type at all stages of maturity and for monitoring proteolysis in the early stages of ripening or throughout the ripening of cheeses that matured slowly, whereas PCA of the RP- or SE-HPLC peak area data was useful for differentiating cheese type at all stages of maturity and for determining maturity throughout the ripening of each cheese type.

7.1 INTRODUCTION

The unique character of the many 'matured' types of cheese produced around the world develops gradually during storage or ripening and reflects the effect of differences in the manufacture and storage of the cheese on the quantity and activity of the enzymes present.

In Chapter 6, it was shown that the protein and peptide patterns of a representative group of mature cheeses could be compared using urea-PAGE of the USF or WSF, RP-HPLC of the WSF, or the SE-HPLC method developed for the analysis of cheese (Ch. 5). That study showed that each technique provided a different pattern of proteolysis for seven cheese varieties and had potential for cheese characterisation and differentiation.

It was decided to extend the study to determine how maturation changed the protein and peptide patterns of typical commercial cheeses of similar composition. The cheese types (Swiss, Elsberg, Cheddar, Mozzarella and Gouda) were selected from the group previously analysed (Ch. 6), and encompassed the range of enzymes activities normally found in hard and semi-hard cheese. That is, they were expected to have combinations of high or low plasmin, chymosin and microbial enzyme activity.

Each of the methods recommended in the previous study separates cheese proteins and peptides into a number of bands or peaks (Ch. 6). The areas of these peaks can be measured and the peak data incorporated in a large data set. Previous authors (see literature review, Ch. 2, section 2.8) have used multivariate statistics to provide objective interpretation of this type of data. This approach has been used to broadly group cheese of similar maturity (with varying success) (e.g. Amantea et al, 1986; Santa-Maria et al, 1986; Alonso et al, 1987; Furtula et al, 1994a,b; Garcia Ruiz et al, 1998), using data from physico-chemical analyses or RP-HPLC peak data. The same approach has been used in attempts to establish proteolysis indices for characterising and differentiating cheese types, with varying degrees of success. For example, Smith and Nakai (1990) used principal component analysis (PCA) and discriminant analysis (DA) to analyse RP-HPLC (C8 columns) peak data. These authors had problems with column stability and the low principal component scores (PC1 accounted for only 13% of the variation in the data) suggested the data sets were unsuitable for PCA. When data from a single column was used, it was possible to differentiate ~90% of cheese samples according to type, but this was reduced to 64% when data from all columns was used. Lopez-Fandiño et al (1994), used cluster analysis to determine the usefulness of various proteolysis indices for characterising and differentiating six types of cheese (each at a different stage of maturation). Their results indicated that the combination of pH, TN, WSN, TCA-N, PTA-N, urea-PAGE and SDS-PAGE was useful for distinguishing the most from the least mature cheese and that the two Swiss-type cheeses (Apenzeller and Comte) appeared to be related. Millán et al (1996a) used DA of physico-chemical data (WSN, NPN, ammonia N, a_w moisture, fat, salt and pH) with limited success to classify 7 varieties of Spanish cheeses from the Canary Islands. (The standard deviations were high between samples of the same type and this would have caused problems with the differentiation).

PCA has not been successfully extended to the objective differentiation of cheese type throughout maturation, and to date, no simpler method of sample analysis has been found to replace RP-HPLC.

In the current study, methods recommended in the previous study are used to analyse five types of cheese throughout maturation and principal components and classification analysis (PCA) is used to provide an objective assessment of the results (peak data).

7.2 OBJECTIVES

This study attempts to characterise proteolysis in selected cheese types and to identify methods that could be used to objectively differentiate the cheese types regardless of age.

In order to determine the relative usefulness of each of the methods recommended in the previous study (Ch. 6) for characterising cheese proteolysis during maturation, to determine the effect of maturity on the differentiation of cheese type, and to identify the best method(s) for the objective determination of cheese type throughout maturation the following steps were taken:

a) the cheese types of interest were identified,
b) cheese of each type was obtained as soon as possible after manufacture,
c) the cheese was stored under appropriate conditions and sampled at regular intervals throughout maturation,
d) cheese samples for analysis were stored at −85°C to avoid changes to the proteins during storage,
e) methods identified as suitable to examine proteolysis in cheese (Ch. 6), namely urea-PAGE, SE-HPLC, RP-HPLC of the WSF and urea-PAGE of the USF, were used to analyse each cheese sample,
f) the integrated absorbance of the bands on the gels was used to create a chromatograph. The area under each of the (absorbance) peaks on the urea-PAGE, SE- and RP-HPLC traces was determined, and a data set that included the five cheese types was obtained for each type of analysis, and
g) appropriate statistical methods were used to analyse the data.

7.3 MATERIALS AND METHODS

7.3.1 Materials

All materials are described in Ch. 4, section 4.1.

7.3.2 Methods

7.3.2.1 Cheese manufacture and maturation

Five of the cheese types (Cheddar, Elsberg, Gouda, Mozzarella and Swiss) selected for the preliminary experiments were again selected for a more detailed study that included ripening of the cheese. The Swiss, Elsberg and Gouda cheese were sourced commercially from Mainland Products Ltd, New Zealand, while the Cheddar and Mozzarella were made at NZDRI.

Details of the manufacture that are known can be found in the following table (see Ch. 4, section 4.2.1 for Cheddar and Mozzarella cheese-making procedure).

Table 7.1 *Cheese manufacture and storage*

Cheese type	Starter and other bacteria	Coagulant mL/100L milk	Cook temperature	Drain pH	Curd treatment	Storage time and temperature
Cheddar (NZDRI)	*Lactococcus lactis* subsp. *cremoris*	calf chymosin 16	38°C	6.1	Cheddared pressed dry-salted	13°C until 6 m
Elsberg	Mesophilic (Flora Danica) *Propionibacterium shermanii*	calf chymosin 17	37°C	6.4	washed pressed brine-salted	12°C/14 d 20°C/10 d 5°C until 6 m
Gouda	Mesophilic (Flora Danica)	calf chymosin 17	38°C	6.4	washed pressed brine-salted	5°C until 6 m
Mozzarella (NZDRI)	*Lactococcus. lactis* subsp. *cremoris*	calf chymosin 4	36°C	5.9	dry-salted stretched at 68°C	13°C until 6 m
Swiss	*Streptococcus thermophilus* *Lactobacillus bulgaricus* *Propionibacterium shermanii*	calf chymosin 17	50°C	6.5	pressed brine-salted	12°C/14 d 20°C/21 d 5°C until 6 m

The commercial cheese (20 - 40 kg of each type) was transported at 4°C to NZDRI within 2 – 3 days of manufacture.

The cheese was ripened at NZDRI using the temperature protocol normally used by the manufacturer (Table 7.1). Representative cheese samples were taken at approximately 1, 14, 28, (45), 60, 90, 120, 150 and 180 d, and stored at -85°C until all the samples were collected. At the end of the ripening period, sets of cheese samples that represented the ripening of each of the five cheese types were available for analysis.

7.3.2.2 Determination of the chemical composition of cheese

The chemical composition (calcium, salt, pH, moisture, fat, TN) of each cheese type was determined as described in Ch. 4, section 4.2.2. NCN and NPN were determined for each cheese sample.

7.3.2.3 Preparation and analysis of the USF and WSF of cheese

Each set of cheese samples was analysed by urea-PAGE of the USF and WSF, and SE-HPLC and RP-HPLC of the WSF according to the methods described in Ch.4, section 4.2.3.1, 4.2.3.3 and 4.2.3.4, respectively.

7.3.2.4 Principal Component Analysis

The peak areas obtained using urea-PAGE, SE-HPLC and RP-HPLC were determined and the results were separately analysed using both the covariance and the correlation forms of PCA (Ch. 4, section 4.2.6.1).

7.4 RESULTS

7.4.1 Cheese chemical composition

The chemical composition (pH, TN, fat, moisture, salt, calcium), and the derived S/M and MNFS of each cheese type is outlined in Table 7.2.

Table 7.2 *Cheese chemical composition*

	Cheddar	Elsberg	Gouda	Mozzarella	Swiss
Fat (% w/w)	35.0	26.0	28.0	20.0	28.5
Moisture (% w/w)	34.2	42.1	44.1	48.0	39.3
Salt (% w/w)	1.73	0.85	0.74	1.43	0.40
Ca (mmoles/kg)	201	221	200	168	239
pH at 1 day	5.26	5.56	5.53	5.48	5.42
TN (% w/w)	3.83	4.15	3.67	4.27	4.36
S/M (%)	5.06	2.02	1.68	2.98	1.02
MNFS (%)	52.62	56.89	61.25	60.00	54.97

Each type of cheese was within the normal (New Zealand) specification and was considered by the manufacturer to be typical of the type.

7.4.2 Cheese protein and peptide analysis

Ripening profiles for each cheese type were established using NCN, NPN, urea-PAGE of the USF and WSF, and SE-HPLC and RP-HPLC of the WSF.

7.4.2.1 NCN and NPN

NCN (pH 4.6 soluble N) (Dahlberg and Kosikowski, 1947) and NPN (12 % TCA-soluble N) (Vakaleris and Price, 1959) were analysed at each stage of sampling of the five types of cheese. Differences in the rates of generation of the NCN and NPN during ripening were evident (Fig. 7.1A and B).

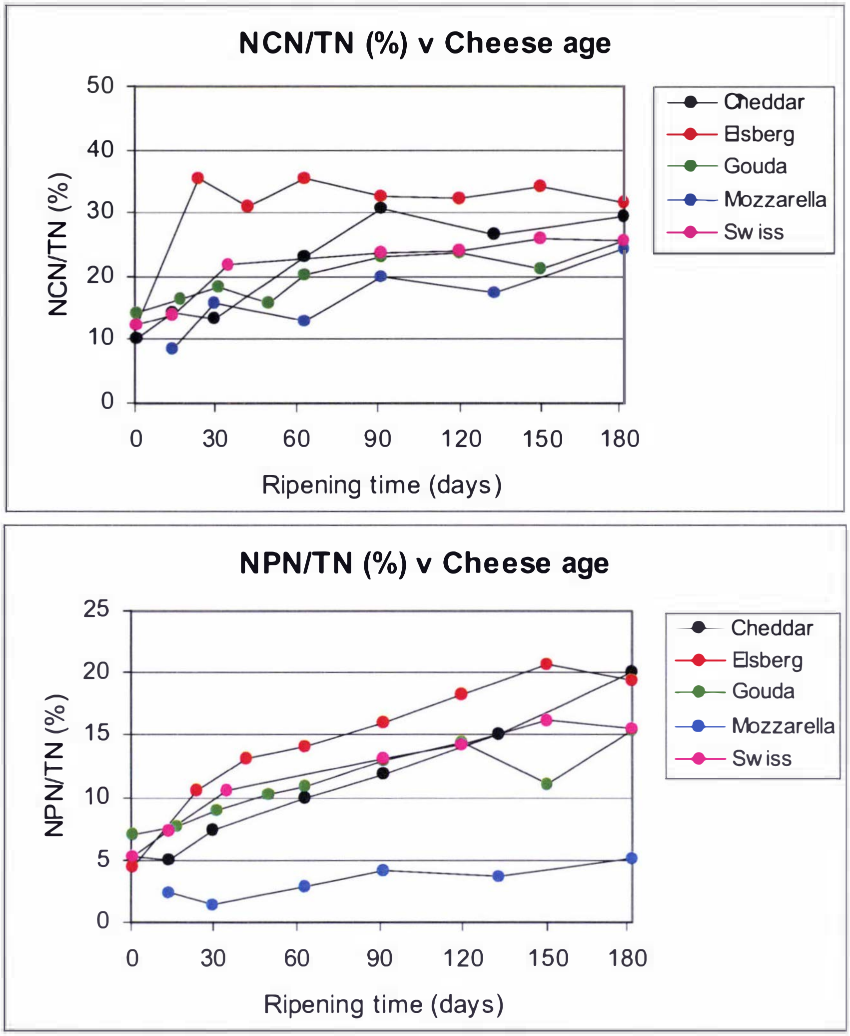


Fig. 7.1A *NCN/TN and NPN/TN - Changes during ripening of five cheese types (Cheddar, Elsberg, Gouda, Mozzarella and Swiss cheese).*

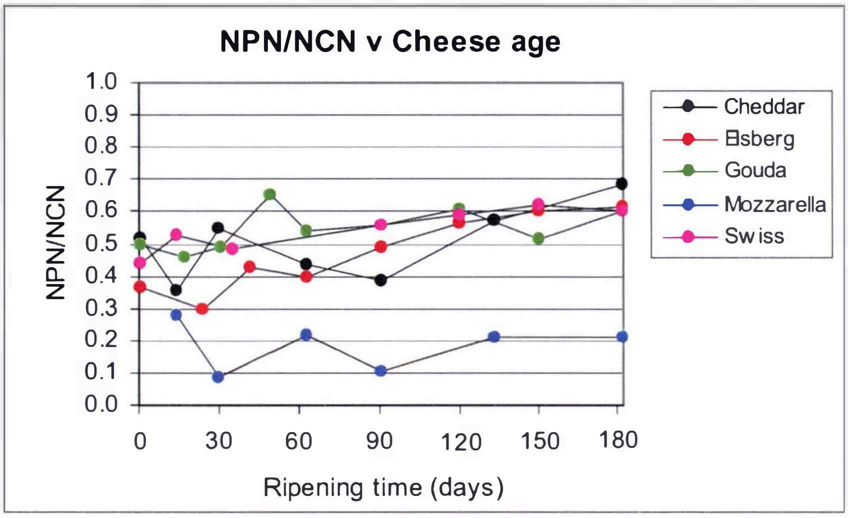


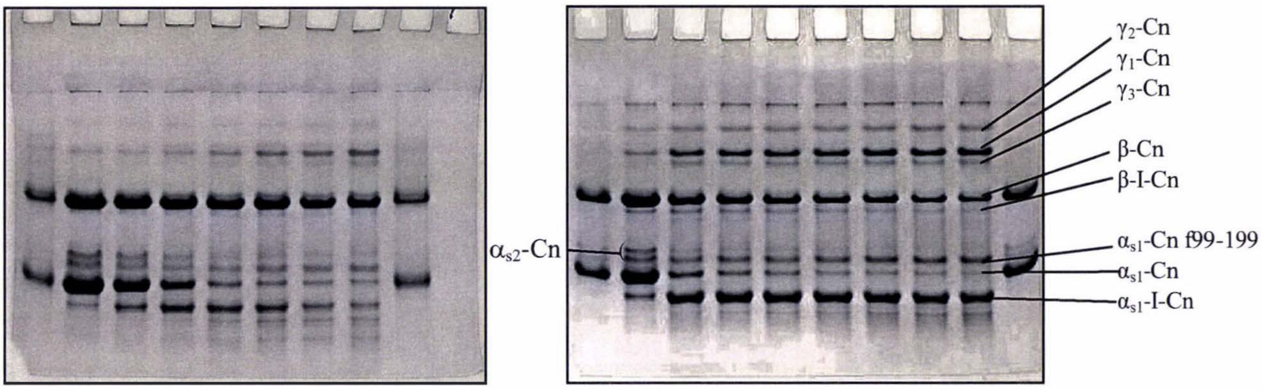
Fig. 7.1B *NPN/NCN - Changes during ripening of five cheese types (Cheddar, Elsberg, Gouda, Mozzarella and Swiss cheese).*

The rates of generation of NCN and NPN reflected the extents of primary and secondary proteolysis in the cheese, respectively. (NPN is a subset of NCN, representing small peptides and amino acids produced mainly by the cheese microflora.) The amounts of NCN and NPN gradually increased at rates that were similar in the Cheddar, Gouda and Swiss cheeses (Fig. 7.1A) and, as could be expected, the ratio of NPN to NCN was also similar (Fig. 7.1B). There was a rapid increase in the amount of NCN in the Elsberg cheese during the early stages of ripening when the cheese was stored at a higher temperature (12°C for 14 d and 20°C for 21 d), but after this period there was no further increase, although the amount of NCN remained greater than in any of the other cheese types (Fig. 7.1A). The amount of NPN also increased at a greater rate than in any of the other cheese types during the period at higher temperature, and it continued to increase for about 150 days (Fig. 7.1A). As a result, the ratio of NPN to NCN was similar to that in the Cheddar, Gouda and Swiss cheeses (Fig. 7.1B). The amount of NCN in the Mozzarella cheese was similar to the amount in the Cheddar, Gouda and Swiss cheeses, but there was only a very small amount of NPN produced in the Mozzarella cheese and this enabled it to be distinguished from the other cheese types. This reflected the effect of the high stretch temperature (68°C) on the low survival of the cheese microflora. The low ratio of NPN to NCN enabled this cheese type to be readily distinguished from the other cheese types.

In summary, the amount of NPN was less variable than the amount of NCN, and provided a reasonable estimation of cheese maturity for the first 120-150 days. The amount of NCN or NPN in the Cheddar, Gouda and Swiss cheeses was similar and only the Elsberg and Mozzarella could be differentiated from the other cheese types on the basis of NCN and/or NPN.

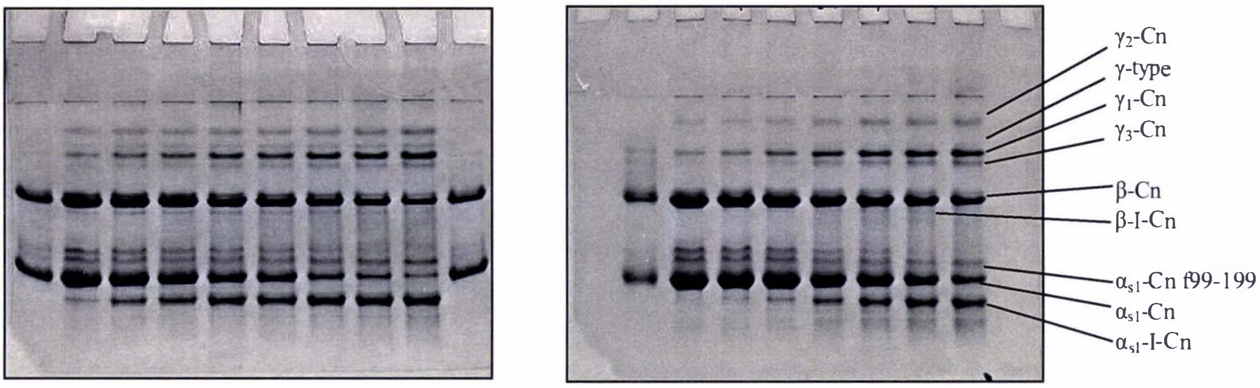
7.4.2.2 Urea-PAGE of the USF

The maturation of each cheese type was examined using urea-PAGE of the USF to establish a ripening profile for each cheese type that provided a picture of primary proteolysis (Fig. 7.2). The gels were scanned and the total dye absorbance for each band was determined using ImageQuant software as described in Ch. 4, section 4.2.3.1.9. The values for β-, γ-, α_{s1}- and α_{s1}-I-casein for each cheese sample were plotted (Fig. 7.3A).



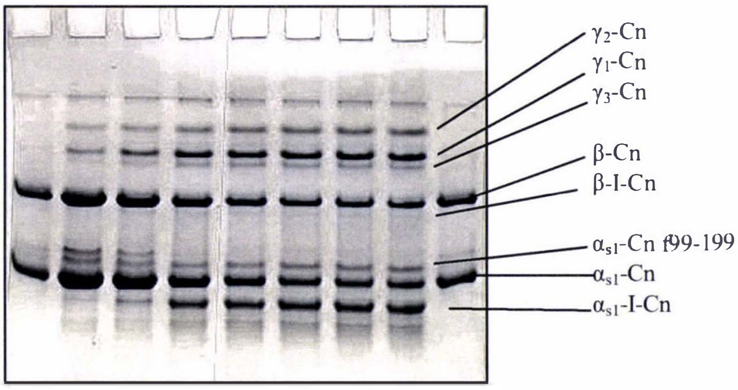
A. Cheddar cheese

B. Elsberg cheese



C. Gouda

D. Mozzarella



E. Swiss

Fig. 7.2 *Urea-PAGE of the USF of each cheese type during ripening* (2% cheese, 5 μL sample volume) - sampled at several time intervals during ripening. Lanes 1 and 10 of the Swiss, Elsberg and Gouda gels, lanes 1 and 9 of the Cheddar gel and lane 2 of the Mozzarella gel contain a rennet casein standard.

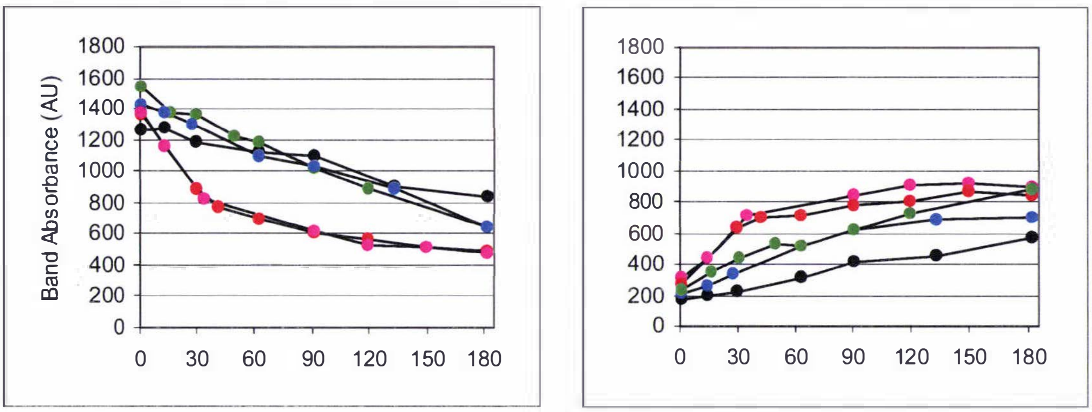
A. Cheddar cheese : Lanes 2 to 8, sampled after 1, 14, 28, 63, 91, 133 and 182 d.

B. Elsberg cheese : Lanes 2 to 9, sampled after 2, 24, 42, 63, 91, 120, 150 and 182 d.

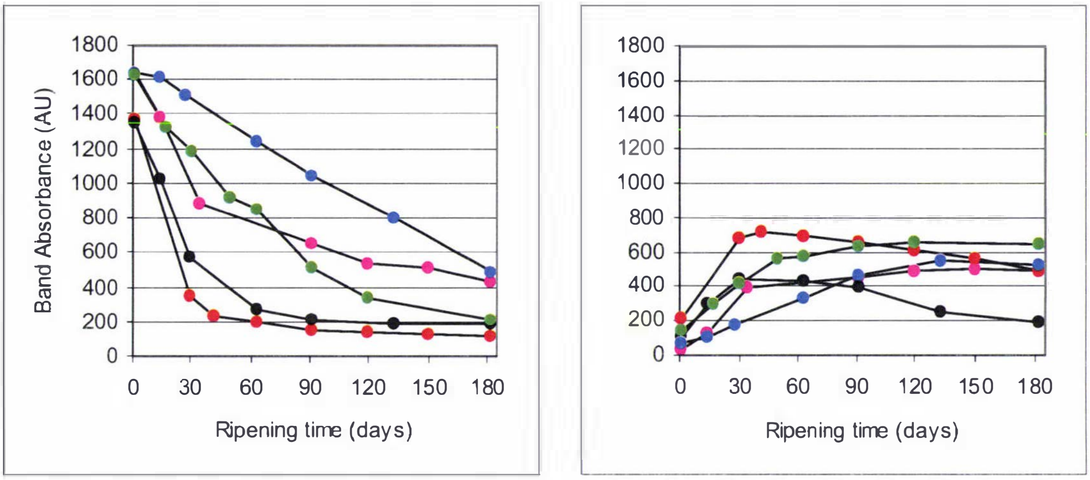
C. Gouda cheese : Lanes 2 to 9, sampled after 1, 17, 31, 50, 63, 91, 120 and 182 d.

D. Mozzarella cheese : Lanes 3 to 9, sampled after 1, 14, 28, 63, 91, 133 and 182 d.

E. Swiss cheese: Lanes 2 to 8, sampled after 1, 14, 35, 91, 120, 150 and 182 d.


β-casein

γ-casein ($\sum \gamma_1$-Cn + γ_2-Cn + γ_3-Cn + γ-type-Cn)


α_{s1}-casein

α_{s1}-I-casein

Fig. 7.3A *Urea-PAGE - Casein breakdown during the ripening of each cheese type.*

--●-- **Cheddar cheese** sampled after maturation for 1, 14, 28, 63, 91, 133 and 182 d.

--●-- **Elsberg cheese** sampled after maturation for 2, 24, 42, 63, 91, 120, 150 and 182 d.

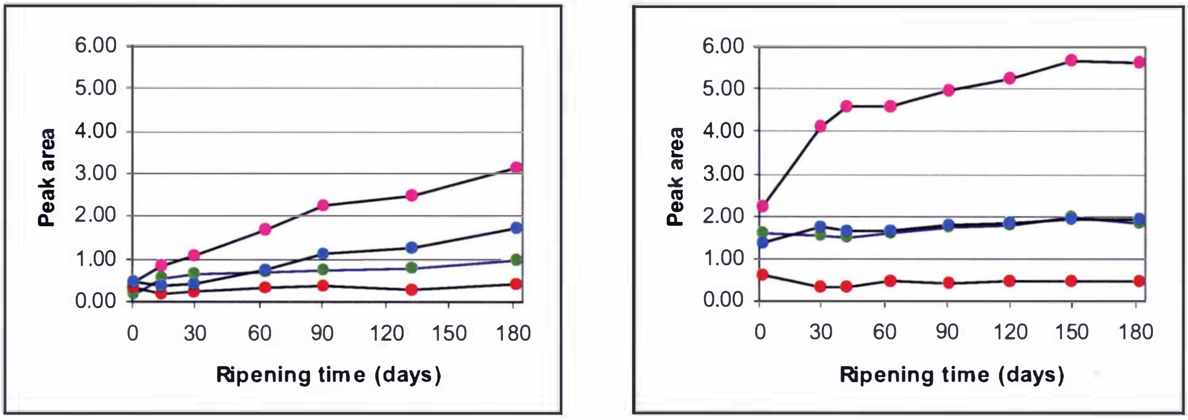
--●-- **Gouda cheese** sampled after maturation for 1, 17, 31, 50, 63, 91, 120, 150 and 182 d.

--●-- **Mozzarella cheese** sampled after maturation for 1, 14, 28, 63, 91, 133 and 182 d.

--●-- **Swiss cheese** sampled after maturation for 1, 14, 35, 91, 120, 150 and 182 d.

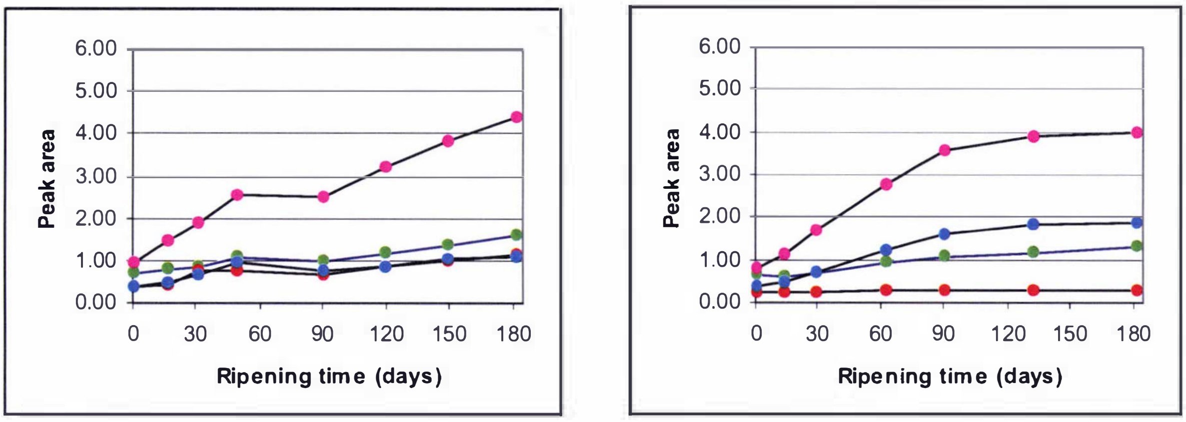
Small quantities of α_{s1}-casein appeared to remain in the mature Cheddar and Elsberg cheese. A 2-dimensional gel electrophoresis study (Coker, 1994) showed that the 'α_{s1}-casein' band in mature Cheddar cheese contained 3 small peptide bands as well as a small quantity of α_{s1}-casein. It is therefore probable that the band labelled α_{s1}-casein contained mainly peptides in the later stages of ripening. In the same study (Coker, 1994) it was shown that in mature Cheddar, the α_{s1}-I-casein band was comprised of 2 bands of similar intensity and a third less intense band. According to Mooney et al (1998), the three bands in this region are α_{s1}-casein f102-199, α_{s1}-casein f102-191 and α_{s1}-casein f24-199 (α_{s1}-I-casein).

β-Casein was broken down to γ_2-, γ-type-, γ_1- and γ_3-casein, which could be separated by urea-PAGE, and these were plotted for each cheese type (Fig. 7.3B).



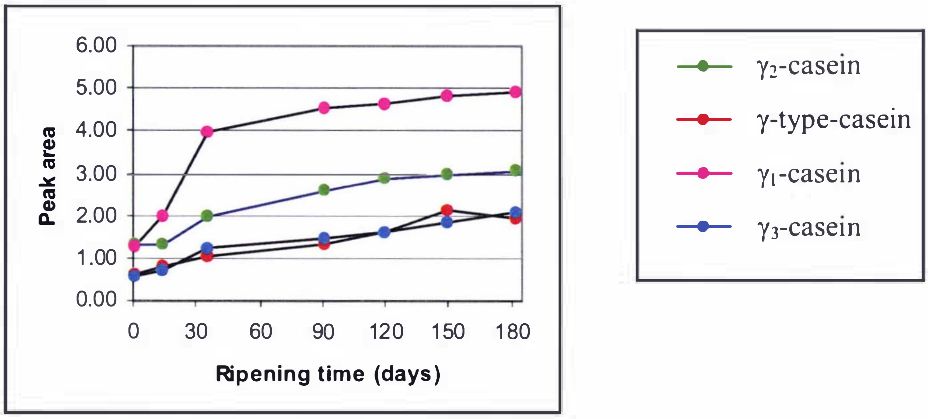
A.Cheddar

B. Elsberg



C. Gouda

D. Mozzarella



E. Swiss

Fig. 7.3B *Urea-PAGE - γ-Casein formation during the ripening of each cheese type.* See Figs. 7.3A and 7.3B for cheese and sampling details.

The trends (Figs. 7.3A and 7.3B) show the effects of the different manufacturing and storage conditions on enzyme activity in each cheese type.

The possibility of using γ-casein/β-casein as a means of classification was suggested by Fox (1993) who showed (qualitatively) that the ratio was different for 13 cheese samples of different types examined using alkaline urea-PAGE. This ratio had earlier been suggested by Haasnoot et al (1989) as a means of determining the age of Gouda. These authors used anion exchange chromatography to examine the urea-soluble fraction of 27 Gouda cheeses of different ages and found that the ratio of γ-casein/β-casein was useful for determining the maturity (age) of Gouda up to 10 months and that the ratio of α_{s1}-I-casein/α_{s1}-casein was useful for determining the maturity (age) of young Gouda cheese of less than 4 weeks. To test these suggestions the ratios of α_{s1}-I-casein/α_{s1}-casein and γ(total)-casein/ β-casein were plotted for all five cheese types at all sampling times throughout their maturation (Fig. 7.3C).

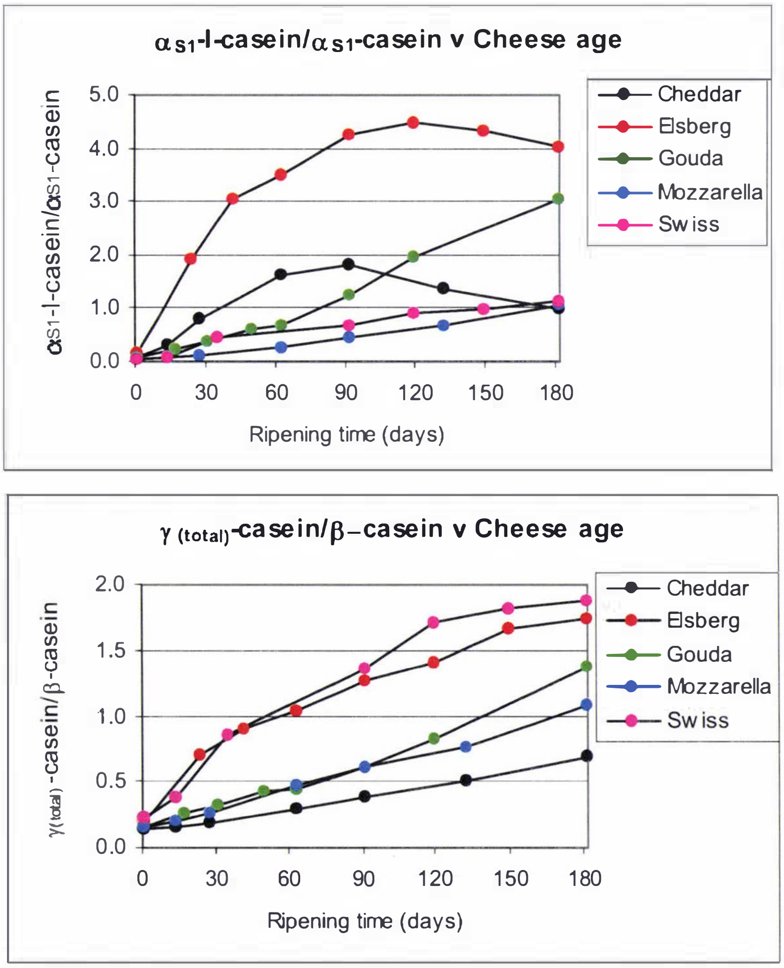


Fig. 7.3C *Urea-PAGE - Changes in α_{s1}-I-casein/α_{s1}-casein and γ-casein/β-casein during ripening.* Cheese ages are shown in the caption of Fig. 7.3A.

The ratio of α_{s1}-I-casein to α_{s1}-casein, proposed by Haasnoot et al (1989) as an indicator of the age of young cheese, proved a reasonable indicator of cheese maturity in the early stages of maturation when α_{s1}-casein decreased and α_{s1}-I-casein increased in concentration. It was therefore useful for determining the age of the Cheddar cheese for 90 d and the Elsberg for 120 d. It was useful for determining the maturity of the Gouda, Swiss and Mozzarella cheese throughout the six month ripening period. However, α_{s1}-casein was almost depleted and the amount of α_{s1}-I-casein had levelled off in the Gouda cheese (Fig. 7.3A), and it was therefore unlikely that the ratio of α_{s1}-I-casein to α_{s1}-casein would prove useful beyond this point in maturation, and this variety is often ripened for longer.

The ratio of γ-casein to β-casein, proposed by Haasnoot et al (1989) as a useful indicator of the age of more mature Gouda cheese, was useful for determining cheese maturity during the period when γ-casein increased most in concentration, and ceased to be useful when the amounts of β- and γ-casein became constant. It therefore proved useful for determining the maturity of the Gouda, Cheddar and Mozzarella cheese which had similar slow rates of plasmin action. The faster rates of plasmin action in the Elsberg and Swiss cheese meant that the ratio of γ-casein to β-casein was only useful as an indicator of age for the first 90–120 days maturation after which the rate of change in the amounts of β- and γ-casein had become small. The trends for β- and γ-casein (Fig. 7.3A) indicate that the ratio of γ-casein to β-casein for Gouda, Cheddar and Mozzarella would eventually cease to be a useful indicator of maturity.

There did not appear to be sufficient differences between cheese types for the ratio of γ-casein to β-casein to be an effective means of characterisation of cheese types as suggested by Fox (1993). It should be remembered that the graphs relate age at the prescribed composition and storage temperature. For each cheese type, it is probably better to think in terms of maturity (ie. at a certain percentage of loss of α_{s1}- or β-casein) rather than age, as this would take into account any differences in composition and storage regimen that would influence the time at which any cheese type reached a given maturity.

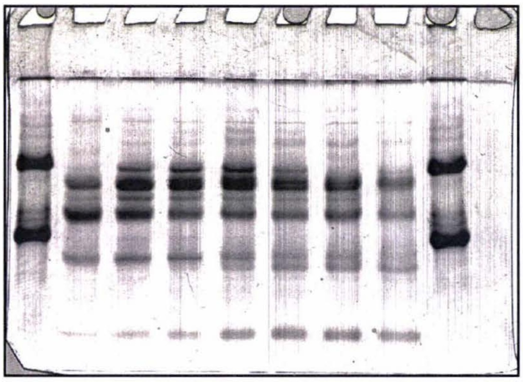
7.4.2.3 Urea-PAGE of the WSF

The WSF of each cheese was examined using urea-PAGE (Fig. 7.4). The WSF was taken from 12.5 times more cheese than the USF to enable the bands to be seen.

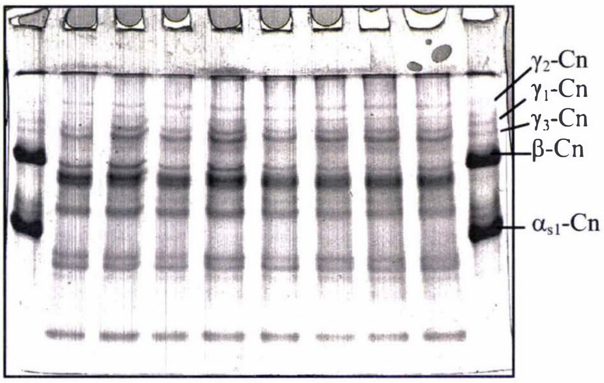
Urea-PAGE of the WSF (Fig. 7.4) gave patterns of peptides that changed with maturation and were generally different for each cheese. However, the peptide patterns of the Gouda and Elsberg were similar and many of the PAGE bands had a moderate stain intensity. By comparison, the patterns for the WSF of Swiss, Cheddar and Mozzarella cheese were different from each other as well as from the Elsberg and Gouda. Mozzarella was the only cheese to show a marked increase in the density of most bands during maturation (Fig. 7.4), reflecting the known low level of peptidases and proteases in this cheese variety.

Within many of the cheese types there was variability in the intensity of the band that eluted in the region of β-casein and it seems possible that some β-casein was water-soluble. Although some peptides increased in concentration during ripening, there were others that initially increased in concentration and then decreased again, which suggests that they were intermediates in the ripening process. A further group of bands were present in varying amounts as the cheese ripened. There are several possible explanations, for example, there may have been more than one peptide present, the peptides may have been only poorly soluble in water, or they may have leached from the gel during staining and destaining.

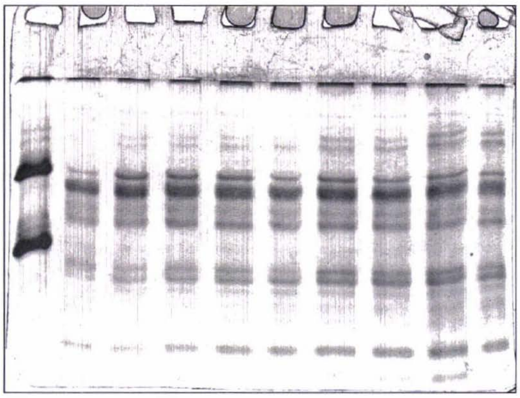
A. Cheddar



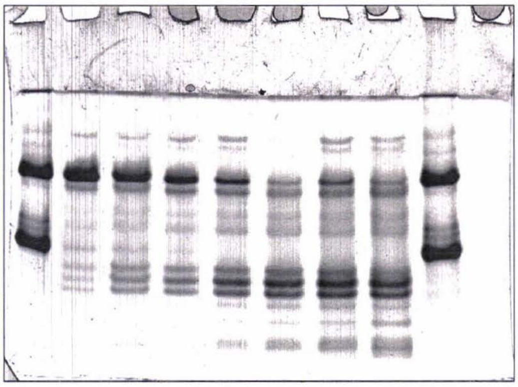
B. Elsberg



C. Gouda



D. Mozzarella



E. Swiss

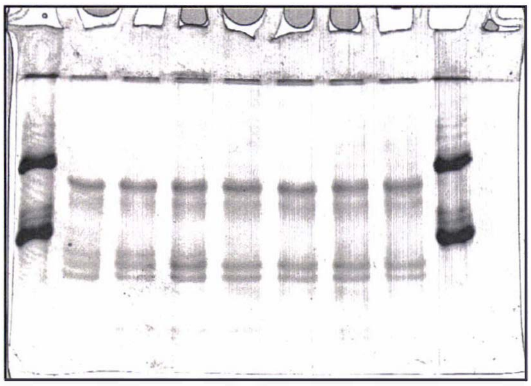


Fig. 7.4 *Urea-PAGE of the WSF of each cheese type during ripening (*10% cheese, diluted 1:1 with urea sample buffer; 25μL sample volume). Scanned images viewed in logarithmic scale.

A. Cheddar cheese: Lanes 2 to 8, sampled after 1, 14, 28, 63, 91, 133 and 182 d maturation. Lanes 1 and 9, rennet casein standard.

B. Elsberg cheese : Lanes 2 to 9, sampled after 2, 24, 42, 63, 91, 120, 150 and 182 d maturation. Lanes 1 and 10, rennet casein standard.

C. Gouda cheese : Lanes 2 to 10, sampled after 1, 17, 31, 50, 63, 91, 120, 150 and 182 d maturation. Lane 1, rennet casein standard.

D. Mozzarella cheese : Lanes 2 to 8, sampled after 1, 14, 28, 63, 91, 133 and 182 d maturation. Lanes 1 and 9, rennet casein standard.

E. Swiss cheese: Lanes 2 to 8, sampled after 1, 14, 35, 91, 120, 150 and 182 d maturation. Lanes 1 and 9, rennet casein standard.

7.4.2.4 SE-HPLC of the WSF

SE-HPLC enables peptides to be grouped according to molecular weight (Chapter 6). The WSF of each cheese sample was examined by SE-HPLC and the traces were overlaid to form a ripening profile depicting the changes in molecular weight distribution during ripening (Figs. 7.5 - 7.9).

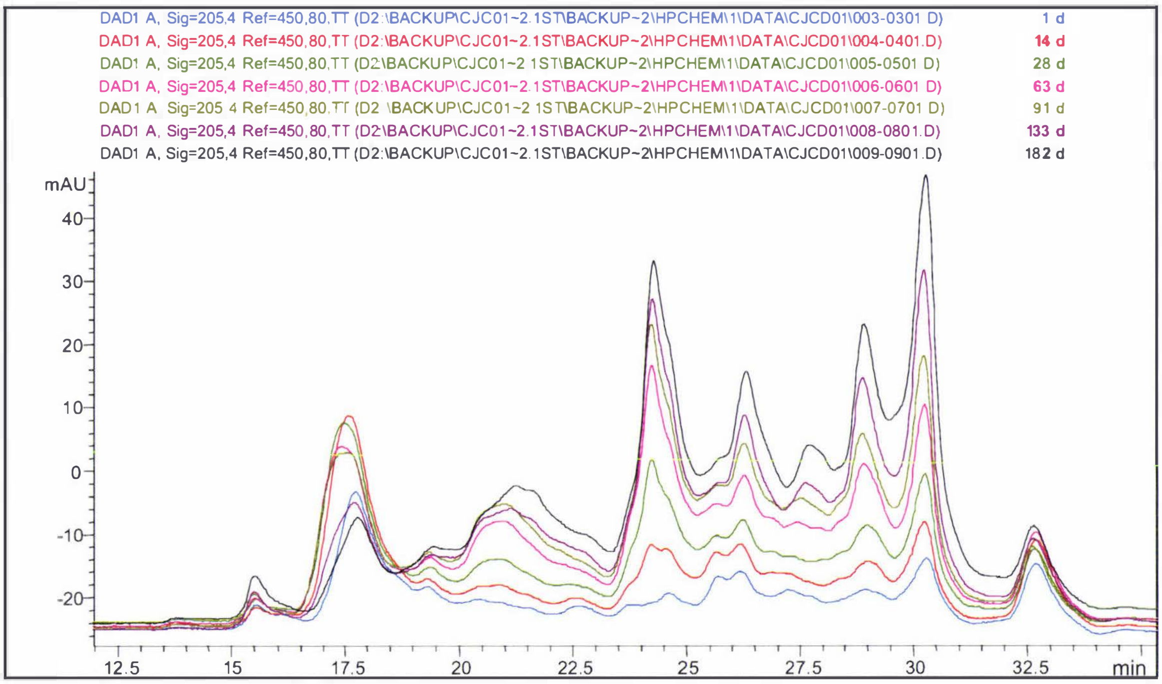


Fig. 7.5 *Cheddar cheese - SE-HPLC molecular weight distribution profiles (205 nm) of the WSF.* The cheese was sampled after 1, 14, 28, 63, 91, 133, and 182 days maturation at 13°C and analysed by SE-HPLC. The WSF (10% cheese) was diluted 1:48 (200 μL with 9.8 mL of the mobile phase (36% acetonitrile, 0.1% TFA)) prior to loading 50 μL on to the column. Flow rate, 0.4 mL/min. The absorbance was measured at 205, 210, 220, 280 and 295 nm but only 205 nm was plotted.

The values on the *y*-axis are expressed in milli absorbance units (mAU) and it is the difference between the minimum and maximum value that is important rather than the absolute values.

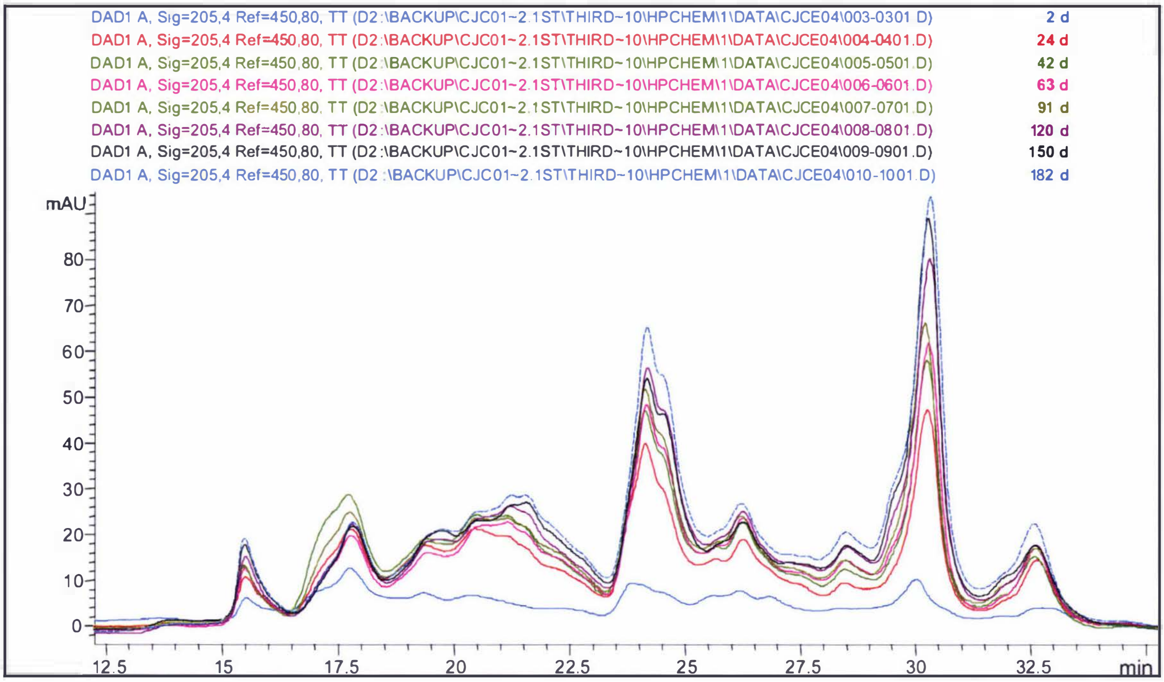


Fig. 7.6 *Elsberg cheese - SE-HPLC molecular weight distribution profiles (205 nm) of the WSF.* The cheese was stored at 12°C for 14 d, 20°C for 10 d and 5°C until it was 182 d old. It was sampled after 2, 24, 42, 63, 91, 120, 150 and 182 days maturation (14 d sample not available) and the WSF was analysed by SE-HPLC. See Fig 7.5 for details of sample preparation and analysis.

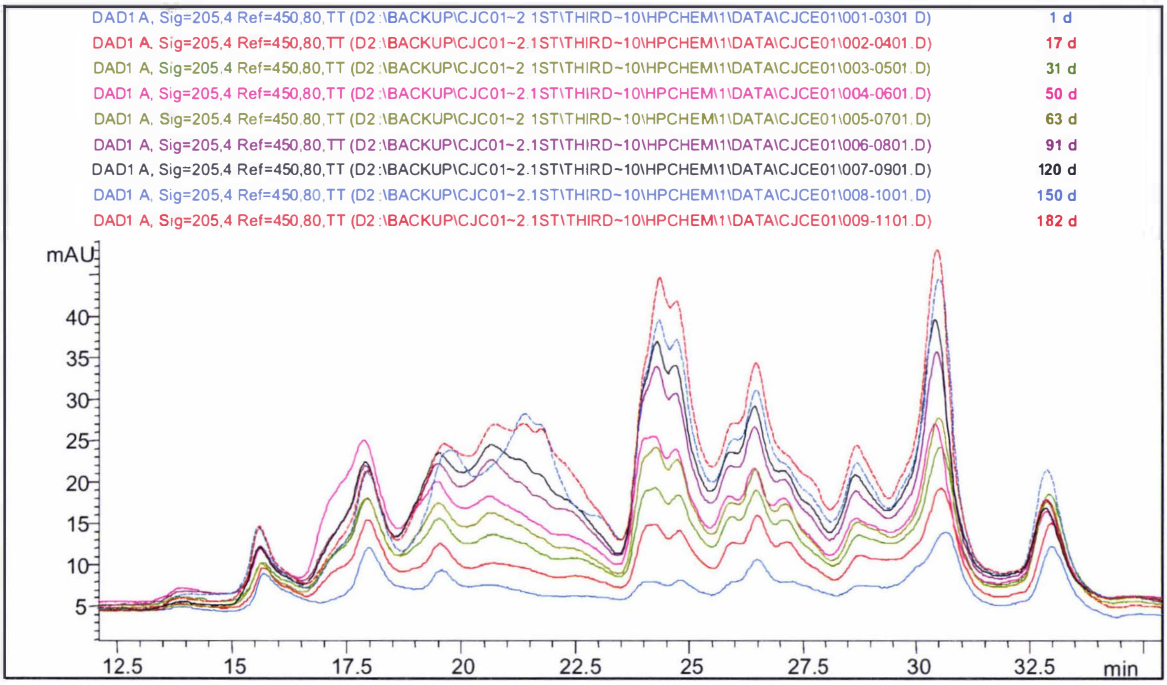


Fig. 7.7 *Gouda cheese - SE-HPLC molecular weight distribution profiles (205 nm) of the WSF.* The cheese was sampled after 1, 17, 31, 50, 63, 91, 120, 150 and 182 days maturation at 6°C. See Fig 7.5 for details of sample preparation and analysis.

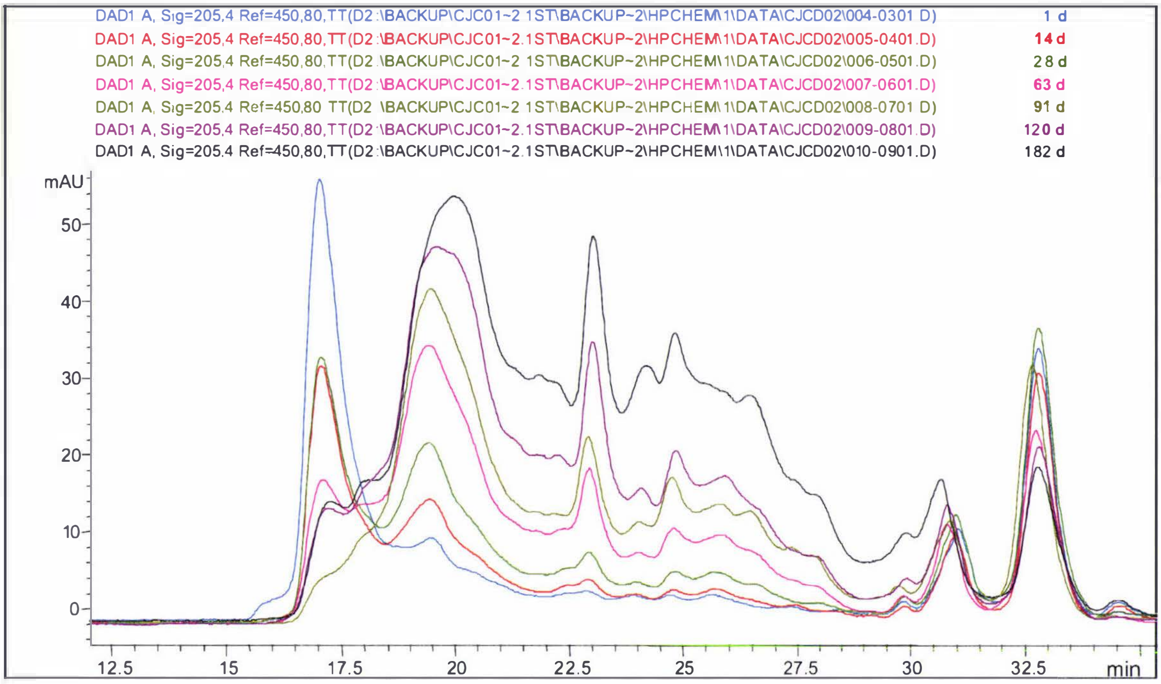


Fig. 7.8 *Mozzarella cheese - SE-HPLC molecular weight distribution profiles (205 nm) of the WSF.* The cheese was sampled after 1, 14, 28, 63, 91, 120 and 182 days maturation at 13°C. See Fig 7.5 for details of sample preparation and analysis.

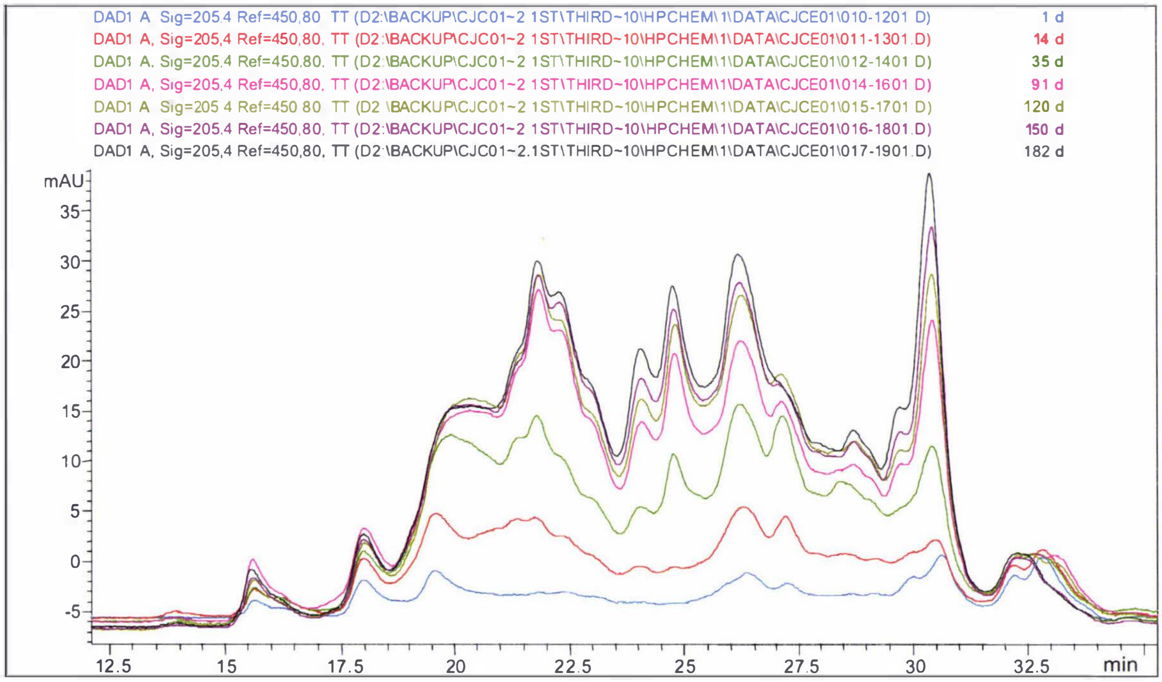


Fig. 7.9 *Swiss cheese - SE-HPLC molecular weight distribution profiles (205 nm) of the WSF.* The cheese was stored at 12°C for 14 d, 20°C for 21 d and 5°C until it was 182 d old. It was sampled after 1, 14, 35, 91, 120, 150 and 182 days maturation. See Fig 7.5 for details of sample preparation and analysis.

The molecular weight distribution profiles of the Cheddar, Mozzarella and Gouda cheese showed a gradual increase in water-soluble material throughout maturation (Figs. 7.5 - 7.9). In the Swiss and Elsberg cheese, the greatest increase in water-soluble material occurred in the early stages of maturation when the cheese was stored at higher ripening temperatures (Table 7.1). Once these cheeses were moved to lower storage temperatures, the amount of water-soluble material increased more slowly. As ripening progressed (in most cheese types), the higher molecular weight peptides were hydrolysed to progressively smaller peptides and amino acids.

The molecular weight distribution profiles for Mozzarella cheese stood out as being very different to those of the other cheese types. A large amount of higher molecular weight material accumulated in the cheese as the result of a small amount of chymosin (or perhaps Cathepsin D) and plasmin activity, and only a relatively small amount of lower molecular weight peptides and amino acids were formed. The absence of lower molecular weight material was attributed to the effect of the high stretch temperature (68°C) on chymosin activity and on the subsequent growth of the cheese microflora and consequent lack of microbial enzyme activity in the cheese.

7.4.2.5 Reverse phase-HPLC of the WSF

RP-HPLC was used to examine the peptides in the WSF of each cheese type (Figs. 7.10 - 7.14). This method separates peptides and amino acids mainly on the basis of hydrophobicity, so that small hydrophilic amino acids and peptides elute first and large hydrophobic peptides elute last (Cliffe et al, 1993).

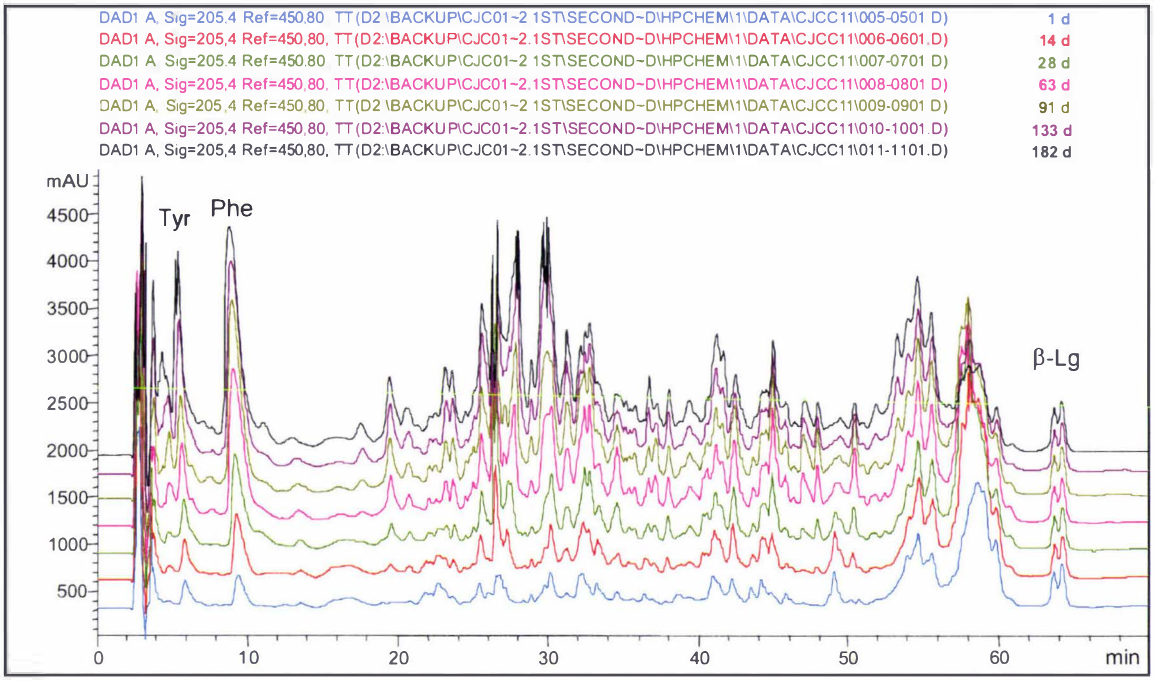


Fig. 7.10 Cheddar cheese - RP-HPLC profiles (205 nm) of the WSF. The cheese was sampled after 1, 14, 28, 63, 91, 133, and 182 days maturation at 13°C. The WSF (10% cheese) was diluted 1:1 (v/v) with water prior to loading 200 µL on to the column. Gradient: 100% solvent A for 5 min; 0 to 100% solvent B over 75 min; 100% solvent A for 15 min. Flow rate, 0.7 ml/min. The absorbance was measured at 205, 210, 220, 280 and 295 nm. Only the 205 nm trace is shown.

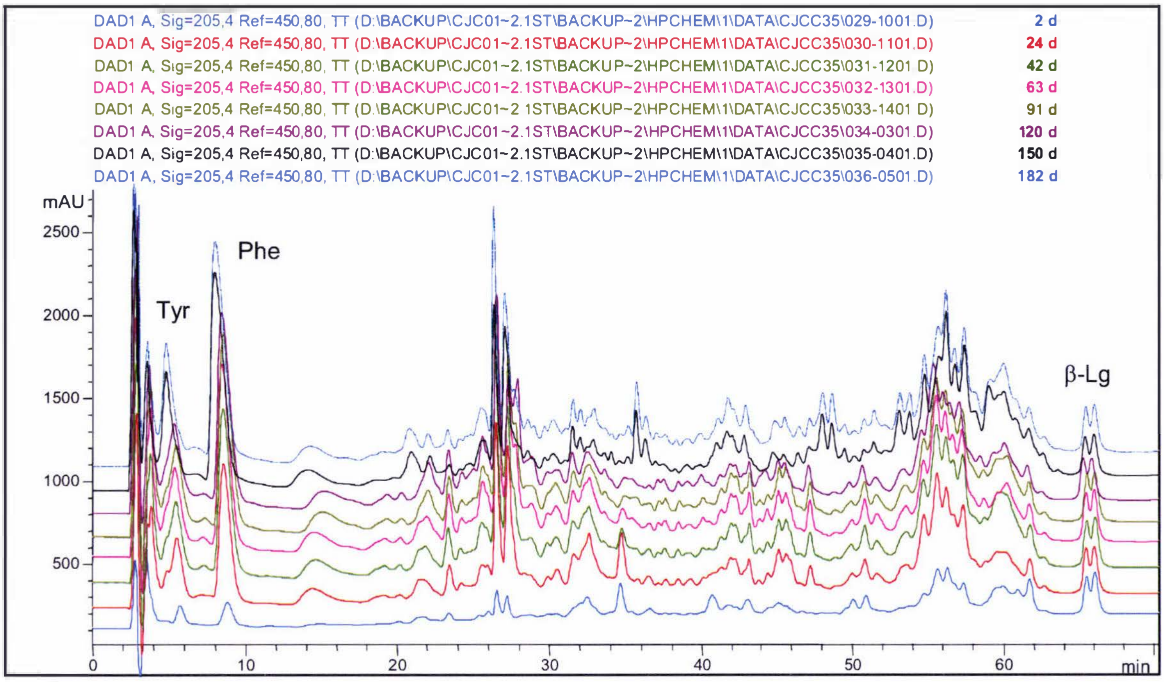


Fig. 7.11 *Elsberg cheese - RP-HPLC profiles (205 nm) of the WSF.* The cheese was stored at 12°C for 14 d, 20°C for 10 d and 5°C until it was 182 d old. It was sampled after 2, 24, 42, 63, 91, 120, 150 and 182 days maturation. See Fig. 7.10 for details of sample preparation and analysis.

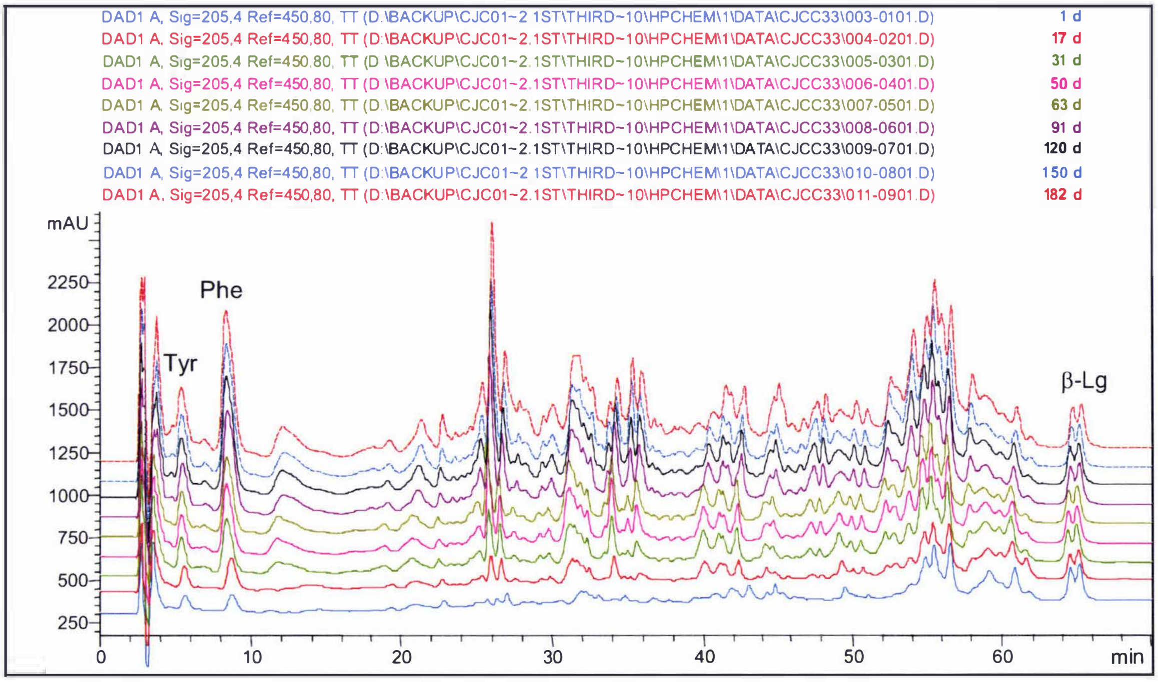


Fig. 7.12 *Gouda cheese - RP-HPLC profiles (205 nm) of the WSF.* The cheese was sampled after 1, 17, 31, 50, 63, 91, 120, 150 and 182 days maturation at 6°C. See Fig. 7.10 for details of sample preparation and analysis.

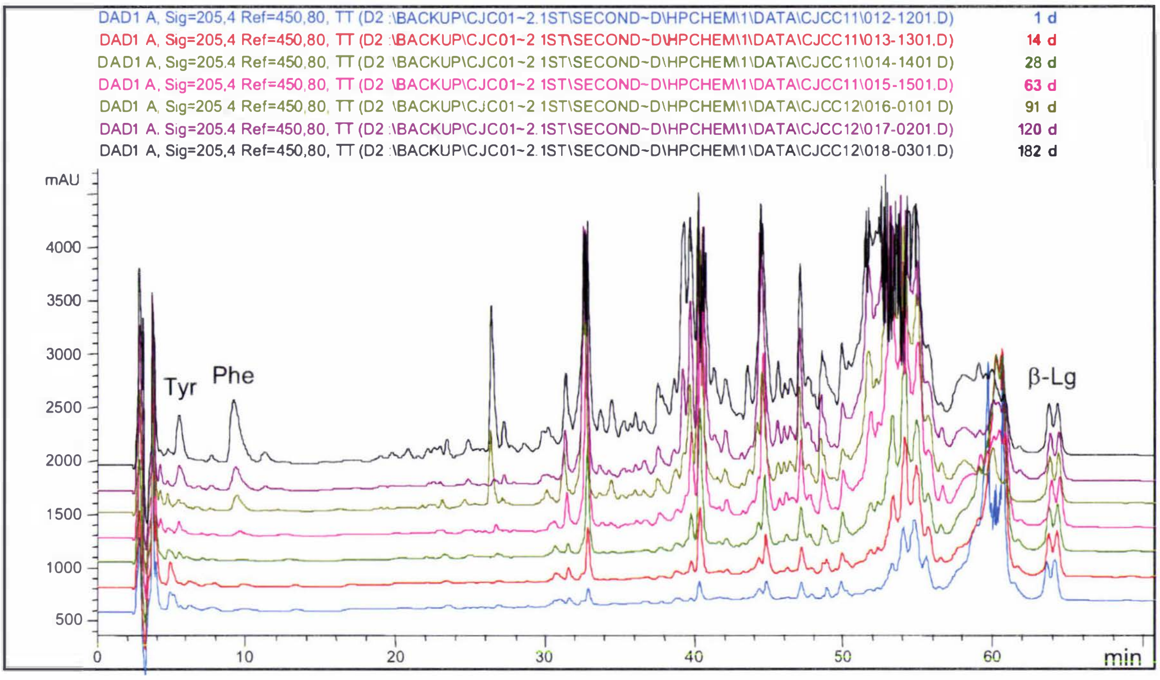


Fig. 7.13 *Mozzarella cheese - RP-HPLC profiles (205 nm) of the WSF.* Cheese sampled after 1, 14, 28, 63, 91, 120 and 182 days maturation at 13°C. See Fig. 7.10 for details of sample preparation and analysis.

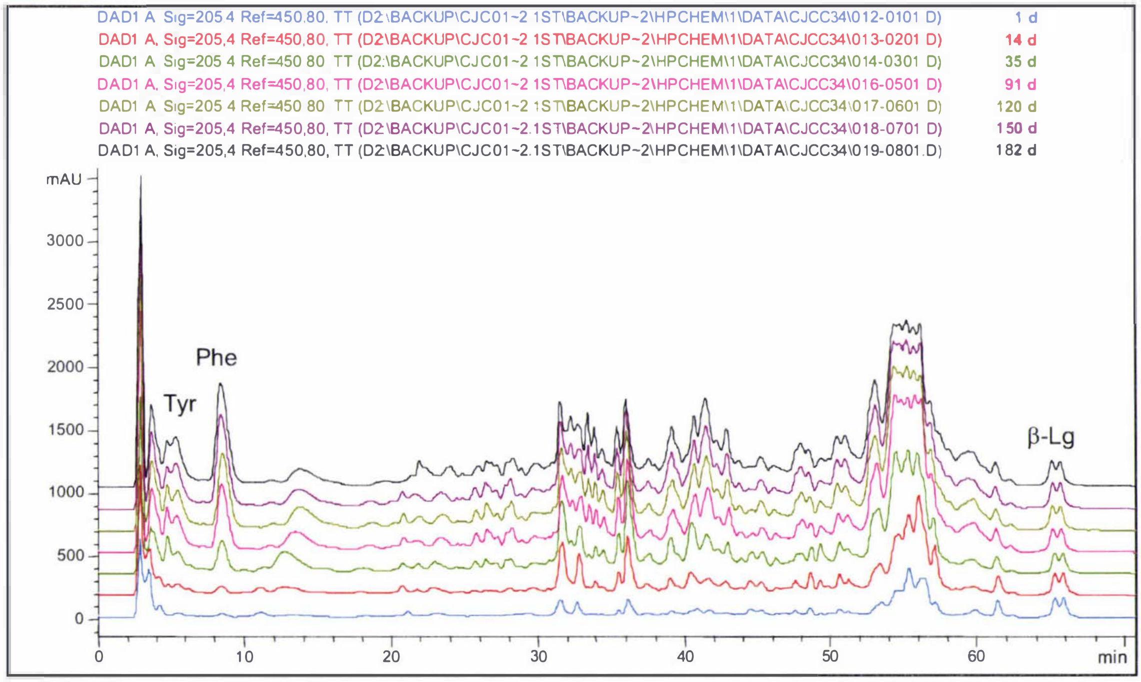


Fig. 7.14 *Swiss cheese - RP-HPLC profiles (205 nm) of the WSF.* The cheese was stored at 12°C for 14 d, 20°C for 21 d and 5°C until it was 182 d old. It was sampled after 1, 14, 35, 91, 120, 150 and 182 days maturation. See Fig. 7.10 for details of sample preparation and analysis.

The RP-HPLC profiles of the cheese WSFs appeared to be sufficiently different to distinguish each cheese, although some cheese types had similar patterns to one another eg, Elsberg and Gouda. The Mozzarella cheese profiles were very different from any of the other cheese profiles. The WSF of this cheese type contained comparatively few lower molecular weight peptides and amino acids, which was in agreement with the results obtained by SE-HPLC and reflected the low level of chymosin activity and small number of microorganisms in that cheese variety.

7.4.3 PCA of data from urea-PAGE, SE-HPLC or RP-HPLC analysis of the WSF

The relative value of each method for characterising and differentiating the cheese types at several stages of maturity was determined. For each method, an electrophoretogram or chromatogram was generated for each sample analysed. The area of each peak was determined and a data set was formed that represented all the peaks in each cheese sample. These large data sets required the application of statistical analysis to compress the data and extract the critical effects in an objective manner. The data were analysed using both the covariance and correlation forms of PCA[1,2] (Statistica 6.0, StatSoft Inc., OK, USA) and the results were expressed as a score plot. The score plot of the first three principal components showed any differentiation achieved and the loading plot showed which peaks or bands were responsible for the differentiation.

1. The correlation form of PCA requires that the peak area data for each peak used in the analysis is transformed so that the mean area for each peak across the sample range is zero (that is, the peak areas sum to zero), with a standard deviation of 1, before the analysis is performed. This means that each peak is standardised to the same variance and each has the same weighting regardless of whether it is a large or small peak.
2. The covariance form of PCA requires that the peak area data for each peak used in the analysis is centred so that the mean area for each peak across the sample range is zero (that is, the peak areas sum to zero) before the analysis is performed. This means that each peak retains its original variance, which may be different from the variance of other peaks, and that those peaks with the greatest variance will be dominant.

7.4.3.1 PCA of data obtained from urea-PAGE of the USF

A gel containing a representative sample of each cheese type (from Ch. 6, Fig. 6.5) was used to set up a system that numerically identified each band across the range of gels in a consistent manner (Fig. 7.15).

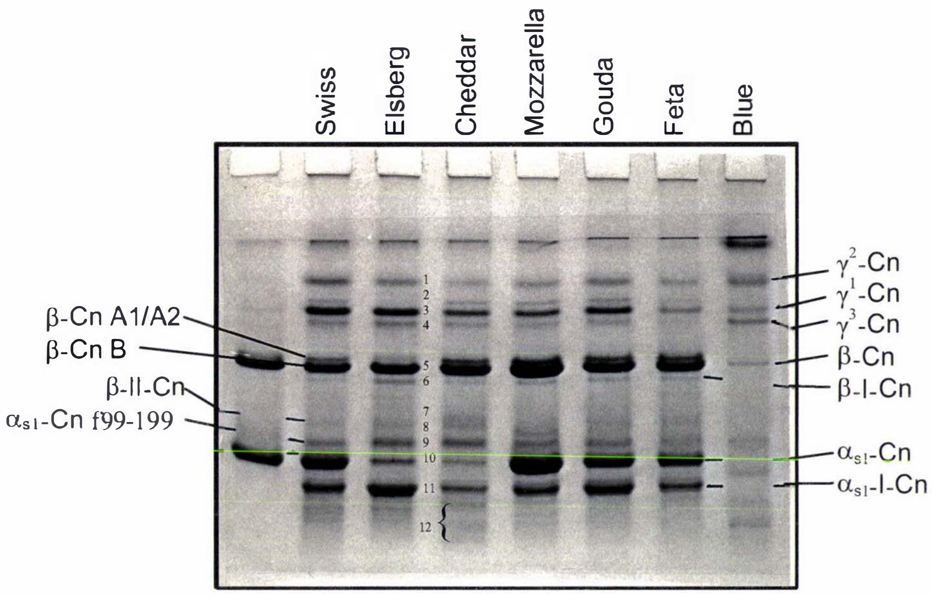


Fig. 7.15 *Urea-PAGE of the USF of several cheese types.* Proteolysis in each cheese type selected at optimum ripeness, as determined by the manufacturer. Lane 1, rennet casein standard; lanes 2 to 8, Swiss, Elsberg, Cheddar, Mozzarella, Gouda, Feta and Blue cheese USF (2% cheese, 5 μL sample volume loaded), respectively.

Samples from each cheese type were analysed in duplicate using urea-PAGE. The gels were scanned, and to make the methods comparable, an electrophoretogram was generated for each gel lane. A line was drawn down each gel lane and the absorbance (at 532 nm) of ~40 pixels on both sides of the line was summed and plotted against the distance along the lane, to give a trace representing the stained PAGE pattern seen in the gel. The peaks on the graph were numbered to match the bands. A baseline was drawn, the peak areas were determined, and the data collated and analysed using the correlation and covariance forms of PCA.

Because 'band volume' (the value obtained from summing the absorbance value of each of the pixels (after subtracting the background value from each) within a defined region) is normally used (see Fig. 7.3A), peak area and 'band volume' were compared. The correlation between the two methods of band or peak analysis was high, with R^2 values >0.99 for α_{s1}- and β-casein, indicating that the methods were directly comparable.

7.4.3.1.1 PCA of the correlation matrix (PAGE/USF)

Fig. 7.16 depicts PCA loading plots of factors 1, 2 and 3, that represent the peaks/bands resulting from PAGE analysis of the USF. Fig. 7.17 depicts 2-dimensional graphical representations of PC1, PC2 and PC3, calculated for each sample. Tables relating to PCA of the correlation matrix, scores for PC1 (56%), PC2 (13%) and PC3 (10%) and a 3-dimensional graph can be found in the appendix (section 7.8.1.1).

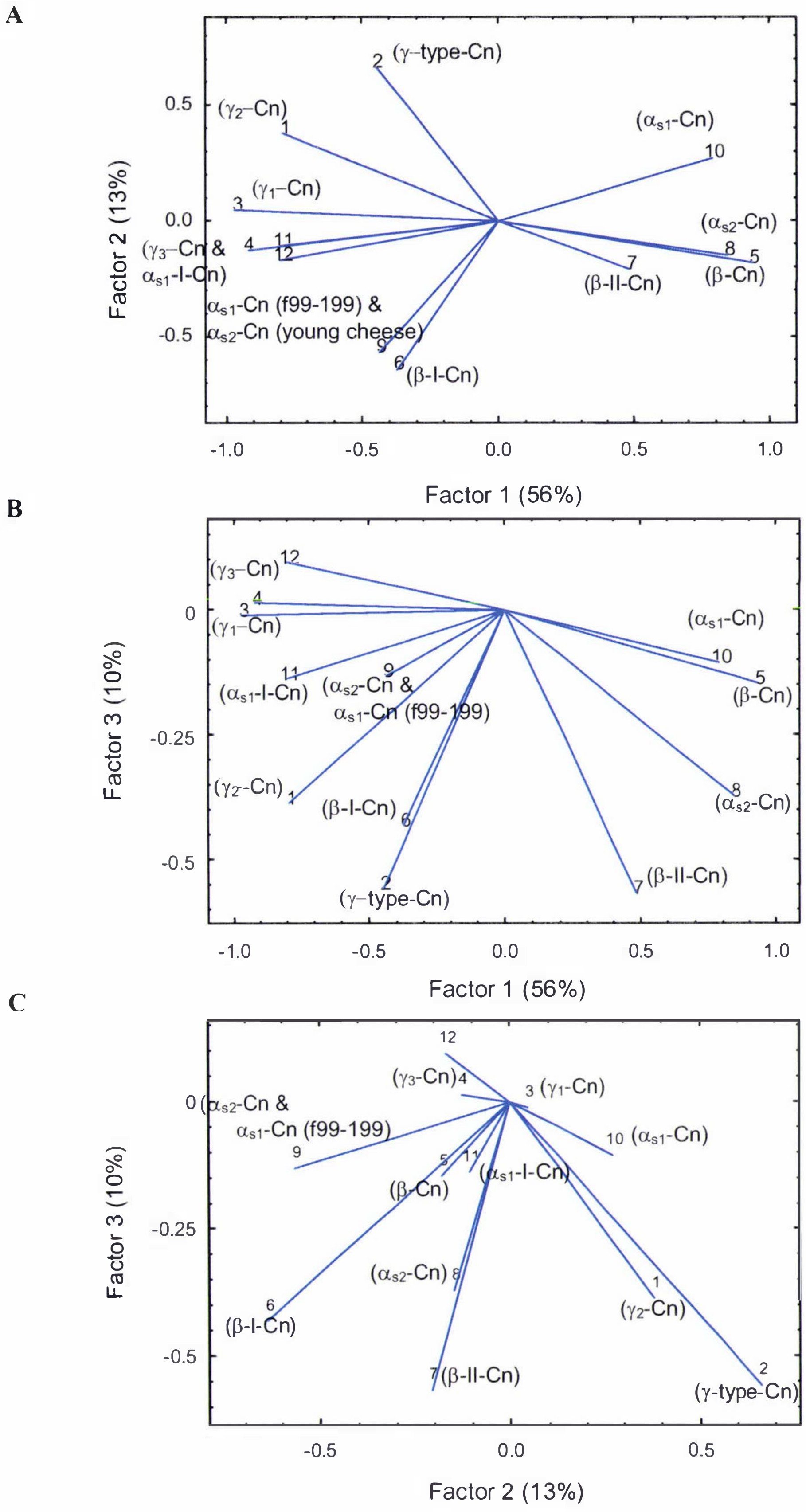


Fig. 7.16 *Urea-PAGE (USF) data analysis using PCA (correlation matrix) - loading plots.* A) factor 1 v factor 2 and B) factor 1 v factor 3 and C) factor 2 v factor 3 for bands/peaks 1-12 from urea-PAGE (USF) of ripening Cheddar, Elsberg, Gouda, Mozzarella and Swiss cheese. Labels identify peak numbers and /or names.

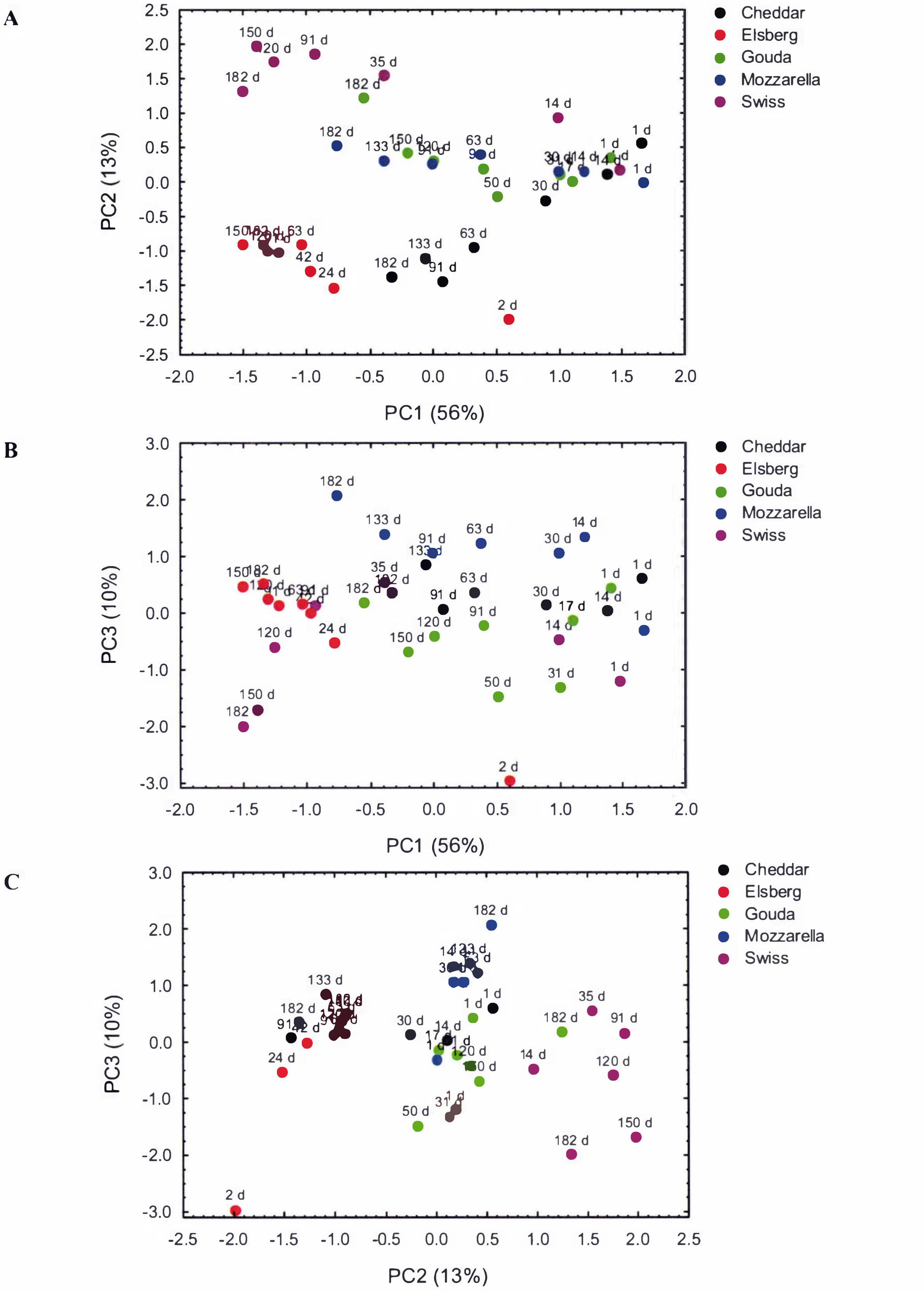


Fig. 7.17 *Urea-PAGE (USF) data analysis using PCA (correlation matrix) - score plots.* A) PC1 v PC2, B) PC1 v PC3 and C) PC2 v PC3 for bands/peaks 1-12 from urea-PAGE (USF) of ripening Cheddar, Elsberg, Gouda, Mozzarella and Swiss cheese. Data point labels indicate ripening period (days). For a 3-D plot, see appendix, section 6.8.1.1

A comparison of the PCA loading and score plots (Fig. 7.16 and 7.17) showed that the first factor or principal component (PC1) accounted for 56% of the variation in the data and was associated with cheese maturity. The second and third factors or principal components (PC2 and PC3) accounted for 13 and 10% of the variation in the data, respectively, and provided the basis for cheese differentiation because they reflected the different activities of the enzymes associated with the maturation of each cheese type.

The direction of increasing maturity was from right to left (PC1 v PC2, Fig. 7.17), with the distance between points being greatest in the early stages of maturation. The loading plot for PC1 and PC2 showed that the peaks projecting to the right were predominantly the primary substrates for enzyme activity, α_{s1}-casein (peak 10), β-casein (peak 5) and α_{s2}-casein (peak 8) (Fig. 7.16). Those projecting to the left, in the direction of maturity, were breakdown products (Fig. 7.3A). The peptide peaks closest to the direction of maturity were the γ-caseins (peaks 3 and 4) produced from β-casein predominantly as a result of plasmin action; a peak (peak 2) likely to arise from β-casein as a result of starter enzyme action; α_{s1}-I-casein (peak 11) produced by chymosin action on α_{s1}-casein, which increased in some cheese types and in others increased and then decreased; and the small peptides that migrated ahead of α_{s1}-I-casein on the gel (peak 12).

Peaks that differentiated the cheese types were most closely associated with PC2 and PC3. The predominant peaks associated with PC2 contained β-I-casein (peak 6), produced by chymosin action on β-casein; α_{s1}-casein f99-199 (peak 9), produced by chymosin action on α_{s1}-casein or α_{s1}-I-casein; and the γ-type casein (peak 2), possibly produced by microbial enzyme action on β-casein. The predominant peaks associated with PC3 contained β-II- (peak 7), α_{s2}- (peak 8), β-I- (peak 6), γ_2- (peak 1) and γ-type-casein (peak 2), respectively.

For each cheese type the combination of the loading and score plot (Figs. 7.16 and 7.17, respectively) provided a reasonable representation of the proteolytic events (Fig. 7.3A, B, C) in the maturing cheese.

The Mozzarella and Gouda cheese pattern of maturation was from right to left through the centre of the graph for PC1 v PC2 (Fig. 7.17A), and was strongly influenced by α_{s1}- and β-casein as well as α_{s1}-I-Cn, peptide peak 12 and the γ-caseins (Fig. 7.16A). In the later stages, the direction of maturation of the Gouda was more to the upper-left, with the change in direction after 50 days corresponding with the levelling off in the amount of α_{s1}-I-casein (Fig. 7.3A) and the continued increase in the amount of each of the γ-caseins. As a result of the continued formation of γ-casein there was no diminution of the gap between the data points. PC3 enabled the Mozzarella to be differentiated from the Gouda cheese from 14 days. An examination of the loading plot for factors

2 and 3 showed that this separation was complex. The predominant peaks influencing factor 3 contained γ_2-, γ-type-, β-I-, β-II- and α_{s2}-casein, respectively. These peaks therefore represent the activity of chymosin, plasmin and the enzymes of the starter bacteria on β- or α_{s2}-casein.

In the early stages of maturation of the Elsberg and Swiss cheese the distances between the data points were greatest (PC1 v PC2, Fig. 7.17A). This reflected the effect of the initial high storage temperature (Table 7.1) on the activity of the proteolytic enzymes, chymosin and plasmin, and the rapid hydrolysis of α_{s1}- and β-casein to α_{s1}-I- and γ-casein, respectively. In the later stages of maturation of the Swiss cheese, the data points were located in the upper-left corner and the distance between them was small (Fig. 7.17A). This reflected a levelling off in the amount of α_{s1}-I- and γ-casein, however from this point, differentiation of maturity was possible on the basis of PC3. The maturity of the Elsberg cheese was only poorly differentiated in the later stages of maturation, because most of the primary proteolytic events had taken place in the early stages of maturation (Fig. 7.3A).

The direction of increasing maturity of the Cheddar cheese (PC1 v PC2, Fig. 7.17A) was from the right to the lower-left, with the direction influenced not only by the hydrolysis of α_{s1}-casein to α_{s1}-I-casein, but also by the formation of α_{s1}-casein f99-199 (peak 9) (Fig. 7.16A) as a result of chymosin action. Differentiation of maturity was more difficult in the later stages of maturity when both α_{s1}-, and α_{s1}-I-casein were depleted.

The coordinates of the point (to the lower right of the plot) (Fig. 7.17A) that represents the 2 day Elsberg sample were different from those of the other young cheeses, and were influenced by the quantities of α_{s1}-I-casein (PC1) and β-I-casein (PC2) present. β-I-Casein was present in the greatest quantities in the young Elsberg cheese and decreased in concentration during maturation (Fig. 7.2B). Because PCA of the correlation matrix was used, this minor component was given the same weighting as the other peaks and it played a key role in the differentiation of this point. As the cheese matured the direction of maturity was towards the centre left and was influenced by the hydrolysis of α_{s1}-casein to α_{s1}-I-casein and α_{s1}-casein f99-199, the hydrolysis of β-casein to β-I- and γ-casein, and the hydrolysis of the small amount of β-I-casein, probably to β-II- and β-III-casein. α_{s1}-Casein f99-199 was present in relatively large quantities in this cheese type.

In summary, urea-PAGE of the USF with data analysis using the correlation form of PCA,

- was suitable for the determination of maturity in the early stages of maturation of most cheese types while the products of primary proteolysis continued to increase in concentration;
- was suitable for the determination of maturity in cheese types such as Gouda and Mozzarella in which primary proteolytic events continued throughout the slow process of maturation;

- was not particularly suitable for the determination of maturity in more rapidly maturing cheeses such as Cheddar where peaks such as α_{s1}-I-casein increased and decreased in concentration during maturation, or Elsberg where the rate of decrease or increase in area of peaks that influenced the assessment of maturity became very slow and caused the data points associated with the later stages of maturity to be closely grouped; and
- provided reasonable differentiation of cheese type, with the combination of PC1 and PC2 enabling the Swiss, Elsberg and Cheddar cheese to be differentiated from one another, and the combination of PC2 and PC3 enabling the Mozzarella to be separated from the Gouda as well as the other cheese types.

7.4.3.1.2 PCA of the covariance matrix (PAGE/USF)

Fig. 7.18 depicts the loading plots for factors 1, 2 and 3, that represent the peaks/bands resulting from PAGE analysis of the USF. Fig. 7.19 depicts 2-dimensional graphical representations of the relationship between PC1, PC2 and PC3, calculated for each sample. Tables relating to PCA of the covariance matrix, scores for PC1 (86%), PC2 (8%) and PC3 (3%) and a 3-dimensional graph can be found in the appendix (section 7.8.1.2).

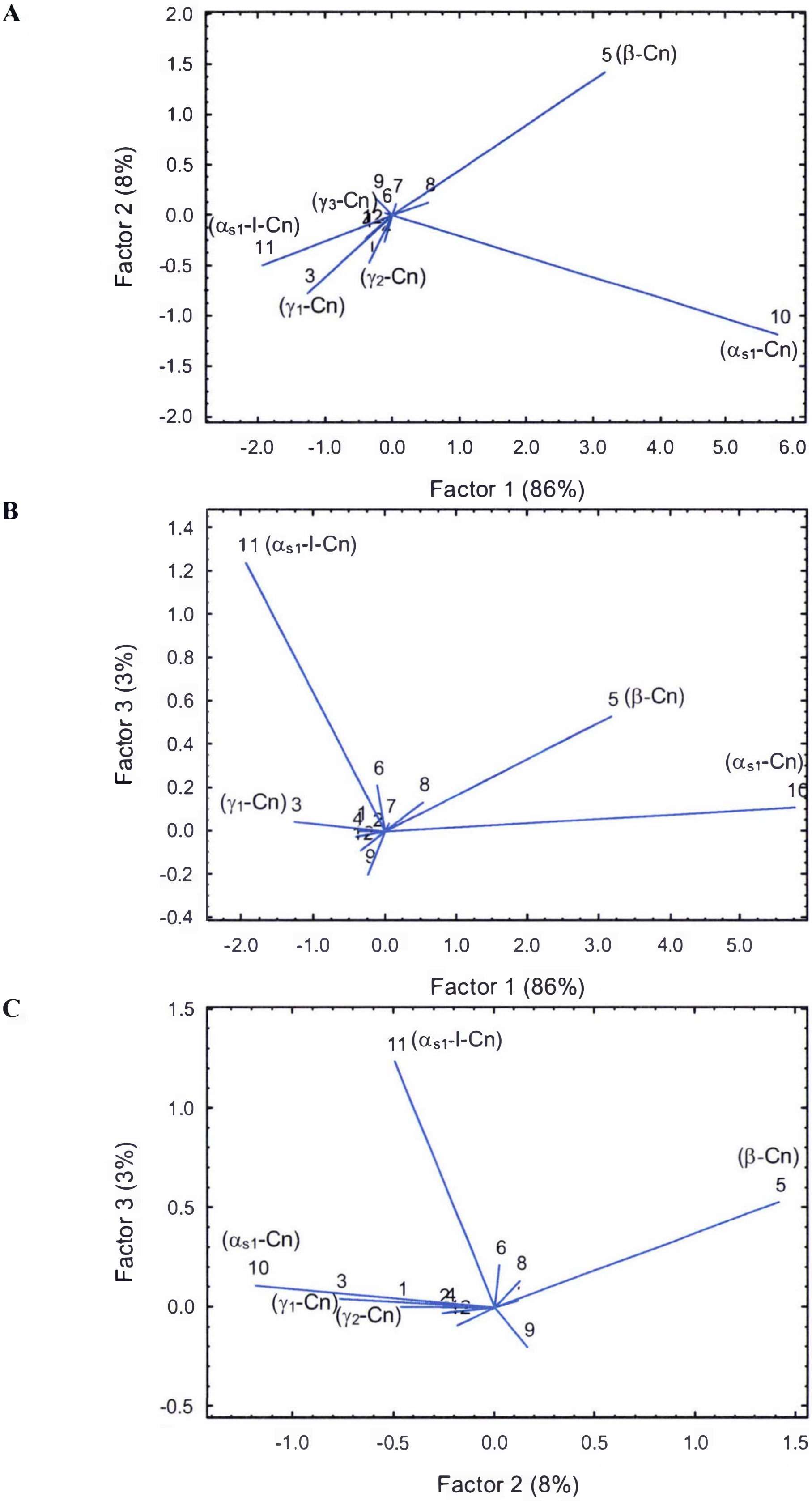


Fig. 7.18 *Urea-PAGE (USF) data analysis using PCA (covariance matrix) - loading plots.* A) factor 1 v factor 2, B) factor 1 v factor 3 and C) factor 2 v factor 3 for bands/peaks 1-12 from urea-PAGE (USF) of ripening Cheddar, Elsberg, Gouda, Mozzarella and Swiss cheese.

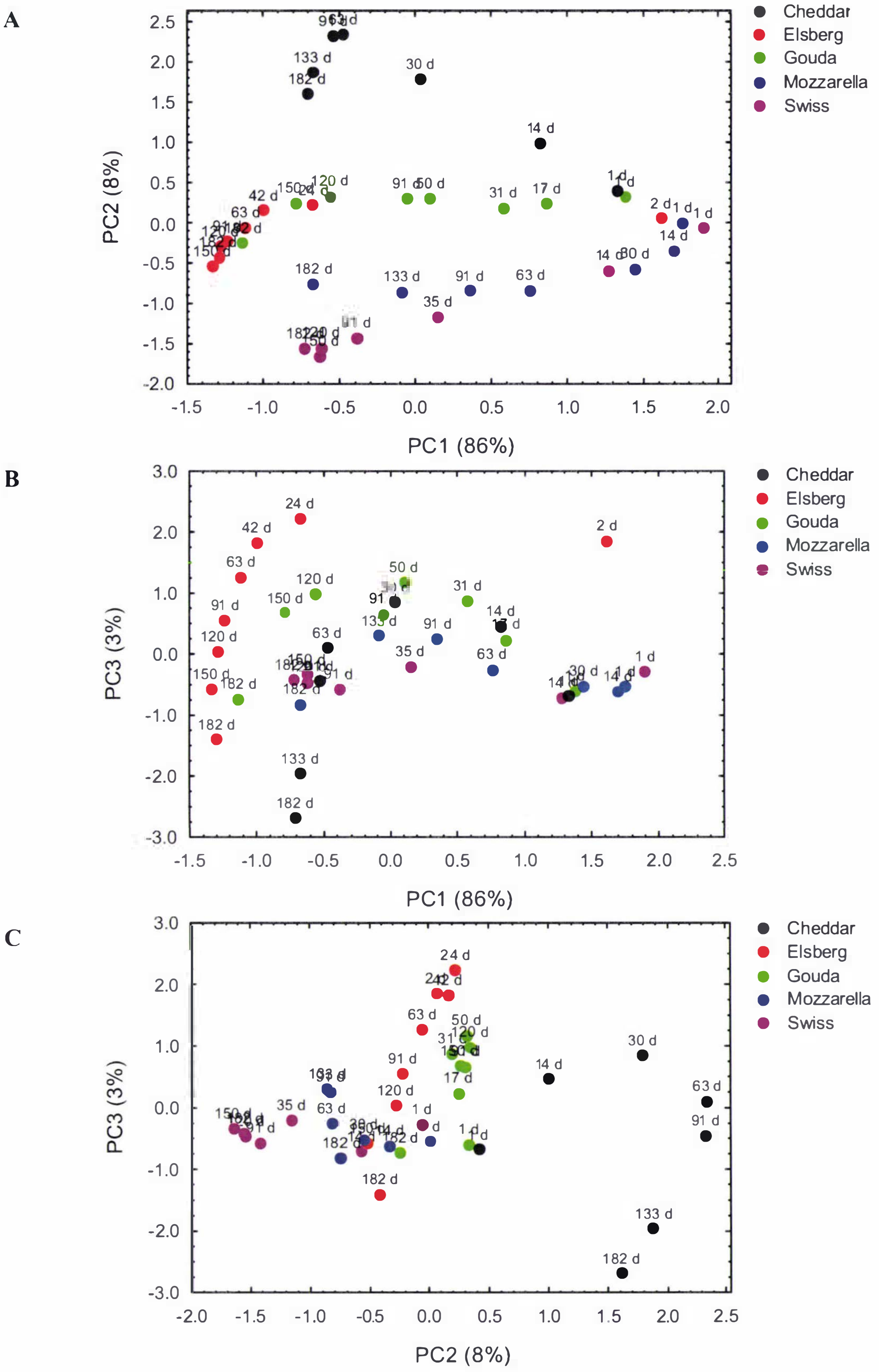


Fig. 7.19 *Urea-PAGE (USF) data analysis using PCA (covariance matrix) - score plots.* A) factor 1 v factor 2, B) factor 1 v factor 3 and C) factor 2 v factor 3 for bands/peaks 1-12 from urea-PAGE (USF) of ripening Cheddar, Elsberg, Gouda, Mozzarella and Swiss cheese. For a 3-D plot, see appendix, section 7.8.1.2.

The covariance form of PCA was the more effective form of analysis of the urea-PAGE (USF) data set, with the first two principal components accounting for 94% of the variation in the data, and with PC1 accounting for 86% of the variation.

Analysis of the loading and score plots (Fig. 7.18 and 7.19) showed that PC1 was associated with cheese maturity, with α_{s1}- and β-casein the predominant peaks, followed by α_{s1}-I- and the γ-caseins. This differed from the correlation form of PCA where each substrate and each of the products had equal weighting because of the standardisation required for the analysis. PC2 and PC3 were associated with both cheese maturity and differentiation of type. PC2 was related mostly to the hydrolysis of β- and α_{s1}-casein and PC3 was related mainly to the formation and loss of α_{s1}-I-casein, with a small contribution from both β-casein and peak 9 (α_{s2}-casein and α_{s1}-casein f99-199).

The direction of increasing maturity was from right to left on the plot of PC1 v PC2 (Fig. 7.19A), with the data points more evenly spaced using the covariance form (than the correlation form) of PCA, which reflected the higher score for PC1 and the fact that PC1 was strongly influenced by the loss of the major substrates, β- and α_{s1}-casein.

Analysis of the score plot (PC1 v PC2, Fig. 7.19A) in conjunction with the loading plot (Fig. 7.18A) showed that differentiation between cheese types was mainly based on the hydrolysis of α_{s1}-casein to α_{s1}-I-casein by chymosin, and the hydrolysis of β-casein to the γ-caseins by plasmin. These interactions enabled the five cheese varieties to be divided into three groups: i) Cheddar, ii) Gouda and Elsberg and iii) Mozzarella and Swiss cheese. PC1 v PC3 (Fig 7.19B) enabled the Elsberg cheese to be separated from the other cheese types.

The direction of increasing maturity of the Cheddar cheese (to the upper left) (Fig. 7.19A, PC1 v PC2) was influenced mostly by the breakdown of α_{s1}-casein and the formation of as α_{s1}-I- casein (Fig. 7.18A). The score plot (PC1 v PC2) showed that most changes took place early in maturation, reflecting the large amount of rennet activity that is typical of this cheese type. From 63 days, the direction of increasing maturity changed (towards the lower left) and this coincided with the point at which α_{s1}-casein reached a minimum (Fig. 7.3A). The score plots of PC2 v PC3 and PC1 v PC3 (Fig. 7.19B and C) reflected the trend for α_{s1}-I-casein which reached a maximum at 30 days and then began to decline in concentration (Fig. 7.3A).

The Gouda and Mozzarella cheese showed a steady shift in data points in the direction of increasing maturity (from right to left) (PC1 v PC2, Fig. 7.19A), reflecting the hydrolysis of α_{s1}- and β-casein and the steady accumulation of α_{s1}-I-casein and γ_3-casein throughout the 6 m ripening period (Fig. 7.3A). The two cheese types could be differentiated because although the rate of β-casein hydrolysis was similar in both, the plot for Mozzarella cheese was less influenced by the hydrolysis of α_{s1}-casein to α_{s1}-I-casein by chymosin. This was in agreement with the values for α_{s1}-, α_{s1}-I, β- and γ-casein obtained by PAGE analysis (Fig. 7.3A). After 120 days the direction of increasing maturity of the Gouda cheese changed (to the lower left), reflecting the depletion of α_{s1}-casein (Fig. 7.3A).

The direction of increasing maturity of the Elsberg cheese (from right to left) (PC1 v PC2, Fig. 7.19A) was strongly influenced by both α_{s1}- and β-casein hydrolysis, as indicated by the central position of the points (PC2 $\approx$ 0). From 42 days, the direction of increasing maturity changed (towards the lower left) and this corresponded with the point at which α_{s1}-casein reached a minimum (Fig. 7.3A). The large gap between the 1 day and 30 day data points reflected the effect of the high initial ripening temperature on the rate of primary proteolysis. The score plots of PC1 v PC3 and PC2 v PC3 (Fig. 7.19B and C) showed a turning point at 30 days, which corresponded approximately with the maxima for α_{s1}-I-casein (Fig. 7.3A). After 30 days, the combination of PC1 and PC3 enabled the Elsberg data points to be separated, and this corresponded with the decrease in concentration of α_{s1}-I-casein as the cheese matured.

The score plot of PC1 and PC2 for Swiss cheese (Fig. 7.19A) showed a similar trend to that of the Elsberg cheese, with the large distance between the initial data points corresponding with rapid changes that took place in the early stages of maturation due to the elevated storage temperature. The trend towards the lower left corner reflected the importance of β- and γ-casein, and plasmin by inference, in the ripening of this cheese type. In the later stages of maturation of the Swiss cheese, the rate of hydrolysis of β-casein to γ_1-casein slowed (see Fig. 7.3A and B) and this seemed to account for the close proximity of the data points after 120 days. There was no decrease in α_{s1}-I-casein (Fig. 7.3A) and this was reflected in the PC1 and PC3 score plot (Fig. 7.19B) for Swiss cheese which, unlike the other cheese types, remained relatively constant with respect to PC3.

In summary, urea-PAGE of the USF with data analysis using the covariance form of PCA,

- placed emphasis on the main proteolytic events, the hydrolysis of α_{s1}-to α_{s1}-I-casein by chymosin, and β- to γ_1-casein by plasmin, as well as the hydrolysis of α_{s1}-I-casein;
- was partially successful for differentiating cheese type, with the Cheddar, Elsberg and Gouda cheese clearly differentiated from the other types and from each other by differences in the rates of chymosin and plasmin action. The Swiss cheese could be separated from the Mozzarella cheese from 35 days, but the data points of the less mature Swiss cheese and the Mozzarella cheese were intermingled;
- was useful for monitoring trends in primary proteolysis. PC1 and PC2 were useful up to the point where the substrates, and particularly α_{s1}-casein, were depleted or the rate of their hydrolysis slowed. After this point, with the exception of the Swiss cheese, the trends could be monitored using PC1 and PC3. Depletion of the substrates was not an issue for the Mozzarella cheese and the method provided useful differentiation of maturity over the entire 6 month period of maturation using the first two principal components; and
- may not be useful for some cheese types that are matured for more than 6 months, because the substrates may be depleted, the products may be transient intermediates, and the end-products may be too small to be retained in the gel or in quantities too small to be seen.

7.4.3.2 PCA of data obtained from urea-PAGE of the WSF

The identification system used for the USF was adapted for the WSF in order to classify each band across the range of gels in a consistent manner. Scanned gel slices containing a representative sample for each cheese type, were aligned and used to assign a number to each discrete band across the range of gels (Fig. 7.20). It is recognised that two or more peptides can have indistinguishable mobilities. The entire width of each gel lane was converted to a graphical image (as described in Ch. 7, section 7.4.3.1) and the peaks on the line graph were numbered to match the bands. The peak areas were determined, and the data collated and analysed using the correlation and covariance forms of PCA. Peak 3, which is particularly prominent in the WSF of Mozzarella cheese, was removed from the analysis as it was identified as β-casein, a protein not normally found in the WSF. The presence of β-casein in the water extract of Mozzarella cheese is dependent on the calcium concentration and pH of the cheese (Coker, unpublished). This particular Mozzarella cheese had a low Ca concentration (168 mM/kg) and a relatively high cheese pH (pH 5.48).

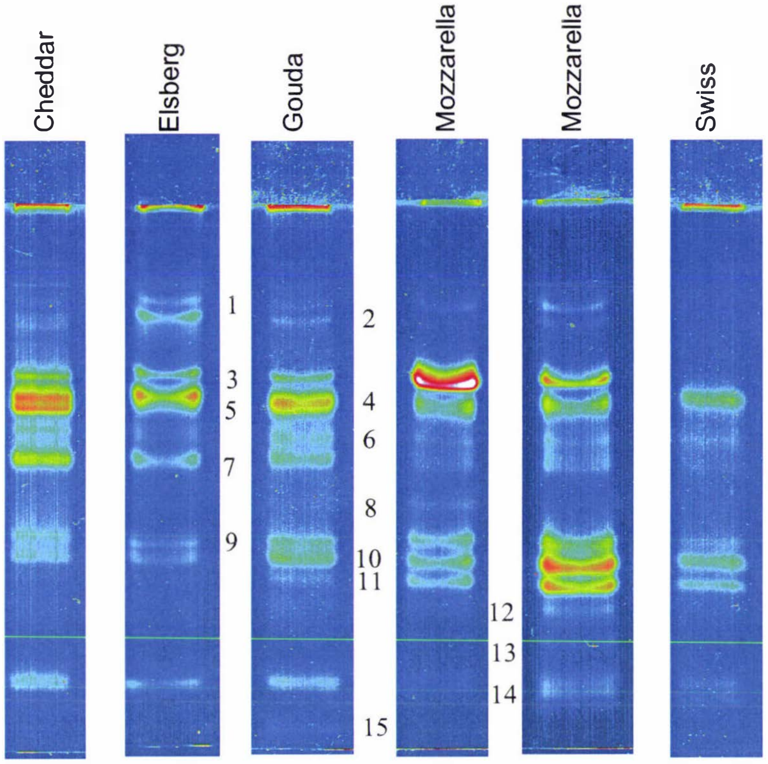


Fig. 7.20 *Urea-PAGE of the WSF - Scanned gel images with bands numbered.* Lanes 1 to 6, Cheddar (91 d), Elsberg (150 d), Gouda (120 d), Mozzarella (14 d), Mozzarella (133 d) and Swiss (120 d) cheese WSF (5% cheese, 25 μl loaded), respectively.

7.4.3.2.1 PCA of the correlation matrix (PAGE/WSF)

Fig. 7.21 depicts PCA loading plots for the peaks/bands resulting from PAGE analysis of the WSF. Fig. 7.22 depicts 2-dimensional graphical representations of the relationship between PC1, PC2 and PC3, calculated for each sample. Tables related to PCA of the correlation matrix, scores for PC1 (32%), PC2 (25%) and PC3 (15%) and a 3-dimensional graph can be found in the appendix (section 7.8.2.1).

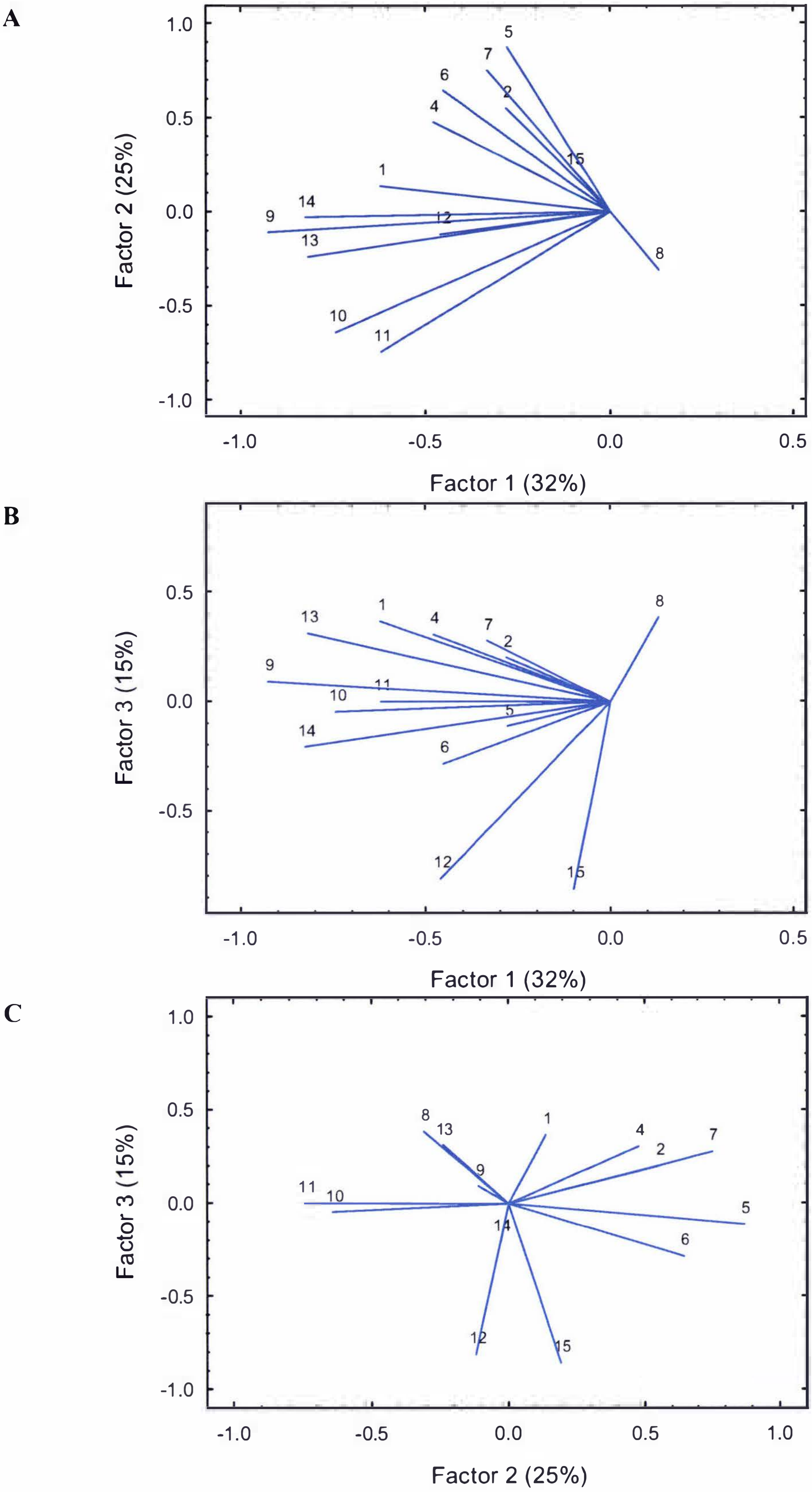


Fig. 7.21 *Urea-PAGE (WSF) data analysis using PCA (correlation matrix) - loading plots.* A) factor 1 v factor 2, B) factor 1 v factor 3 and C) factor 2 v factor 3 for peaks/bands 1-15 from urea-PAGE (WSF) of ripening Cheddar, Elsberg, Gouda, Mozzarella and Swiss cheese.

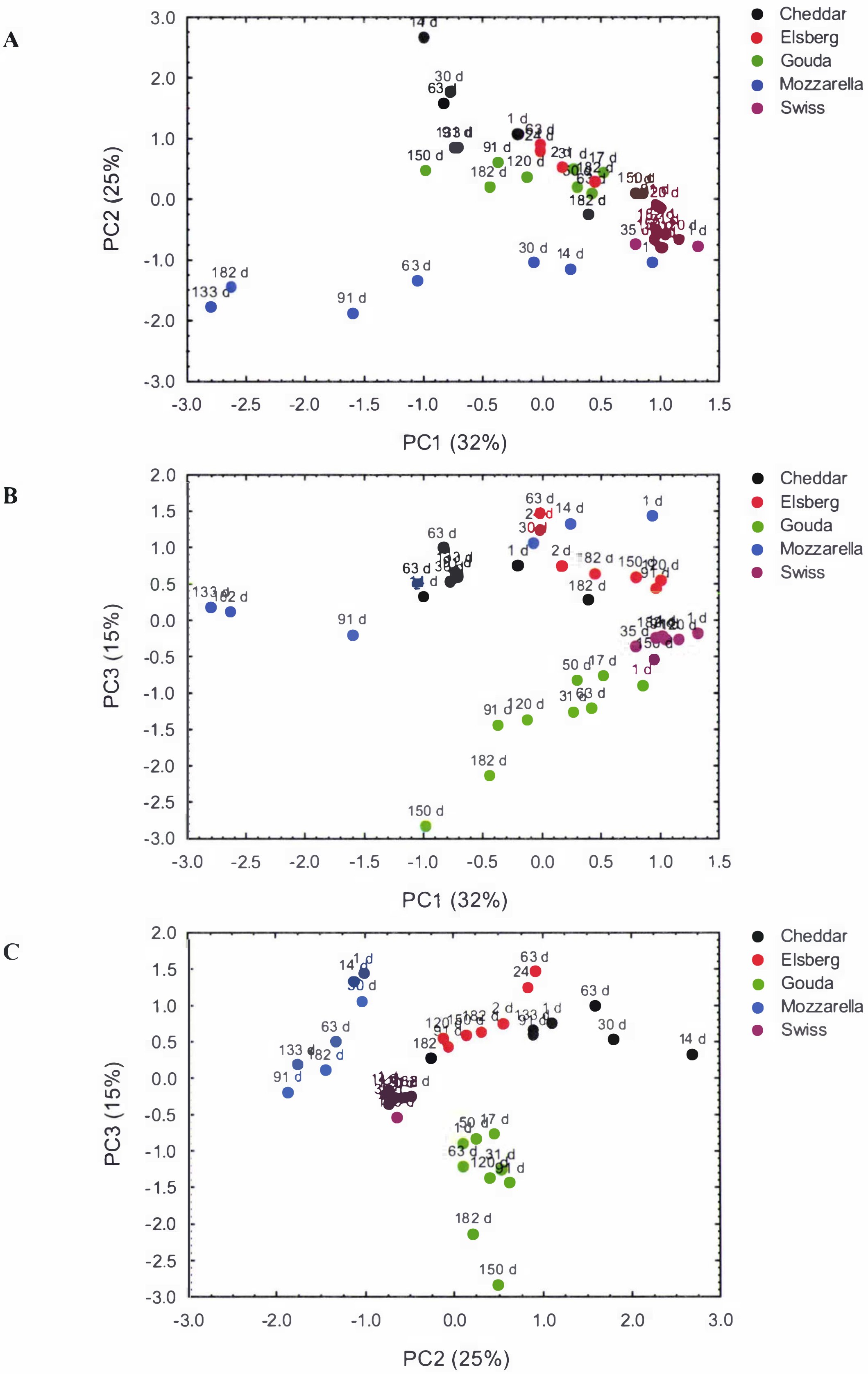


Fig. 7.22 *Urea-PAGE (WSF) data analysis using PCA (correlation matrix) - score plots.* A) PC1 v PC2, B) PC1 v PC3 and C) PC2 v PC3 for bands/peaks 1-15 from urea-PAGE (WSF) of ripening Cheddar, Elsberg, Gouda, Mozzarella and Swiss cheese. For a 3-D plot, see appendix, section 7.8.2.1.

Analysis of the urea-PAGE (WSF) data using the correlation form of PCA resulted in 57% of the variation in the data set being accounted for by the first two principal components, with PC1 accounting for 34%, PC2 for 23% and PC3 for 15%. The value for PC1 was markedly less than the value (56%) obtained when this form of PCA was used to analyse the data from the USF.

An important feature of urea-PAGE analysis of the WSF was that all the peptides captured in the gel were intermediates in the proteolysis pathway, and there was no clear start or end point common to all cheese types. As well, many peptides increased and then decreased in concentration (see appendix, section 7.8.2).

An examination of the loading and score plots (Figs. 7.21 and 7.22) showed that differentiation of maturity was only possible for the Mozzarella cheese. In this cheese type, slow proteolysis meant that many peptides steadily increased in concentration throughout ripening. PCA captured this progression (PC1 v PC2, Fig. 7.22A), with the direction of maturity (from centre right to lower left) and distance between data points being most closely associated with the relative increase in area of peaks 10 and 11 (Fig. 7.21A and Ch. 7, section 7.8.2). After 133 days ripening, peptides within these peaks decreased in concentration and as a result, the distance between the 133 day and 182 day data points was small. As Mozzarella is not usually stored for this length of time, this was not considered important.

Although there was no common starting point, the general trends for the Cheddar, Elsberg and Gouda cheese (from the centre right to the upper left) (PC1 v PC2, Fig. 7.22A) were associated with peaks 2, 4, 5, 6 and 7 (Fig. 7.21A). Because peptides within these peaks increased and then decreased in concentration as the cheese ripened (see Ch. 7, section 7.8.2), the maturity trends were not linear and the direction of the points was eventually reversed. The combination of PC1 and PC3 (Fig. 7.22B) highlighted a different trend for the Gouda cheese (from centre right to lower left). This trend was associated with peptides in peaks 12 and 15 (Fig. 7.21B), which increased in concentration for the first 150 days. The decrease in concentration of these peptides was responsible for the position of the 182 day sample point.

Most peaks in the Swiss cheese increased and decreased in area during ripening (Ch. 7, section 7.8.2) and there was no maturity trend evident (Fig. 7.22A, B and C).

Despite low principal component scores, the correlation form of PCA proved useful for differentiating most cheese types on the basis of PC2 and PC3 (Fig. 7.22C). Peaks influencing the separation were; peaks 8, 10 and 11 for the Mozzarella cheese, and peaks 12 and 15 for the Gouda (Fig. 7.21C). Some peptides, present in one cheese type, were apparently absent in others and this, in conjunction with the increase and decrease in concentration of many peptides, is believed to have provided the basis for segregation of the other cheese types. The apparent absence of some peptides may, in some cases, have been related to their low concentration.

In summary, analysis of the urea-PAGE (WSF) data using the correlation form of PCA,

- provided reasonable differentiation of each cheese type; and
- was unsuitable for determining stage of maturity of all except the Mozzarella cheese.

7.4.3.2.2 PCA of the covariance matrix (PAGE/WSF)

Fig. 7.23 depicts PCA loading plots for the peaks/bands resulting from urea-PAGE analysis of the WSF of the cheese. Fig. 7.24 provides 2-dimensional graphical representations of the relationship between PC1, PC2 and PC3, calculated for each sample. Tables related to PCA of the covariance matrix, scores for PC1 (57%), PC2 (28%) and PC3 (7%) and a 3-dimensional graph can be found in the appendix (section 7.8.2.2).

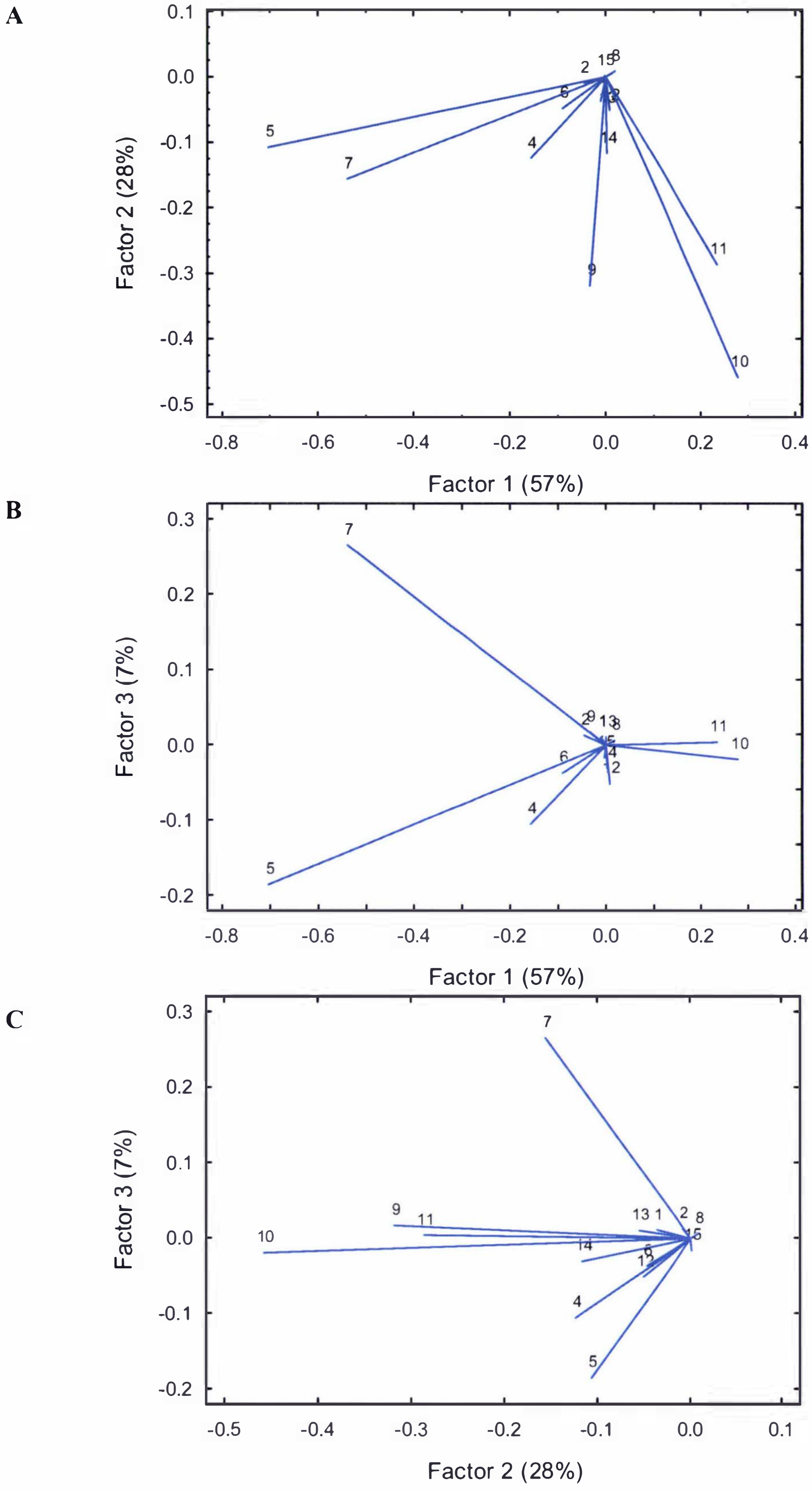


Fig. 7.23 *Urea-PAGE (WSF) data analysis using PCA (covariance matrix) - loading plots.* A) factor 1 v factor 2, B) factor 1 v factor 3 and C) factor 2 v factor 3 for peaks/bands 1-15 from urea-PAGE (WSF) of ripening Cheddar, Elsberg, Gouda, Mozzarella and Swiss cheese.

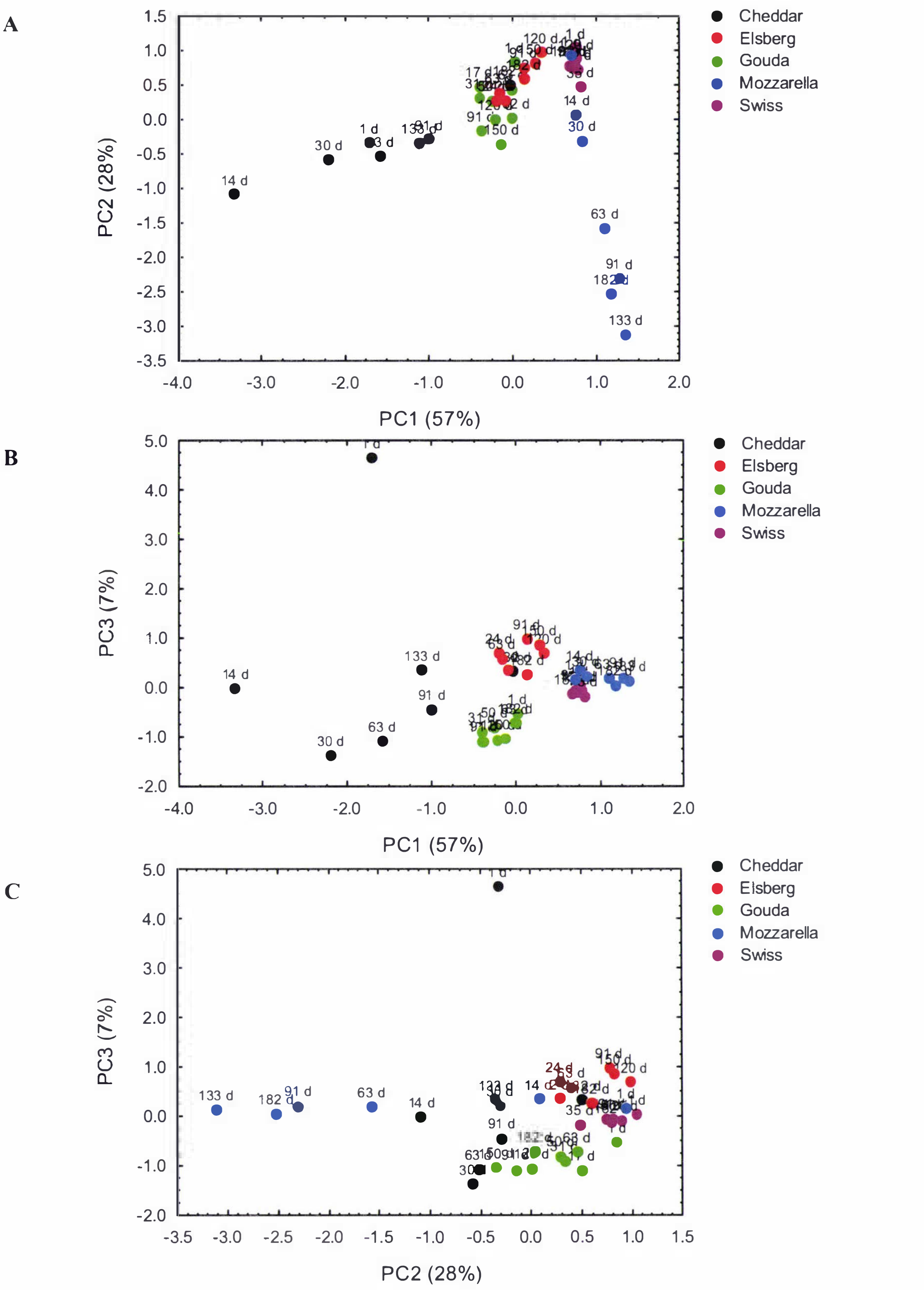


Fig. 7.24 *Urea-PAGE (WSF) data analysis using PCA (covariance matrix) - score plots.* A) PC1 v PC2, B) PC1 v PC3 and C) PC2 v PC3 of peaks/bands 1-15 from urea-PAGE (WSF) of ripening Cheddar, Elsberg, Gouda, Mozzarella and Swiss cheese. For a 3-D plot, see appendix, section 7.8.2.2.

The covariance form of PCA provided effective analysis of the urea-PAGE (WSF) peak data, with the first two principal components accounting for 85% of the variation in the data and the third principal component accounting for a further 7%. This was much greater than the correlation form of PCA in which the first two principal components accounted for only 57% of the variation in the data. However, it was clear from the results that, although PCA of the covariance matrix accounted for most of the variation in the data and provided reasonable differentiation of most cheese types (Fig. 7.24), there was no clear direction for cheese maturity associated with factors 1, 2 or 3 (Fig. 7.23) for most cheese types.

PC1 v PC2 (Fig. 7.24A) enabled clear differentiation of the Mozzarella cheese from the other cheese types, with peaks 9, 10 and 11 (PC2) influencing the differentiation and direction of the maturity trend for the first 133 days. After this time, the concentration of peptides in these peaks diminished and the method ceased to be useful for determining maturity. However, as 133 days was outside the normal storage time for Mozzarella this was not considered important.

Cheddar cheese could be differentiated from the other cheese types for the first 133 days on the basis of PC1 v PC2 and PC1 v PC3 (Fig. 7.24A and B), with peaks 5 and 7 responsible for the differentiation. As Cheddar cheese is often stored for much longer periods, the lack of differentiation after 133 days was considered important. Peak 5, a peptide intermediate, increased in area for the first 14 days and then decreased, and consequently the maturity trend was complex and not useful.

The data points for the Swiss, Elsberg and Gouda cheese samples, formed distinct clusters that were separated from each other as well as from the Cheddar (except the 182 day old cheese) and Mozzarella data points on the basis of the first three principal components (PC1, PC2 and PC3, Fig. 7.24A, B and C). The main peaks responsible for the (complex) differentiation of maturity of the Cheddar cheese, peaks 5 and 7, increased and then decreased in area (see Ch. 7, section 7.8.2) in the Swiss (peak 5 absent), Elsberg and Gouda cheese, and there was therefore no clear maturity trend for any of these cheese types based on PC1, PC2 or PC3.

In summary, analysis of the urea-PAGE (WSF) data using the covariance form of PCA,

- provided reasonable differentiation of the cheese types using the combination of PC1, PC2 and PC3;
- was adequate for determining the stage of maturity of the Mozzarella cheese for the first 133 days, because the important peaks contained peptides that increased steadily in concentration; and

- was inadequate for determining the maturity of the Cheddar, Elsberg, Gouda and Swiss cheeses because most peaks present on the gel contained peptides that were intermediates in the proteolytic process and their concentration increased and then decreased during ripening.

7.4.3.3 PCA of data obtained from SE-HPLC of the WSF

Subjective analysis of the ripening profiles for each cheese type (Fig. 7.5 – 7.9) showed that they were different from one another and that there were consistent changes in the molecular weight distribution of the peptides during ripening. It was therefore considered likely that statistical analysis of the data from each of the peaks would provide an objective assessment of maturity and highlight the suitability of the SE-HPLC method for differentiating stage of maturity and cheese type.

A systematic approach was taken to labelling the peaks, so that there was consistency across the range of cheeses at all stages of maturity (Fig. 7.25). Firstly the traces of the Cheddar cheese were overlaid and the peaks determined. Then, for each remaining cheese type the traces were overlaid along with representative traces of the young, medium and mature Cheddar cheese. In this way, the peaks on the Cheddar traces were used as a reference so that peak identification was consistent across the range of cheese types. The areas of each of the 14 peaks obtained for each cheese sample were determined. The data were analysed using the correlation and covariance forms of PCA.

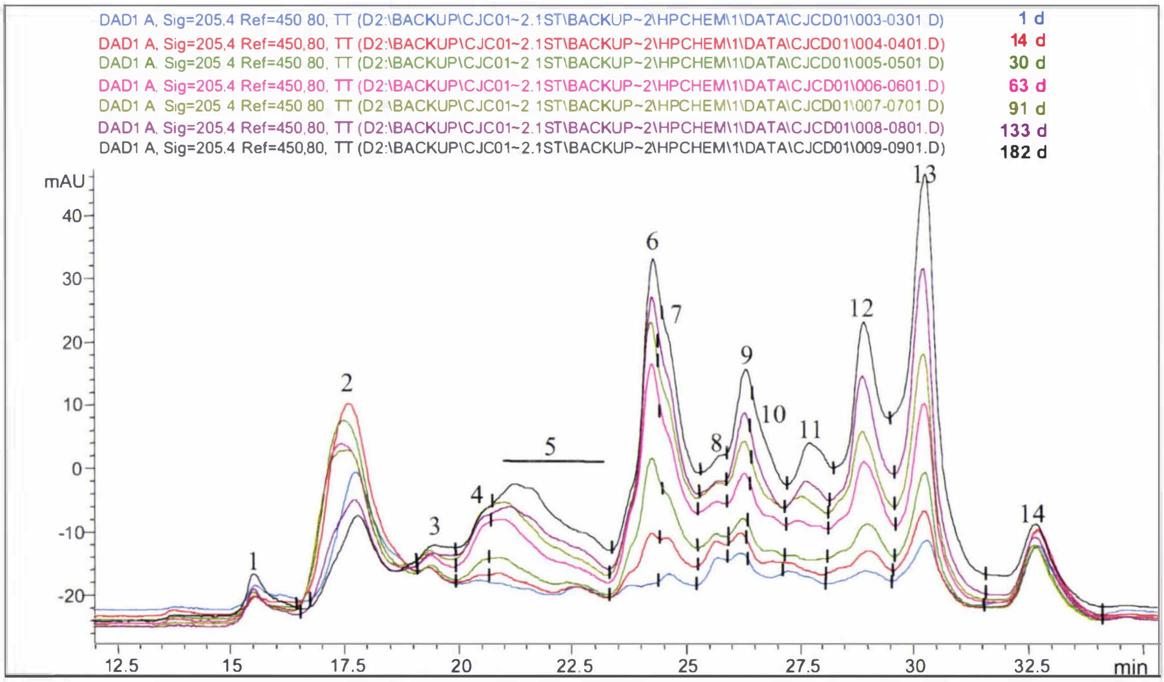


Fig. 7.25 *SE-HPLC traces of the WSF of Cheddar cheese with the 14 peaks labelled.*

7.4.3.3.1 PCA of the correlation matrix (SE-HPLC/WSF)

Fig. 7.26 depicts PCA loading plots for factors 1, 2 and 3 derived from the peak areas resulting from SE-HPLC analysis of the WSF of the cheese. Fig. 7.27 provides a 2-dimensional graphical representation of the relationship between PC1, PC2 and PC3 calculated for each sample. Tables related to PCA of the correlation matrix, scores for PC1 (50%), PC2 (17%) and PC3 (15%) and a 3-dimensional graph can be found in the appendix (section 7.8.3.1).

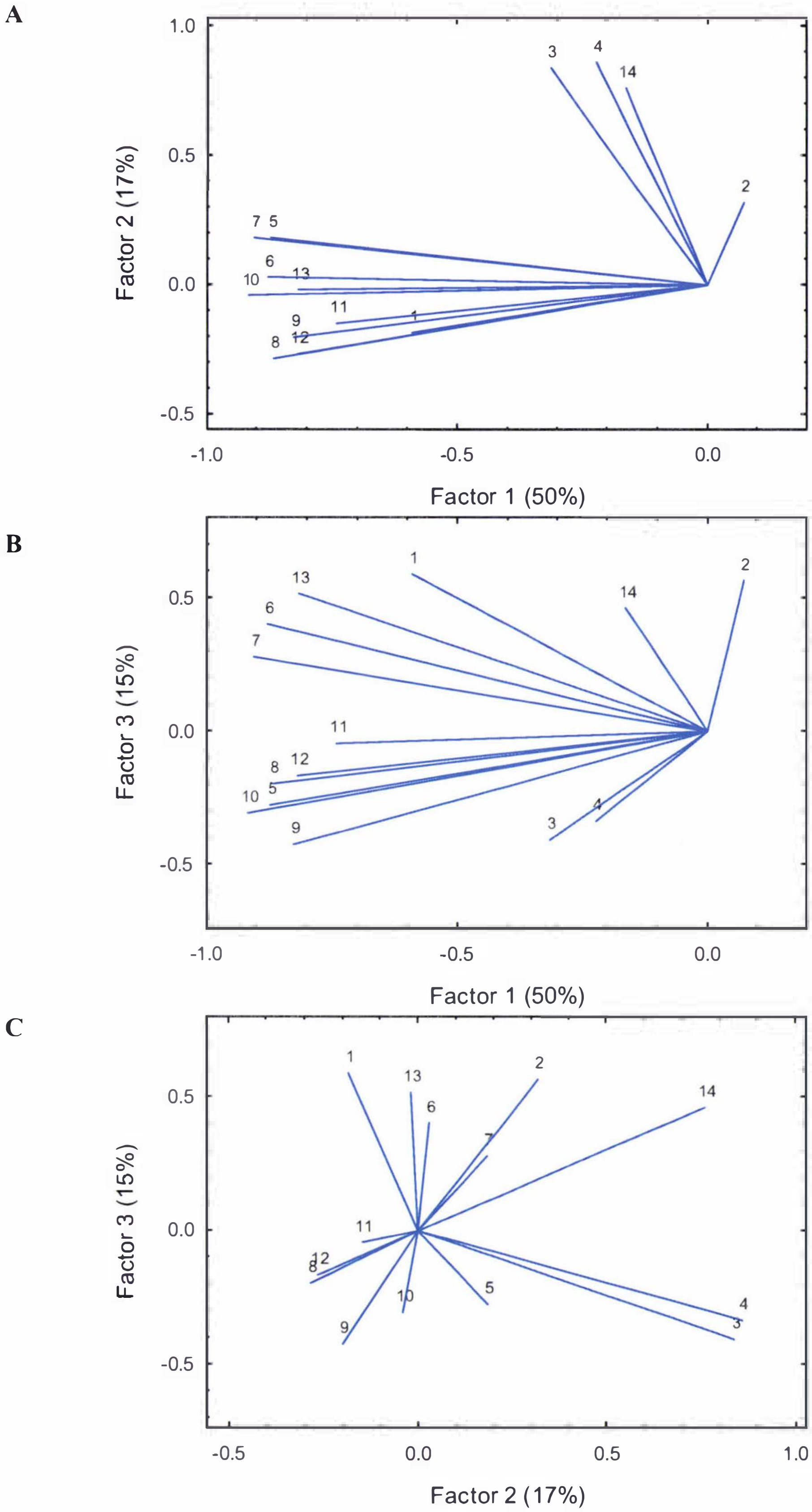


Fig. 7.26 *SE-HPLC (WSF) data analysis using PCA (correlation matrix) - loading plots.* A) factor 1 v factor 2, B) factor 1 v factor 3 and C) factor 2 v factor 3 for peaks 1-14 from SE-HPLC (WSF) of ripening Cheddar, Elsberg, Gouda, Mozzarella and Swiss cheese.

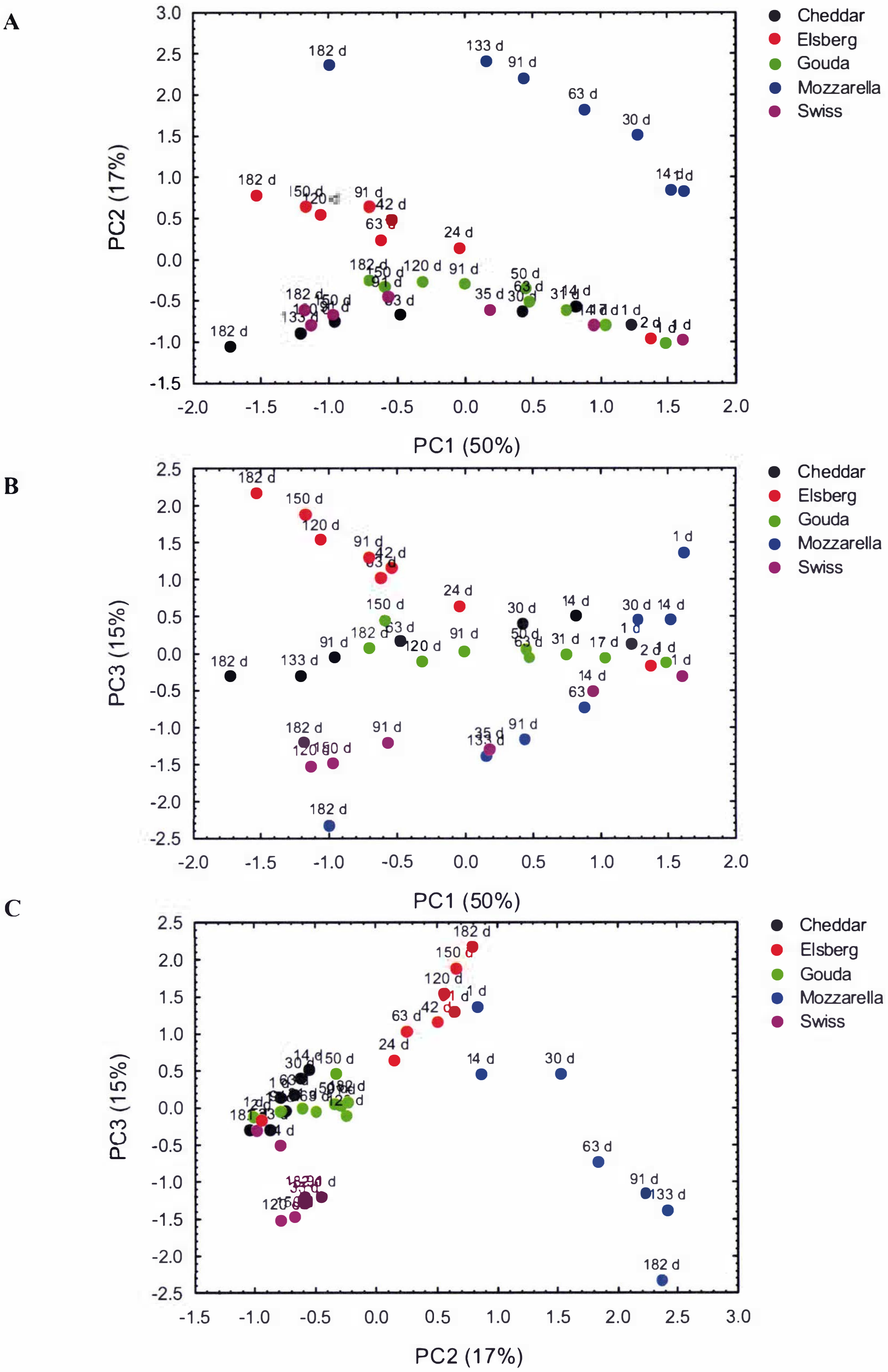


Fig. 7.27 *SE-HPLC (WSF) data analysis using PCA (correlation matrix) - score plots.* A) PC1 v PC2, B) PC1 v PC3 and C) PC2 v PC3 for peaks 1-14 from SE-HPLC (WSF) of ripening Cheddar, Elsberg, Gouda, Mozzarella and Swiss cheese. For a 3-D plot, see appendix, section 7.8.3.1.

The correlation form of PCA accounted for 82% of the variation in the data within the first three principal components. Examination of the loading plots (Fig. 7.26) showed clear divisions of peaks into those influencing factor 1 (peaks 1, 5, 6, 7, 8, 9, 10, 11, 12 and 13) and those influencing factor 2 (peaks 3, 4 and 14).

PC1 (Fig.7.27A and B) was associated mainly with cheese maturity and influenced by the majority of peaks that increased in area throughout maturation. Maturity trends were clearly evident for the Cheddar, Elsberg and Mozzarella cheese throughout ripening and the Gouda and Swiss cheese during the earlier stages of ripening. After 120 days ripening, the data points of the Gouda and Swiss cheese were clustered, indicating that the combination of SE-HPLC of the WSF and the correlation form of PCA was unsuitable for monitoring maturation in these types beyond 4 months of age.

PC2 (Fig. 7.27A and C) provided differentiation of cheese type and was associated with the some of the highest molecular weight peptide peaks that usually increased in area as a result of primary proteolysis in the early stages of maturation and then decreased, as they became substrates for the peptidolytic enzymes of the starter, secondary starter and adventitious microorganisms. Peak 14, believed to contain species such as pyroglutamic acid, N-acetyl glutamic acid and N-acetyl glutamine (R. Motion, unpublished) was the exception to this and was present in different concentrations in each cheese type, with no obvious trends in its amount. The PC2 scores enabled the Mozzarella cheese, which contained high concentrations of higher molecular weight peptides (peaks 2, 3 and 4), to be clearly separated from the other cheese types. The Elsberg cheese data points prior to 30 days were intermingled with those of the Cheddar and Gouda cheese on the plot of PC1 v PC2 (Fig. 7.27A). However the Elsberg cheese was clearly differentiated from the other cheese types after 30 days on the basis of both the PC2 and PC3 scores (Fig. 7.27C). The Gouda, Swiss and Cheddar cheeses were closely associated on the basis of their PC1 and PC2 scores, but the Swiss cheese could be differentiated from the others on the basis of its PC3 scores. The Cheddar and Gouda cheese were unable to be differentiated from one another.

A comparison of the loading and score plots suggested that differentiation of the more closely related cheese types, Cheddar, Elsberg, Gouda and Swiss would be more achievable if Mozzarella cheese, which was clearly different and accounted for most of the variation in area of some peaks (eg. peaks 3, 4, and 14 (Fig. 7.26)), was removed from the data set. The effect of removing the Mozzarella cheese data was tested and the resultant loading and score plots are depicted in Figs. 7.28 and 7.29. Tables related to PCA of the correlation matrix, scores for PC1 (58%), PC2 (19%) and PC3 (13%) and a 3-dimensional graph can be found in the appendix (section 7.8.3.2).

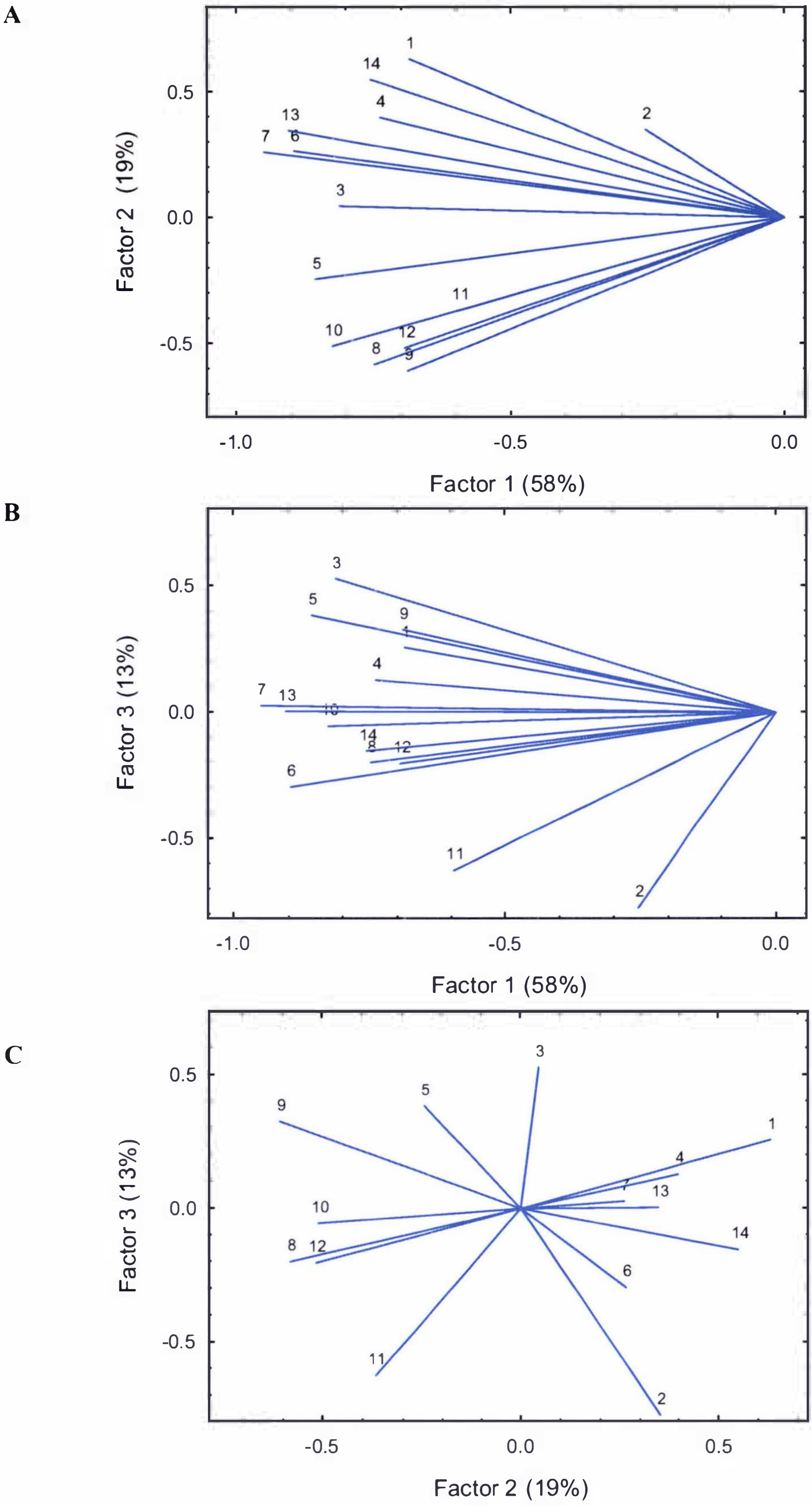


Fig. 7.28 *SE-HPLC (WSF) data (minus Mozzarella) analysis using PCA (correlation matrix) - loading plots.* A) factor 1 v factor 2, B) factor 1 v factor 3 and C) factor 2 v factor 3 for peaks 1-14 from SE-HPLC (WSF) of ripening Cheddar, Elsberg, Gouda and Swiss cheese.

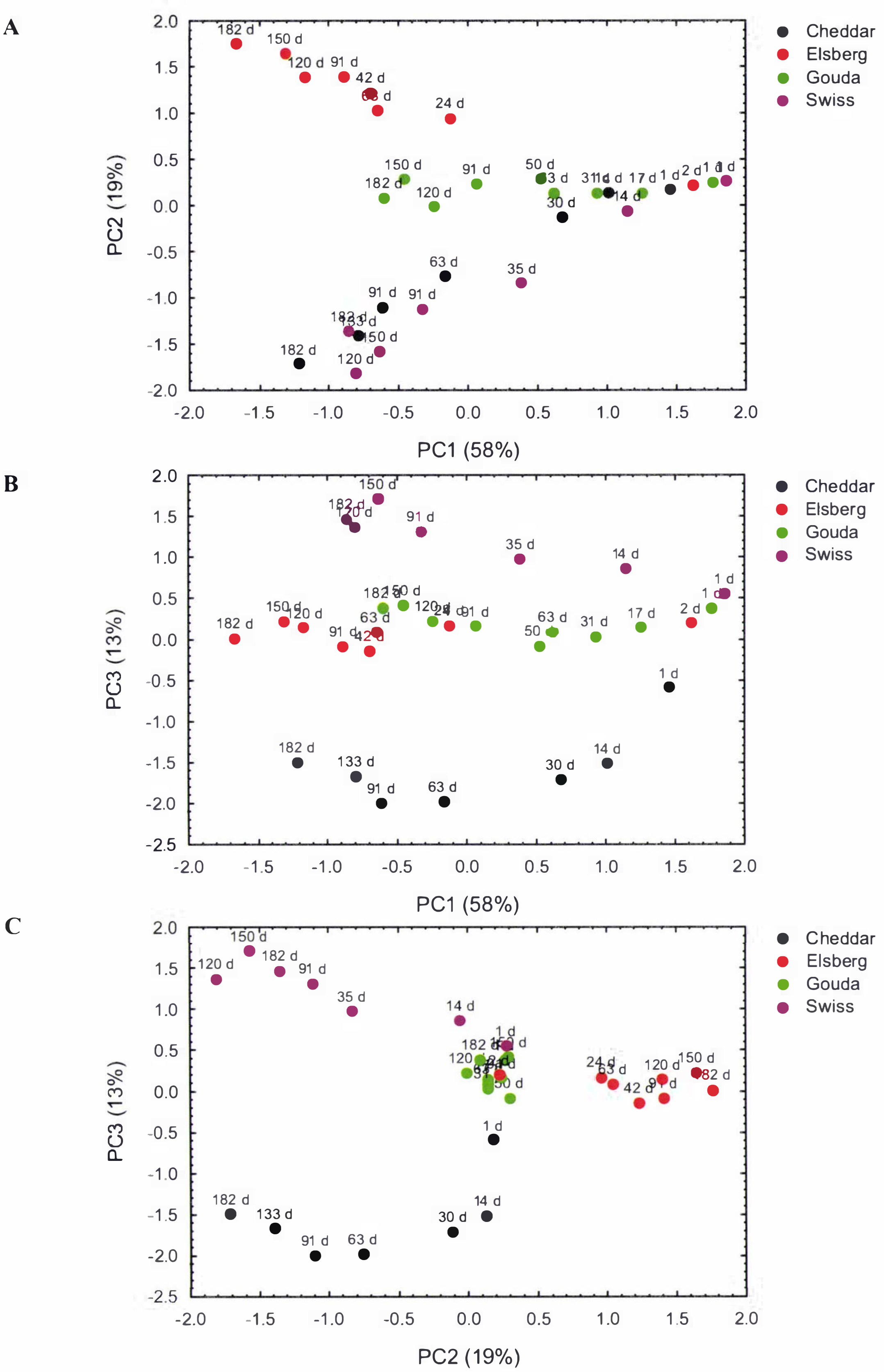


Fig. 7.29 *SE-HPLC (WSF) data (minus Mozzarella) analysis using PCA (correlation matrix) - score plots.* A) PC1 v PC2, B) PC1 v PC3 and C) PC2 v PC3 for peaks 1-14 from SE-HPLC (WSF) of ripening Cheddar, Elsberg, Gouda and Swiss cheese. For a 3-D plot, see appendix, section 7.8.3.2.

Good differentiation of both cheese maturity (with the exception of the more mature Swiss cheese) and type was achieved when the Mozzarella cheese data was removed from the analysis, with peaks associated with factors 1 and 2 (Fig. 7.28A) influencing the direction of maturity, and peaks associated with factors 2 and 3 (Fig. 7.28 A, B and C) influencing cheese differentiation. The plots of PC1 v PC3 and PC2 v PC3 (Fig. 7.29A and C) show clear differentiation the cheese types.

The score plot of PC1 v PC3 (Fig. 7.29B) was similar to that of PC1 v PC2 obtained when the USF was analysed by urea-PAGE (Fig 7.19A). However, in that case the peak/band data was analysed using the covariance form of PCA, which gave greatest weighting to the largest peaks, the casein substrates and first large breakdown products. This finding suggests that differentiation by SE-HPLC of the WSF gave similar overall conclusions to differentiation by urea-PAGE of the USF, which provided a direct demonstration of primary proteolysis. It would seem that chymosin and plasmin activities were inextricably linked to the peptides formed and extracted into the WSF.

In summary, when PCA results in one subgroup that is quite different from the remainder of the subgroups, a second round of analysis is useful. In the present situation, analysis of the SE-HPLC (WSF) data using the correlation form of PCA,

- provided very good differentiation of all the cheese types, although this required a stepwise approach with removal of the Mozzarella cheese data in order to capture the variation in peak area of the other cheese types, and
- although adequate for differentiation of the maturity of the Cheddar, Mozzarella, Gouda and Elsberg, was inadequate for the Swiss cheese in the later stages of maturation.

7.4.3.3.2 PCA of the covariance matrix (SE-HPLC/WSF)

Fig. 7.30 depicts PCA loading plots for the peak areas resulting from SE-HPLC analysis of the WSF. Fig. 7.31 shows 2-dimensional graphical representations of the relationship between PC1, PC2 and PC3 calculated for each sample. Tables related to PCA of the covariance matrix, scores for PC1 (55%), PC2 (22%) and PC3 (13%) and a 3-dimensional graph can be found in the appendix (section 7.8.3.3).

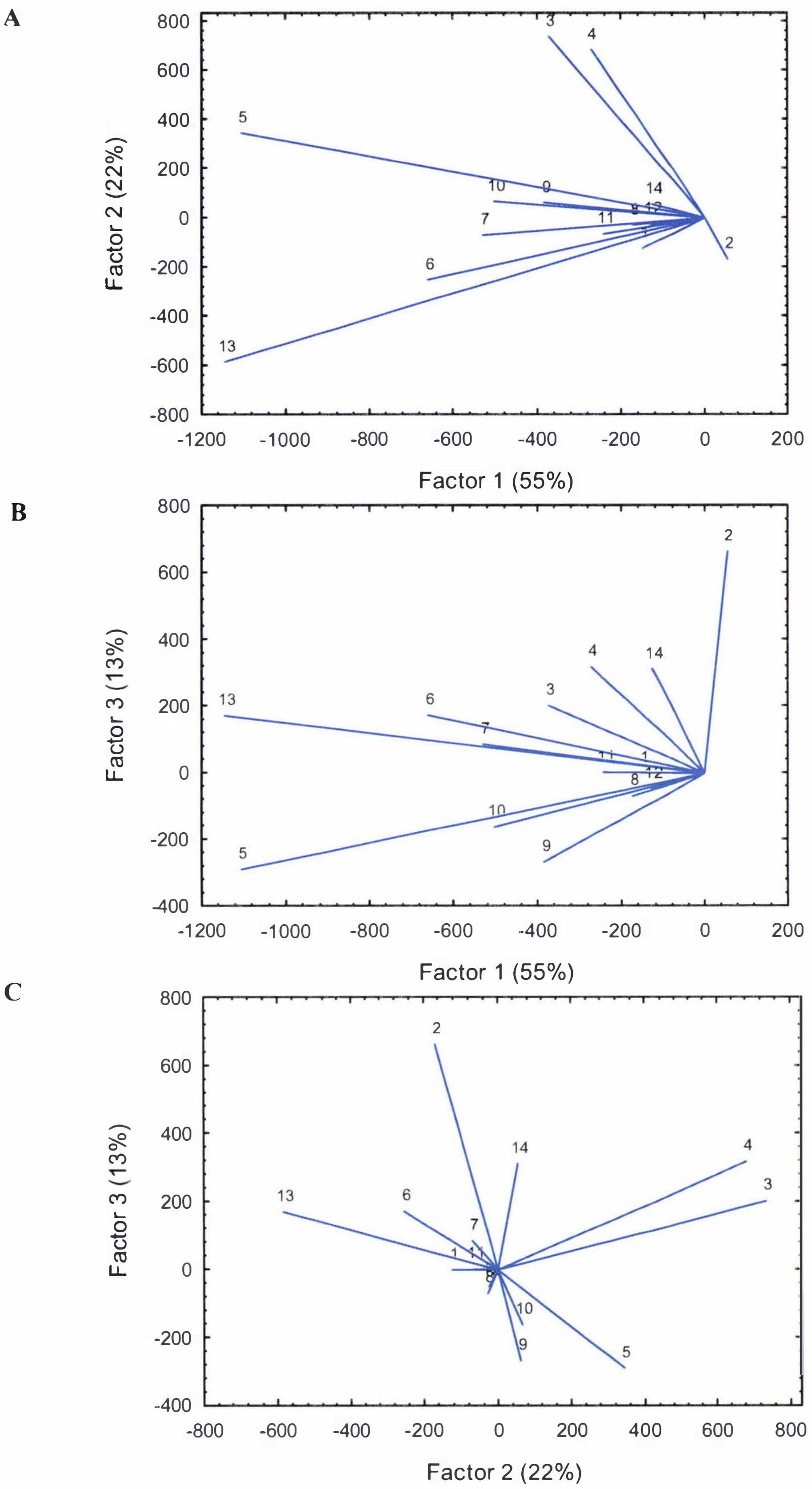


Fig. 7.30 *SE-HPLC (WSF) data analysis using PCA (covariance matrix) - loading plots.* A) factor 1 v factor 2, B) factor 1 v factor 3 and C) factor 2 v factor 3 for peaks 1-14 from SE-HPLC (WSF) of ripening Cheddar, Elsberg, Gouda, Mozzarella and Swiss cheese.

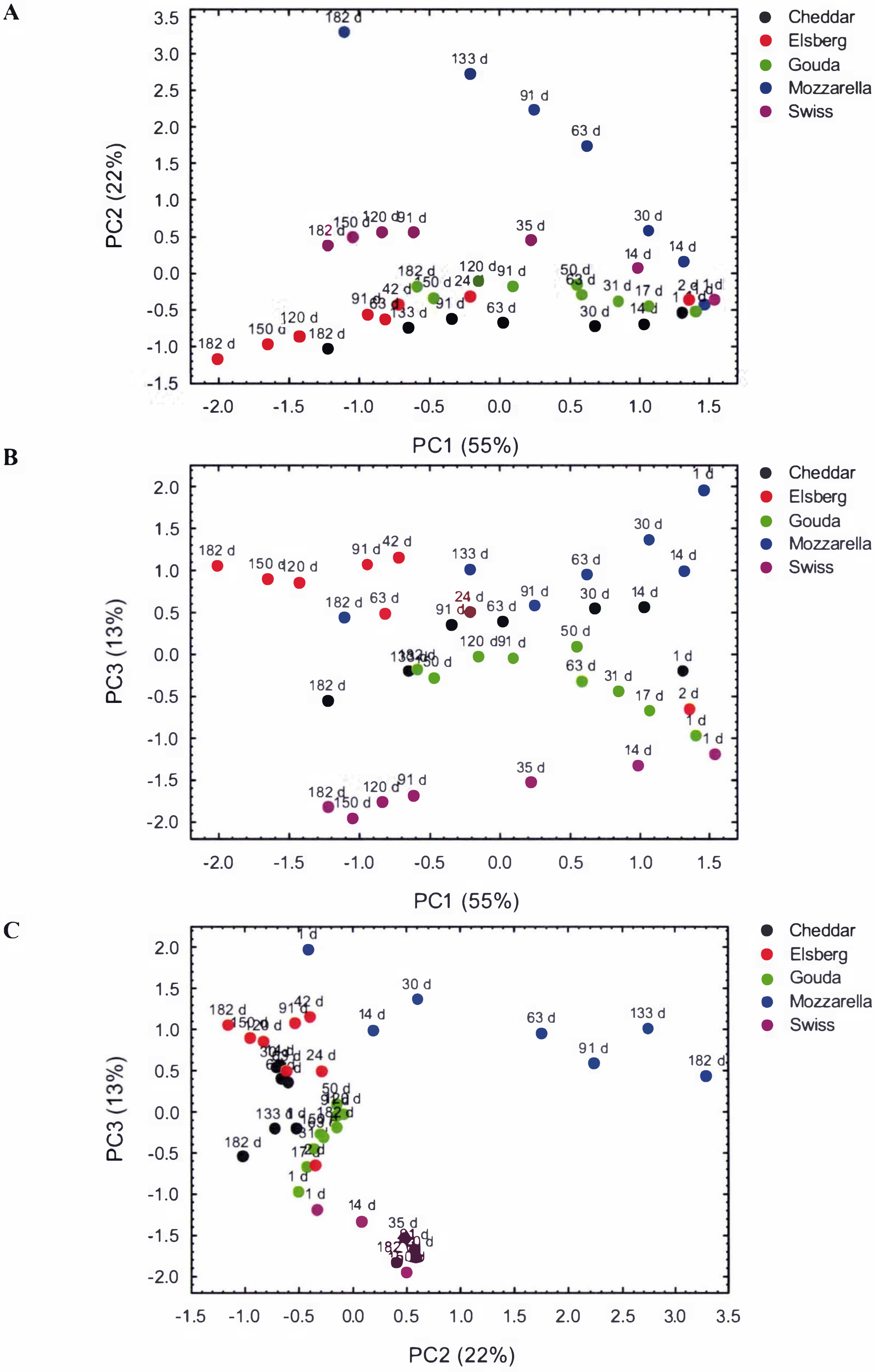


Fig. 7.31 *SE-HPLC (WSF) data analysis using PCA (covariance matrix) - score plots.* A) PC1 v PC2, B) PC1 v PC3 and C) PC2 v PC3 for peaks 1-14 from SE-HPLC (WSF) of ripening Cheddar, Elsberg, Gouda, Mozzarella and Swiss cheese. For a 3-D plot, see appendix, section 7.8.3.3.

The SE-HPLC profiles were distinctive (Fig. 7.5 - 7.9) and PCA of the covariance matrix provided an effective method of data analysis that accounted for 90% of the variation in the data within the first three principal components.

PC1 was closely associated with maturity (Fig. 7.31A), with the direction of maturity strongly influenced by peak 5 (Fig. 7.30A), considered to be a product of primary proteolysis, and peak 13, which contained amino acids and was therefore an end product of proteolysis. Peak 5, comprised of peptides of between approximately 5000 and 8000 Da in molecular weight was likely to be an intermediate, but increased in concentration at a rate greater than any decrease that may have occurred as a result of peptidolytic enzyme activity. This was particularly noticeable in the Mozzarella cheese where the peak increased substantially in area throughout maturation (Fig. 7.8). Other peaks associated with maturity were peaks 6, 7, 9 and 10, which accumulated in each cheese to a different degree throughout maturation.

The differentiation of maturity was excellent for most cheese types (Fig. 7.31A), with peaks associated with maturity gradually increasing in area throughout maturation (Fig. 7.5 – 7.9). The distance between data points increased in the later stages of maturation of the Cheddar, Mozzarella and Elsberg cheese, and remained constant for the Swiss cheese, making this a very good method for tracking the maturity of each of these cheese types. There was a slight anomaly in the distribution of data points in the Gouda cheese at 50 and 63 days, which was consistent with irregularities in the SE-HPLC profiles (Fig. 7.7). Each Gouda cheese sample was taken from a separate cheese round from the same batch and it is possible that small differences in composition were responsible for this irregularity.

Peaks 3 and 4 as well as peaks 5, 13 and 6 (Fig. 7.30A and C) accounted for the largest differences in PC2 (Fig. 7.31 A). These peaks showed small incremental changes in area (see the appendix, section 7.8.3) that were responsible for the gradual change in the slope of the trend line of most cheese types. The Mozzarella and Swiss cheeses were well separated from the other cheese types on the basis of PC2, but the Cheddar, Elsberg and Gouda were closely grouped. With the exception of the very young cheeses, PC3 provided reasonable differentiation of the Gouda and Elsberg cheeses, but there was an overlap of data points of both these cheese types with those of the Cheddar cheese.

In summary, SE-HPLC of the WSF with analysis of the data set using the covariance form of PCA,

- provided very good differentiation of cheese maturity with no bunching of the data points later in maturity, and
- provided reasonable differentiation of the cheese types.

It seemed likely that the large quantities of very high molecular weight peptides (peaks 3 and 4), that caused the Mozzarella cheese to be well differentiated from the other cheese types, were unduly influencing differentiation of the remaining cheese type. This was investigated by removing the Mozzarella cheese data from the analysis.

The resultant loading and score plots are depicted in Fig. 7.32 and 7.33. Tables related to PCA of the covariance matrix, scores for PC1 (70%), PC2 (18%) and PC3 (7%) and a 3-dimensional graph can be found in the appendix (section 7.8.3.4).

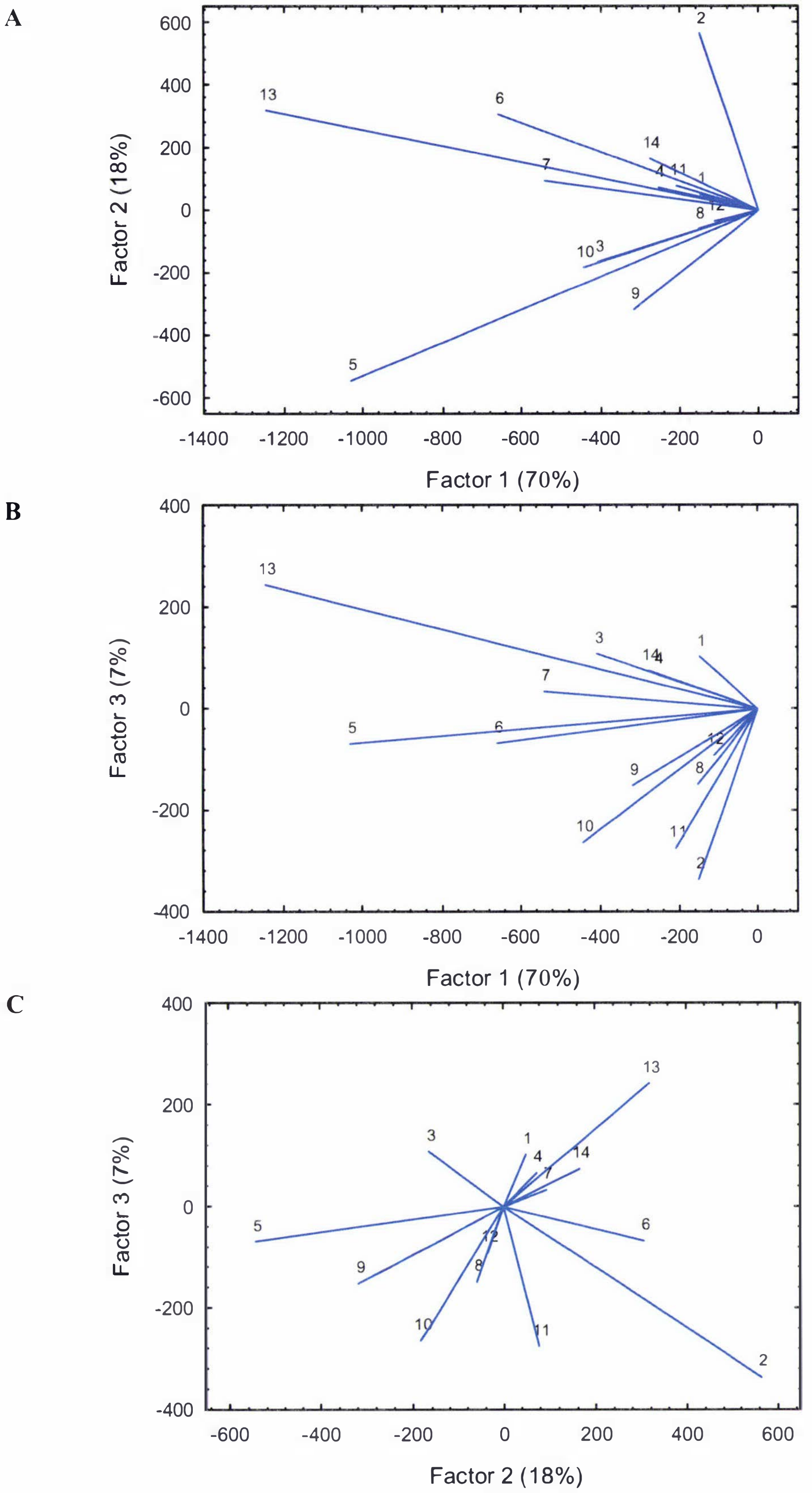


Fig. 7.32 *SE-HPLC (WSF) data (minus Mozzarella) analysis using PCA (covariance matrix) - loading plots.* A) factor 1 v factor 2, B) factor 1 v factor 3 and C) factor 2 v factor 3 for peaks 1-14 from SE-HPLC (WSF) of ripening Cheddar, Elsberg, Gouda and Swiss cheese.

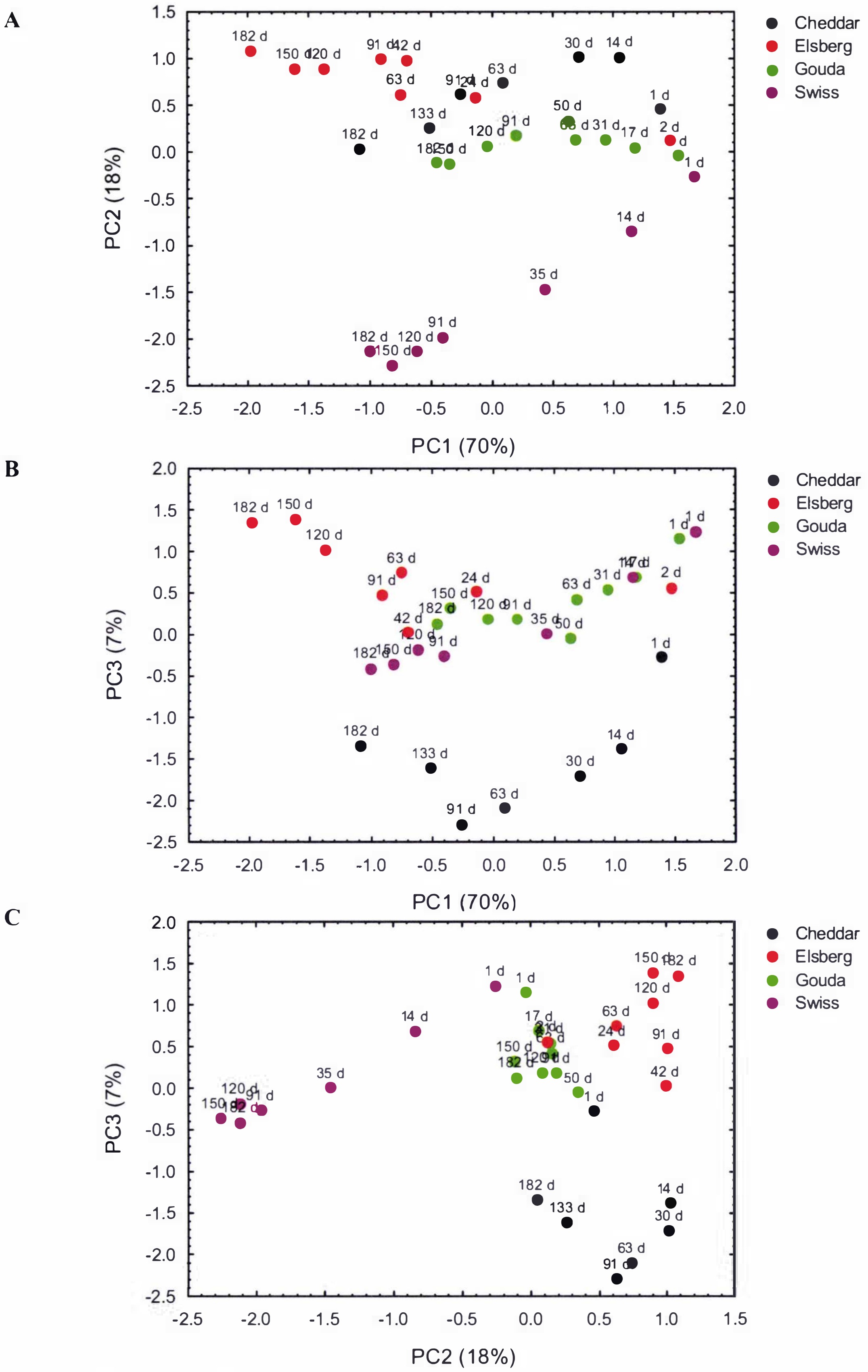


Fig. 7.33 *SE-HPLC (WSF) data (minus Mozzarella) analysis using PCA (covariance matrix) - score plots.* A) PC1 v PC2, B) PC1 v PC3 and C) PC2 v PC3 for peaks 1-14 from SE-HPLC (WSF) of ripening Cheddar, Elsberg, Gouda and Swiss cheese. For a 3-D plot, see appendix, section 7.8.3.4.

The new loading plot (PCl v PC2, Fig. 7.32A) was very similar to that obtained when all the cheese was analysed (PCl v PC2, Fig. 7.30A), with many of the same peaks dominant. Peaks 3 and 4 were particularly large in the Mozzarella cheese and the removal of the Mozzarella cheese data removed their dominance and altered the separation (Fig 7.33A) of the other cheese types (Fig. 7.30A and C).

A comparison of the loading and score plots (Fig. 7.32 and 7.33) showed that PCl was closely associated with cheese maturity, with peaks 5 and 13 continuing to dominate the assessment of maturity following the removal of the Mozzarella cheese data. For each cheese type the data points were well spread out in accordance with the ripening interval and storage history, although the final 150 and 182 day points for the Gouda cheese were close together.

The separation of each of the four cheese types was improved with the removal of the Mozzarella cheese data. The combinations of PC1, PC2 and PC3 (Fig. 7.33A, B and C) provided very good separation of the cheese types (see Fig. 7.33 and 3-D plot, appendix 4.8.3.4), with peaks 2 and 5 (Fig. 7.32) being particularly important. PC1 and PC2 provided reasonable separation of each cheese type (Fig. 7.33A), with the Swiss cheese being particularly well separated from the other cheese types on the basis of the PC2, as it was when Mozzarella cheese data was present (Fig. 7.30). The Cheddar cheese was well separated from the other cheese types on the basis of PC3 (Fig. 7.33B).

In summary, SE-HPLC of the WSF with data analysis using the covariance form of PCA:

- provided excellent differentiation of cheese maturity, and
- very good differentiation of cheese type, although it was helpful to use a stepwise approach in which data from cheese that was clearly separated from the other cheese types was removed from the analysis.

7.4.3.4 PCA of data obtained from RP-HPLC of the WSF

The data from the RP-HPLC ripening profiles (Fig. 7.10 – 7.14) were subjected to statistical analysis to ascertain the suitability of the RP-HPLC profiles for objectively determining stage of maturity or cheese type. A systematic approach was taken to labelling the peaks (or regions) to provide consistency across the range of cheeses at all stages of maturity. The RP-HPLC chromatogram was divided into 48 peaks or regions (Fig. 7.34). In some instances a peak eventually became so large that it engulfed one or two of the surrounding peaks and in this case that area was defined as a region. In this eventuality, the peaks in that region in the earlier traces were summed to enable the changes in the region to be measured over the ripening period. In each case, all of the peaks that were summed had gradually increased during ripening.

The area of each of the 48 peaks or regions obtained for each cheese sample was determined and the data analysed using the correlation and covariance forms of PCA.

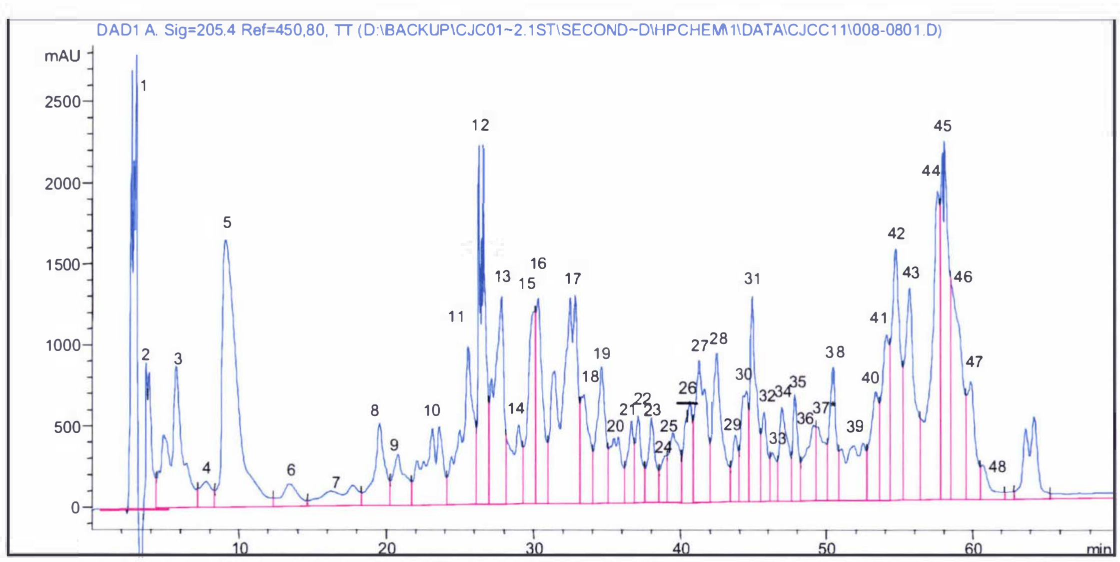


Fig. 7.34 *RP-HPLC trace of the WSF of Cheddar cheese with the 48 peaks labelled.*

7.3.3.4.1 PCA of the correlation matrix (RP-HPLC/WSF)

Fig. 7.35 depicts PCA loading plots for the peaks or regions resulting from RP-HPLC analysis of the WSF. Fig. 7.36 shows 2-dimensional graphical representations of the relationship between PC1, PC2 and PC3 for each sample. Tables related to PCA of the correlation matrix, scores for PC1 (54%), PC2 (22%) and PC3 (7%) and a 3-dimensional graph can be found in the appendix (section 7.8.4.1).

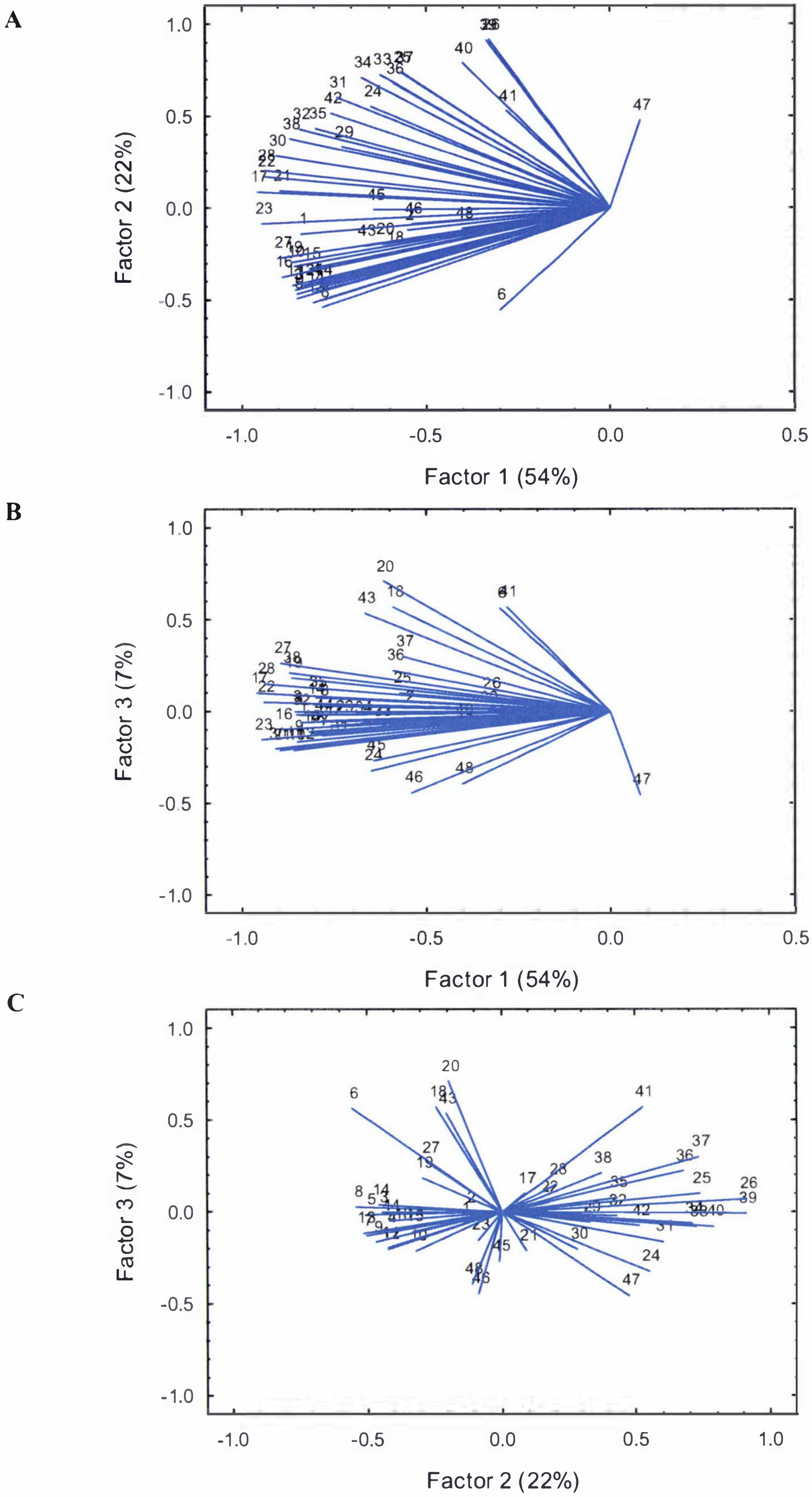


Fig. 7.35 *RP-HPLC (WSF) data analysis using PCA (correlation matrix) - loading plots.* A) factor 1 v factor 2, B) factor 1 v factor 3 and C) factor 2 v factor 3 for peaks/regions 1-48 from RP-HPLC (WSF) of ripening Cheddar, Elsberg, Gouda, Mozzarella and Swiss cheese.

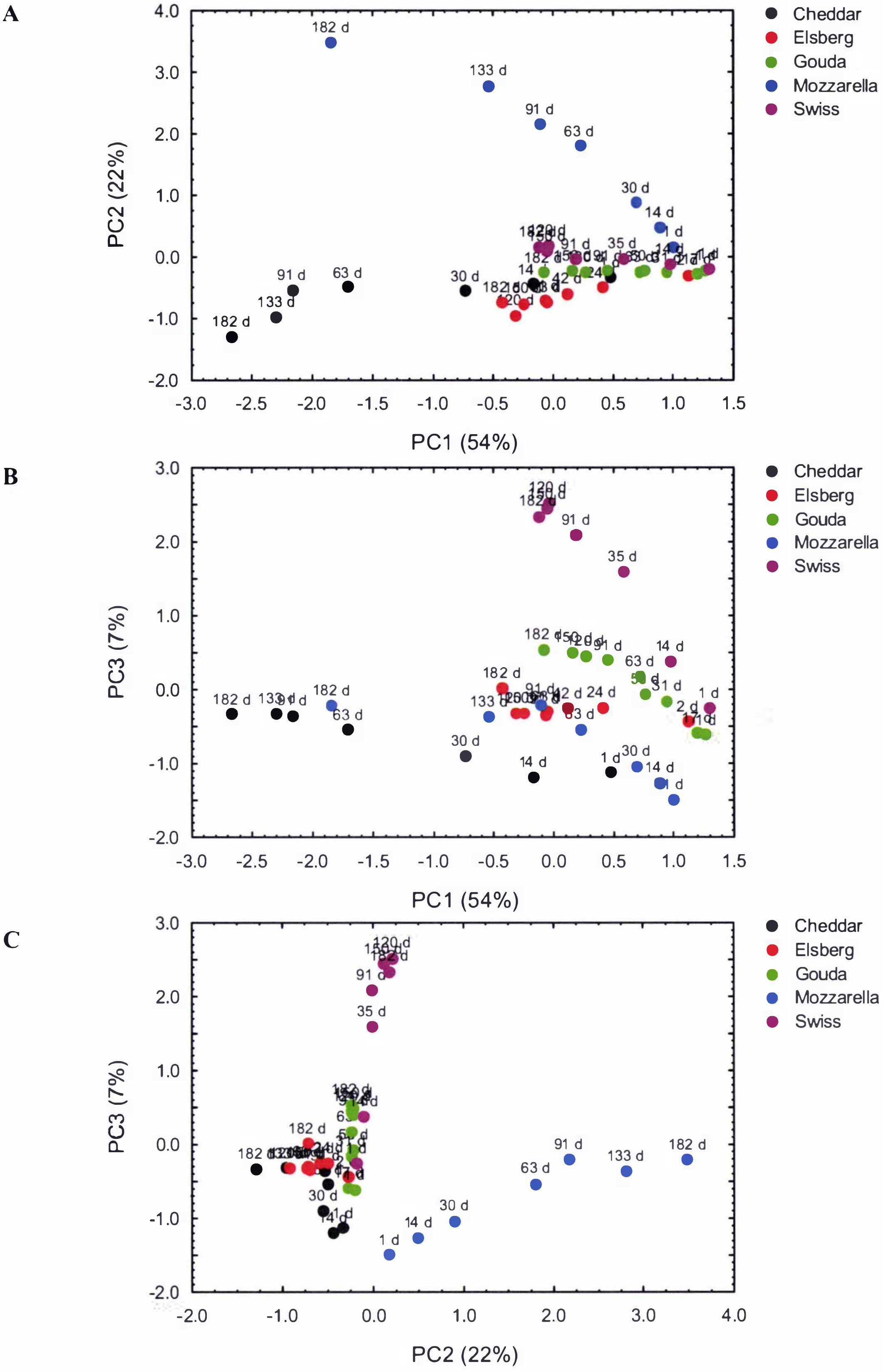


Fig. 7.36 *RP-HPLC (WSF) data analysis using PCA (correlation matrix) - score plots.* A) PC1 v PC2, B) PC1 v PC3 and C) PC2 v PC3 for peaks/regions 1-48 from RP-HPLC (WSF) of ripening Cheddar, Elsberg, Gouda, Mozzarella and Swiss cheese. For a 3-D plot, see appendix, section 7.8.4.1.

The combination of RP-HPLC analysis of the WSF and data analysis using the correlation form of PCA accounted for 76% of the variation in the data within the first two principal components, with the third principal component contributing a further 7%. A comparison of the loading and score plots (Figs. 7.35 and 7.36) showed that PC1 (54%) was associated with maturity, with most peaks contributing. PC2 (22%) was associated with the maturity of the Mozzarella cheese, as well as differentiation of the Mozzarella from the other cheese types, with the peaks being approximately divided into two groups, that contained early and later-eluting peaks, respectively. PC3 (7%) was most closely associated with the early stages of maturation of the Swiss cheese, as well as the differentiation of some cheese types.

Differentiation of maturity was very good for both the Mozzarella and Cheddar cheese, with the data points being well separated throughout ripening. PC1 and PC3 to a lesser extent (Fig. 7.36A and B) provided differentiation of the maturity of the Cheddar cheese, with most peaks contributing (Fig. 7.35A). The combination of PC1 and PC3 also provided good differentiation of the maturity of the Gouda cheese, but the data points were closer together than the Cheddar data points. PC1 and PC2 provided differentiation of the maturity of the Mozzarella cheese, with many of the later-eluting peaks, peaks 24-26 and 29-42, influencing the separation.

The correlation form of PCA was less useful for determining the maturity of the Elsberg or Swiss cheese. In these cheese types, maturity trends were only evident in the early stages of maturation, (Fig. 7.36A, B and C), and in the later stages the data points were close together. An examination of the peak data (Ch. 7, section 7.8.4) showed that most peaks closely associated with the Elsberg maturity, peaks 3-9 and 11-13 (Fig. 7.35A), or Swiss cheese maturity, peaks 6, 18, 20, 41 and 43 (Fig. 7.36B and C), increased rapidly in area for the first 120-150 days and then remained constant or decreased in area.

The combination of PC1 and PC2 enabled clear separation of the Mozzarella cheese from the other cheese types, which were closely grouped (Fig. 7.36A). Separation of the Mozzarella cheese was influenced mostly by later-eluting peaks, as could be expected in a cheese with little microbial enzyme activity. The other cheese types were closer together, with their position influenced by the early-eluting peaks comprised of amino acids and small hydrophilic peptides, as could be expected in cheeses with more extensive enzyme activity. The combination of PC1 and PC3 (Fig. 7.36B) enabled clear differentiation of the Swiss cheese, with peaks 6, 18, 20, 41 and 43 influencing the differentiation. The Gouda and Elsberg cheeses were less well separated from one another. Fig. 7.36 and the 3-D plot (see appendix, section 4.8.4.1) show reasonable differentiation of cheese type in the later stages of maturation.

In summary, analysis of the RP-HPLC (WSF) data using the correlation form of PCA,

- provided differentiation of the maturity of the Cheddar, Mozzarella and Gouda cheese, and the Swiss and Elsberg cheese in the early stages of maturation, and
- provided reasonable differentiation of cheese type, although the Gouda and Elsberg cheese were close to one another.

The clear segregation of the Mozzarella cheese from the other cheese types (Fig. 7.36 A and C) and the large variation in the area of some influential Mozzarella peaks, suggested that the Mozzarella data was influencing the results for the other cheese types. In order to determine the effect of the Mozzarella data on the separation of the other cheese types it was removed from the analysis.

Fig. 7.37 depicts the loading plot for factors 1 and 2, while Fig. 7.38 depicts the score plots of the first three principal components. Tables related to PCA of the correlation matrix, scores for PC1 (66%), PC2 (12%) and PC3 (6%) and a 3-dimensional graph can be found in the appendix (section 7.8.4.2).

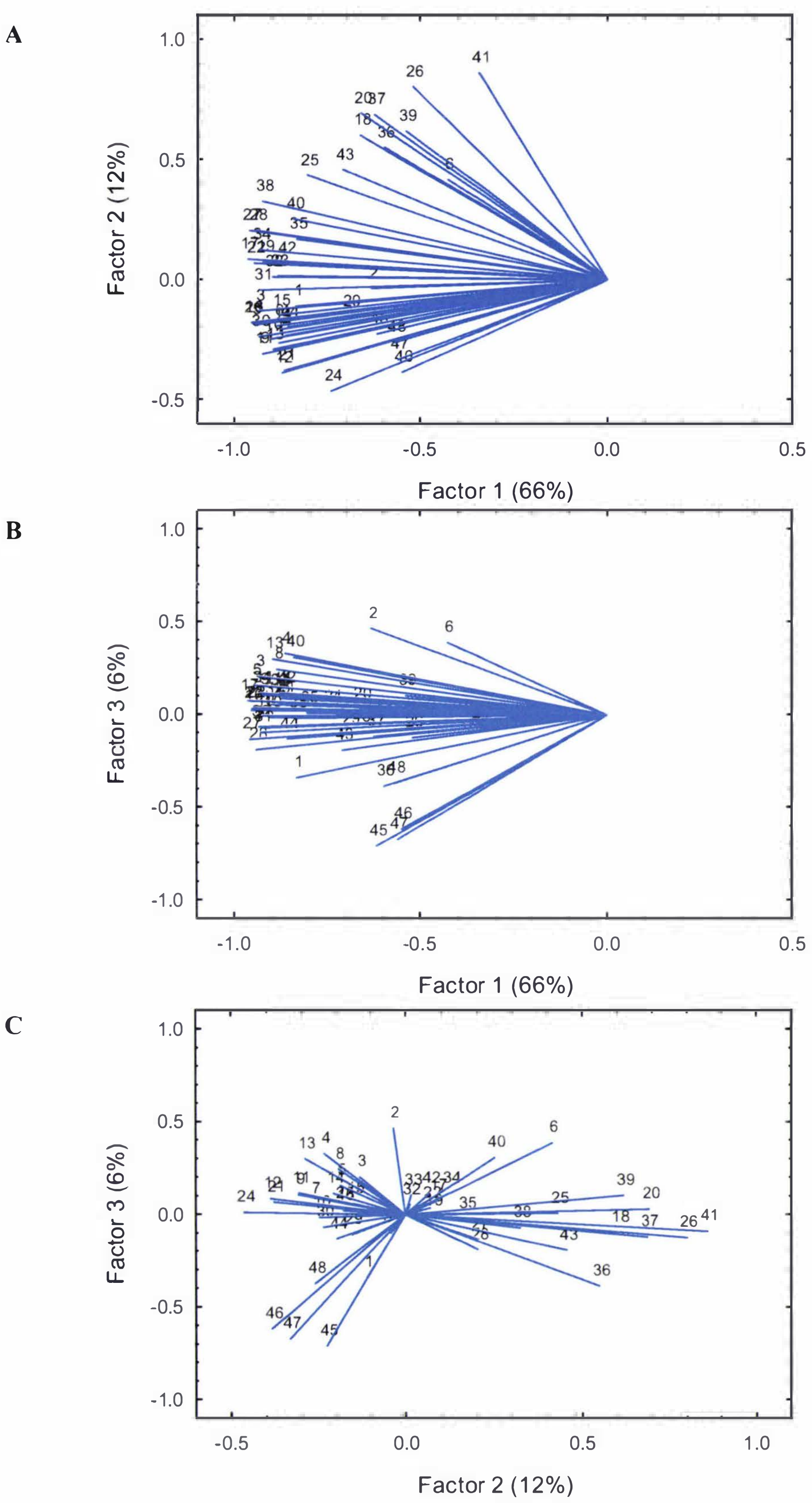


Fig 7.37 *RP-HPLC (WSF) data (minus Mozzarella) analysis using PCA (correlation matrix) - loading plots.* A) factor 1 v factor 2, B) factor 1 v factor 3 and C) factor 2 v factor 3 for peaks/regions 1-48 from RP-HPLC (WSF) of ripening Cheddar, Elsberg, Gouda and Swiss cheese.

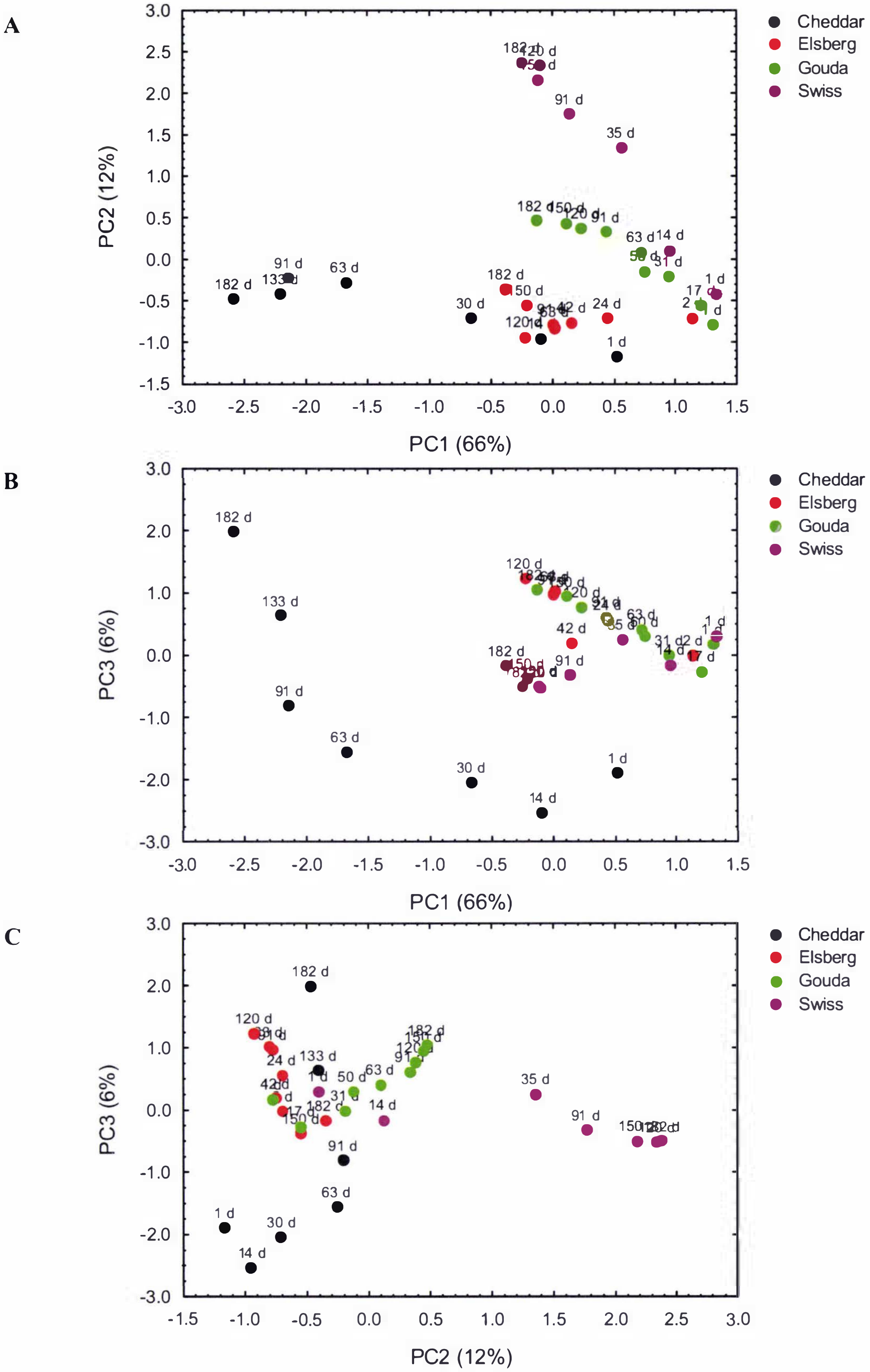


Fig. 7.38 *RP-HPLC (WSF) data (minus Mozzarella) analysis using PCA (correlation matrix) - score plots.* A) PC1 v PC2 B) PC1 v PC3 and C) PC2 v PC3 for peaks/regions 1-48 from RP-HPLC (WSF) of ripening Cheddar, Elsberg, Gouda and Swiss cheese. For a 3-D plot, see appendix, section 7.8.4.2.

The score plots (Fig. 7.38) showed that PC1 (66%) was associated with cheese maturity, while PC2 (12%) and PC3 (6%) were associated with both maturity and differentiation according to cheese type.

Removal of the Mozzarella data had only a small effect on differentiation of cheese maturity. In the previous analysis (Fig. 7.36A), peaks that influenced PC2 had the greatest variation in the Mozzarella cheese. Removal of the Mozzarella peak data resulted in a plot of PC1 v PC2 (Fig. 7.38A) that was very similar to the original plot of PC1 v PC3 (Fig. 7.36B). PC1 v PC3 (Fig. 7.38B) provided good differentiation of the Cheddar cheese maturity. PC1 v PC2 (Fig. 7.38A) provided reasonable differentiation of the Gouda cheese maturity and enabled the Swiss cheese maturity to be determined in the early stages of maturation. Peaks 6, 18, 20, 26, 36, 37, 39 and 41 were closely associated with Swiss cheese maturity, and during the first 120 days they increased in area markedly and the data points were well separated. After either 120 days or 150 days, most of these peaks decreased or remained constant in area and the data points were close to one another (Fig. 7.38).

PC1 and PC2 provided reasonably good differentiation of cheese type (Fig. 3.38A), with the Cheddar and Swiss cheese being well separated from the Gouda and Elsberg cheese. This separation was improved when all three principal components were taken into consideration.

In summary, after removal of the Mozzarella cheese data, analysis of the remaining RP-HPLC (WSF) data using the correlation form of PCA provided:

- reasonable differentiation of cheese type; and
- was only suitable for determining the maturity of Swiss cheese in the early stages of maturation, but the combination of PC1 and PC3 provided a good assessment of the maturity of Cheddar and Gouda cheese. Differentiation of the maturity of Elsberg cheese was poor.

7.3.3.4.2 PCA of the covariance matrix (RP-HPLC/WSF)

Fig. 7.39 depicts PCA loading plots for peaks and regions resulting from RP-HPLC analysis of the WSF of each cheese. Fig. 7.40 shows 2-dimensional graphical representations of the relationship between PC1, PC2 and PC3 calculated for each sample. Tables related to PCA of the covariance matrix, scores for PC1 (47%), PC2 (30%) and PC3 (8%) and a 3-dimensional graph can be found in the appendix (section 7.8.4.3).

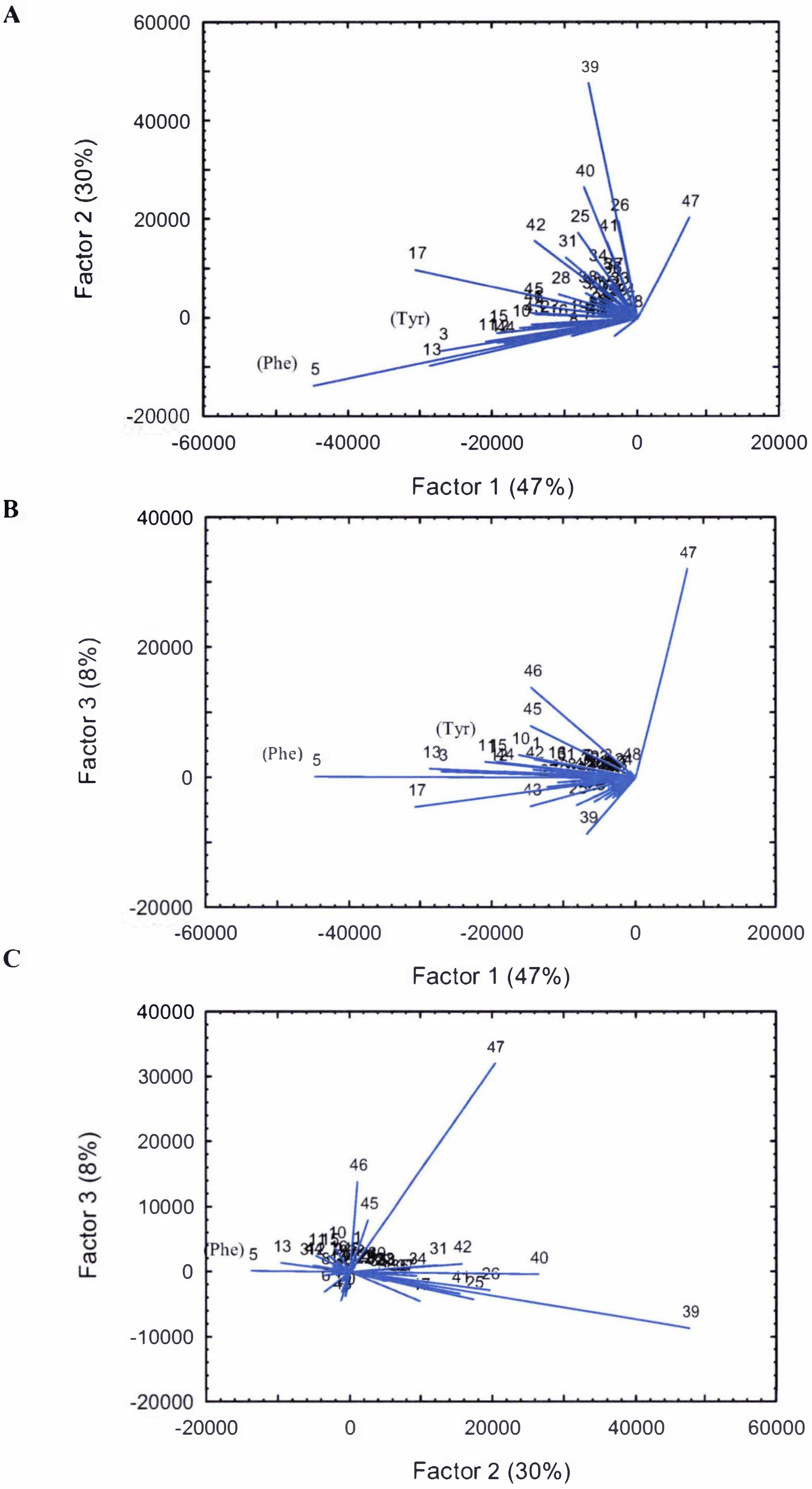


Fig. 7.39 *RP-HPLC (WSF) data analysis using PCA (covariance matrix) - loading plots.* A) factor 1 v factor 2, B) factor 1 v factor 3 and C) factor 2 v factor 3 for peaks/regions 1-48 from RP-HPLC (WSF) of ripening Cheddar, Elsberg, Gouda, Mozzarella and Swiss cheese.

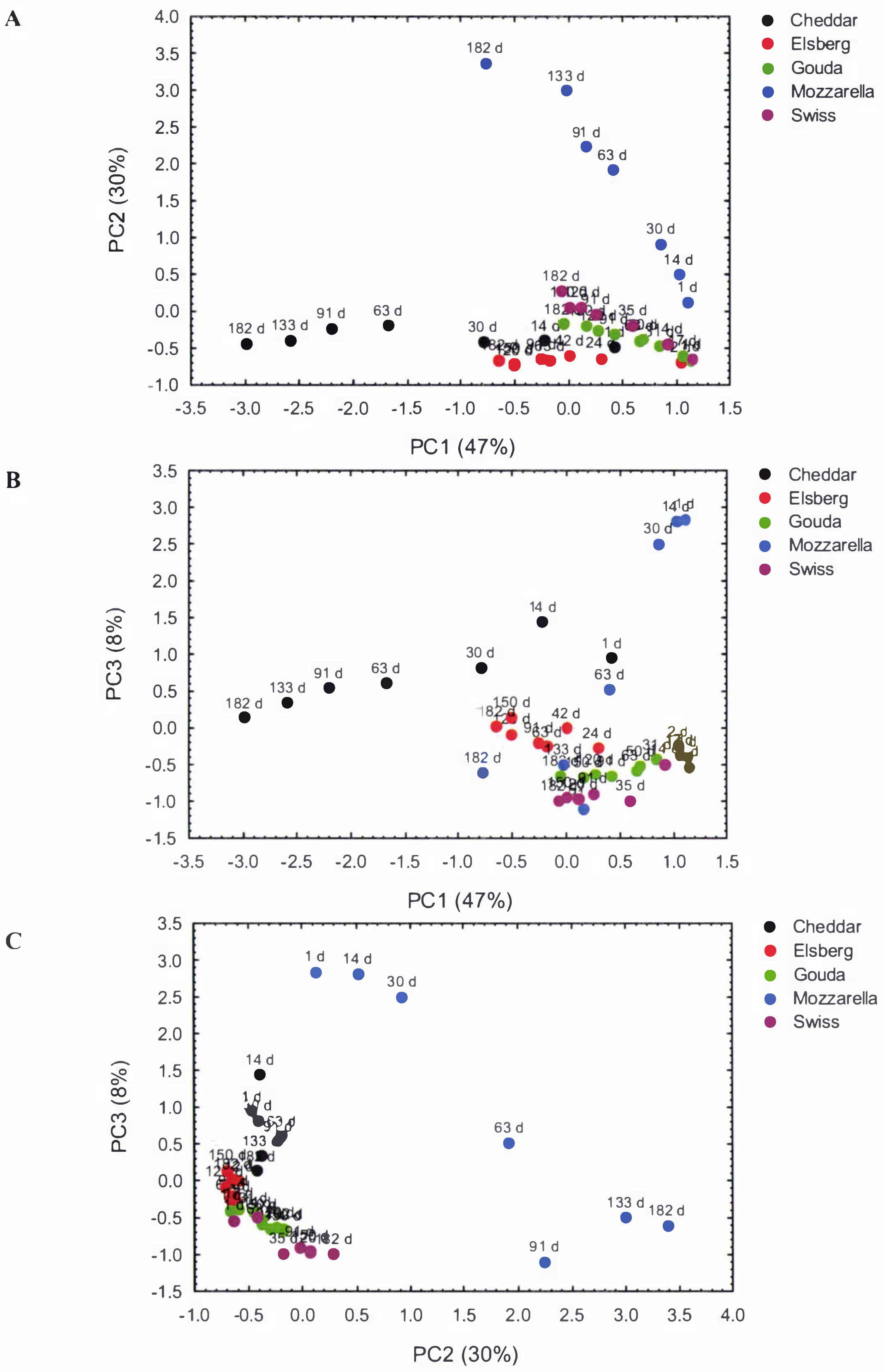


Fig. 7.40 *RP-HPLC (WSF) data analysis using PCA (covariance matrix) - score plots.* A) PC1 v PC2, B) PC1 v PC3 and C) PC2 v PC3 for peaks/regions 1-48 from RP-HPLC (WSF) of ripening Cheddar, Elsberg, Gouda, Mozzarella and Swiss cheese. For a 3-D plot, see appendix, section 7.8.4.3.

Analysis of the RP-HPLC (WSF) data set using the covariance form of PCA provided an effective analysis, with the first three principal components accounting for 85% of the variation in the data. PC1 (47%) was closely associated with the maturity of each cheese type (Fig. 7.40A), with the predominant peaks being 3 (Tyr), 5 (Phe), 13 and 17 (Fig. 7.39A), with Phe the most dominant. Tyr and Phe are the end-products of proteolysis in cheese where starter and adventitious bacteria are present. PC2 (30%) was associated with the maturity of the Mozzarella cheese and differentiation of the Mozzarella from the remaining cheese types (Fig. 7.40A), with the predominant peaks being peaks 25, 26, 39--42 and 47. PC3 (8%) was also associated mainly with Mozzarella cheese maturity and differentiation (Fig. 7.40C).

Differentiation according to maturity was very good for the Cheddar and Mozzarella cheese with the data points well spread in accordance with the stage of maturity (Fig. 7.40A). PC1 played the major role in the differentiation of the maturity of the Cheddar and Elsberg cheese. PC1, with a minor contribution from PC2, influenced the direction of maturity of the Gouda and Swiss cheese, while PC1, PC2 and PC3 influenced the direction of maturity of the Mozzarella cheese. The data points for the Gouda, Swiss and Elsberg cheese were close together in the later stages of maturity, when the rate of production of key peptides slowed. The small distance between the data points of the maturing Gouda, Swiss or Elsberg cheese, was also influenced by the large variation in area of key peaks (peaks 3, 5, 13 and 17) in the Cheddar cheese that were associated with maturity.

Mozzarella, Elsberg and Cheddar cheese could be differentiated according to type on the basis of PC1 and PC2 (Fig. 7.40A), although the Cheddar differentiation was best on the basis of PC2 and PC3 (Fig. 7.40C). Peak 5 (Phe), was largest in the Cheddar cheese (see Fig. 7.10 – 7.14 for chromatographs and Ch. 7, section 7.8.4 for peak data) and this accounted for its separation from the other cheese types. The rate of decrease of α_{s1}- and α_{s1}-I-casein (Fig. 7.3A) suggested there was greatest chymosin activity in the Cheddar cheese. Chymosin exposes Phe, at the C-terminus (and sometimes the N-terminus) of many of the peptides that result from its action, to the enzymes of the cheese microflora. Differentiation of the Gouda and Swiss cheese was not possible on the basis of PC1, PC2 or PC3 (Fig. 7.40A, B and C).

In summary, RP-HPLC of the WSF with data analysis using the covariance form of PCA,

- was useful for differentiating the maturity of the Mozzarella and Cheddar cheese throughout maturation, and the Gouda, Elsberg and Swiss cheese in the early stages of maturation; and
- was able to differentiate the Mozzarella, Cheddar and Elsberg cheese from the other cheese types, but provided only poor differentiation of the Gouda and Swiss cheese.

The dominance of peak 39 in the loading plot (Fig. 7.39A) and the Mozzarella cheese in the PCA score plots (Fig. 7.40 A and C) suggested the possibility of a clearer division of the other cheese types if the Mozzarella cheese data was removed from the analysis.

Fig. 7.41 depicts loading plots for factors 1, 2 and 3, while Fig. 7.42 shows 2-dimensional graphical representations of the relationship between PC1, PC2 and PC3 calculated for each sample after the Mozzarella peak data was removed. Tables related to PCA of the covariance matrix, scores for PC1 (73%), PC2 (10%) and PC3 (8%) and a 3-dimensional graph of PC1, PC2 and PC3 can be found in the appendix (section 7.8.5.4).

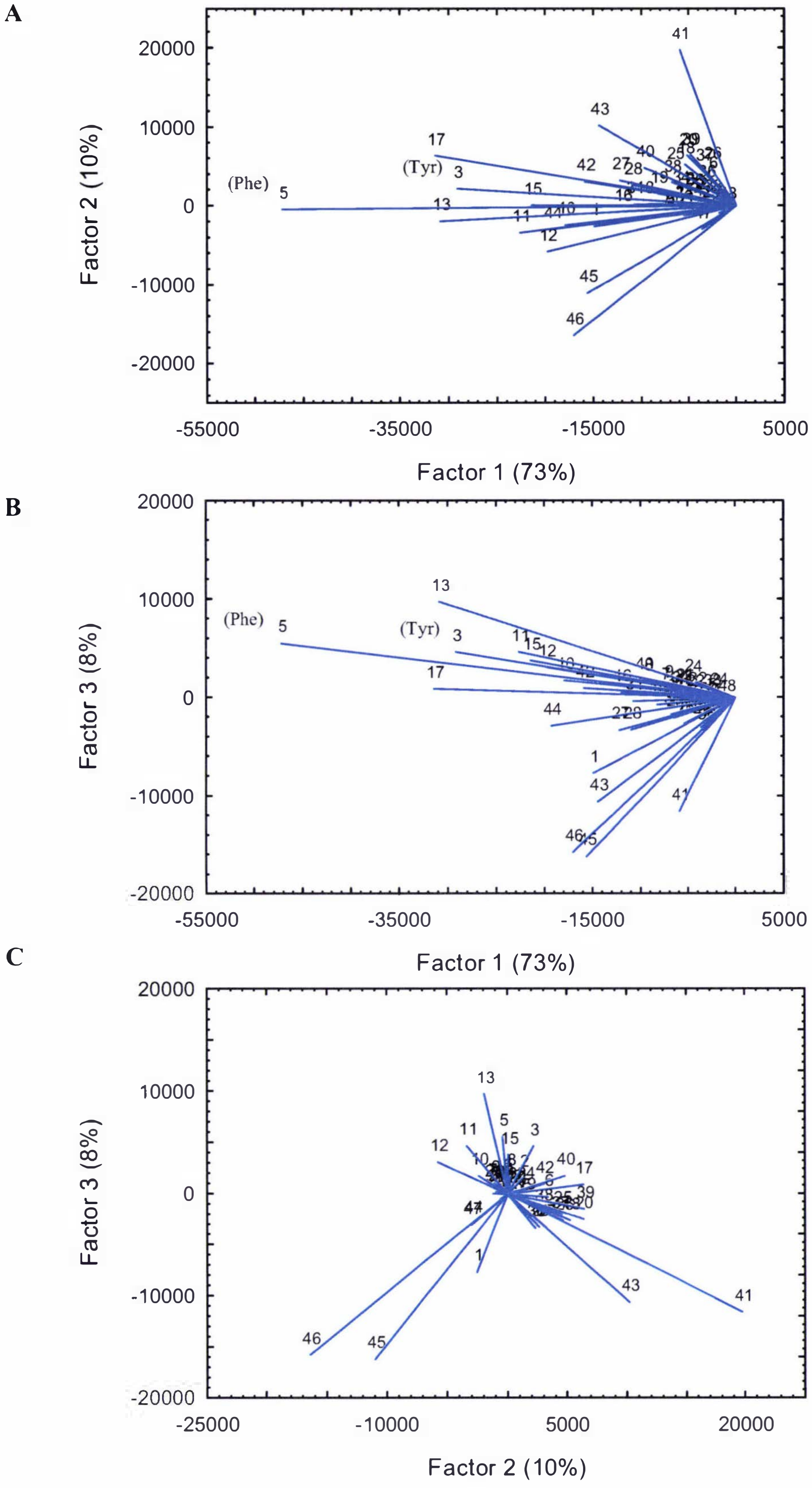


Fig. 7.41 *RP-HPLC (WSF) data (minus Mozzarella) analysis using PCA (covariance matrix) – loading plots.* A) factor 1 v factor 2, B) factor 1 v factor 3 and C) factor 2 v factor 3 for peaks/regions 1-48 from RP-HPLC (WSF) of ripening Cheddar, Elsberg, Gouda and Swiss cheese.

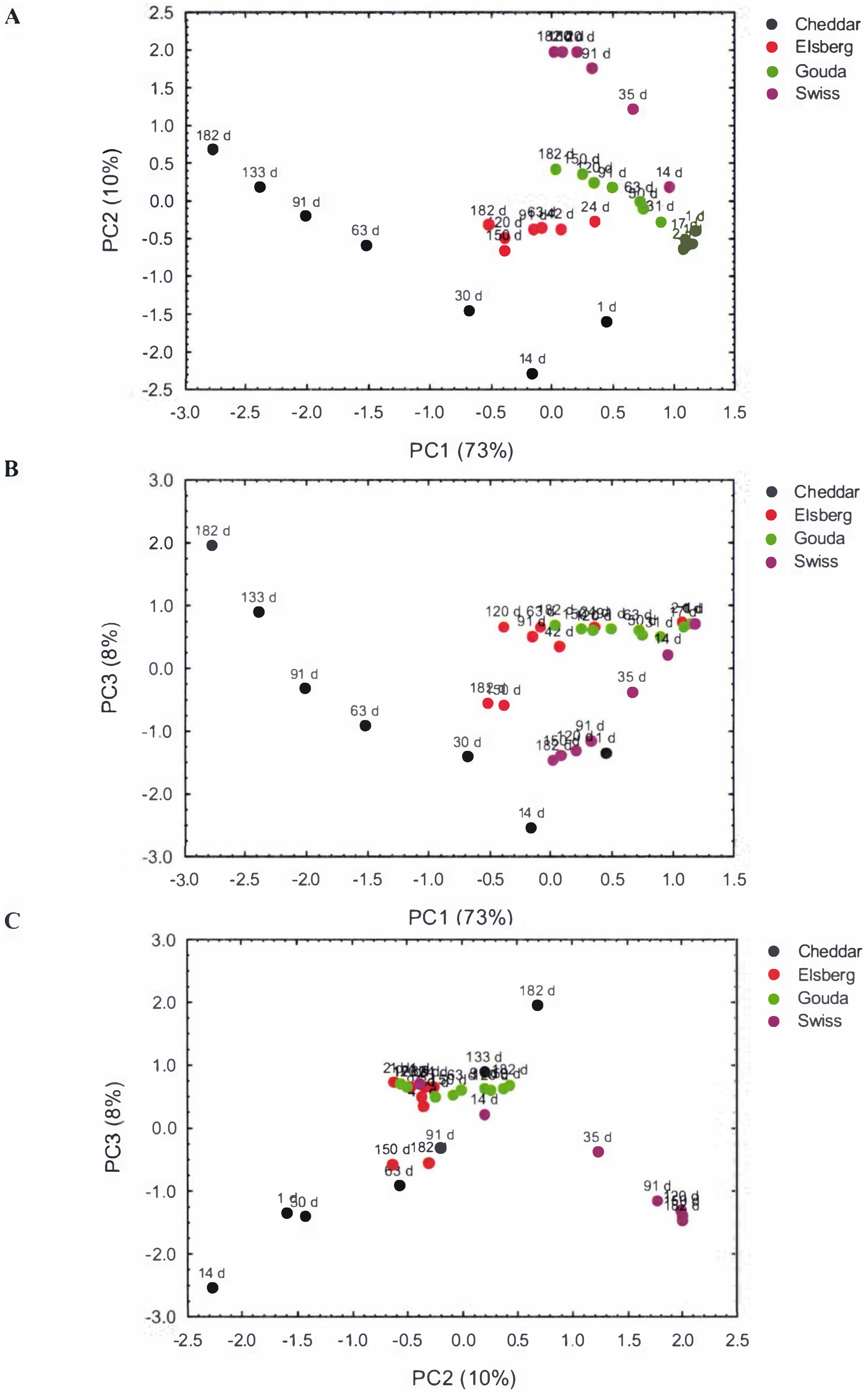


Fig. 7.42 *RP-HPLC (WSF) data (minus Mozzarella) analysis using PCA (covariance matrix) – score plots.* A) PC1 v PC2, B) PC1 v PC3 and C) PC2 v PC3 for peaks/regions 1-48 from RP-HPLC (WSF) of ripening Cheddar, Elsberg, Gouda and Swiss cheese. For a 3-D plot, see appendix, section 7.8.4.4.

The removal of the Mozzarella cheese data removed the dominance of peak 39, which because of its size in the Mozzarella cheese, was the major peak influencing PC2 and differentiation of the other cheese types.

The combination of PC1 and PC2 enabled clear differentiation of the Cheddar, Elsberg, Gouda (after 17 days), and Swiss cheese (Fig. 7.42A), with peaks 41 and 43 (Fig. 7.41A) responsible for differentiation of the Swiss cheese.

The main peaks associated with maturity remained unchanged and were peaks 5 (Phe), 3 (Tyr), 17 and 13 (Fig. 7.41A). However, removal of the Mozzarella data removed the influence of peak 39, a particularly large peak in the Mozzarella cheese, and a major influence on PC2. This enabled PC2 to play a larger role in differentiation of maturity of the Cheddar, Gouda and Swiss cheese. The combination of PC1 and PC2 (Fig. 7.42A) enabled clear differentiation of the Cheddar and Gouda maturity, clear differentiation of the Swiss cheese maturity for the first 120 days, and did not alter the Elsberg maturity trend.

The Cheddar was clearly separated from the other cheese types (Fig. 4.42B), and had comparatively high PC1 scores (Fig. 4.42A). PC1 was influenced mainly by peak 5 (Phe) (Fig. 4.41A and B), which was particularly large in the Cheddar cheese. It seemed likely that the removal of the Cheddar data would improve the differentiation of maturity of the remaining cheese types.

The Elsberg, Gouda and Swiss cheese peak data were analysed using PCA of the covariance matrix. Peak 5 (Phe) remained the main determinant of cheese maturity (PC1 59%) (Fig. 7.43A and B) and, in the absence of the Cheddar data, provided clear maturity trends for each cheese type (Fig. 4.44A). Differentiation of the Swiss cheese maturity and type was assisted by PC2 (27%) (Fig. 4.44A) and influenced by peaks 41 and 43 (Fig. 4.43A). Differentiation of the Gouda cheese maturity was assisted by PC3 (8%) (Fig. 4.44B) and influenced by peaks 17 and 42 (Fig. 4.43B). As well, each cheese type was clearly differentiated from the other cheese types (Fig. 4.44A).

In summary, a stepwise approach to analysis of the RP-HPLC peak data using the covariance form of PCA, with removal of data pertaining to cheeses clearly differentiated from the remaining cheese types,

- improved the differentiation of maturity for the remaining cheese types and enabled clear maturity trends to be established.
- enabled the clear differentiation of cheese type.

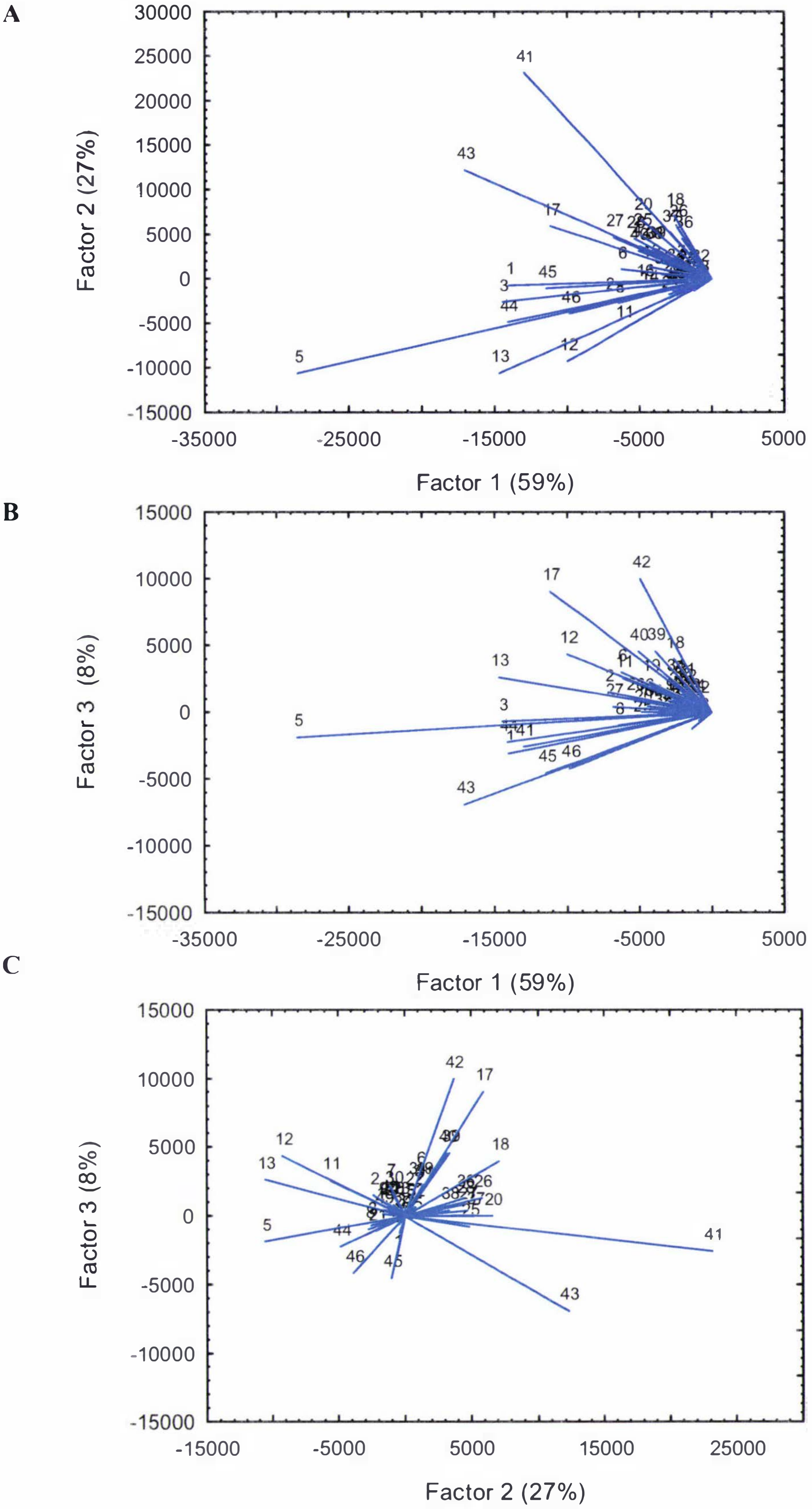


Fig. 7.43 *RP-HPLC (WSF) data (minus Mozzarella and Cheddar) analysis using PCA (covariance matrix) – loading plots.* A) factor 1 v factor 2, B) factor 1 v factor 3 and C) factor 2 v factor 3 for peaks/regions 1-48 from RP-HPLC (WSF) of ripening Elsberg, Gouda and Swiss cheese.

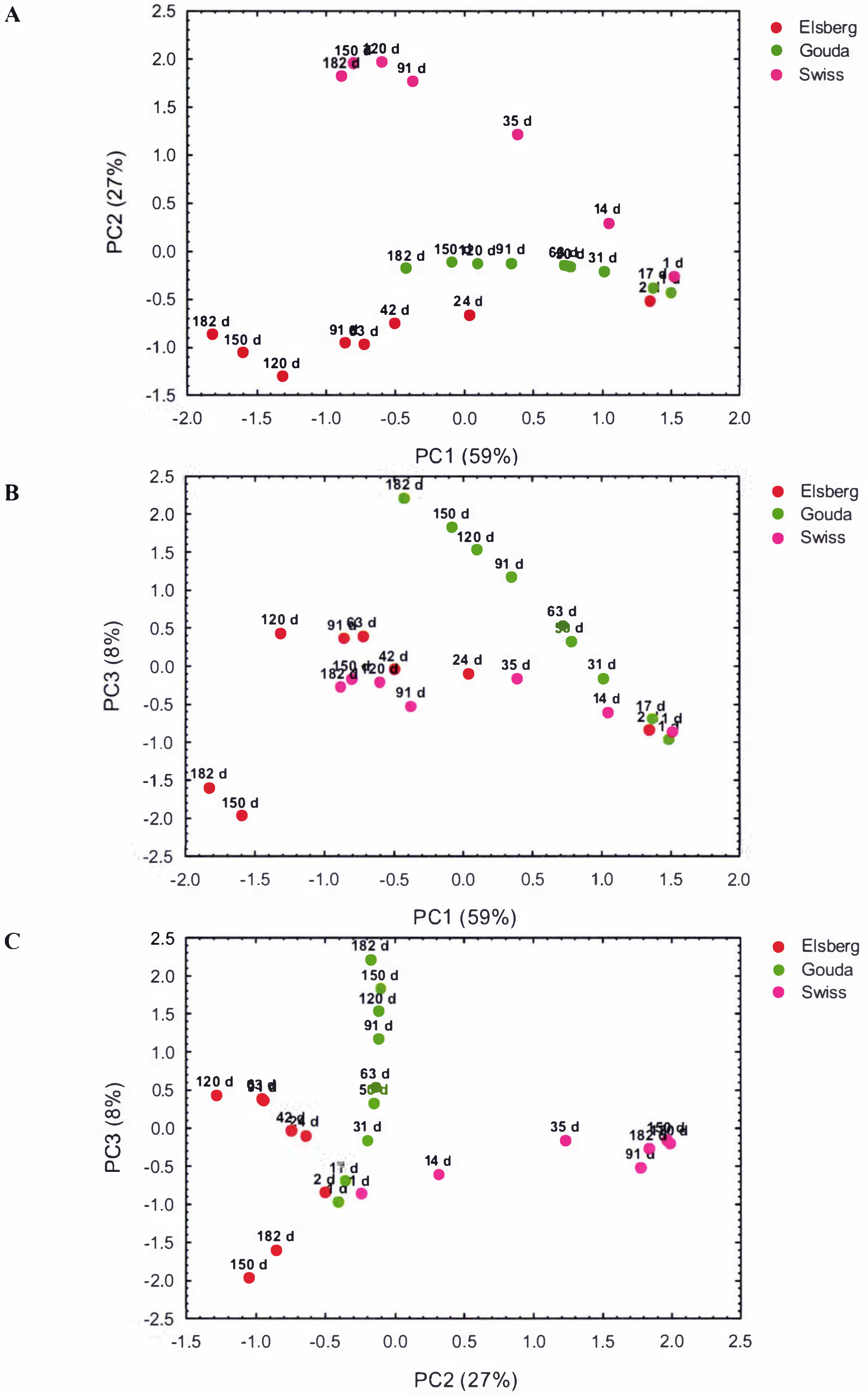


Fig. 7.44 *RP-HPLC (WSF) data (minus Mozzarella and Cheddar) analysis using PCA (covariance matrix) – score plots.* A) PC1 v PC2, B) PC1 v PC3 and C) PC2 v PC3 for peaks/regions 1-48 from RP-HPLC (WSF) of ripening Elsberg, Gouda and Swiss cheese. For a 3-D plot, see appendix, section 7.8.4.5.

7.5 DISCUSSION

Each of the five cheese types examined in the current study, Cheddar, Elsberg, Gouda, Mozzarella and Swiss, has a unique combination of flavour, texture and appearance that develops during ripening and differentiates it from the other cheese types. This differentiation is initiated by the cheese-maker who manipulates manufacturing variables (Table 7.1) to achieve a particular cheese composition (protein, fat, salt, moisture, S/M, lactose and mineral contents and pH) (Table 7.2) and differences in the complement and concentration of the enzymes present (rennet, indigenous enzymes such as plasmin and Cathepsin D, and the enzymes of the starter, secondary microflora and NSLABs). During ripening, the action of these enzymes is modulated by cheese composition and storage regimen and is largely responsible for the development of cheese texture and the production of amino acids which may add to cheese flavour or be precursors of cheese flavour compounds.

7.5.1 Differentiation of cheese maturity and type

The methods used to monitor the transformation from caseins to progressively smaller peptides and amino acids have different modes of protein and peptide separation and capture different parts of the proteolytic process (see Fig. 7.45).

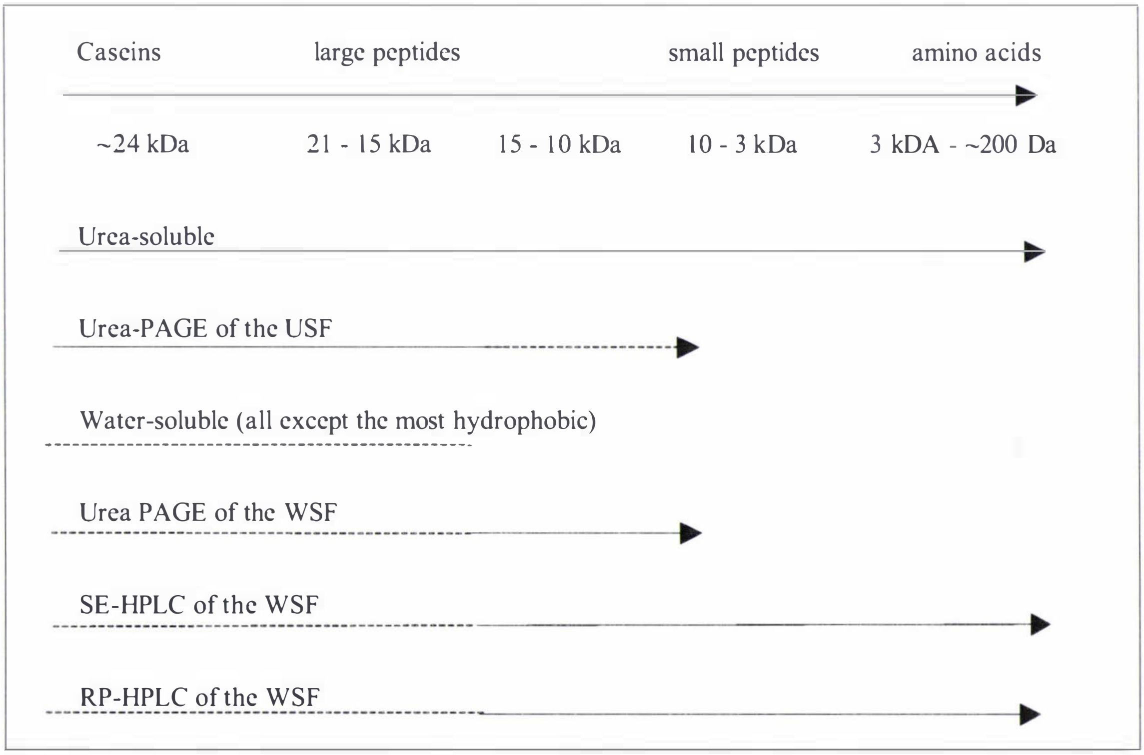


Fig. 7.45 *Methods for monitoring the hydrolysis of caseins to peptides and amino acids* - a generalised diagram.

Two cheese fractions were analysed (Fig. 7.45), the USF, which contains all of the cheese proteins, peptides and amino acids, and the WSF, recommended by Kuchroo and Fox (1982) and Bütikofer et al (1996), which contains those peptides soluble in water under the particular extraction conditions. The samples from each cheese type were extracted in the same way, with the quantity of cheese, and the time and temperature of extraction strictly adhered to. Each of these factors influences the amount of material extracted, although only the casein to water ratio has a large effect (Polychroniadou, 1999). According to Bütikofer et al (1992), the amount of material extracted into the WSF is not influenced by pH provided the cheese pH is less than 6.0.

7.5.1.1 Urea-PAGE of the USF with peak data analysis using PCA

Urea-PAGE of the USF (Fig. 7.2) provided a very good assessment of primary proteolysis in each cheese type throughout maturation. This method enabled the chymosin-mediated hydrolysis of α_{s1}-casein to α_{s1}-I-casein and α_{s1}-casein f99-199, and β-casein to β-I- and β-II-casein, and the plasmin-mediated hydrolysis of β-casein to γ_1-, γ_2- and γ_3-casein to be monitored during ripening (Fig. 7.2). Graphs depicting trends in casein hydrolysis and peptide formation (Fig. 7.3A,B) showed both similarities and differences between cheese types. The order of greatest chymosin activity was Elsberg > Cheddar > Gouda ≈ Swiss > Mozzarella cheese. The order of greatest plasmin activity was Elsberg ≈ Swiss > Gouda ≈ Mozzarella > Cheddar cheese.

The results were complex and it was difficult to subjectively differentiate the cheese with confidence. In fact, McGoldrick and Fox (1999) had found, after subjective analysis, that urea-PAGE of the water-insoluble fraction of cheese was not well-suited to differentiating random samples of Cheddar and a range of British-, Dutch-, Swiss- and Italian-type cheeses.

Objective analysis of the urea-PAGE peak area data was provided by PCA. The correlation and covariance forms of PCA (Figs. 7.17 and 7.19, respectively) provided effective analysis of the data, with the first three principal components accounting for 79% (PC1 56%, PC2 13% and PC3 10%) and 97% (PC1 86%, PC2 8% and PC3 3%) of the variation in the data, respectively. The scores for PC1 decreased with maturity and enabled a maturity trend line to be established for each cheese type. The direction of each maturity trend line was influenced by particular peaks associated with PC2 and PC3.

Standardisation of the peaks to the same variance prior to analysis using the correlation form of PCA enabled the twelve peaks to contribute equally to the analysis and differentiation, whereas the use of a mean-centred data set for the covariance form of PCA meant that the original peak area variance was retained and β-, α_{s1}-, α_{s1}-I- and γ_1-casein, which had the greatest variance, contributed most to the analysis and differentiation.

The correlation form of PCA (Figs. 7.16 and 7.17) provided a reasonable assessment of the maturity of young cheese and cheese in which proteolysis was slow, and provided differentiation of each cheese type after the first few days of maturation. Differentiation of maturity was based on events associated with primary proteolysis and was possible for cheese types such as Gouda and Mozzarella that had undergone limited proteolysis during the ripening period (Fig. 7.17), and Cheddar, Elsberg and Swiss cheeses during the early stages of ripening. However as proteolysis in the latter cheese types progressed, the substrates were depleted and the rate of formation of some peptides slowed or they were hydrolysed (e.g. α_{s1}-I-casein). This caused the sample points to be close to one another, making the method unsuitable for monitoring maturity. The divergence of the maturity trend lines (from PC2 = 0) (Fig. 7.17) was influenced by peaks associated with PC2, and provided differentiation of cheese type. The rapid plasmin-mediated hydrolysis of β-casein and formation of the γ-caseins was responsible for the direction of the Swiss cheese maturity trend, while the rapid chymosin-mediated hydrolysis of α_{s1}-casein and formation of α_{s1}-casein-I and α_{s1}-casein f99-199 was responsible for the direction of the maturity trends of the Cheddar. The Gouda and Mozzarella maturity trends were clustered about the point where PC2 = 0 because both plasmin and chymosin played an important role in their ripening. These cheese types were differentiated by peaks contributing to PC3.

The covariance form of PCA (Figs. 7.18 and 7.19) (PC1 86%) enabled very good maturity trend lines to be established during the early stages of maturation of the Cheddar, Elsberg and Swiss cheese and throughout the maturation of the Mozzarella and Gouda cheese. The distance between sample points could be explained in terms of the influence of the cheese environment on the action of chymosin on α_{s1}-casein and plasmin on β-casein in the cheese. This finding was similar to the more recent finding of Pripp et al (2000a) who used the covariance form of PCA to demonstrate changes related to incubation time that were attributed mainly to the hydrolysis of α_{s1}-casein to α_{s1}-I-casein and α_{s1}-casein f102-199 in sodium caseinate solutions treated with chymosin and extracts of different lactococcal strains. In the later stages of maturation, following the depletion of α_{s1}-casein, the primary products (α_{s1}-I- and γ-casein) were particularly important for separating the data points and resulted in a curve in the maturity trend lines of the Cheddar and Elsberg cheese (and the 182 day old Gouda). The data points of the Swiss cheese were clustered from 120 days, reflecting the relatively small amount of proteolytic activity occurring in the cheese after this point, and the inability of the method to detect any of the small peptides normally produced in this cheese type. The directions of the maturity trend lines were dictated by differences between cheese types in the rates of hydrolysis of α_{s1}-casein by chymosin and β-casein by plasmin (Fig. 7.3A). The (+ve) divergence of the Cheddar maturity trend line (from PC2 = 0) was associated mainly with the rapid hydrolysis of α_{s1}-casein; the central location of the Elsberg trend line was associated with the rapid hydrolysis of both α_{s1}-casein and β-casein, with the initial wide spread of the sample points resulting from the rapid hydrolysis of these caseins during the period the cheese was ripened at high temperatures; the central location of the Gouda trend line was associated with more moderate rates of hydrolysis of both α_{s1}- and β-casein; the (-ve) divergence of the Swiss and Mozzarella cheese (from PC2 = 0) were mainly associated with the hydrolysis of β-casein. A small number of key peaks were important in all cheese types, making the method less suitable than the correlation form of PCA for differentiating cheese type. The Cheddar, Elsberg and Gouda cheese were clearly separated from each other as well as the other cheese types, but the Swiss and Mozzarella cheese was less well separated.

The method of sample analysis rather than the method of data analysis provided a limitation to the effectiveness of urea-PAGE and PCA for assessing cheese maturity. That is, urea-PAGE is well-suited to monitoring primary proteolysis in each cheese type, but ceases to be useful in the later stages of maturation of cheese types in which secondary proteolysis is an important feature. And in fact, this is the reason such techniques as RP-HPLC or urea-PAGE of the WSF are used for cheese ripening studies (e.g. Aston and Creamer, 1986; McSweeney et al 1993a; Bütikofer et al, 1998).

7.5.1.2 Urea-PAGE of the WSF with peak data analysis using PCA

Urea-PAGE of the WSF (Fig. 7.4) is used for separating the water-soluble peptides in cheese, and many water-soluble peptides have been identified in Cheddar (Law et al, 1993; Singh et al, 1995, 1997). Although peptides with a large net negative charge and those below about 5000 Da pass rapidly through the gel during electrophoresis and are lost or do not stain, this method was proposed by Fox (1993), and a similar method that uses urea-PAGE to examine the ethanol-insoluble fraction of the WSF of cheese was proposed by McGoldrick and Fox (1999), for differentiating cheese type.

An examination of the urea-PAGE gels of the WSF of each cheese type (Fig. 7.4) showed that many peptides captured in the gels were transient intermediates in the proteolytic process and did not accumulate for long. Therefore, the quantities of peptides present on the gel did not adequately represent either the hydrolysis of the initial substrates or the accumulation of final products. As well, there was no common starting point for each cheese type. Therefore, this method of sample analysis did not provide the information necessary to achieve good differentiation of the maturity of most cheese types.

PCA provided a good description of the variation in the data set, with the correlation form (Fig. 7.22) accounting for 72% (PC1 32%, PC2 25% and PC3 15%) and the covariance form (Fig. 7.24) for 92% of variation in the data set (PC1 57%, PC2 28% and PC3 7%). Both forms of PCA showed that urea-PAGE of the WSF provided poor differentiation of cheese maturity and reasonable differentiation of cheese type, in agreement with the visual analysis of Fox (1993) and McGoldrick and Fox (1999).

For most cheese types, the transience of the peptides made it impossible to differentiate cheese maturity using PCA of either the correlation or the covariance matrix (Fig. 7.22 and 7.24). Mozzarella cheese was the exception, with some peptides able to accumulate in the cheese and influence the direction of the maturity trend (peaks 9, 10 and 11 (covariance matrix) and 9-15 (correlation matrix)) because of the low level of proteolytic activity in this cheese type.

The correlation form of PCA provided better differentiation of cheese type than the covariance form of PCA because standardisation of the peak areas to the same variance allowed minor peaks associated with differences between cheese types to influence the differentiation. This form of PCA provided differentiation of the Mozzarella cheese (Fig. 7.22A and C) with peaks 10 and 11 largely responsible, differentiation of the Gouda cheese (Fig. 7.22B andC) with peaks 12 and 15 responsible, and differentiation of the Elsberg and Swiss (Fig. 7.22C) with several peaks contributing, while differentiation of the Cheddar required the combination of PC1, PC2 and PC3 (Fig. 7.22A, B and C) and the 182 day sample could not be differentiated from the Elsberg cheese. Relatively few peaks (peaks 5, 7, 8, 10 and 11) contributed when the covariance form of PCA was used, and differentiation of cheese type was less effective. Peaks 9, 10 and 11 provided differentiation of the Mozzarella cheese and peaks 5 and 7 provided differentiation of the Cheddar cheese (with the exception of the 182 day sample) (Fig. 7.24A). The remaining cheese types were differentiated by the combination of PC1, PC2 and PC3 (Fig. 7.24A, B and C).

This method could be considered reasonable for differentiating cheese type, but it is a method that should be used with caution because of the difficulties of capturing and measuring small quantities of peptides with any degree of accuracy. This method requires a well-defined experimental protocol and very good experimental technique.

7.5.1.3 SE-HPLC of the WSF with peak data analysis using PCA

SE-HPLC is used for monitoring the hydrolysis of whey proteins and caseins but it has not been widely used for the same purpose in cheese. In fact, Haasnoot et al (1989), in search of methods suitable for the assessment of Gouda cheese maturity, reported that SE-HPLC of the WSF (using a TSK G-3000SW and G-2000SW in series with a solvent comprised of 0.1 M sodium sulphate and 0.01 M sodium dihydrogenphosphate (pH6.8)) was unsuitable for examining the WSF of cheese. Wilkinson et al (1992) had more success with SE-HPLC and used it to demonstrate the effects of different commercial enzyme preparations on the peptide profiles of Cheddar cheese. They used an FPLC Superose-12HR column (Pharmacia), and the solvent composition (not published) was 30%

acetonitrile and 0.1% TFA in water (M. Wilkinson, personal communication, 2000). The particular method used in the present study has not previously been used for the study of cheese.

SE-HPLC of the WSF enabled the continuum of the proteolytic process to be represented in a series of peaks that showed the molecular weight distribution of water-soluble peptides in the cheese. These profiles of molecular weight distribution were different for each cheese type and changed in a consistent manner as the cheese matured (Fig. 7.5 – 7.9).

Objective analysis of the peak area data using PCA of either the correlation or the covariance matrix (Fig. 7.27 and 7.31) provided good differentiation of cheese type, with the sample points well spread in accordance with stage of maturation. SE-HPLC therefore provided a significant improvement on urea-PAGE (USF) which did not detect changes in some cheese types in the later stages of maturation.

The covariance form of PCA provided superior data analysis, with 90% of the variation in the data accounted for by the first three principal components (PC1 55%, PC2 22% and PC3 13%) compared with 82% (PC1 50%, PC2 17% and PC3 15%) for the correlation form of PCA. PCA of the covariance matrix resulted in smooth maturity trend lines, with the space between data points reflecting the proteolytic events in the life of each cheese type. The Mozzarella cheese data dominated both types of analysis and segregated the peak vectors on the loading plots (Fig. 7.26 and 7.30) into two groups, those associated with Mozzarella cheese (mostly the very high molecular weight peptides) and those associated with the remainder of the cheese types. Despite this, reasonable separation of the cheese types was achieved at all stages of maturation when the covariance form of PCA was used. Differentiation was improved when data for the Mozzarella cheese, which was clearly segregated, was removed from the analysis. Analysis of the reduced data set using both the correlation and covariance forms of PCA (Fig. 7.29 and 7.33) enabled each remaining cheese type to be differentiated. This stepwise approach could be used to differentiate diverse cheese types.

In summary, the new SE-HPLC method provided excellent differentiation of maturity and very good differentiation of cheese type. The single mode of separation that groups peptides of similar molecular weight is believed to be the key to the success of the method. The single solvent system means that there is no change in peptide structure during elution. The SE-HPLC peaks collectively represent a wide range of molecular weights, and individually represent the fractionation of the WSN into groups of peptides based on molecular weight. This equates approximately to separation of the WSN into the various fractions (e.g. TCA-N, NPN, PTA-N) that have been found by others to be useful when used in combination for determining cheese maturity (Santa-Maria et al, 1986;

Lopez-Fandiño et al, 1994 and Garcia Ruiz et al, 1998). In comparison, the current SE-HPLC method provides a simple and very effective method for measuring maturity and, because of the choice of column and solvent, the method is better than SE-HPLC methods used in the past (M Wilkinson, personal communication, 2000).

7.5.1.4 RP-HPLC of the WSF with peak data analysis using PCA

RP-HPLC, although not necessarily quantitative because of slow desorption, has been combined with multivariate analysis to differentiate first grade from downgraded cheese (Amantea and Nakai, 1984), to classify random samples of Cheddar, Edam, Gouda, Swiss or Parmesan cheeses according to type (Smith and Nakai, 1990), and to compare standard Cheddar cheese with cheese incorporating enzyme extracts of lactic bacteria (Furtula et al, 1994a,b). More recently, Pripp et al (2000a) used PCA to analyse RP-HPLC (ethanol-soluble fraction of the WSF) peak height data and demonstrated that sodium caseinate solutions incubated with chymosin and one of six lactococcal cell extracts could be differentiated according to incubation time and divided into three groups believed to be related to the presence of P_I, $P_{I/III}$ or P_{III} lactococcal cell-envelope proteases.

RP-HPLC of the WSF provided a distinctive peptide pattern that changed in a reasonably consistent manner during maturation (Figs. 7.10 - 7.14). The formation of a data set that adequately represented all cheese types at all stages of maturity proved to be a difficult and time-consuming exercise because of the large number of peaks, minor shifts in their retention time, and because in the later stages of maturation, what had previously been two or three distinct peaks sometimes became one large peak. This meant that the particular collection of peaks needed to be summed in all of the cheese samples. Nevertheless, a good data set containing 48 peaks was obtained, and PCA enabled 83% (correlation matrix) or 85% (covariance matrix) of the variation in the data to be accounted for by the first three principal components. This was a significant improvement on the work of Smith and Nakai (1990) who required 17 principal components to account for 74% of the variation in their data.

PCA of the correlation or covariance matrix (Fig. 7.36 and 7.40) provided similar results (Fig. 7.36A and 7.40A), with PC1 associated with maturity, and PC2 as well as PC3 associated more with differentiation of cheese type. However, the maturity trend lines were more regular and the data points better spaced using PCA of the covariance matrix. The exception was Mozzarella cheese, where the data standardisation required for PCA of the correlation matrix enabled both PC1 and PC2 to be involved in the assessment of maturity and provided a better maturity trend line than PCA of the covariance matrix.

PCA of the covariance matrix was most useful for establishing Cheddar cheese maturity and provided a maturity trend line with well-spaced data points (Fig. 7.40A). Four key peaks were associated with PC1 or cheese maturity, peaks 3 (Tyr), 5 (Phe), 13 and 17 – all peaks linked to the combination of primary and secondary proteolysis and particularly dominant in the Cheddar cheese (Fig. 7.39A). The slow rate of production of these peptides and amino acids in the Swiss, Elsberg and Gouda cheese meant that differentiation of maturity was poorer, and in the later stages of maturation the data points became clustered when production of these peptides and amino acids slowed even further. There were six key peaks (peaks 25, 26, 39, 40, 41 and 47) closely associated with PC2 and these peaks dominated the Mozzarella profile, enabling it to be well differentiated from the other cheese types. As a consequence, the remaining cheese types were closely grouped. Removal of the Mozzarella cheese data from the analysis meant that the variance of the peaks in the remaining cheese types could be captured. In particular, the importance of peak 41 for the differentiation of the Swiss cheese from the other cheese types and for the differentiation of Swiss cheese maturity could be demonstrated, although the data points representing the more mature Swiss cheese remained closely grouped (Fig. 7.42A). Peaks 3 (Tyr), 5 (Phe), 13 and 17 remained the dominant peaks associated with Cheddar, Elsberg and Gouda cheese maturity and the Cheddar data continued to account for a large part of the variance of each of these peaks. Removal of the Cheddar data from the analysis enabled the variance of peaks 3 (Tyr), 5 (Phe), 13 and 17 in the remaining cheese types to be captured and improved both the differentiation of cheese maturity and type.

These findings suggested that different forms of PCA suited each cheese type when diverse cheese types were analysed at the same time. The correlation form of PCA, where all peaks were given equal weighting and many peaks were associated with PC1 suited the assessment of maturity in Mozzarella cheese and resulted in a more uniform trend line with better-spaced data points than the covariance form of PCA. Conversely, in cheese types such as Cheddar, many peptides were transient, and only three or four peptides strongly influenced the assessment of maturity. In this case, the covariance form of PCA provided a better assessment of maturity and a smoother trend line, at least for the first 6 months.

Although these findings suggested that cheese maturity could be assessed by measuring the concentration of key peptides or amino acids, this is unlikely to be effective because in many cheese types the concentration of these peptides levels off or decreases as they are hydrolysed, and the amino acids may eventually decrease in concentration as they are catabolised (Fig. 7.45). That is, although the amino acids are end-products of proteolysis, they are also intermediates in the biochemical processes associated with cheese ripening. Methods that capture several peaks involved in the assessment of maturity are therefore of more use. These methods may include free amino acid analysis in which the concentration of all the amino acids present in the cheese could be measured.

RP-HPLC with data analysis using PCA was less efficient than SE-HPLC with data analysis using PCA for differentiating cheese maturity and type. Effective differentiation involved the stepwise removal of influential Mozzarella data to improve differentiation of cheese type, and removal of influential Cheddar data to improve differentiation of the maturity of the remaining cheese types (Coker et al, 2002, see appendix, section 7.8.5). This approach could be taken when analysing diverse cheese samples. The combination of RP-HPLC and PCA could also be useful for locating peaks most closely associated with particular cheese characteristics. The determination of maturity does not necessarily require the identification of peptides, but the method would lend itself to combination with sensory analysis and it may be useful to identify peaks associated with particular sensory attributes.

7.6 CONCLUSIONS

Three methods of protein and peptide analysis were identified as having the potential to differentiate both cheese type and maturity: urea-PAGE of the USF depicted chymosin- or plasmin-mediated hydrolysis of α_{s1}-casein and β-casein and the formation of the first breakdown products during the ripening of each cheese type; SE-HPLC provided patterns of the molecular weight distribution of water-soluble peptides that were different for each cheese type and changed throughout ripening; and RP-HPLC demonstrated large numbers of peaks containing water-soluble peptides and amino acids that were present in different ratios in each cheese type and changed during ripening.

PCA provided an objective interpretation of the peak area data. PCA of the urea-PAGE data provided useful differentiation of the maturity of young cheese or cheese in which primary proteolysis was slow and reasonable differentiation of cheese type; PCA of the SE-HPLC data provided very good differentiation of each cheese type and excellent differentiation of maturity throughout the ripening period of each cheese type; and PCA of the RP-HPLC data, with stepwise removal of influential data pertaining to cheese types that were clearly different, enabled good differentiation of each cheese type and maturity throughout the ripening period.

SE-HPLC was the simplest of the methods used in this study. It was easy to use, the analysis time was short, the peak area data was relatively simple to obtain, and analysis using the covariance form of PCA provided the best differentiation of cheese type and maturity.

7.7 RECOMMENDATIONS

The SE-HPLC method is recommended for differentiation of diverse cheese samples. A database containing peak areas of cheese types from different sources and analysed at different times throughout maturation should be established. PCA should be used to compare peak data from unknown samples with peak data of samples in the database. In this case, the database should include data from cheese types that are the same or very similar to the unknown cheese. In this way, it may be possible to objectively identify a cheese sample and estimate its maturity.

It is also recommended that the combination of SE-HPLC or RP-HPLC and PCA be used to examine and compare a large number of New Zealand-made cheeses of one type to determine homogeneity within a cheese type and to develop a tool for the objective assessment of cheese maturity.

8.0 OBJECTIVE ASSESSMENT OF CHEDDAR CHEESE MATURITY

ABSTRACT

A wide range of New Zealand cheese of one type (Cheddar) was assessed using methods established in the previous chapter for determining cheese type. Multivariate statistical analysis of the data provided the means to demonstrate the diversity of the cheese, to develop models that explained the relationship between instrumental results and the maturity assessment of a trained sensory panel, and to establish an objective maturity index.

First grade mild to mature commercial Cheddar cheeses (77 cheeses) that had been ripened at 5, 10 or 13°C for 3, 6, 12 or 15 months were chosen from cheese produced in 8 NZ factories. Each cheese was provided with a sensory maturity score (SMS) by a trained taste panel (standard error of determination = ±0.35 on a scale of 1.00 to 9.00).

MLR analysis showed that the correlation between the SMSs and most simple measures of cheese maturity (Maturity Index (the product of maturation time (months) and temperature (°C)), NPN, WSN, RP-HPLC (amino acid) 'peak' 1, Tyr, Phe and RP-HPLC 'peak' 17) was reasonable. Inclusion of TN in the regression equation compensated for the effect of TN on the perception of cheese maturity and improved the correlation with the SMS. Although the standard error (SE) of prediction of sensory maturity (always reported on a scale of 1.00 - 9.00) was improved, it was still relatively large in each instance (SE of prediction = 0.61- 0.76).

The accuracy of the prediction of the SMS was improved using the more complex data sets provided by reverse phase high performance liquid chromatography (RP-HPLC) or size exclusion-HPLC (SE-HPLC). PCA of the data highlighted the diversity of cheese made at most factories and showed the considerable overlap between cheese from most sources. The first principal component indicated the maturity trend for cheese from all sources. The scores for the first three principal components provided by PCA of the covariance matrix (using more principal components had little effect) correlated well with sensory maturity (RP-HPLC: R = 0.9368, R^2 (adj) = 0.8708, SE of prediction = 0.58 for PC1, PC2, PC3 and TN (Model G); SE-HPLC: R = 0.9224, R^2 (adj) = 0.8425, SE of prediction = 0.64 for PC1, PC2, PC3 and TN (Model I).

Further improvements to the prediction of maturity were made by using the PCA factor loading scores to select the peaks that contributed most to the predicted maturity scores and using MLR to relate the area of these peaks to the SMSs. Peaks not contributing significantly to the regression equation were removed. The selected peaks from RP- or SE-HPLC analysis were highly correlated with sensory maturity (RP-HPLC: R = 0.9944, R^2 (adj) = 0.9803, SE of prediction = 0.23 for 32

peaks plus TN (Model K); SE-HPLC: R = 0.9585, R^2 (adj) = 0.8970, SE of prediction = 0.52 for the 15 and TN (Model M).

The equations were validated and therefore provided an objective index of maturity that encompassed the diversity of NZ Cheddar cheese. The maturity index based on SE-HPLC peak area data, although less accurate than the maturity index based on RP-HPLC peak area data, is more practical because differentiation of the peaks is unambiguous and straightforward and is better suited to mechanical or electronic processing.

8.1 INTRODUCTION

Of the 270,000 MT of cheese produced in New Zealand, approximately 60,000 MT is sold as 'consumer' (ready-to-eat) cheese, with the remaining 210,000 MT being sold as an ingredient cheese for reprocessing. The cheese is sold to both market sectors on the basis of its maturity.

Some market sectors, perhaps accustomed to cheese sourced from regions with a nearly uniform rate of production throughout the year, equate age with cheese maturity.

New Zealand has a seasonal milk supply of varying composition. Milk production commences in mid-winter and is at its greatest during the spring and summer months, a time when most of the cheese is made. This is followed by a period of at least 3 months, when the cows are 'dried-off' and there is no milk available for cheese manufacture. The geographic isolation of New Zealand adds a further complication. Approximately 90% of the cheese produced in New Zealand is destined for the international marketplace, and there are several weeks between the time the cheese leaves controlled temperature storage facilities in New Zealand and reaches the customer. To ensure there is no break in the supply of cheese and that the customer receives a uniform product, it is necessary to alter the time taken for the cheese to reach maturity by holding cheese made in the summer at different temperatures.

The development of cheese maturity is complex and may involve glycolysis, proteolysis and lipolysis as well as secondary catabolic processes. Of these, proteolysis is the most complex and is considered the most important (Fox et al, 2000) because it has been linked to changes in cheese texture (Creamer and Olson, 1982) and flavour (Aston and Creamer, 1986).

The use of different ripening temperatures influences the rate of activity of the various proteolytic enzymes (rennet, plasmin, Cathepsin D and the enzymes of the starter and non-starter lactic acid bacteria (NSLAB)) present in the cheese. In New Zealand, either calf rennet or microbial rennet (e.g. Hannilase XL (Chr. Hansen, Hørsholm, Denmark) or Fromase XL (Royal Gist-Brocades, The Netherlands) from *Rhizomucor meihei*) are used in the manufacture of cheese. These enzymes differ not only in their specificities, but at a concentration that gives the same coagulation rate, their rate of hydrolysis of α_{s1}- and β-casein in cheese is different (Johnston et al, 1994) and may influence the rate of maturation. As well, the starter bacterial strains differ between factories, as does the NSLAB population (Williams and Banks, 1997; Crow et al, 2001). These bacteria possess different enzymes with different specificities and may also influence the rate and type of proteolysis in the cheese and the consequent development of maturity.

The perception of cheese maturity is subjective and results from the combination of aroma, flavour and texture experienced by the consumer. In the New Zealand industry, cheese maturity is determined by experienced graders, and for research purposes a trained sensory panel is used to predict the consumer's assessment of the maturity of a particular cheese. Continuity between panels or graders is difficult to achieve over a prolonged period because of changes in graders or panels and training parameters. It would therefore be desirable to have objective instrumental means available for determining cheese maturity.

Two major studies have linked the sensory assessment of cheese flavour or texture with instrumental measures. The first, by Banks et al (1995), used multivariate statistics, including principal component regression (PCR), to demonstrate the close correlation between aspects of sensory flavour (flavour intensity, and acid and bitter flavours) and components of the volatile fraction of Cheddar cheese. The second, by Noël et al (1998), used multivariate statistics to correlate sensory-assessed texture and data from instrumental analysis (compositional analysis, polyacrylamide gel electrophoresis (PAGE), reverse phase-high performance liquid chromatography (RP-HPLC) of the water-soluble fraction (WSF), free amino acid analysis and rheological analysis) of Appenzeller and Parmigiano-Reggiano cheese. The study concluded that 'sensory data can be related to instrumental data' and, of the parameters studied, 'the composition of free amino acids and peptide mapping were the most promising tools for predicting sensory texture'.

Examination of the WSF of cheese seemed an appropriate starting point for developing instrumental maturity indices for Cheddar cheese. It has typical Cheddar flavour (Aston and Creamer, 1986), components of the WSF can be correlated with sensory texture (Noël et al, 1998) and water-soluble nitrogen (WSN), a component of the WSF, correlates with sensory-assessed Cheddar cheese maturity (Barlow et al, 1986) and cheese age (García Ruiz et al, 1998). The WSF contains at least 200 peptides (McSweeney and Fox, 1997) and amino acids as well as organic acids and their salts (Aston and Creamer, 1986; McSweeney and Fox, 1997), and many of these are flavour components or precursors of flavour components.

RP-HPLC analysis of the WSF (or some soluble fraction) had previously been used to measure age-related changes due to proteolysis in different cheese types (e.g. Pham and Nakai, 1984; Aston and Creamer, 1986; Cliffe and Law, 1991; Gonzalez de Llano et al, 1991; McGoldrick and Fox, 1999). Analysis of the RP-HPLC data has usually been subjective, but a range of statistical techniques has been used to provide objective analysis. For example, analysis of RP-HPLC (WSF) peak height data using the covariance form of principal component analysis (PCA) was used to differentiate Emmental cheese made with and without *L. casei* subsp. *casei* and to demonstrate age-related changes (Bütikofer et al, 1998). A combination of PCA and discriminant analysis (DA) was used to compare the sensory assessment of cheese graders with total free amino acid concentration and RP-HPLC peak areas of 60 Cheddar cheese samples that varied in age and quality (O'Shea et al, 1996). This study was not particularly successful, with 82-84% of the variation in data explained by 19 or 20 principal components. Correct classification into mild, mature and extra-mature ranged from 33 to 48% using RP-HPLC peak area data, and 70% using free amino acid concentration, although the value for mature cheese was only 20%. The authors suggested 'objective assessment of Cheddar cheese quality could be performed if a suitable 'model' is designed' and 'based on a uniform grader's assessment (all samples assessed by one grader)'. (An examination of traces in this paper showed that peak selection was not consistent.)

The work of Pham and Nakai (1984), Noël et al (1998) and Bütikofer et al (1998) had demonstrated that the peptides in cheese (analysed by RP-HPLC of the WSF) could be used to broadly group cheese on the basis of age or to demonstrate age-related changes. In addition, the work of Noël et al (1998) had demonstrated that sensory textural data could be statistically related to instrumental data. It therefore seemed likely that this type of objective approach could be refined and used to analyse and objectively compare instrumental data from a range of Cheddar cheese with an overall sensory assessment of cheese maturity. In this way an objective index of maturity that encompassed the continuum of Cheddar cheese maturities could be developed.

In Chapter 7, it was shown that SE-HPLC, with peak area data analysis using PCA, could be used to differentiate cheese types at all stages of maturity, and to provide a maturity trend for each cheese type. However in that study, age and maturity were highly correlated because only a single batch of cheese of each type was used. The simplicity (and reproducibility) of the SE-HPLC method (Chapter 5), in comparison with instrumental measures previously used for determining the maturity of cheese, made it a desirable method to pursue for examining the diversity and determining the maturity of a wider range of Cheddar cheeses.

8.2 OBJECTIVES

The objectives of this study are:

- to use chemical and instrumental methods established in the previous chapter to examine the diversity of NZ Cheddar cheese,
- to use these methods to determine the effect of factory, ripening temperature and time on differentiation of the maturity of a large range of Cheddar cheese,
- to explore and model the relationship between the sensory-assessed maturity of Cheddar cheese and chemical or instrumental measurements,
- to establish accurate objective indices of sensory maturity that would encompass the diversity of NZ Cheddar cheese, and
- to provide NZ Cheddar cheese with a maturity score that would provide the customer with an assurance of maturity.

To achieve these objectives it was necessary:

a) to obtain Cheddar cheese from each of the New Zealand manufacturing sites, that represented each of the storage regimes used by the industry,
b) to subject the cheese to appropriate chemical analyses that included measurement of WSN and NPN,
c) to accurately assess the maturity of the same cheese using a trained sensory panel,
d) to analyse the WSF of the cheese using SE-HPLC and RP-HPLC,
e) to establish an appropriate protocol for identifying peaks or regions on the RP-HPLC and SE-HPLC traces,
f) to establish the relationship between the results of the chemical and instrumental analyses and the sensory-assessed maturity using statistical methods,
g) to identify potentially useful models for predicting cheese maturity,
h) to validate selected models, and
i) to construct equations for predicting the sensory maturity of NZ Cheddar cheese.

8.3 MATERIALS AND METHODS

8.3.1 Materials

Most materials are described in Ch. 4, section 4.1

8.3.2 Methods

Most methods are fully described in Ch. 4, section 4.2.

8.3.2.1 Cheddar cheese manufacture

A representative range of (77) export Cheddar cheeses that were 3, 6, 12 or 15 months of age, and had been ripened at 5°C, 10°C or 13°C, were supplied from the available stocks in the factory stores of the 8 New Zealand cheese factories. Only relatively small quantities of Cheddar cheese are matured for long periods and therefore there was less of this cheese available.

Cheddar cheese was made to New Zealand Cheddar Specification 30 standard and each cheese was of the highest grade (Quality Control 1). The cheese fell into 2 subgroups within that specification. Cheese from 7 of the 8 factories had a target composition of 37% fat, 33.8% moisture, 24.0% protein and 1.7% salt. Cheese from the one remaining factory (Marlborough) had a target composition of 35% fat, 32.5% moisture, 26.8% protein and 1.75% salt. The cheese was made using either calf rennet ("Australian Double Strength" calf rennet (280 IMCU/mL, Dairy Meats, New Zealand) or microbial rennet (Fromase XL or Hannilase XL) as the coagulant. The cheese was made over a period of two dairy seasons and the starter strains varied with season and from factory to factory. The cheeses (77 x 20 kg blocks) were brought to NZDRI and sub-sampled for sensory and instrumental analyses.

8.3.2.2 Determination of chemical composition of cheese

Each cheese was analysed for pH, TN, WSN and NPN using methods described in Ch. 4, section 4.4.2. For the purposes of the present study it was considered unnecessary to obtain (from the Analytical Services Group, Food Science Section, NZDRI) the complete chemical composition of every cheese.

8.3.2.3 Determination of cheese sensory maturity

The cheese was assessed by 12 members of a trained sensory panel who evaluated duplicate samples of each cheese according to the protocol outlined in the appendix (section 8.8.1). The panel assessed the intensity of 3 texture attributes, 16 flavour attributes and an overall maturity attribute. A SMS was determined for each cheese sample.

8.3.2.4 Statistical analysis of the chemical composition data

The chemical composition data were collated using Microsoft Excel 7.0 software (Microsoft Corporation, CA, USA) and transferred to a Statistica 6.0 spreadsheet for statistical analysis and graphing. Linear regression analysis was used to determine the relationship between data for each compositional parameter and the SMS of the cheese. MLR was used to relate various combinations of TN, WSN, NPN or pH to the SMS.

8.3.2.5 Preparation and analysis of the WSF of cheese

The WSF of each cheese was prepared and analysed in a single batch using SE-HPLC and RP-HPLC methods described in Ch. 4, sections 4.2.3.3 and 4.2.3.4. Deviations from these methods are described below.

Prior to RP-HPLC analysis, the WSF was diluted 1:1 with RP-HPLC solvent A (5% acetonitrile, 0.1% TFA in water) and allowed to stand at room temperature (22°C) for 1 h after which time it was filtered using a 0.45 μm filter (Gelman Sciences) to remove any precipitate. The material removed was comprised of small quantities of casein and high molecular weight peptides that were present in varying concentrations as a result of any differences in cheese salt concentration (N White and C Coker, unpublished). Unless removed, this material can precipitate on the column and shorten its life.

The WSF prepared for RP-HPLC analysis was also used for SE-HPLC analysis after being diluted 1:24 with SE-HPLC mobile phase (36% acetonitrile, 0.1% TFA in water).

8.3.2.6 Preparation and analysis of standard sets A and B

Two sets of molecular weight standards were prepared according to the method described in Ch. 5, section 8.3.2.3. Each solution (50μL) was examined separately using SE-HPLC, according to the method described in Ch. 4, section 4.2.3.3.

8.3.2.7 Statistical analysis of SE-HPLC and RP-HPLC data

SE-HPLC and RP-HPLC peak area data were collated (separately) using Microsoft Excel 7.0 software and transferred to a Statistica 6.0 spreadsheet for statistical analyses and graphing. Linear regression analysis was used to relate individual RP-HPLC peak areas to the SMS. PCA of both the correlation and covariance matrices (see Ch. 4, section 4.2.6.1) was used to separately analyse the SE-HPLC and RP-HPLC peak area data. MLR analysis was used to determine the relationship between the principal component scores and the SMSs. PCA factor loading scores were used to select RP-HPLC or SE-HPLC peaks that contributed significantly to the analysis, and for each method of sample analysis, MLR was used to determine the relationship between the selected peak area data and the SMSs.

8.4 RESULTS

8.4.1 SMS and its relationship with cheese age, ripening temperature and Maturity Index

Details of the age and storage temperature of each cheese, the Maturity Index (MI; the product of cheese age, in months and storage temperature, in °C), and the results from the sensory analysis (SMS) and chemical analyses (pH, TN, WSN and NPN) (discussed later) of each cheese sample are included in the appendix (section 8.8.2).

8.4.1.1 Distribution of sensory maturity scores

The distribution of the SMSs of the 77 samples (see appendix, section 8.8.1, column 6) was determined and is depicted in Fig. 8.1.

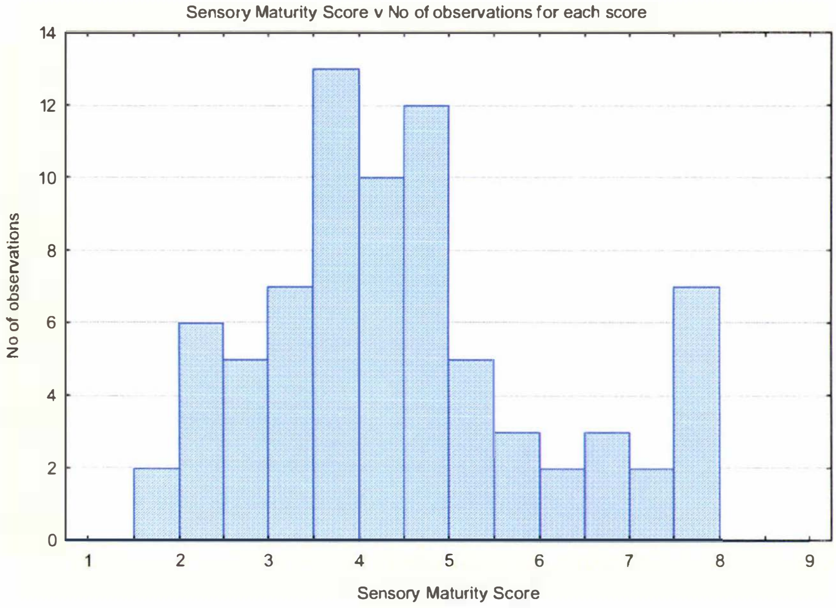


Fig. 8.1 *Distribution of SMSs.* SMS v the number of observations (samples) for each score.

The disproportionate number of samples with a SMS of 7.5 – 8.0 (Fig. 8.1), and the lack of samples with a SMS of > 8.0 may have been caused by a reluctance of the sensory panel members to allocate the highest scores (> 8.0), despite their training (personal communication, Dr R Crawford, Senior Statistician, NZDRI).

8.4.1.2 Relationship between SMS and Maturity Index, age, or temperature

The distribution of MI scores (see appendix, section 8.8.2) was determined and is shown in Graph 1 (Fig. 8.2). The relationship between SMS and MI, age or temperature was determined and is shown in graphs 2 – 4 (Fig. 8.2).

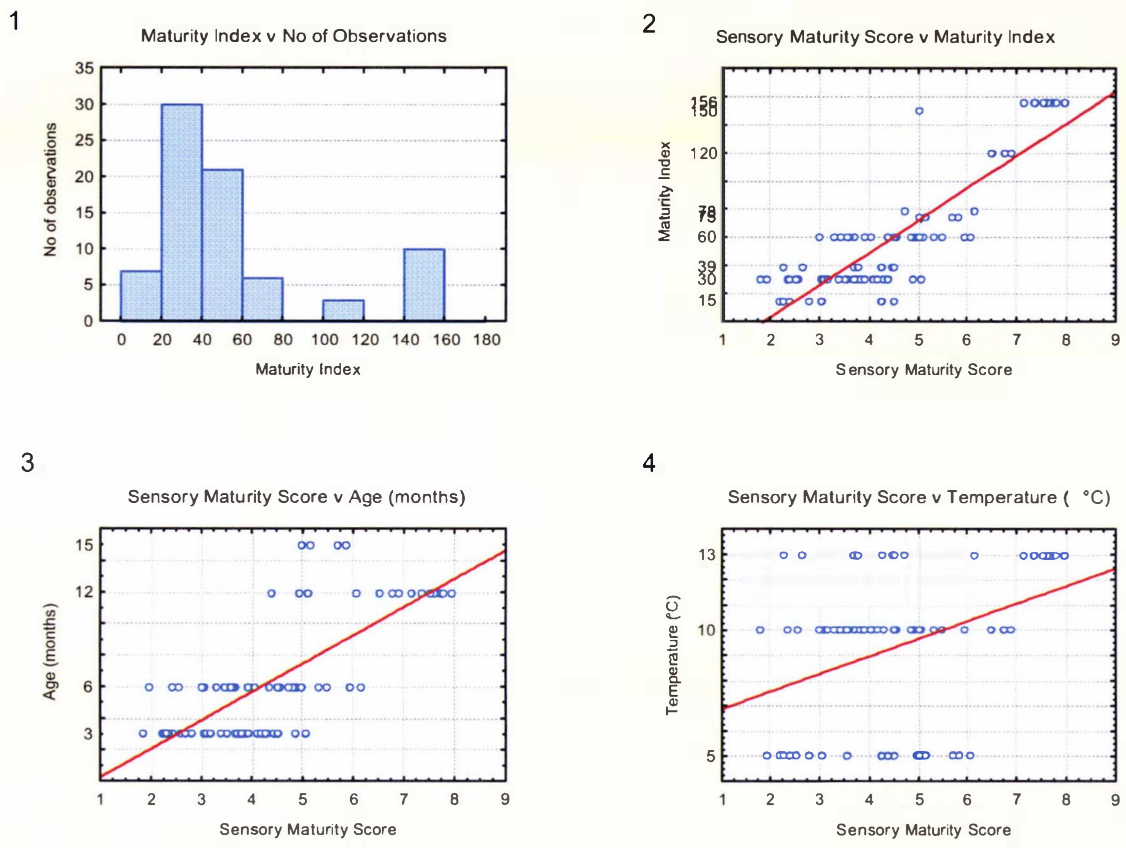


Fig. 8.2 *Distribution of MI values and the relationship between SMS and MI, cheese age and ripening temperature.* MI v the number of observations (samples) for each score (graph 1) and SMS v MI (graph 2), age (graph 3) or ripening temperature (graph 4). Lines of best fit (a linear regression) are included on graphs 2 – 4.

The distribution of MI values (Fig. 8.2, graph 1) was similar to the distribution of SMSs (Fig. 8.1) and provided a better indicator of NZ Cheddar cheese sensory maturity (graph 2) than either cheese age (graph 3) or ripening temperature (graph 4).

MI is used in New Zealand to provide a "rule of thumb" guide to the maturity of cheese destined for use as an ingredient in processed cheese. Graph 2 (SMS v MI) shows that for any given MI value, the SMS can vary by as much as 3 on the scale of 1.00 – 9.00 for SMS. Despite this, and the high correlation between SMS and MI, (R = 0.8602), MI is not a suitable predictor for maturity because of the wide variation in SMS for each MI value ($R^2 = 0.7401$, $p < 0.0001$, standard error

(SE) of prediction of maturity = 0.8284). MI only takes into account the ripening temperature and time and does not encompass other effects (due to small differences in composition as a result of manufacture or seasonal and lactational differences in milk composition, or differences in starter and NSLAB populations).

8.4.2 SMS and its relationship with simple measures of proteolysis

The 77 cheese samples were analysed for TN, WSN, NPN and pH. The results, as well as the age, storage temperature, MI and SMS of the samples, are presented in a table in the appendix (section 8.8.2). WSN and NPN are usually expressed in relation to TN, and accordingly, these values were quoted in the table.

The values for WSN, WSN/TN, NPN, or NPN/TN were regressed against the SMS (using Linear Regression analysis (LR)) and the values for R, R^2, p and the SE of prediction are shown in Table 8.1. The values for MI are included for comparison.

Table 8.1 *Comparison of MI, WSN, WSN/TN, NPN and NPN/TN as predictors of sensory maturity.* Regression statistics (R, R^2, p and SE of prediction of sensory maturity) for SMS v MI, WSN, WSN/TN, NPN or NPN/TN.

Method	MI	WSN	WSN and TN	NPN	NPN and TN
LR	SMS v	SMS v	SMS v	SMS v	SMS v
	MI	WSN	WSN/TN	NPN	NPN/TN
R	0.8603	0.8556	0.8749	0.8026	0.8319
R^2	0.7401	0.7320	0.7654	0.6441	0.6920
p-level	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
SE of prediction	0.8284	0.8411	0.7869	0.9694	0.9018

WSN, WSN/TN, NPN and NPN/TN all increased as the cheese matured and provided a reasonable assessment of cheese maturity. WSN/TN was the most highly correlated with SMS with R = 0.8749 (Table 8.1). The coefficient of determination (R^2) (Table 8.1) was used to make the following comparisons. WSN/TN (R^2 of 0.7654), provided a better indication of cheese maturity than MI, WSN, NPN or NPN/TN. However, the SE of the prediction of maturity of 0.7869 (on a scale of 1.00 to 9.00)) was reasonably large, and more than twice the error of determination of sensory maturity of 0.3464 by the sensory panel (R. Crawford, personal communication, 2002).

The effect of TN as well as pH on the SMS was determined, and the relationship between SMS and WSN/TN or NPN/TN was examined and graphs plotted (Fig. 8.3).

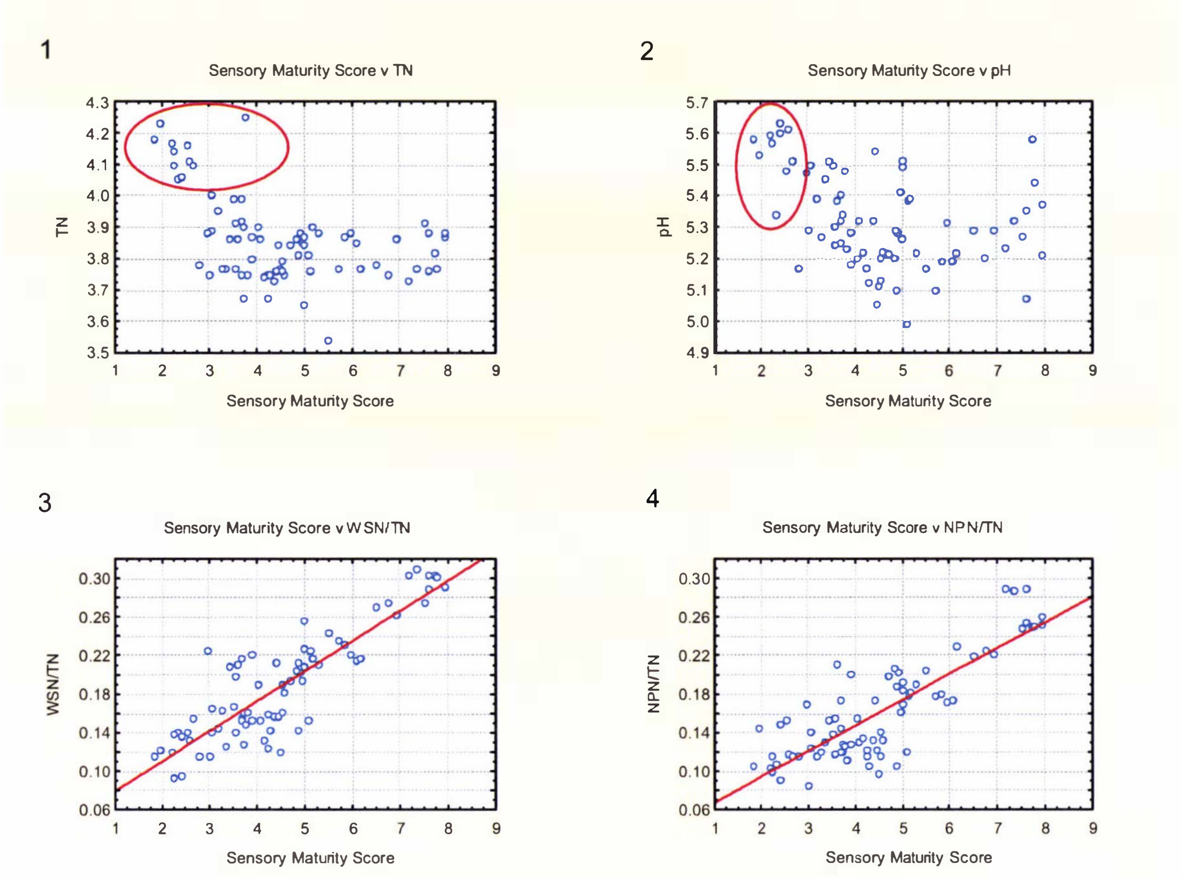


Fig. 8.3 *Relationship between SMS and TN, pH, WSN/TN and NPN/TN.* SMS v TN (graph 1), pH (graph 2), WSN/TN (graph 3) and NPN/TN (graph 4). The area circled (graphs 1 and 2) highlights the high TN and pH of cheese from one of the 8 factories. Lines of best fit (using a linear regression) are included on graphs 3 and 4.

The two cheese specifications were clearly differentiated by TN (Fig. 8.3, graph 1). The cheese with the greatest amount of TN (circled; TN target = 26.8%) was made at a single factory (Marlborough), was the least mature and had the highest pH (~5.5 – 5.6). This cheese was manufactured to be mild by adjusting the composition (e.g. pH), as well as the ripening time (Fig. 8.2, graph 3) and temperature (Fig. 8.2, graph 4). There was a linear relationship between SMS and WSN/TN (Fig. 8.3, graph 3) and SMS and NPN/TN (Fig. 8.3, graph 4).

There seemed to be a non-linear relationship between cheese pH and SMS (Fig. 8.3, graph 2). A linear fit provided an R^2 of 0.1689, whereas a 2nd order polynomial fit provided an R^2 of 0.4169, but there was a wide spread of points, particularly around pH 5.15 to 5.30. If the trend in pH was a real effect, it could reasonably be expected that there would be a pH dependent effect on WSN or NPN. To examine this, pH was plotted against WSN/TN or NPN/TN (Fig. 8.4, graphs 1 and 2).

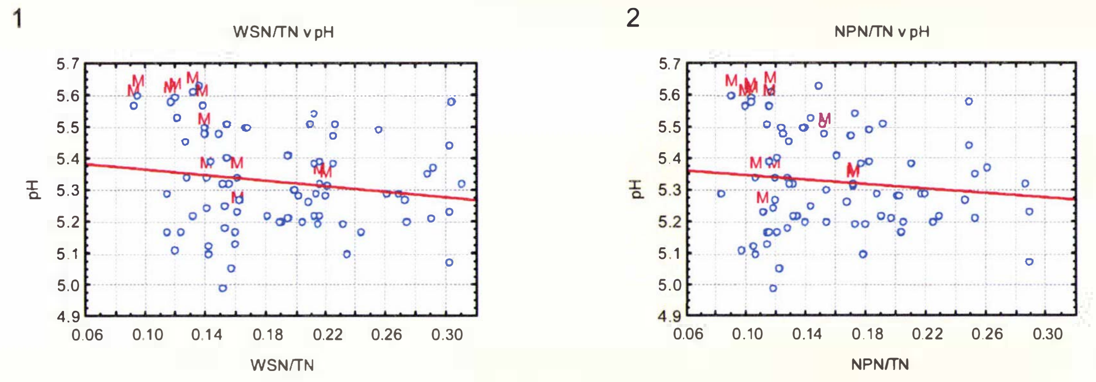


Fig. 8.4 *Effect of pH on WSN/TN and NPN/TN.* WSN/TN v pH (graph 1) and NPN/TN v pH (graph 2). The areas circled (graphs 1 and 2) highlight the high TN and pH of cheese from one of the 8 factories. Marlborough cheese is labelled (M).

With the exception of the high pH Marlborough cheese, there was a wide distribution of (pH) sample points for WSN/TN (Fig. 8.4, graph 1) and NPN/TN (Fig. 8.4, graph 2) at each pH. This indicated that pH and WSN/TN or NPN/TN were not correlated at the stage of maturation of these cheeses.

It therefore seems likely that the Marlborough cheese, with its high pH and low SMS, was creating the appearance of a polynomial distribution. It should perhaps be remembered that very marked effects of pH on enzyme activity occur very early in the life of the cheese and probably before most of the current cheese was sampled. The pH of Cheddar cheese usually decreases rapidly during cheese manufacture as lactose is converted to lactic acid and then increases during maturation as the lactate is catabolised. The hydrolysis of proteins and the release of amino acids also influences pH, as does the catabolism of amino acids. These changes are associated with the production of flavour compounds (discussed later). As well, a lower pH is associated with increased acid flavour and the perception of cheese maturity. The high pH of immature cheese from Marlborough may be a consequence of early salt addition to the curd and the resultant inhibitory effect on starter growth and utilisation of lactose.

The combination of the regression statistics (Table 8.1) and graphs (Fig. 8.3) indicated that WSN/TN provided a better indication of maturity than NPN/TN, but was a poor predictor of SMS.

8.4.2.1 SMS, WSN, TN and the prediction of cheese maturity

Of the predictors examined, NPN/TN and WSN/TN had provided the best prediction of cheese sensory maturity. Another way of looking at the relationship between SMS, WSN and TN or SMS, NPN and TN would be to consider WSN, NPN and TN as separate variables and use MLR to determine the relationship with SMS. Linear models that predicted SMS were estimated using values for two independent variables: TN and MI, TN and NPN, or TN and WSN (see Ch. 4, section 4.2.6.2). The predicted values for the SMS are referred to as the predicted maturity score. The results of these regression analyses are included in Table 8.2 and compared with the previous results, as well as the combination of MI and TN.

Table 8.2 *Comparison of the combination of NPN and TN or WSN and TN as predictors of cheese sensory maturity.* Regression statistics (R, R^2, R^2 (adj), p and SE of prediction of sensory maturity) for SMS v NPN and TN, and SMS v WSN and TN. The statistics for SMS v MI, SMS v NPN/TN and SMS v WSN/TN are included for comparison. (R^2 (adj) is a modified version of the coefficient of determination (R^2) that is used to compare equations with different numbers of independent variables (Hair et al, 1998).)

Model				**Model A**	**Model B**	**Model C**
Method	MI	TN and NPN	TN and WSN	TN and MI	TN and NPN	TN and WSN
LR or MLR	SMS v MI	SMS v NPN/TN	SMS v WSN/TN	SMS v TN and MI	SMS v TN and NPN	SMS v TN and WSN
R	0.8603	0.8319	0.8749	0.9006	0.8814	0.8963
R^2	0.7401	0.6920	0.7654	0.8111	0.7768	0.8033
R^2 (adj)	0.7366	0.6879	0.7623	0.8060	0.7708	0.7980
p-level	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
SE of prediction	0.8284	0.9018	0.7869	0.7109	0.7728	0.7255

The statistical results (Table 8.2) (greater R and R^2 (adj) values and decreased values for SE of prediction), show that this approach provided an improvement in the prediction of cheese sensory maturity. The combinations of TN and MI (Model A), TN and NPN (Model B) or TN and WSN (Model C) were highly correlated with sensory maturity (R = 0.9006, 0.8814 and 0.8963, respectively). The combination of TN and MI or TN and WSN provided the best prediction of SMS, with R^2 (adj) values (of 0.8060 and 0.7980, respectively) that were higher and the SE of prediction values (of 0.7109 and 0.7255, respectively) that were lower than the values for the other analyses.

Graphs that show the relationship between SMS, TN and MI, SMS, TN and WSN and SMS, TN and NPN and the residual scores (the difference between the sensory maturity score and the maturity score predicted by data for the independent variables (predicted maturity score)) were plotted (Fig. 8.5).

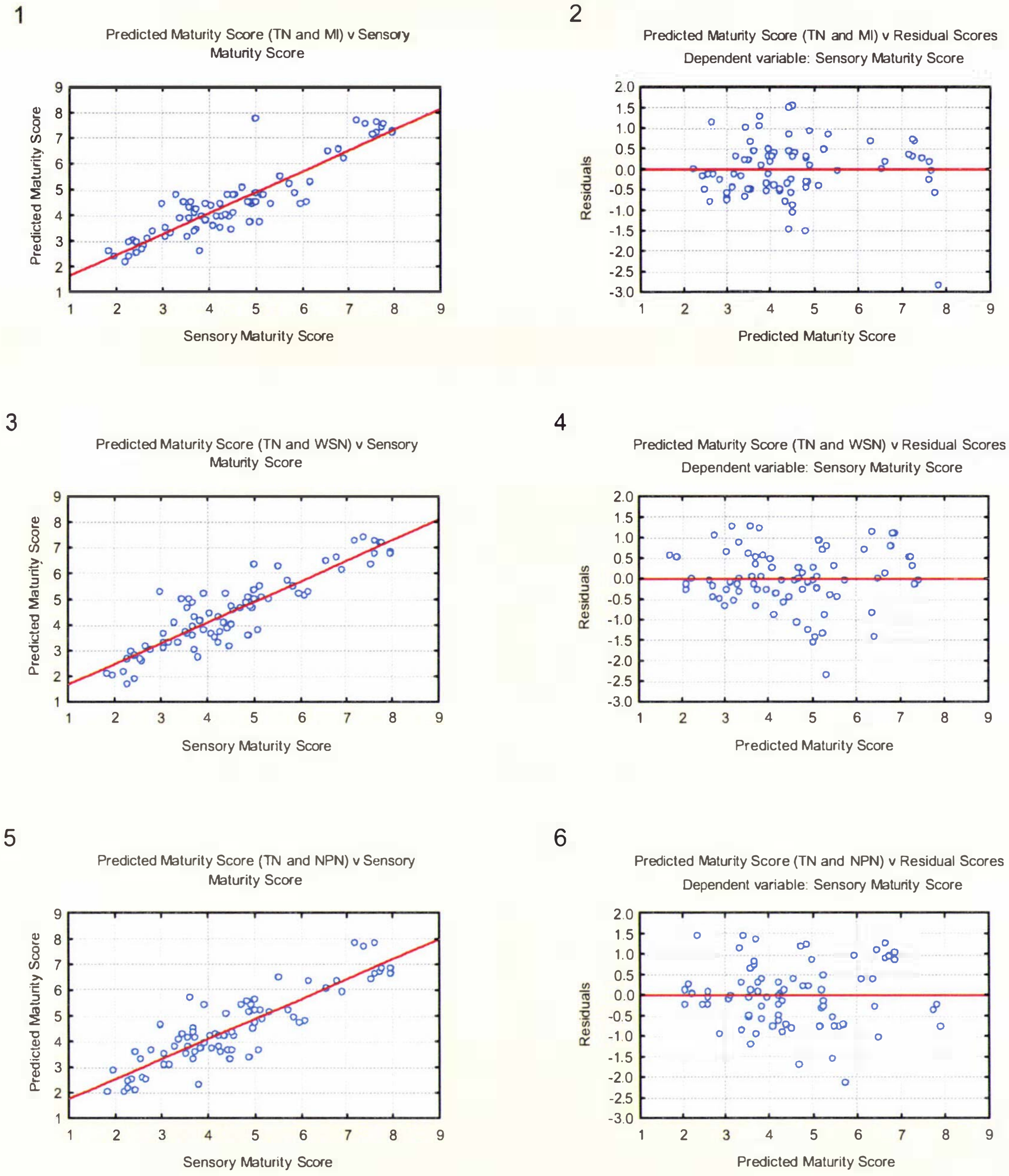


Fig. 8.5 *Relationship between SMS, TN and MI, SMS, TN and WSN, and SMS, TN and NPN.* SMS v TN and MI (graph 1), and the residuals (graph 2), SMS v TN and WSN (graph 3), and the residuals (graph 4), SMS v TN and NPN (graph 5) and the residuals (graph 6). Lines of best fit (using a linear regression) are included on graphs 1, 3 and 5.

The graphs (Fig. 8.5) show the spread of sample data points around the regression line for SMS v TN and MI (graphs 1 and 2), SMS v TN and WSN (graphs 3 and 4), and SMS v TN and NPN (graphs 5 and 6). Each set of graphs showed the high degree of correlation between SMS and the predicted maturity scores. The residual scores (graphs 2, 4 and 6) were evenly spread for the range of SMSs, but were less widely spread for the combinations of TN and MI (graph 2) and TN and

WSN (graph 4), indicating that these provided a better prediction of sensory maturity over the SMS range. Inclusion of TN as a separate variable compensates for its effect on flavour release experienced by the sensory panel (see section 8.5.1).

The following equations describe the relationship between SMS, TN and MI (Model A), SMS, TN and NPN (Model B) and SMS, TN and WSN (Model C):

$$\text{Cheddar cheese maturity} = 15.00833 - 3.17650\ \text{TN} + 0.02934\ \text{MI} \tag{Equation 1}$$

$$\text{Cheddar cheese maturity} = 17.04143 - 4.23210\ \text{TN} + 6.15570\ \text{NPN} \tag{Equation 2}$$

$$\text{Cheddar cheese maturity} = 12.53148 - 3.18184\ \text{TN} + 5.88124\ \text{WSN} \tag{Equation 3}$$

8.4.3 RP-HPLC and the maturity of Cheddar cheese

The RP-HPLC separations used two of the new steel pep-RPC C2/C18 HPLC columns in series, whereas the separations reported in Chapters 5, 6 and 7 used the earlier glass pep-RPC columns that were originally designed for FPLC. Although both the old and new columns have the same matrix, the peptide separation was a little different.

RP-HPLC traces were generated for each of the 77 cheese samples. Examples of RP-HPLC traces of Cheddar cheese made at the Waitoa factory at different times over two seasons and ripened at different temperatures and for different lengths of time are depicted in Fig. 8.6. Each sample was from a different batch of cheese.

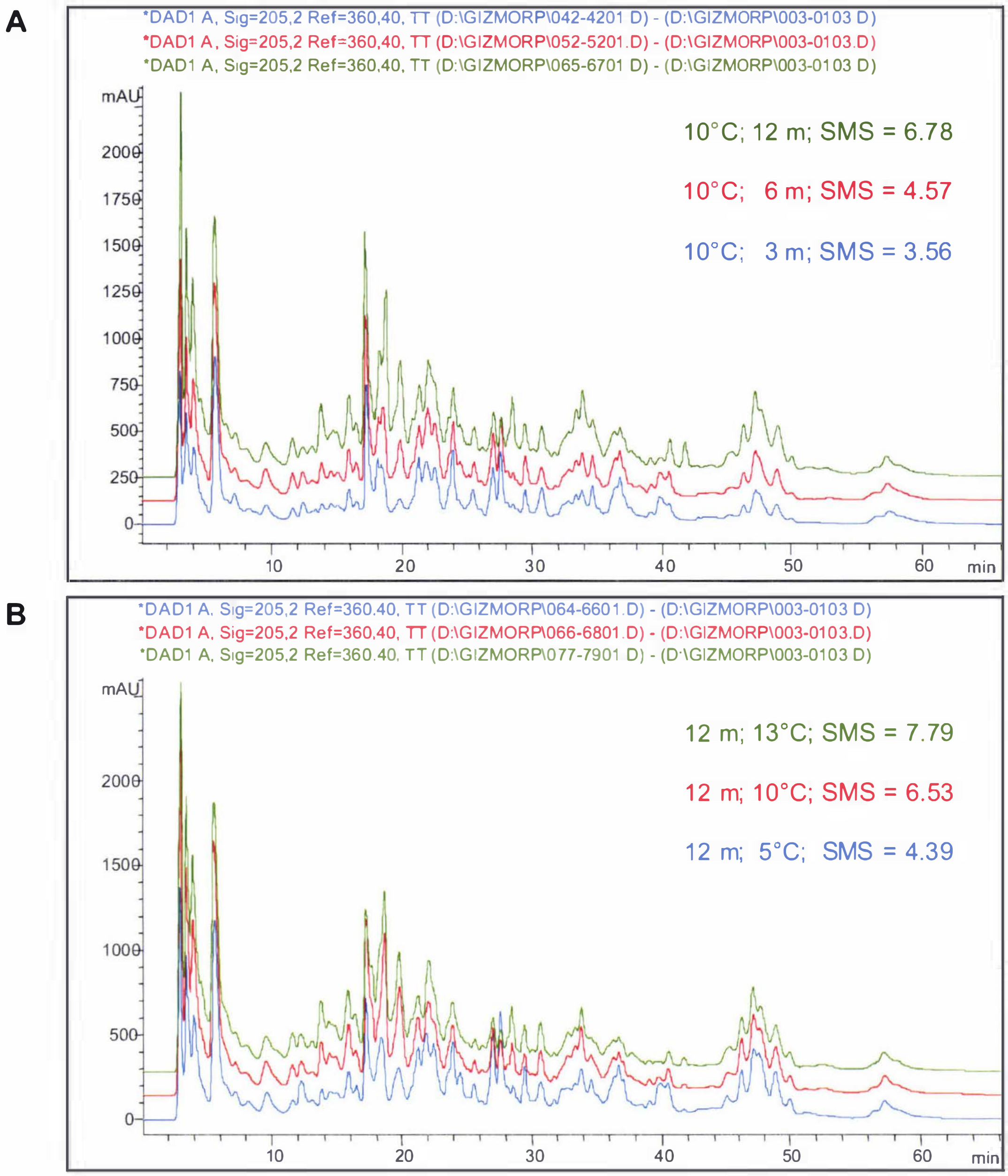


Fig. 8.6 *RP-HPLC traces of the WSF of Cheddar cheese.* Cheese made at different times of the year at the same factory (Waitoa) and ripened A) at 10°C for 3, 6 or 12 m and B) 5, 10 or 13°C for 12 m. Samples are from separate batches and do not represent a time or temperature course for one cheese. Details of the cheese age, ripening temperature and SMS are colour coded to correspond to the chromatograph. The WSF was diluted 1:1 (v/v) with solvent A and filtered (0.45 μm filter) prior to loading a 100 μL aliquot on to the column. Gradient: 100% solvent A for 5 min; 0 to 100% solvent B over 75 min; 100% solvent A for 15 min. Flow rate, 0.7 mL/min. The absorbance was measured at 205, 210, 220, 280 and 295 nm. Only the 205 nm traces are shown.

Each of the 77 RP-HPLC traces was integrated, and 54 peaks or regions (in some instances) were identified and numbered according to the labelled chromatograph (Fig. 8.7). Within a particular region, there may have been two peaks at a particular stage of ripening, and as ripening progressed, one may have increased in size to a point where it engulfed the neighbouring peak (often initially present as a shoulder). Conversely, a particular peak may have developed a shoulder at some stage during ripening. In these instances, it was necessary to combine the peaks to provide uniformity of peak selection across the range of samples. The location of the Tyr, Phe and Trp peaks was found by the characteristic absorbance spectra of these amino acids (Fig. 8.7 insets).

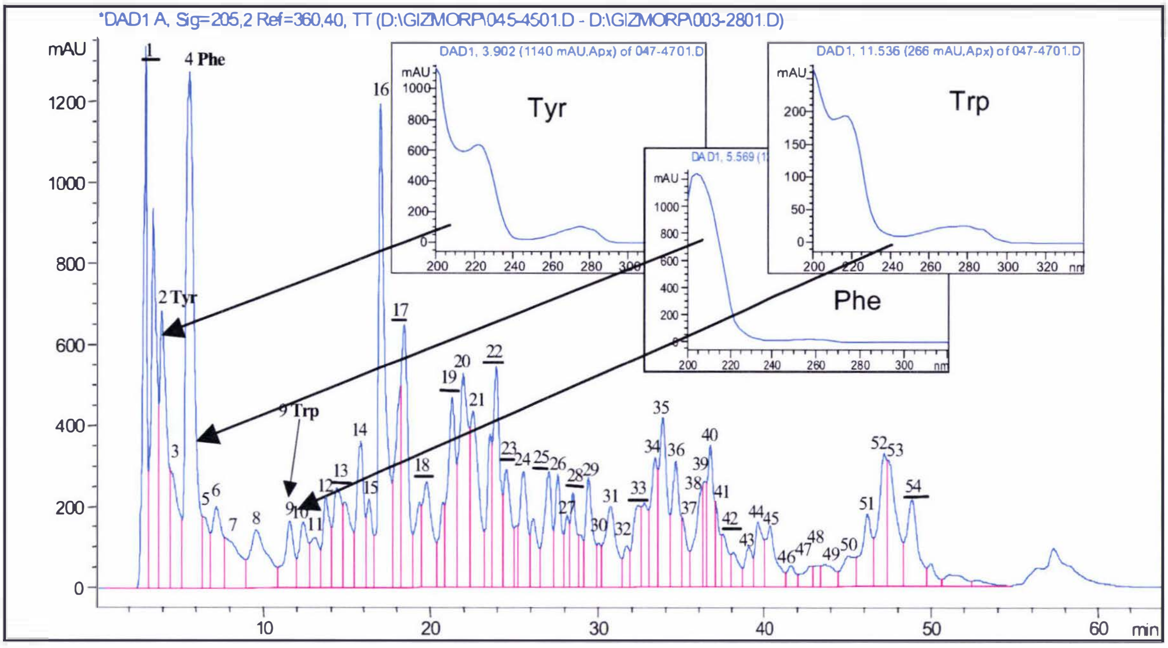
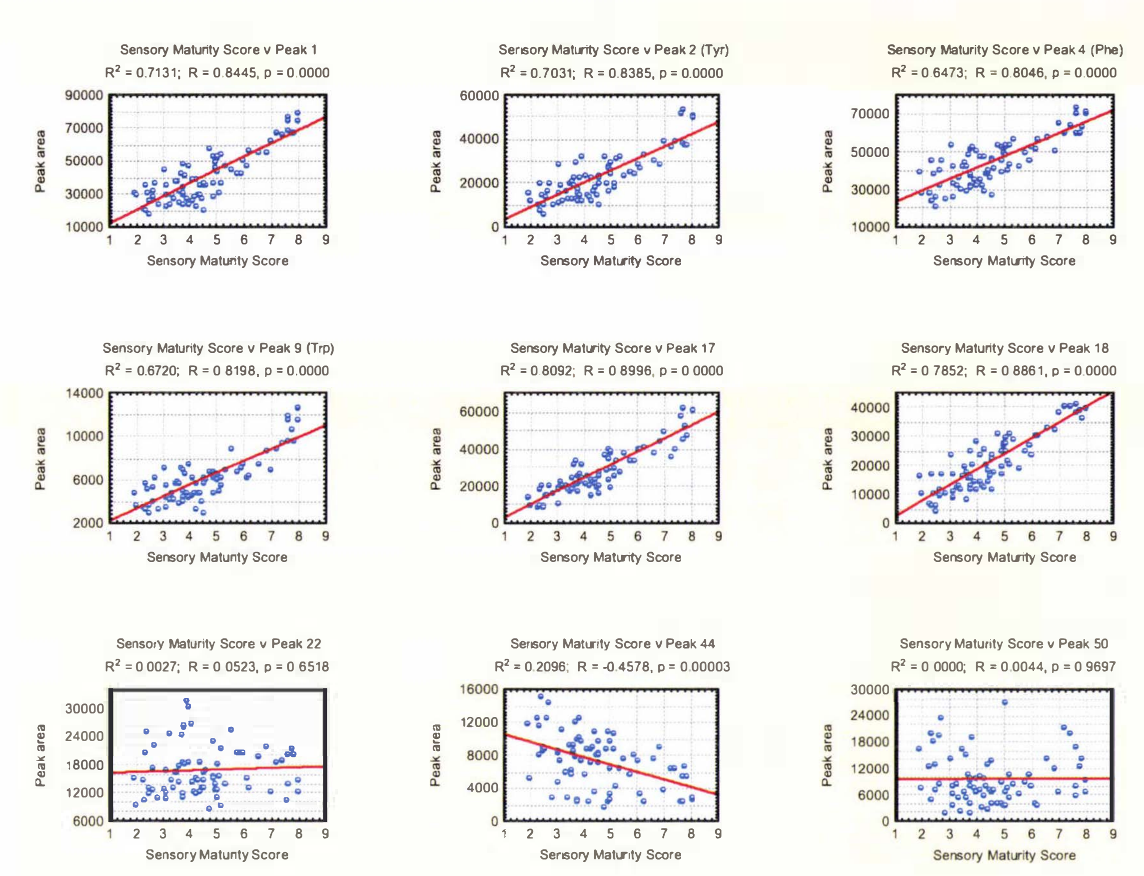


Fig. 8.7 *RP-HPLC trace of the WSF of Cheddar cheese (made at Lichfield and ripened at 10°C for 6 m) with peaks numbered.* The presence of a bar beneath a number indicates that the peak areas within that region were summed prior to further analysis. The absorbance spectra for the Tyr, Phe and Trp peaks (inset) were used to confirm the location of these peaks. See Fig. 8.6 caption for details of analysis.

The area of each of the peaks (includes regions shown in Fig. 8.7) was determined and the data incorporated into a large data matrix, comprised of 77 samples (cases) and 54 peaks (variables), within a spreadsheet (see appendix, section 8.8.3.1). The values obtained for each peak were plotted against the SMS. Many of the graphs were very similar and nine representative graphs are depicted in Fig. 8.8. The remaining graphs are included in the appendix (section 8.8.3.2).


Sensory Maturity Score v Peak 1
$R^2 = 0.7131$; $R = 0.8445$, $p = 0.0000$

Sensory Maturity Score v Peak 2 (Tyr)
$R^2 = 0.7031$; $R = 0.8385$, $p = 0.0000$

Sensory Maturity Score v Peak 4 (Phe)
$R^2 = 0.6473$; $R = 0.8046$, $p = 0.0000$

Sensory Maturity Score v Peak 9 (Trp)
$R^2 = 0.6720$; $R = 0.8198$, $p = 0.0000$

Sensory Maturity Score v Peak 17
$R^2 = 0.8092$; $R = 0.8996$, $p = 0.0000$

Sensory Maturity Score v Peak 18
$R^2 = 0.7852$; $R = 0.8861$, $p = 0.0000$

Sensory Maturity Score v Peak 22
$R^2 = 0.0027$; $R = 0.0523$, $p = 0.6518$

Sensory Maturity Score v Peak 44
$R^2 = 0.2096$; $R = -0.4578$, $p = 0.00003$

Sensory Maturity Score v Peak 50
$R^2 = 0.0000$; $R = 0.0044$, $p = 0.9697$

Fig. 8.8 *Cheese SMS v the area of RP-HPLC peaks 1, 2 (Tyr), 4 (Phe), 9 (Trp), 17, 18, 22, 44 and 50.*

Graphs of the 54 peaks (SMS v peak area) fell into three categories, those with a positive slope, those with a negative slope and those with only a very small slope. The amount of scatter of the points varied for each peak. The peaks that increased most in size during ripening were the early-eluting peaks (peaks 1 - 5, 8 - 9 and 11 - 20). These peaks were comprised of the very small peptides as well as amino acids; the end-products of proteolysis. The remainder of the peaks that increased in size as the cheese matured, did so at a slower rate, indicating that they were probably intermediates that were being produced at a rate that was greater than the rate of their hydrolysis. Conversely, the negative slope of some peaks (24, 26, 27, 36, 38 - 40, 42 - 44 and 46 - 48) indicated that they were intermediates that were being hydrolysed at a rate that was greater than the rate they were being formed.

The results from Ch. 7 and the graphs of peaks 1, 2 and 4 above (Fig. 8.8), suggested that the amino acids ('peak' 1) including Tyr (peak 2) and perhaps Phe (peak 4) as well as 'peak' 17 were good indicators of cheese maturity. Phe in particular, and Tyr to a lesser extent, are located at the N- or C-terminus of many of the peptides produced by chymosin and are cleaved from these peptides by the aminopeptidases of the starter bacteria. The values for these peaks were regressed against the SMSs. The R^2 value for 'peak' 1 was 0.7131 ($p < 0.0001$), for Tyr it was 0.7030 ($p <$

0.0001), for Phe it was 0.6473 ($p < 0.0001$) and for 'peak' 17 it was (0.8092, $p < 0.0001$). These values were markedly improved if they were considered in relation to TN (Table 8.3).

Table 8.3 *Comparison of TN and 'peak' 1, TN and Tyr, TN and Phe and TN and 'peak' 17 as predictors of cheese sensory maturity.* Regression statistics (R, R^2, R^2 (adj), p and SE of prediction of sensory maturity) for SMS v 'peak' 1 and TN (Model D), SMS v Tyr and TN, SMS v Phe and TN, and SMS v 'peak' 17 and TN (Model E). The statistics for SMS v MI and TN (Model A) and SMS v WSN and TN (Model C) are included for comparison.

Model Method MLR	**Model A** TN and MI SMS v TN and MI	**Model C** TN and WSN SMS v TN and WSN	**Model D** RP-HPLC SMS v TN and 'Peak' 1	 RP-HPLC SMS v TN and Tyr	 RP-HPLC SMS v TN and Phe	**Model E** RP-HPLC SMS v TN and 'Peak' 17
R	0.9006	0.8963	0.9130	0.9072	0.8858	0.9278
R^2	0.8111	0.8033	0.8336	0.8231	0.7846	0.8609
R^2 (adj)	0.8060	0.7980	0.8291	0.8183	0.7788	0.8571
p-level	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
SE of prediction	0.7109	0.7255	0.6673	0.6880	0.7592	0.6102

There was good correlation between SMS, TN and 'peak' 1 (R = 0.9130); SMS, TN and Tyr (R = 0.9072); SMS, Phe and TN (R = 0.8858); and SMS, 'peak' 17 and TN (R = 0.9278) (Table 8.3). The larger values for R^2 (adj) and the smaller values for the SE of prediction indicated that the combinations of TN and 'peak' 17 (Model E), TN and 'peak' 1 (Model D) or TN and Tyr were better predictors of sensory maturity than the combination of TN and MI (Model A) or TN and WSN (Model C).

Graphs depicting the relationship between SMS, 'peak' 1 and TN (Model D) or SMS, 'peak' 17 and TN (Model E), and the residual scores for both analyses were plotted alongside one another to allow comparisons to be made (Fig. 8.9).

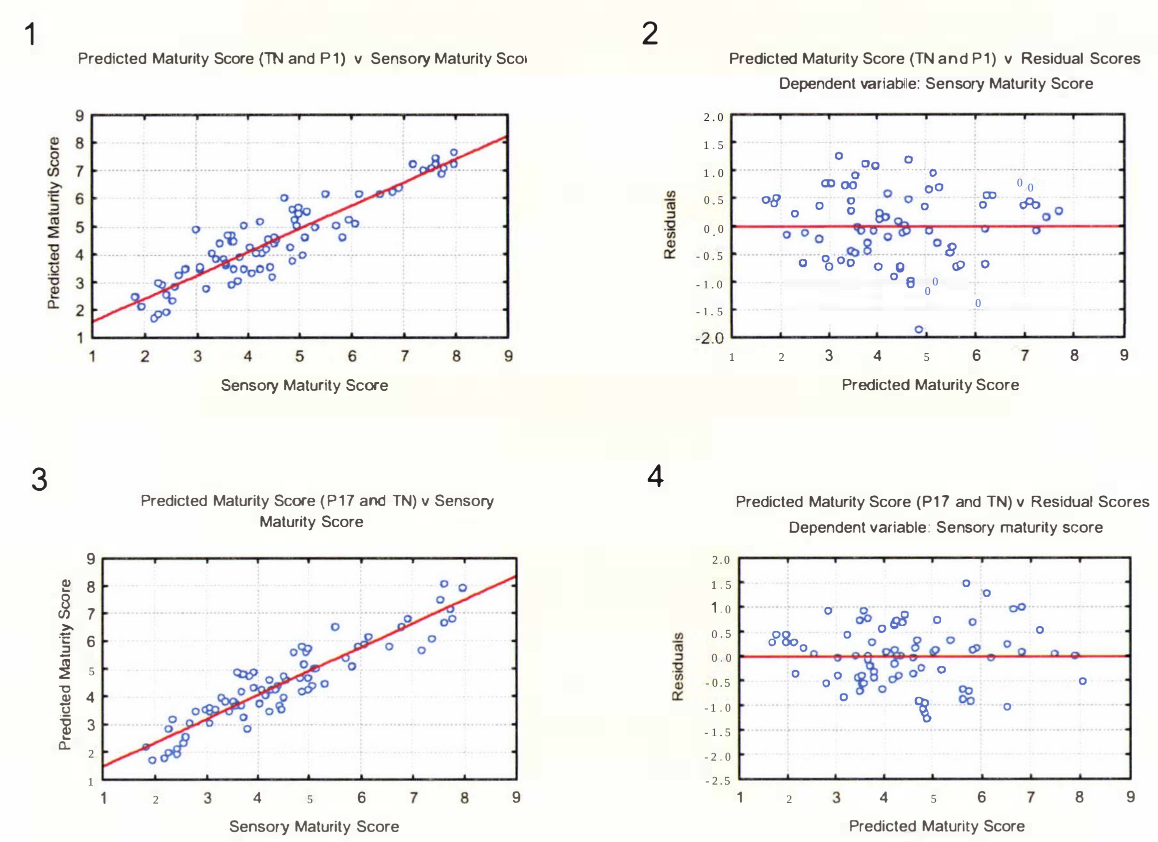


Fig. 8.9 *Comparison of the relationship between SMS, TN and the area of 'peak' 1 (Model D) and SMS, TN and the area of 'peak' 17 (Model E).* SMS v TN and the area of 'peak' 1 (graph 1) and the residuals (graph 2), and SMS v TN and the area of 'peak' 17 (graph 3) and the residuals (graph 4). A line of best fit (using a linear regression) was included on graph 1.

The graphs (Fig. 8.9) show the close association between SMS, TN and 'peak' 1 (Model D) and SMS, 'peak' 17 and TN (Model E) (Fig. 8.9). The residual scores were evenly spread across the range of SMSs (graphs 2 and 4). The graphs were similar to those obtained when SMS was plotted against TN and WSN (Model C) (Fig. 8.5, graphs 3 and 4), but the sample points were closer to the regression line (the range of residual scores was narrower). Models D and E predicted a higher maturity score for most mature cheese samples, and provided a better match with the SMSs than scores predicted by Model C.

The following regression equations describe the relationship between SMS, TN and 'peak' 1 (Model D) and SMS, TN and 'peak' 17 (Model E), respectively:

$$\text{Cheddar cheese maturity} = 16.7962906 - 4.0409530\ \text{TN} + 0.0000819\ \text{P1} \qquad \textit{(Equation 4)}$$

$$\text{Cheddar cheese maturity} = 12.135368 - 2.736957\ \text{TN} + 0.000104\ \text{P17} \qquad \textit{(Equation 5)}$$

8.4.3.1 PCA of the RP-HPLC peak areas

The previous results (Ch. 7) had suggested that PCA was an appropriate method of analysis of the RP-HPLC peak area data. In that chapter, PCA was used to reduce multi-dimensional data to two or three principal components that explained most of the variation within the data set. These principal components were used to group the samples according to differences common to each grouping (PCA of the correlation matrix) and to rank samples within each group according to maturity (PCA of the covariance matrix). In the present situation, PCA is used to examine cheese diversity, to determine whether cheese can be differentiated according to factory, to determine whether maturity trends are evident, and (if maturity trends are evident) to relate the principal component scores to the SMSs.

8.4.3.1.1 PCA of the correlation matrix (RP-HPLC)

The large data matrix resulting from RP-HPLC analysis of the cheese samples was analysed using PCA of the correlation matrix (Statistica 6.0), with the sensory maturity scores included as a supplementary variable. Supplementary variables do not influence the analysis, but their inclusion enables their relativity to the other variables (peaks) to be determined. The tables related to PCA of the correlation matrix, the scores for PC1, PC2 and PC3 can be found in the appendix (section 8.8.3.3).

Approximately 74% of the variation in the data could be explained by the first three principal components, with PC1 accounting for 39%, PC2 for 26% and PC3 for 9%. Fig. 8.11 depicts plots of the principal component factor loadings (or coordinates) for each of the variables peaks, with a vector for each. Peak vectors in the top left-hand corner are closely aligned with the sensory maturity vector and are likely to be more closely related to the development of sensory maturity than vectors relating to the remaining peaks.

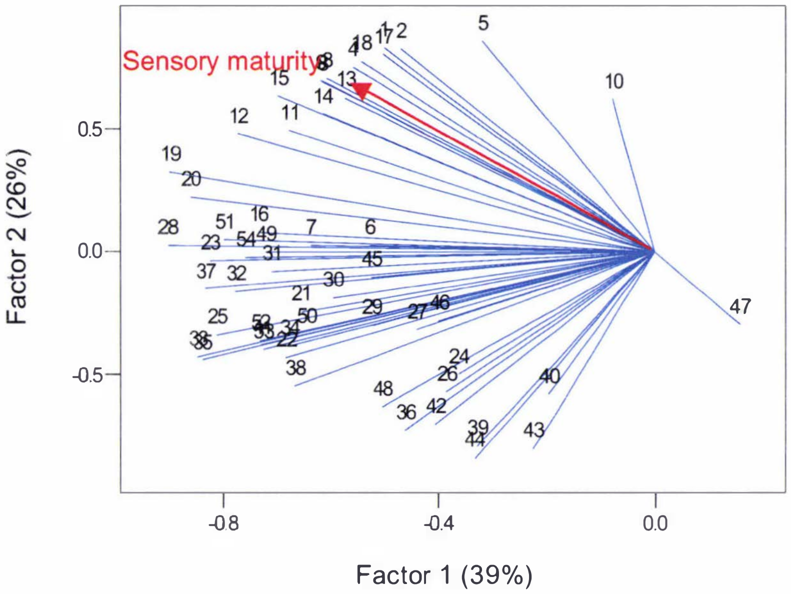


Fig. 8.11 *RP-HPLC (WSF) data analysis using PCA (correlation matrix) - loading plot.* Factors 1 and 2 for peaks 1-54 from RP-HPLC analysis of the WSF of Cheddar cheese ripened at 5, 10 or 13°C for 3, 6, 12 or 15 months. The red arrow depicts the direction of sensory maturity and its length is relative to the length of the peak vectors. Labels identify peak numbers.

Figs. 8.12 and 8.13 depict plots of the principal component scores (PC1, PC2 and PC3) for each of the samples. The data points have been labelled so that the effects of storage temperature and time (Fig. 8.12) and factory (Fig. 8.13) are clear. The direction of the maturity vector was taken from the loading plots (Fig. 8.11). The length of the vector is not relative to the position of the sample points.

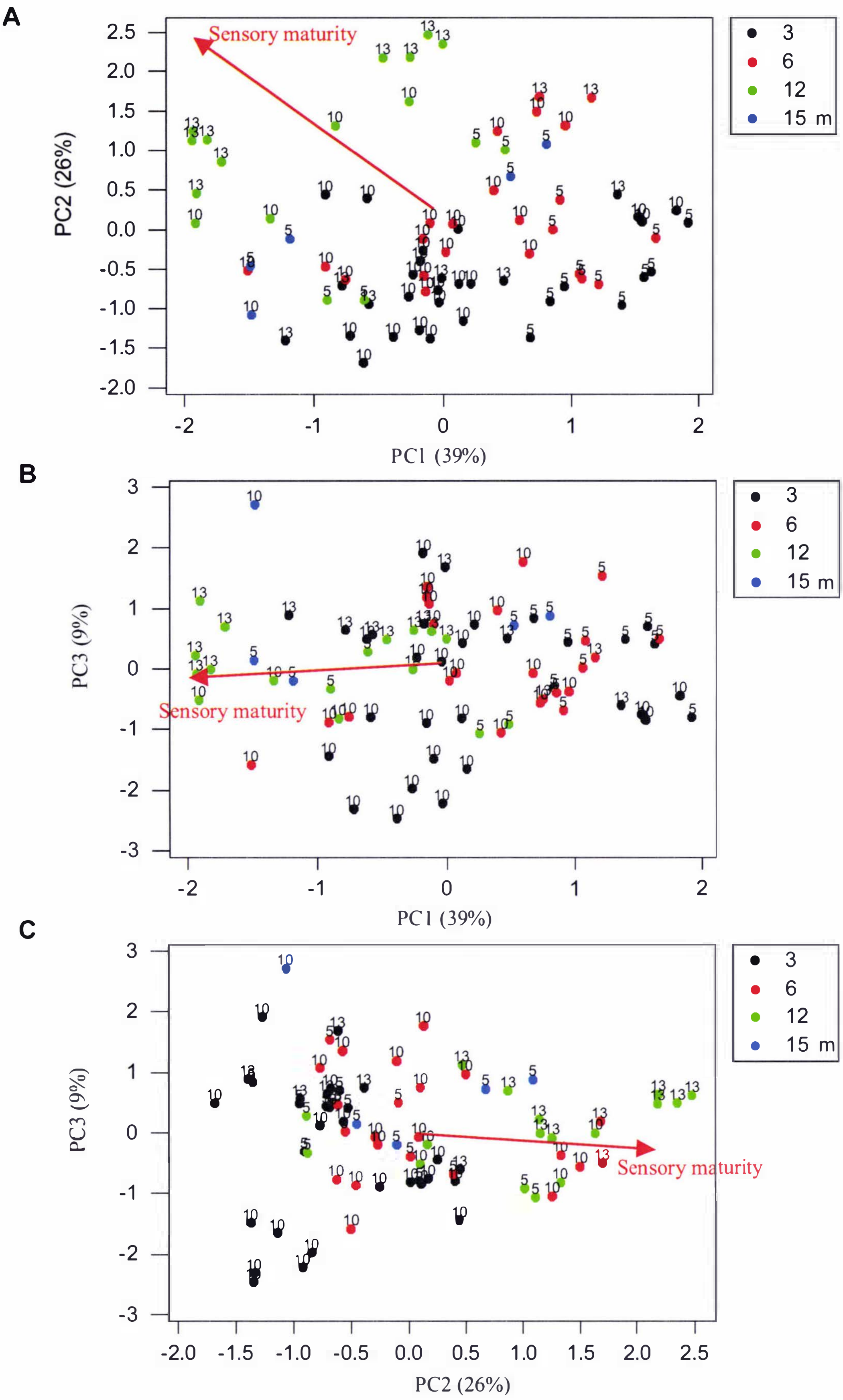


Fig. 8.12 *RP-HPLC (WSF) data analysis using PCA (correlation matrix) – score plot.* A) PC1 v PC2, B) PC1 v PC3 and C) PC2 v PC3 for peaks 1-54 from RP-HPLC analysis of the WSF of Cheddar cheese ripened at 5, 10 or 13°C for 3 (●), 6 (●), 12 (●) or 15 (●) months and labelled accordingly. Arrows indicate the direction of maturity.

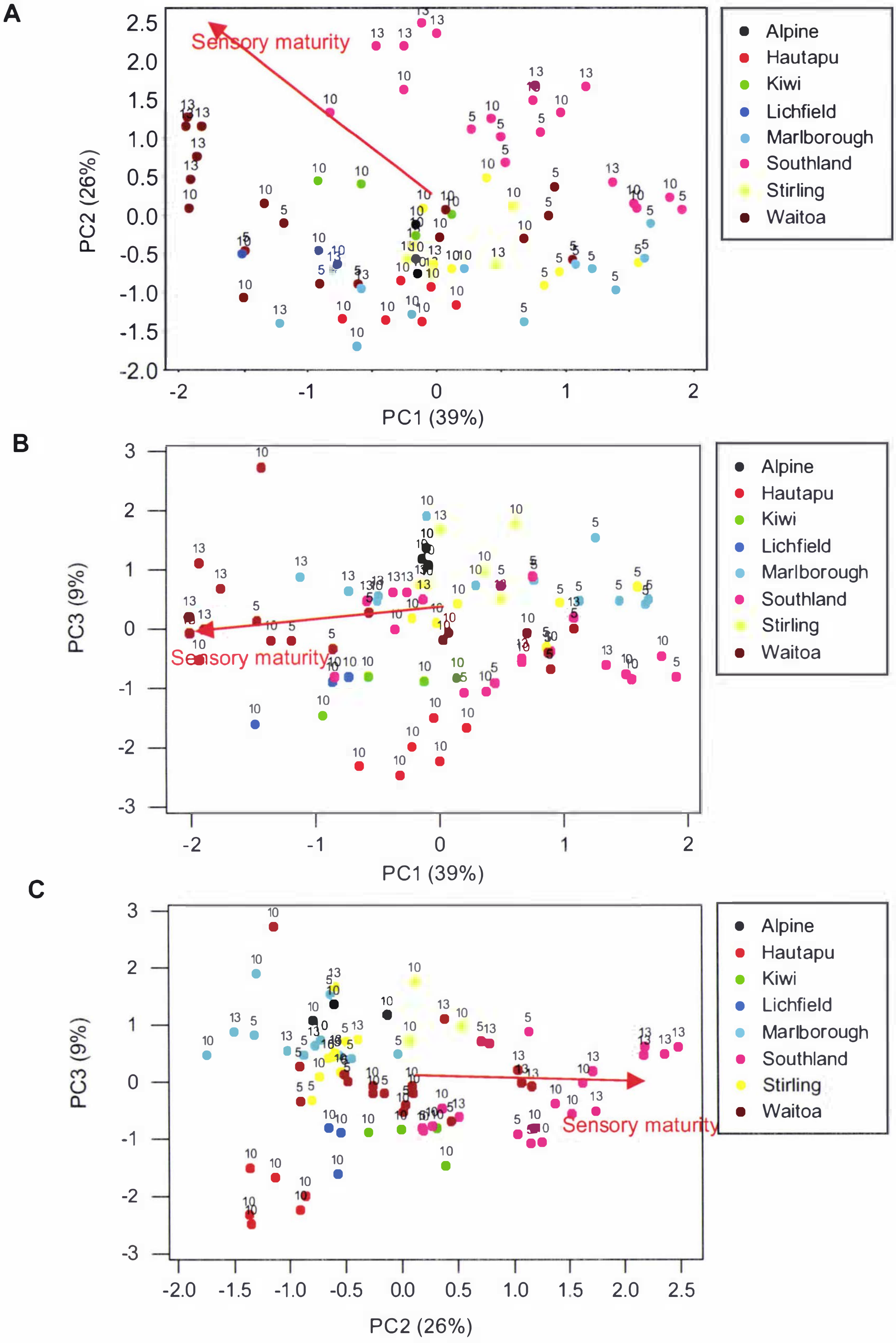


Fig. 8.13 *RP-HPLC (WSF) data analysis using PCA (correlation matrix) – score plot.* A) PC1 v PC2, B) PC1 v PC3 and C) PC2 v PC3 for peaks 1-54 from RP-HPLC analysis of the WSF of Cheddar cheese ripened at 5, 10 or 13°C for 3, 6, 12 or 15 months and labelled accordingly, but coloured according to cheese source (factory). (Note the data points for Fig. 8.12 and Fig. 8.13 are the same).

The direction of the maturity vector (Fig. 8.11) and the trends in the distribution of samples on the principal component plots (Fig. 8.12), were generally in accordance with the temperature and length of time of storage, with some influence from the factory. In particular, most cheese from Southland was maturer than would be expected on the basis of time and temperature alone. This indicates that factors other than ripening temperature and time are important for the development of mature flavour in Cheddar cheese.

MLR was used to relate the scores for the first three principal components (PC1, PC2 and PC3) to the SMSs. These three principal components were collectively highly correlated with the SMS (R = 0.8986, R^2 = 0.8074, R^2 (adj) = 0.7995, $p < 0.0001$, SE of prediction of maturity = 0.7227. The importance of TN to the relationship between SMS and WSN had previously been noted (Model C). RP-HPLC peaks are components of the WSN, and when TN was included as a variable in the analysis, the correlation with SMS was improved (R = 0.9341). Table 8.4 shows the results from MLR analysis of the scores for PC1, PC2 and PC3 and TN versus the SMSs (Model F).

Table 8.4 *Comparison of four models as predictors of sensory maturity.* Regression statistics (R, R^2, R^2 (adj), p and SE of prediction of sensory maturity) for SMS v WSN and TN (Model C), SMS v RP-HPLC 'peak' 1 and TN (Model D), SMS v RP-HPLC 'peak' 17 and TN (Model E), and SMS v TN and PC1, PC2 and PC3 of the (RP-HPLC) correlation matrix (Model F).

Model	**Model C**	**Model D**	**Model E**	**Model F**
Method	TN and WSN	RP-HPLC	RP-HPLC	RP-HPLC
PCA				Corr. matrix
MLR	SMS v TN and WSN	SMS v TN and 'Peak' 1	SMS v TN and 'Peak' 17	SMS v TN, PC1, PC2 and PC3
R	0.8963	0.9130	0.9278	0.9341
R^2	0.8033	0.8336	0.8609	0.8726
R^2 (adj)	0.7980	0.8291	0.8571	0.8655
p-level	< 0.0001	< 0.0001	< 0.0001	< 0.0001
SE of prediction	0.7255	0.6673	0.6102	0.5920

MLR analysis of the SMS and the combination of TN and the first three principal components resulting from RP-HPLC and PCA of the correlation matrix provided an R^2 (adj) of 0.8655 and a SE of prediction of 0.5920 (Table 8.4). This result indicated that RP-HPLC, when used in conjunction with PCA, provided a good indication of cheese maturity. Although it provided an improvement on the use of WSN and TN (R^2 (adj) = 0.7980) (Model C), it was only a little better than using the combination of RP-HPLC 'peak' 17 and TN (R^2 (adj) = 0.8571) (Model E) as the indicator of cheese maturity.

The regression equation for Model F is:

Cheddar cheese maturity = 16.79540 - 3.16884 TN - 0.79332 PC1 + 1.07824 PC2 + 0.04068 PC3 (*Equation 6*)

A graph of the observed sensory maturity versus the predicted sensory maturity was derived from the regression equation (Fig. 8.14, graph 1). The residuals for each of the samples are shown in Fig. 8.14 (graph 2).

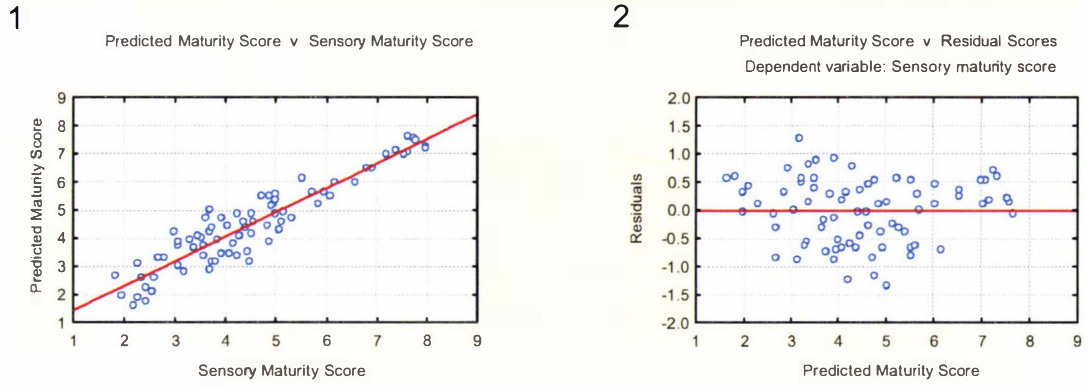


Fig. 8.14 *Predicted Cheddar cheese maturity, based on PCA of the correlation matrix (RP-HPLC) (Model F).* Graph 1) SMS v TN, PC1, PC2 and PC3, and graph 2) predicted maturity score v the residuals.

The graphs (Fig. 8.14) show that Model F provided a very good prediction of cheese sensory maturity and that the prediction was best for the more mature cheese, with a SMS of > 5. The prediction of maturity was likely to be related to the early-eluting peaks, as indicated by their close proximity to the maturity vector on the loading plot (Fig. 8.11). These peaks were the smallest peptides and amino acids that formed the end products of proteolysis and were therefore more abundant in the later stages of proteolysis. The wider distribution of residual scores for samples with predicted maturity scores of < 5 (Fig. 8.14, graph 2) seemed to be influencing the regression, and the residual scores for the samples with SMSs > 5 were mostly positive. The samples with lower SMSs came from the full range of factories, whereas those with higher scores represent fewer factories. The model is therefore providing a better prediction of maturity for some samples than for others. This was in agreement with the results of the previous chapter (Ch. 7), which showed that PCA of the correlation matrix was better for differentiation of cheese samples.

8.4.3.1.2 PCA of the covariance matrix (RP-HPLC)

The results of the previous chapter (Ch. 7) indicated that the covariance form of PCA was best for predicting cheese maturity because the original variance of the peaks was retained in the analysis.

The large data matrix resulting from RP-HPLC analysis of each cheese was analysed using the covariance form of PCA, with the SMSs included as a supplementary variable. Fig. 8.15 depicts the PCA loading plot, and Fig. 8.16 the PCA score plot with the maturity vector displayed. The data points have been labelled so that the effects of storage temperature and time (Fig. 8.16), as well as factory (Fig. 8.17) are clear. The tables related to PCA of the covariance matrix, the scores for PC1 (66%), PC2 (16%) and PC3 (6%) and the data and equations related to regression analysis can be found in the appendix (section 8.8.3.4).

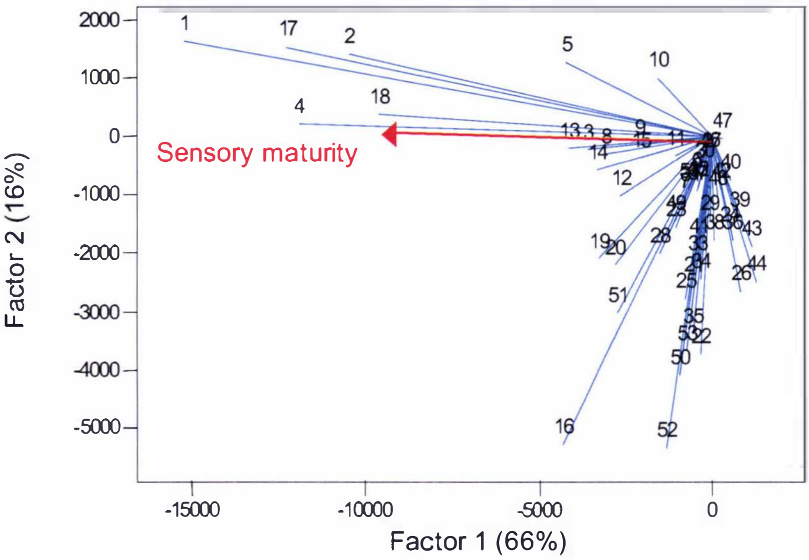


Fig. 8.15 *RP-HPLC (WSF) data analysis using PCA (covariance matrix) - loading plot.* Factors 1 and 2 for peaks 1-54 from RP-HPLC analysis of the WSF of Cheddar cheese ripened at 5, 10 or 13°C for 3, 6, 12 or 15 months. The red arrow depicts the direction of sensory maturity. (The length of the arrow has no significance when PCA of the covariance matrix is used).

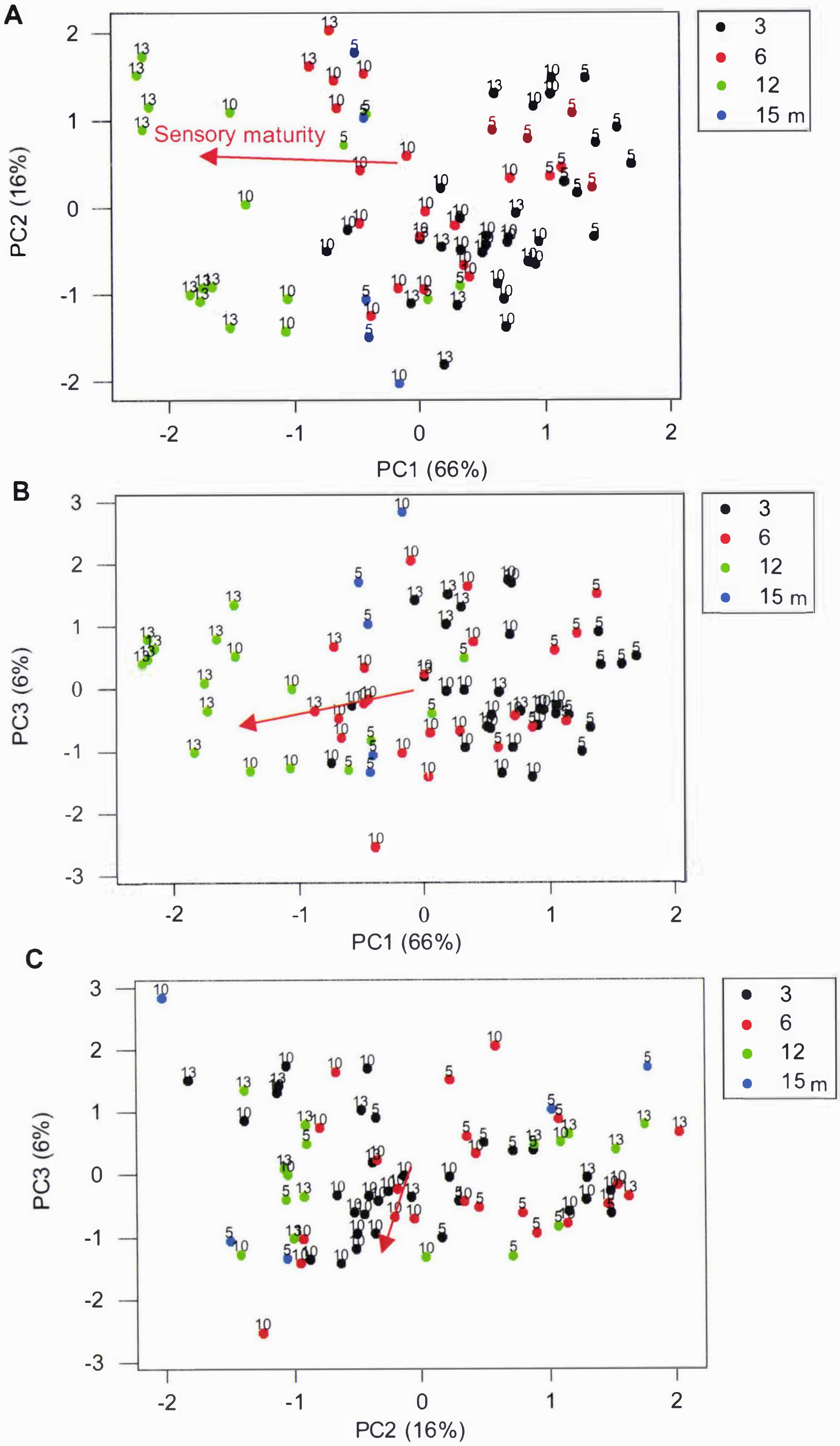


Fig. 8.16 *RP-HPLC (WSF) data analysis using PCA (covariance matrix) – score plot.* A) PC1 v PC2, B) PC1 v PC3 and C) PC2 v PC3 for peaks 1-54 from RP-HPLC analysis of the WSF of Cheddar cheese ripened at 5, 10 or 13°C for 3 (●), 6 (●), 12 (●) or 15 (●) months and labelled accordingly.

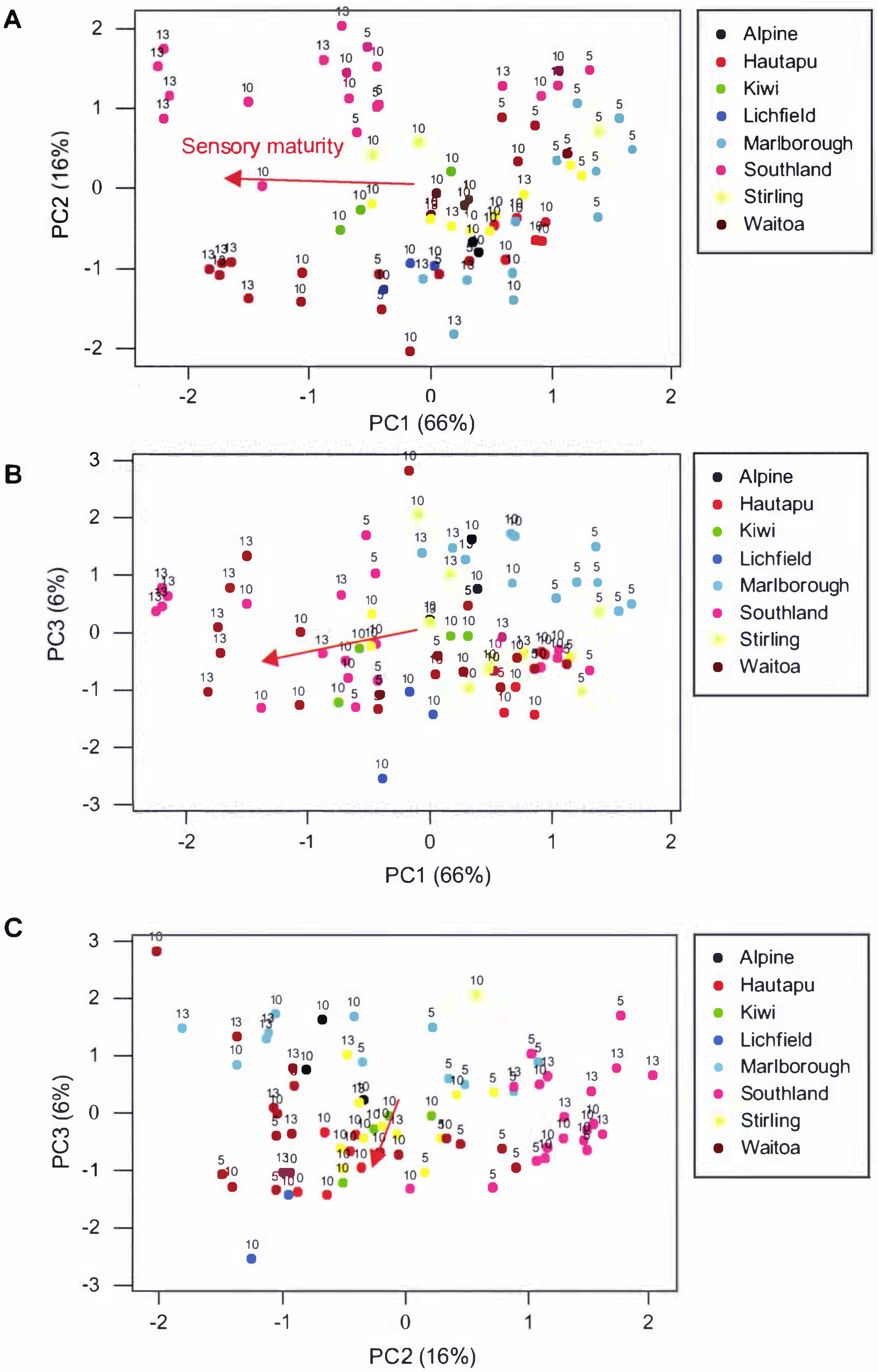


Fig. 8.17 *RP-HPLC (WSF) data analysis using PCA (covariance matrix) – score plot.* A) PC1 v PC2, B) PC1 v PC3 and C) PC2 v PC3 for peaks 1-54 from RP-HPLC analysis of the WSF of Cheddar cheese ripened at 5, 10 or 13°C for 3, 6, 12 or 15 months and labelled accordingly, but coloured according to cheese source (factory).

The covariance form of PCA accounted for 88.7% of the variation in the data within the first three principal components. PC1 (Figs. 8.16A) was closely associated with increased ripening temperature and time, as well as the direction of the sensory maturity vector (in agreement with the results in Ch.7). The loading plot (Fig. 8.15) showed that specific peaks (1, 2, 3, 4, 8, 9, 11, 13, 14, 15, 17 and 18) were closely associated with cheese maturity. Peaks 1-4 and 8-9 were comprised of amino acids, with peaks 2, 4 and 9 representing Tyr, Phe and Trp., while the remaining peaks, were likely to be comprised of very small peptides. The principal component plot (Fig. 8.17) showed that cheese from Southland was again differentiated from cheese from the other factories, mainly by the scores for PC2.

MLR of the principal component scores for PC1, PC2 and PC3 of the (RP-HPLC) covariance matrix with the SMS showed that these three principal components were well correlated with SMS (R = 0.9149, R^2 = 0.8370, R^2 (adj) = 0.8303, p = < 0.0001, SE of prediction = 0.6649). The correlation was further improved when TN was included as a variable (Model G) (Table 8.5).

Table 8.5 *Comparison of five models as predictors of sensory maturity.* Regression statistics (R, R^2, R^2 (adj), p and SE of prediction of sensory maturity) for SMS v WSN and TN (Model C), SMS v RP-HPLC 'peak' 1 and TN (Model D), SMS v RP-HPLC 'peak' 17 and TN (Model E), SMS v TN and PC1, PC2 and PC3 of the (RP-HPLC) correlation or covariance matrices (Models F and G, respectively).

Model	**Model C**	**Model D**	**Model E**	**Model F**	**Model G**
Method	TN and WSN	RP-HPLC	RP-HPLC	RP-HPLC	RP-HPLC
PCA				Corr. matrix	Cov. matrix
MLR	SMS v TN and WSN	SMS v TN and 'Peak' 1	SMS v TN and 'Peak' 17	SMS v TN, PC1, PC2 and PC3	SMS v TN, PC1, PC2 and PC3
R	0.8963	0.9130	0.9278	0.9341	0.9368
R^2	0.8033	0.8336	0.8609	0.8726	0.8776
R^2 (adj)	0.7980	0.8291	0.8571	0.8655	0.8708
p-level	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
SE of prediction	0.7255	0.6673	0.6102	0.5920	0.5802

The regression equation for Model G is:

Cheddar cheese maturity = 15.14657 – 2.74229 TN - 1.34888 PC1 + 0.02216 PC2 + 0.18720 PC3

(Equation 7)

A graph of the observed sensory maturity v the predicted sensory maturity was derived from the regression equation (Fig. 8.18, graph 1). The residuals for each of the samples are shown in Fig. 8.18 (graph 2).

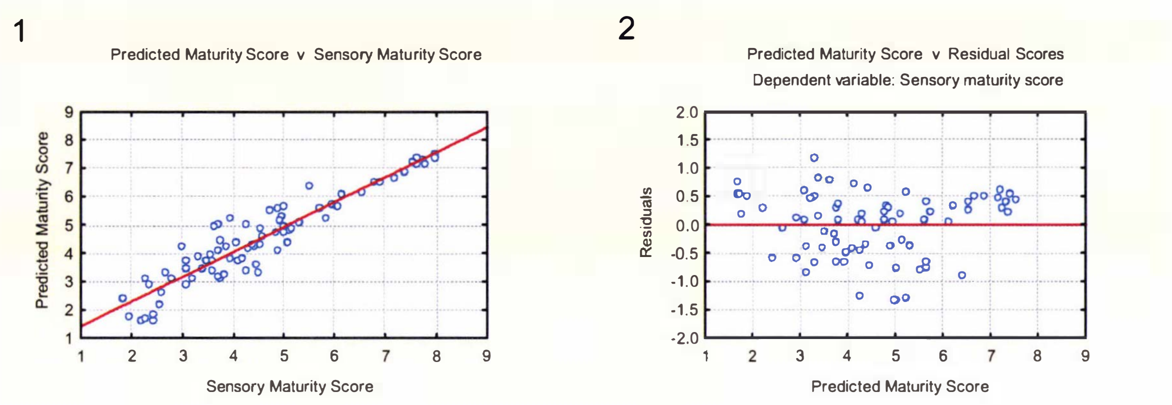


Fig. 8.18 *Predicted Cheddar cheese maturity, based on PCA of the covariance matrix (RP-HPLC) (Model G).* Graph 1) SMS v TN, PC1, PC2 and PC3 and graph 2) predicted maturity score v the residual scores.

The results (Table 8.5 and Fig. 8.18) showed that the RP-HPLC peak areas, when analysed with the covariance form of PCA, provided a good indication of cheese maturity, with MLR analysis of the SMSs and the scores of the first three principal components and TN (Model G) providing a value for R^2 (adj) of 0.8708. This value was greater than for any of the previous models (Table 8.5). The distribution of residual scores was fairly uniform (Fig. 8.18, graph 2), and the residual scores were smaller, indicating that this model provided a better representation of the samples and a better prediction of maturity than any previous model. The SE of prediction of 0.5802 was smaller than for the earlier models (Table 8.5). The graph (Fig. 8.18, graph 1) showed that the model provided a better prediction of the maturity of cheese with a SMS of > 5.

These results were consistent with those of the previous chapter (Ch. 7) which indicated that PCA of the covariance matrix was the most appropriate form of PCA for the assessment of cheese maturity.

8.4.4 SE-HPLC and the maturity of Cheddar cheese

SE-HPLC was used to obtain a simple chromatogram of the components of the WSF of cheese (Ch. 5). As well, it was demonstrated that the molecular weight distribution or peptide profile of a cheese changed with time for a range of cheese types (Ch. 7). SE-HPLC provides a much simpler peptide profile than RP-HPLC, with comparatively few peaks to be analysed (Ch. 7). These factors suggested that SE-HPLC could be developed to provide an objective index of cheese maturity.

The column used for the analysis of the samples was a new TSK G-2000 SWXL column with improved resolution of the peptide peaks when compared with the column used previously (Ch. 6 and Ch.7). Although there were some minor differences in the molecular weight distribution profiles for Cheddar cheese, the profiles were remarkably similar considering they were of Cheddar cheese made in a different year, at a different factory, and analysed on a different column using a different HPLC.

SE-HPLC traces were generated using each of the 77 cheese samples, and the 15 peak regions were numbered according to the labelled chromatograph (Fig. 8.19). The 'peaks' could be divided into four groups, those entering the aqueous phase and being subsequently hydrolysed (peaks 1 to 5, >5000 Da), peptide intermediates (peaks 6 to 10, 5000 to 1000 Da), peptide and amino acid end-products (peaks 11 to 13, <1000 Da) and a collection of low molecular weight peaks representing some products of amino acid catabolism as well as any salts present in the cheese that absorb light at 205 nm (peaks 14 and 15).

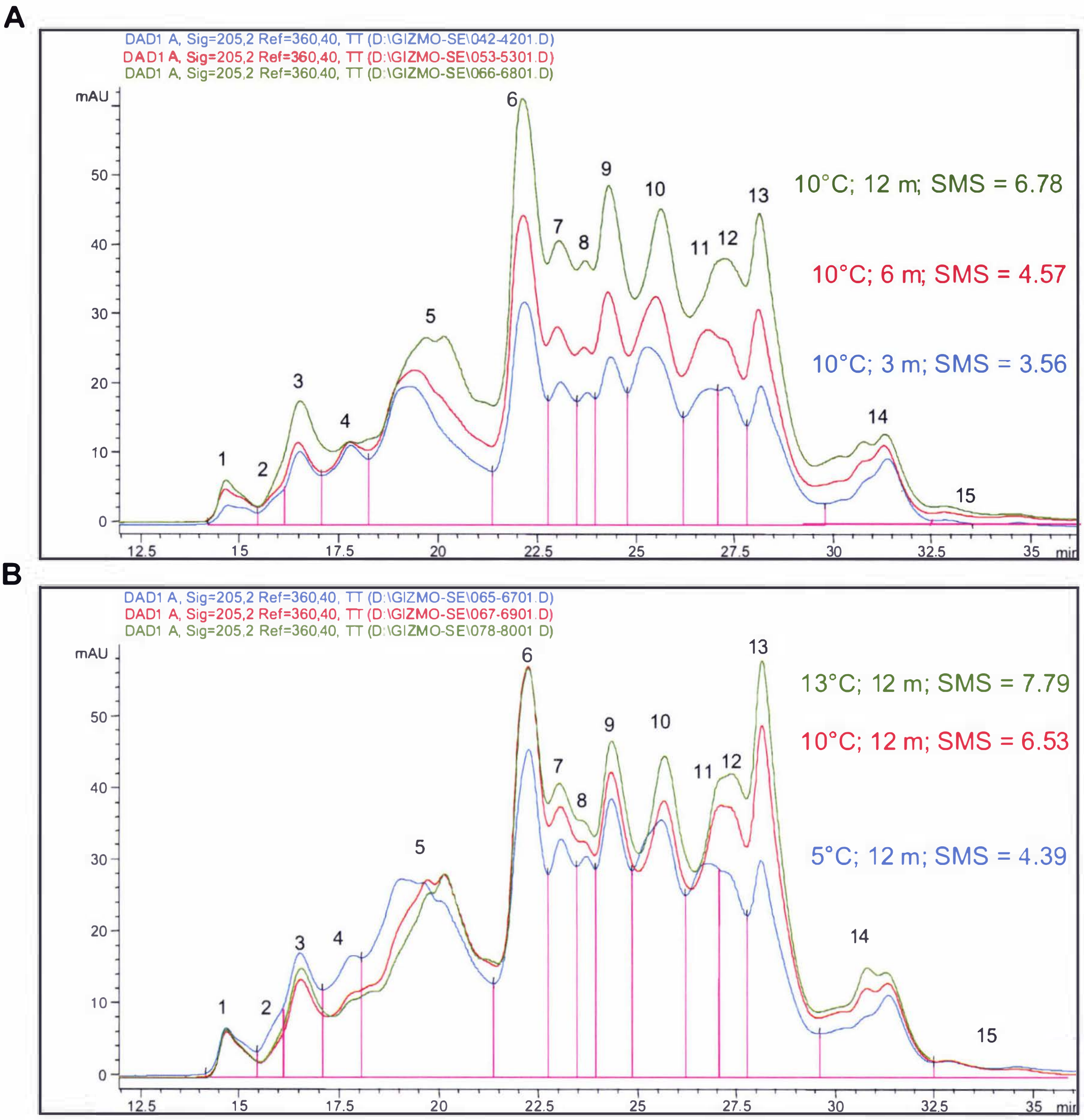
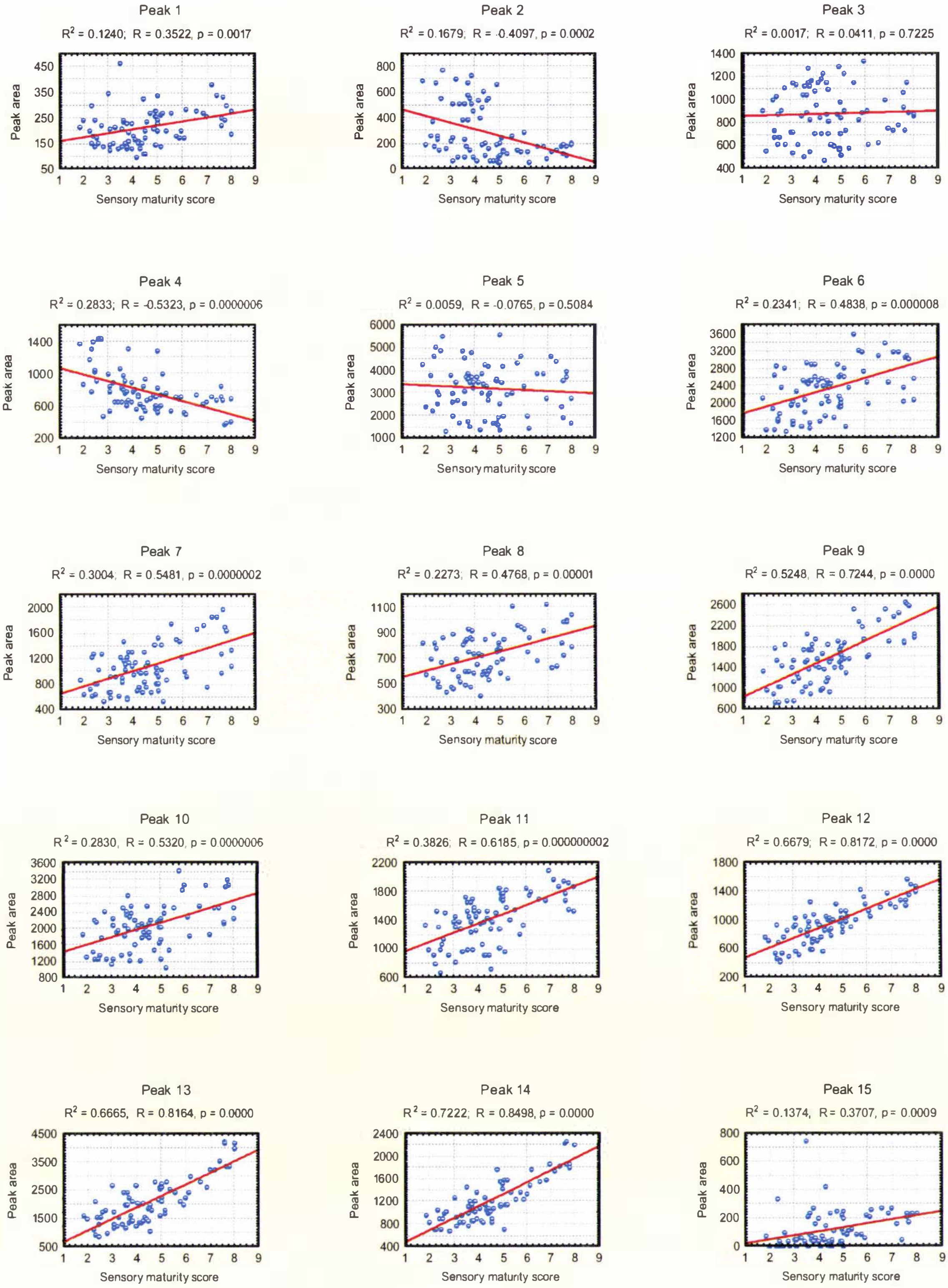


Fig. 8.19 *SE-HPLC peptide profiles of the WSF of Cheddar cheese.* Cheddar cheese made at different times of the year at the same factory (Waitoa) and ripened A) at 10°C for 3, 6 or 12 m and B) 5, 10 or 13°C for 12 m. The samples that had been prepared for RP-HPLC were diluted (100 μL with 2.4 mL of the mobile phase (36% acetonitrile, 0.1% TFA)) prior to loading a 50 μL aliquot on to the column. The eluent flow rate was 0.4 mL/min. The absorbance was measured at 205, 210, 220, 280 and 295 nm but only the 205 nm absorbance was plotted. The SMS is recorded alongside each corresponding chromatograph.

It was evident that as the cheese matured there was a gradual increase in the area of the later-eluting peaks. These peaks are comprised of di- and tri-peptides, plus basic and other amino acids (Ch. 5, Table 5.1).

The area of each of the 'peaks' was determined and the data incorporated in a spreadsheet (see appendix, section 8.8.4.1). This provided a large data matrix comprised of 77 samples and 15 'peak' areas. A series of graphs that demonstrates the relationship between the SMS and each of the 'peak' areas is depicted in Fig. 8.20.



Peak 1
$R^2 = 0.1240$; $R = 0.3522$, $p = 0.0017$

Peak 2
$R^2 = 0.1679$; $R = -0.4097$, $p = 0.0002$

Peak 3
$R^2 = 0.0017$; $R = 0.0411$, $p = 0.7225$

Peak 4
$R^2 = 0.2833$; $R = -0.5323$, $p = 0.0000006$

Peak 5
$R^2 = 0.0059$, $R = -0.0765$, $p = 0.5084$

Peak 6
$R^2 = 0.2341$; $R = 0.4838$, $p = 0.000008$

Peak 7
$R^2 = 0.3004$; $R = 0.5481$, $p = 0.0000002$

Peak 8
$R^2 = 0.2273$; $R = 0.4768$, $p = 0.00001$

Peak 9
$R^2 = 0.5248$, $R = 0.7244$, $p = 0.0000$

Peak 10
$R^2 = 0.2830$, $R = 0.5320$, $p = 0.0000006$

Peak 11
$R^2 = 0.3826$; $R = 0.6185$, $p = 0.000000002$

Peak 12
$R^2 = 0.6679$; $R = 0.8172$, $p = 0.0000$

Peak 13
$R^2 = 0.6665$, $R = 0.8164$, $p = 0.0000$

Peak 14
$R^2 = 0.7222$; $R = 0.8498$, $p = 0.0000$

Peak 15
$R^2 = 0.1374$, $R = 0.3707$, $p = 0.0009$

Fig. 8.20 *Cheese SMS v the area of each of the SE-HPLC 'peaks' (1 – 15).*

There was a linear relationship between the area of ‘peaks’ 1, 6, 7, 8, 9, 10, 11, 12, 13 and 14 and SMS, with the ‘peaks’ of the more mature cheese having the greatest area. Many ‘peaks’ were likely to be comprised of peptide intermediates that increased in concentration at a greater rate than they decreased. ‘Peaks’ 12, 13 and 14 were most highly correlated with sensory maturity (R^2 = 0.6679, 0.6665 and 0.7222, respectively) and these were comprised mainly of amino acids and other small water-soluble compounds (Ch. 5, Table 5.1). ‘Peak’ 4 was negatively correlated with SMS and decreased in size, as its components were hydrolysed as the cheese matured. ‘Peaks’ 2, 3, 5 and 15 showed no particular relationship with cheese maturity. ‘Peaks’ 2, 3 and 5 differed most between the various samples analysed. These were the highest molecular weight peptides and it is most likely that they continued to be produced and hydrolysed throughout the observed stages of maturation.

8.4.4.1 PCA of the SE-HPLC peak areas

The results from the previous chapter (Ch. 7) had suggested that PCA was an appropriate method of analysis of the SE-HPLC 'peak' area data. PCA of the correlation matrix provided differentiation of cheese samples, while PCA of the covariance matrix provided cheese maturity trends. Both forms of PCA were used to analyse the SE-HPLC 'peak' area data set.

8.4.4.1.1 PCA of the correlation matrix (SE-HPLC)

The SE-HPLC data were analysed using the correlation form of PCA to verify the previous findings (Ch. 7) and to determine whether the cheese samples could be differentiated on the basis of factory of origin.

Fig. 8.21 depicts the PCA loading plot, and Fig. 8.22 the PCA score plots with the maturity vector displayed. The data points have been labelled so that the effects of storage temperature and time (Fig. 8.22), as well as factory (Fig. 8.23) are clear. The tables related to PCA of the correlation matrix, the scores for PC1, PC2 and PC3 and the data and equations related to the regression analysis can be found in the appendix (section 8.8.4.2).

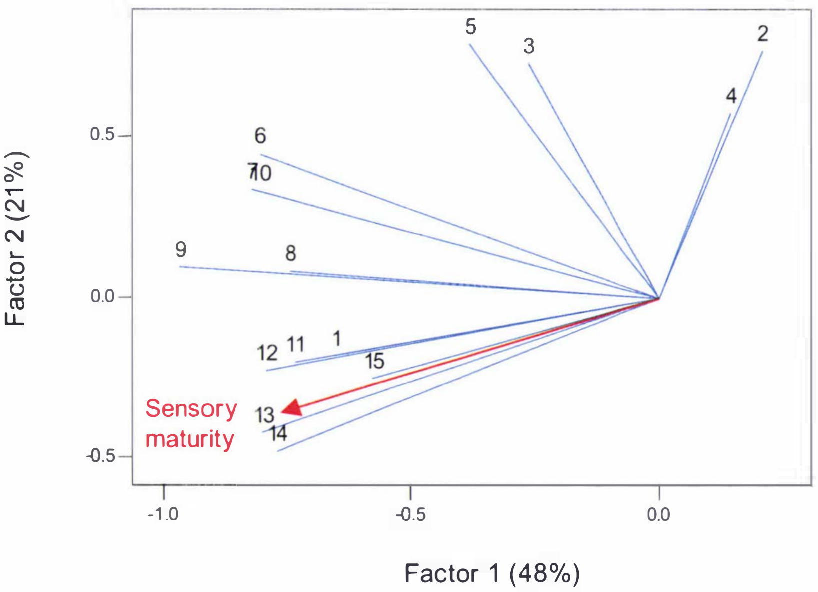


Fig. 8.21 *SE-HPLC (WSF) data analysis using PCA (correlation matrix) – loading plot.* Factors 1 and 2 for 'peaks' 1-15 from SE-HPLC analysis of the WSF of Cheddar cheese (77 samples) ripened at 5, 10 or 13°C for 3, 6, 12 or 15 months.

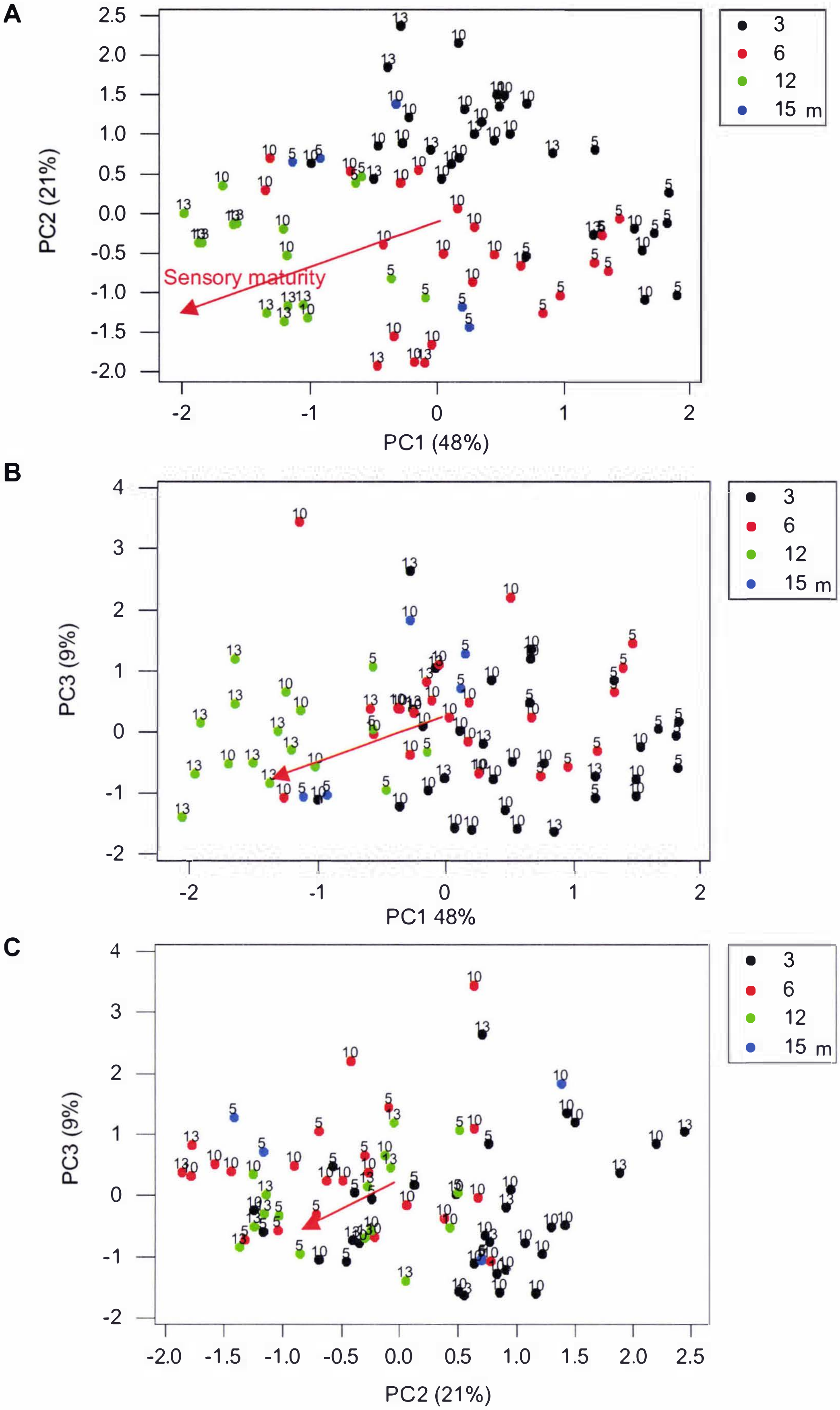


Fig. 8.22 *SE-HPLC (WSF) data analysis using PCA (correlation matrix) – score plots.* A) PC1 v PC2, B) PC1 v PC3 and C) PC2 v PC3 for 'peaks' 1- 15 from SE-HPLC analysis of the WSF of Cheddar cheese ripened at 5, 10 or 13°C for 3 (●), 6 (●), 12 (●) or 15 (●) months and labelled accordingly.

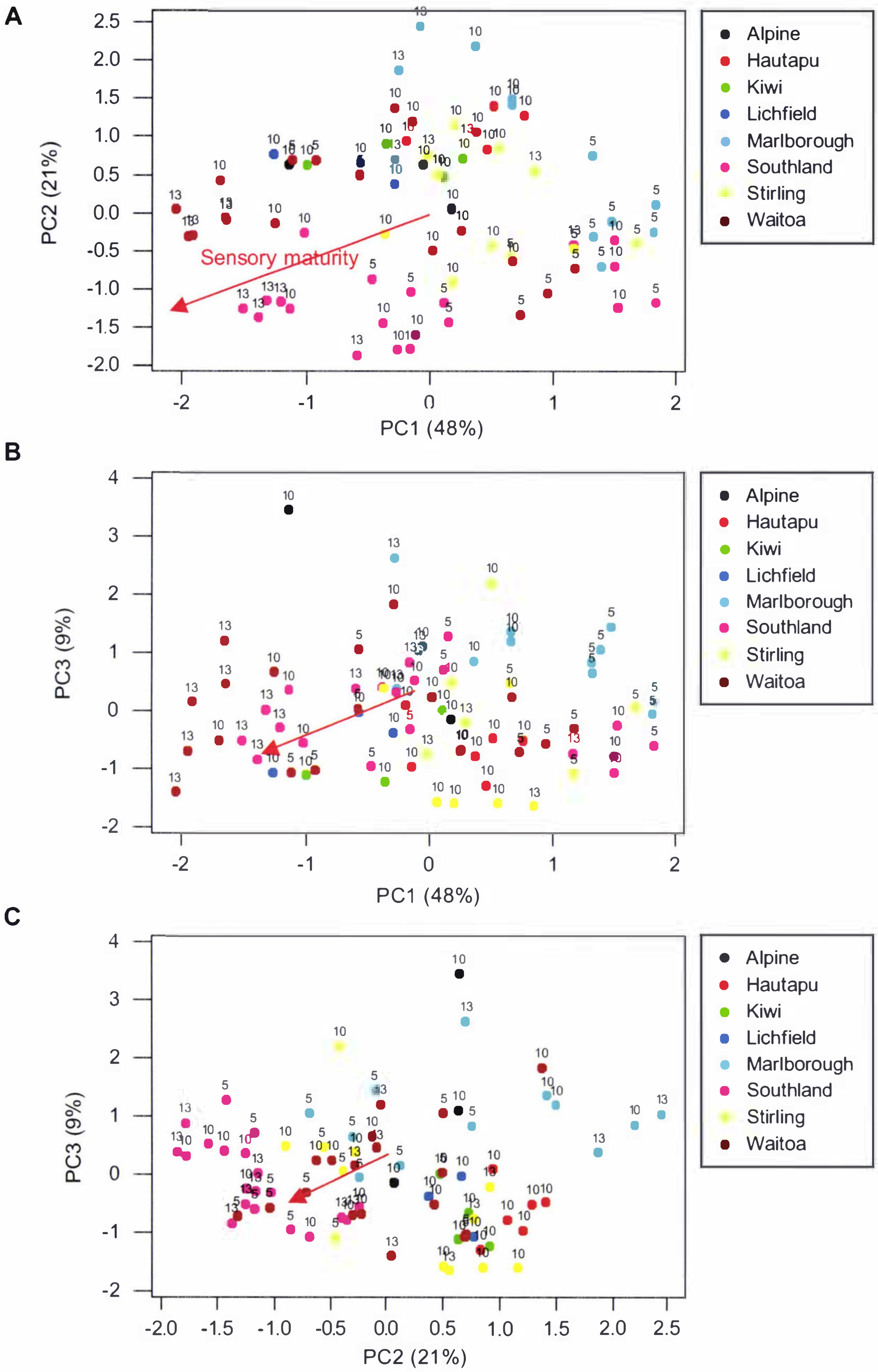


Fig. 8.23 *SE-HPLC (WSF) data analysis using PCA (correlation matrix) – score plots.* A) PC1 v PC2, B) PC1 v PC3 and C) PC2 v PC3 'peaks' 1-15 from SE-HPLC analysis of the WSF of Cheddar cheese ripened at 5, 10 or 13°C for 3, 6, 12 or 15 months, and coloured according to cheese source (factory).

The correlation form of PCA provided a good description of the data with 78% of the variation accounted for within the first three principal components. PC1 was most closely associated with cheese sensory maturity (Fig. 8.21). This was consistent with trends in the distribution of samples on the principal component plots (Figs. 8.22A and 8.23A), which were generally in accordance with the temperature and storage time. Cheese from Southland was differentiated from cheese from the other factories on the basis of the scores for PC2 and PC3 (Fig. 8.23C).

MLR analysis of the principal component scores for PC1, PC2 and PC3 with the SMS (see appendix, section 8.8.4.2) showed that the principal component scores correlated well with SMS (R = 0.9016, R^2 = 0.8129, R^2 (adj) = 0.8053, $p < 0.0001$ and SE of prediction = 0.7123). This was improved with the incorporation of TN in the model (Model H). The statistics for Model H are compared with those of previous models (Table 8.6).

Table 8.6 *Comparison of six models as predictors of sensory maturity.* Regression statistics (R, R^2, R^2 (adj), *p* and SE of prediction of sensory maturity) for SMS v WSN and TN (Model C), SMS v RP-HPLC 'peak' 1 and TN (Model D), SMS v RP-HPLC 'peak' 17 and TN (Model E), SMS v TN and PC1, PC2 and PC3 of the (RP-HPLC) correlation or covariance matrices (Models F and G, respectively), and SMS v TN and PC1, PC2 and PC3 of the (SE-HPLC) correlation matrix (Model H).

Model Method PCA MLR	**Model C** TN and WSN SMS v TN and WSN	**Model D** RP-HPLC SMS v TN and 'Peak' 1	**Model E** RP-HPLC SMS v TN and 'Peak' 17	**Model F** RP-HPLC Corr. matrix SMS v TN, PC1, PC2 and PC3	**Model G** RP-HPLC Cov. matrix SMS v TN, PC1, PC2 and PC3	**Model H** SE-HPLC Corr. matrix SMS v TN, PC1, PC2 and PC3
R	0.8963	0.9130	0.9278	0.9341	0.9368	0.9098
R^2	0.8033	0.8336	0.8609	0.8726	0.8776	0.8277
R^2 (adj)	0.7980	0.8291	0.8571	0.8655	0.8708	0.8181
p-level	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
SE of prediction	0.7255	0.6673	0.6102	0.5920	0.5802	0.6883

The regression equation for Model H is:

$$\text{Cheddar cheese maturity} = 10.72339 - 1.59841\ \text{TN} - 1.19119\ \text{PC1} - 0.54234\ \text{PC2} + 0.41501\ \text{PC3}$$

(Equation 8)

A graph of the observed sensory maturity versus the predicted sensory maturity score was derived from the regression equation (Fig. 8.24).

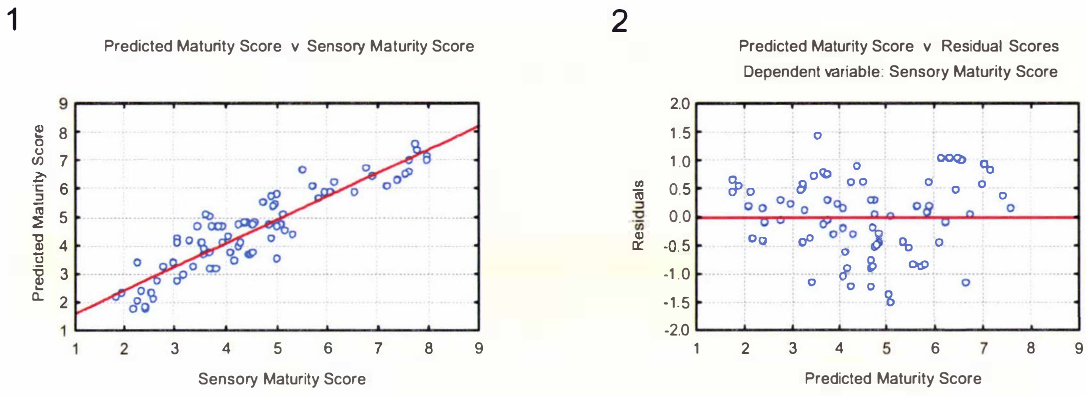


Fig. 8.24 *Predicted Cheddar cheese maturity, based on PCA of the correlation matrix (SE-HPLC).* Graph 1) SMS v TN, PC1, PC2 and PC3, and graph 2) predicted maturity score v the residual scores.

The results (Table 8.6) showed that the SE-HPLC peak data, when analysed using the correlation form of PCA produced principal component scores that when combined with TN (Model H), correlated well with SMS (R = 0.9098) and provided a good indication of cheese maturity. The regression of SMS versus the combination of TN, PC1, PC2 and PC3 provided an R^2 (adj) of 0.8181. This was less than the value obtained when the combination of TN and the first three principal components of the (RP-HPLC) correlation matrix (0.8655) was used (Model F, Table 8.6). However, the distribution of sample points on the graphs of the predicted maturity score and SMS (Fig. 8.24, graph 1) and the residuals (Fig. 8.24, graph 2) were very similar to the distribution of points on the graphs obtained when TN and the first three principal components of the (RP-HPLC) correlation matrix was used (Fig. 8.14, graphs 1 and 2) (Model F). This indicates that the principles that enabled the differentiation of maturity were similar for both methods. An examination of the loading plot (Fig. 8.22) showed that the later-eluting material ('peaks' 11 - 15) was most closely associated with sensory maturity. These 'peaks' were comprised mainly of peptides of less than 1000 Da and amino acids and were therefore the end-products of proteolysis. As Cheddar cheese matures the size of these 'peaks' increases and in the later stage of maturation their increase in size is marked.

8.4.4.1.2 PCA of the covariance matrix (SE-HPLC)

In Ch. 7, it was shown that when SE-HPLC peak data were analysed using PCA of the covariance matrix, trends related to cheese maturity were produced. In the current chapter, it was shown that this form of PCA provided the best form of assessment of the RP-HPLC peak area data (section 8.4.3.1.2). The covariance form of PCA was therefore used to analyse the SE-HPLC data set of 77 samples and 15 'peaks'. The scores for PC1, PC2 and PC3 were regressed against the SMS to determine the relationship between the SE-HPLC peak areas and sensory maturity.

Fig. 8.26 depicts the PCA loading plot, and Fig. 8.27 the PCA score plots with the sensory maturity vector displayed. The data points have been labelled so that the effects of storage temperature and time (Fig. 8.27), as well as factory (Fig. 8.28) are evident. The tables related to PCA of the covariance matrix, the scores for PC1, PC2 and PC3 and the data and equations related to the regression analysis can be found in the appendix (section 8.8.4.3).

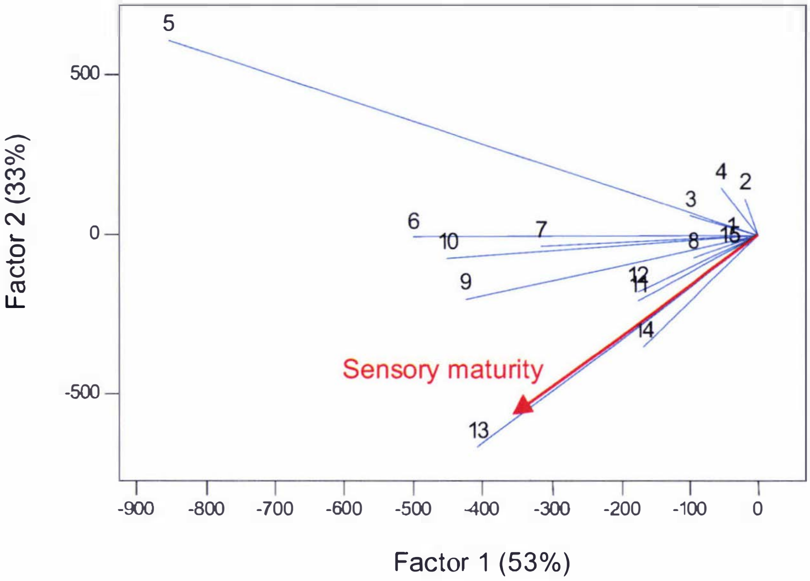


Fig. 8.26 *SE-HPLC (WSF) data analysis using PCA (covariance matrix) – loading plot.* Factors 1 and 2 for 'peaks' 1-15 from SE-HPLC analysis of the WSF of Cheddar cheese ripened at 5, 10 or 13°C for 3, 6, 12 or 15 months.

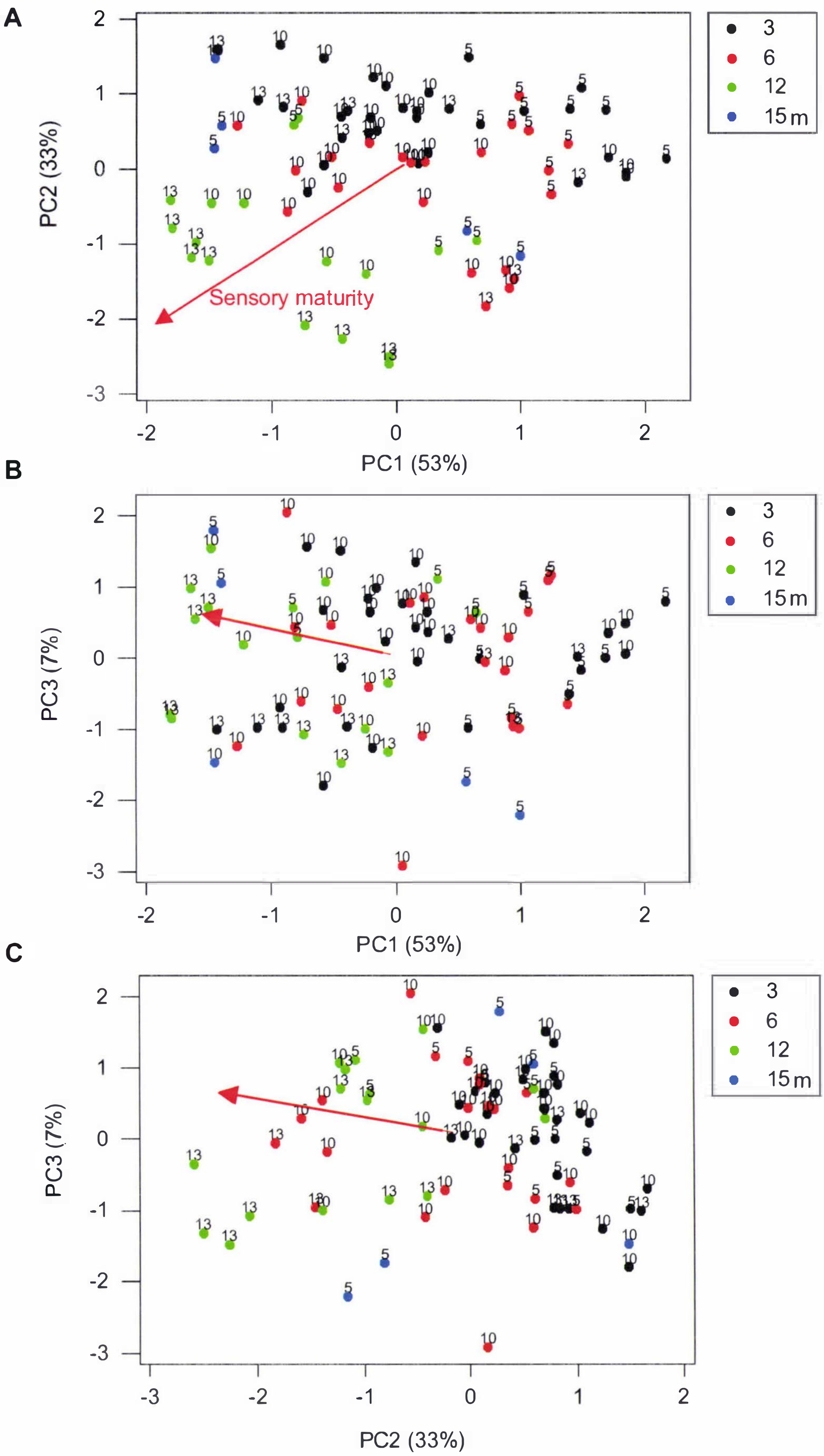


Fig. 8.27 *SE-HPLC (WFS) data analysis using PCA (covariance matrix) – score plots.* A) PC1 v PC2, B) PC1 v PC3 and C) PC2 v PC3 for 'peaks' 1- 15 from SE-HPLC analysis of the WSF of Cheddar cheese ripened at 5, 10 or 13°C for 3 (●), 6 (●), 12 (●) or 15 (●) months and labelled accordingly.

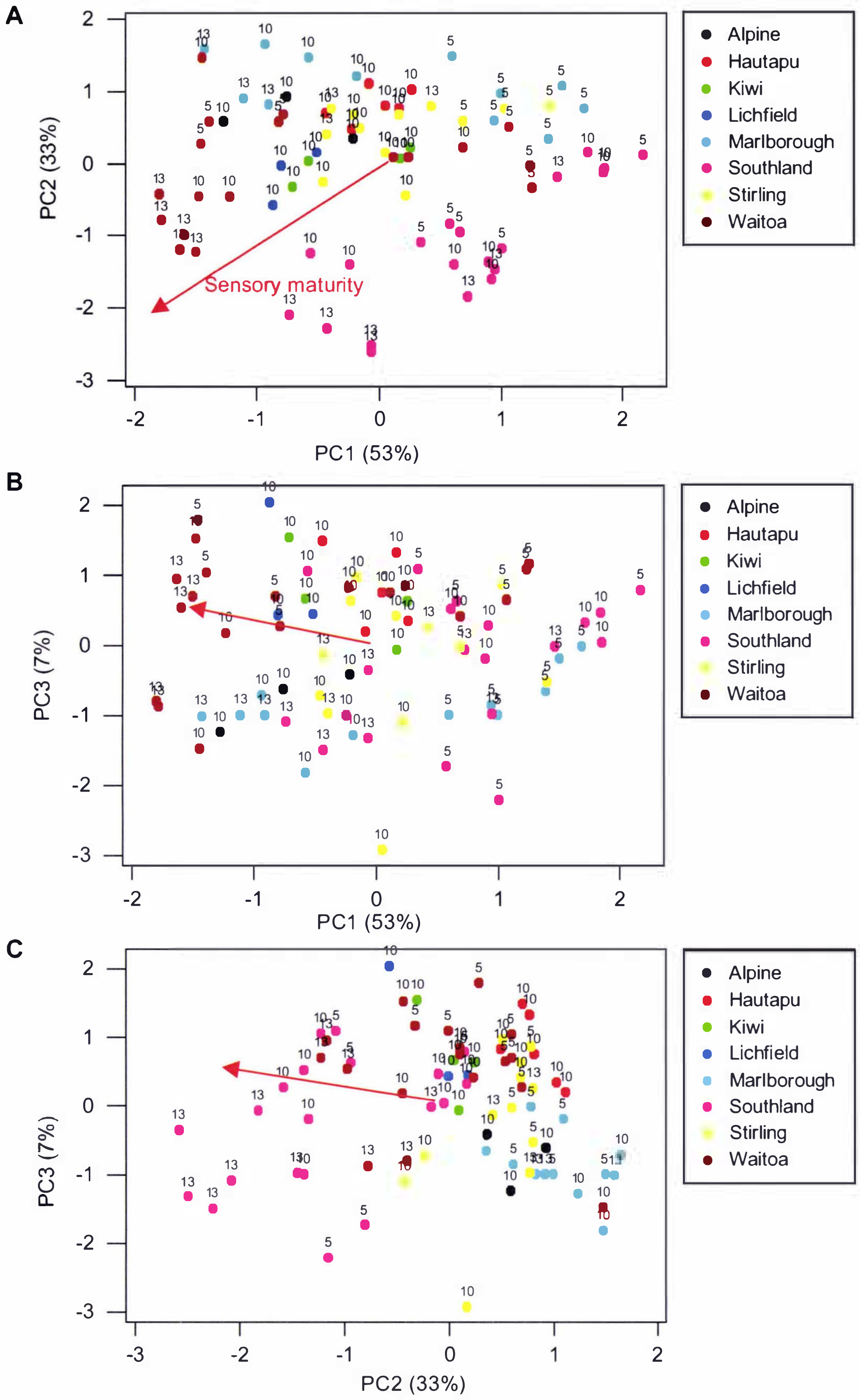


Fig. 8.28 *SE-HPLC (WFS) data analysis using PCA (covariance matrix) – score plots.* A) PC1 v PC2, B) PC1 v PC3 and C) PC2 v PC3 for 'peaks' 1- 15 from SE-HPLC analysis of the WSF of Cheddar cheese ripened at 5, 10 or 13°C for 3, 6, 12 or 15 months, and coloured according to cheese source (factory).

The covariance form of PCA provided a very good assessment of the data, with 93% accounted for in the first three principal components. The direction of the sensory maturity vector mainly reflected the combination of storage time and temperature (Fig. 8.27) with some factory influence (Fig. 8.28). The predominant 'peaks' that influenced the direction of maturity were 'peaks' 11 - 14 (Fig. 8.26), which were comprised of peptides of less than 1000 Da, amino acids, and other end products of proteolysis.

MLR analysis showed that the principal component scores for PC1, PC2 and PC3 were highly correlated with SMS ($R = 0.9024$, $R^2 = 0.8143$, R^2 (adj) $= 0.8067$, $p < 0.0001$, SE of prediction = 0.7097). This correlation was further improved when TN was incorporated in the model (Model I) (Table 8.7).

Table 8.7 *Comparison of seven models as predictors of sensory maturity.* Regression statistics (R, R^2, R^2 (adj), p and SE of prediction of sensory maturity) for SMS v WSN and TN (Model C), SMS v RP-HPLC 'peak' 1 and TN (Model D), SMS v RP-HPLC 'peak' 17 and TN (Model E), SMS v TN and PC1, PC2 and PC3 of the (RP-HPLC) correlation or covariance matrices (Models F and G, respectively), and SMS v TN and PC1, PC2 and PC3 of the (SE-HPLC) correlation and covariance matrices (Models H and I, respectively).

Model	**Model C**	**Model D**	**Model E**	**Model F**	**Model G**	**Model H**	**Model I**
Method	TN and WSN	RP-HPLC	RP-HPLC	RP-HPLC	RP-HPLC	SE-HPLC	SE-HPLC
PCA				Corr. matrix	Cov. matrix	Corr. matrix	Cov. matrix
MLR	SMS v TN and WSN	SMS v TN and 'Peak' 1	SMS v TN and 'Peak' 17	SMS v TN, PC1, PC2 and PC3	SMS v TN, PC1, PC2 and PC3	SMS v TN, PC1, PC2 and PC3	SMS v TN, PC1, PC2 and PC3
R	0.8963	0.9130	0.9278	0.9341	0.9368	0.9098	0.9224
R^2	0.8033	0.8336	0.8609	0.8726	0.8776	0.8277	0.8508
R^2 (adj)	0.7980	0.8291	0.8571	0.8655	0.8708	0.8181	0.8425
p-level	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
SE of prediction	0.7255	0.6673	0.6102	0.5920	0.5802	0.6883	0.6406

The regression equation for Model I is:

$$\text{Cheddar cheese maturity} = 14.62095 - 2.60719\ \text{TN} - 0.71571\ \text{PC1} - 1.12658\ \text{PC2} - 0.08739\ \text{PC3}$$

(Equation 9)

A graph of the observed sensory maturity versus the predicted sensory maturity was derived from the regression equation (Fig. 8.29).

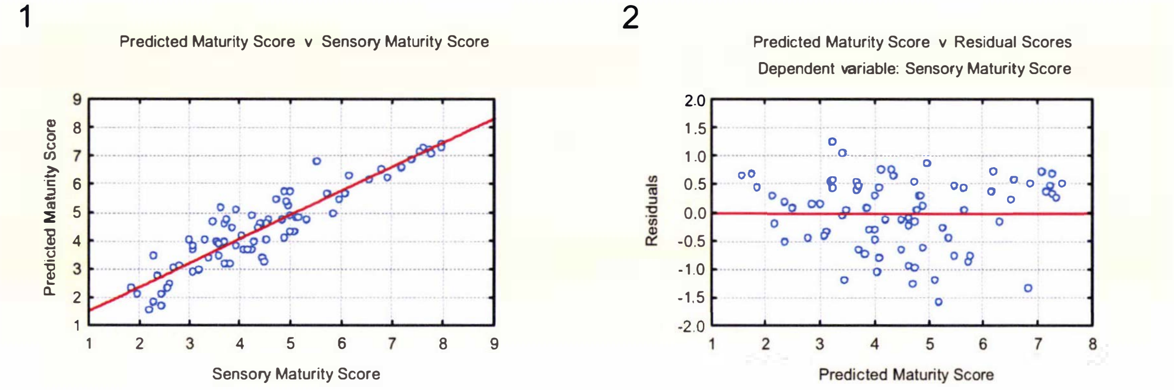


Fig. 8.29 *Predicted Cheddar cheese maturity, based on PCA of the covariance matrix (SE-HPLC) (Model I).* Graph 1) SMS v TN, PC1, PC2 and PC3 and graph 2) predicted maturity score v the residual scores.

The combination of TN and the scores for PC1, PC2 and PC3 of the (SE-HPLC) covariance matrix was highly correlated with SMS (R = 0.9224). The value for R^2 (adj) of 0.8425 (SE of prediction = 0.6406) (Model I) provided an improvement on the value obtained when the combination of TN and PC1, PC2 and PC3 of the correlation matrix was used (R^2 (adj) = 0.8181) (Model H).

The value for R^2 (adj) of 0.8425 (Model I) was a little less than the value obtained when SMS was regressed against TN and RP-HPLC 'peak' 17 (R^2 (adj) = 0.8571) (Model E). However, for Model E, the distribution of sample points around the regression trend line was greater for the more mature cheese (Fig 8.10, graphs 3 and 4), indicating that the model with only 2 variables (Model E) was probably less useful.

The value for R^2 (adj) of 0.8425 (Model I) was less than the value obtained when RP-HPLC and PCA of the covariance matrix (0.8708) (Model G) was used. However, the SE of prediction of 0.6406 was similar to that of Model G (0.5802). The graph of the predicted maturity score and observed SMS (Fig. 8.29, graph 1) was very similar to that obtained by RP-HPLC and PCA of the covariance matrix (Fig. 8.18, graph 1). The differentiation of maturity was mainly based on the concentration of the later-eluting 'peaks' (Fig. 8.26) that were comprised of the end products of proteolysis, and this was consistent with the RP-HPLC result. As with RP-HPLC, the prediction of cheese maturity was best for the more mature cheese with a SMS of > 5 (Fig. 8.29, graphs 1 and 2), which contained greater concentrations of the end-products of proteolysis.

The results showed that SE-HPLC was a good method that compared well with RP-HPLC for assessing the maturity of cheese. SE-HPLC had several advantages over RP-HPLC. It provided a simpler form of analysis, there were fewer peaks, the retention time of the peaks was more consistent and it was therefore easier to align the peaks for the 77 cheeses and to assess the 'peak' area data. For both methods, the assessment of maturity was based mainly on the small peptides and amino acids that are the end-products of proteolysis. The regression statistics were similar for both methods of sample analysis.

8.4.5 MLR of sensory maturity scores and selected RP- and SE-HPLC 'peak' areas

The rationale for using PCA in the first instance was to enable the data set to be reduced to a few principal components that could give a better estimate of sensory maturity than any single component. However, the PCA factor loading plots (Figs. 8.11, 8.15, 8.22 and 8.26) showed that some 'peaks' were more closely associated with sensory maturity than others. This suggested the possibility of using the PCA factor loading scores to select a group of key 'peaks' related to cheese maturity. It is possible that these few 'peaks' could collectively provide a good indication of cheese maturity, and obviate the need to determine the area of all the peaks on the chromatogram in order to provide an objective Cheddar cheese maturity score. The area of the 'peaks' most closely associated with maturity could then be used to form a simplified data set that could be regressed against sensory maturity to test the hypothesis and find a better predictive model.

8.4.5.1 MLR of RP-HPLC peaks selected from PCA (correlation matrix) factor loading scores

The factor loading scores from PCA of the correlation matrix were plotted (Fig. 8.30).

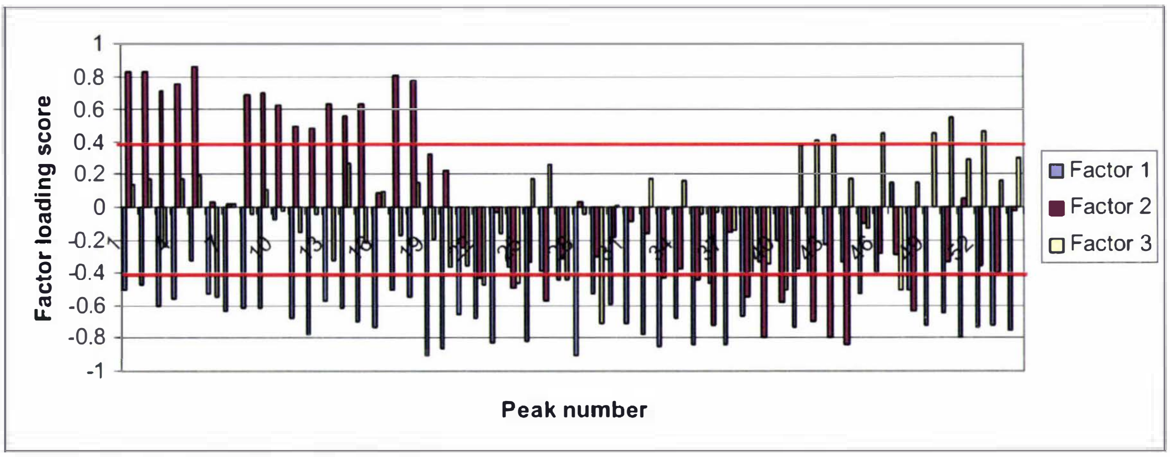


Fig. 8.30 *Factor loading scores for PC1, PC2 and PC3 of the RP-HPLC correlation matrix.* Peak number v factor loading score.

Each peak (Fig. 8.30) had factor loading scores > 0.4 or < –0.4, the cut-off point for determining whether or not a peak was important in the (PCA) analysis. This was not unexpected, because when the correlation form of PCA is used, all peaks are standardised to the same variance, and therefore have equal weighting. It was impossible to eliminate any 'peaks' prior to using MLR for correlating peak areas and SMSs.

As the amount of protein hydrolysis is related to the total protein present, and this varied according to manufacturing site, and was likely to be influenced by seasonal and lactational factors, it was important to include TN as a variable in the data set. As well, TN was proven to be an important variable in Models A – I. For comparison, the complete RP-HPLC peak data set was regressed against SMS, and TN was then included as a variable and the regression analysis was re-applied. The results are compared in Table 8.8.

Table 8.8 *Comparison of RP-HPLC peak areas and the combination of TN and RP-HPLC peak areas as predictors of cheese sensory maturity.* Regression statistics (R, R^2, R^2 (adj), *p*, SE of prediction, degrees of freedom (d.f.) and F-ratio (F)) from MLR of SMS v peaks 1-54, and SMS v TN and peaks 1-54 (Model J). The statistics for Models C, G and I are included for comparison.

Model	**Model C**	**Model G**	**Model I**		**Model J**
Method	TN and WSN	RP-HPLC	SE-HPLC	RP-HPLC	RP-HPLC
PCA		Cov. matrix	Cov. matrix		
MLR	SMS v TN and WSN	SMS v TN, PC1, PC2 and PC3	SMS v TN, PC1, PC2 and PC3	SMS v peaks 1 - 54	SMS v TN and peaks 1 – 54
R	0.8963	0.9368	0.9224	0.9932	0.9961
R^2	0.8033	0.8776	0.8508	0.9865	0.9922
R^2 (adj)	0.7980	0.8708	0.8425	0.9533	0.9717
p-level	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
SE of prediction	0.7255	0.5802	0.6406	0.3487	0.2714
d.f.	2, 74	4, 72	4, 72	54, 22	55, 21
F	151.09	129.04	102.63	29.75	48.48

One of the problems with having such a large number of variables (55) for the number of samples (77) analysed, is that the degrees of freedom (d.f.; the total number of observations – minus the number of estimated parameters (includes the intercept)) is small and the value for R^2, which is influenced by the number of parameters (variables), can become close to one. In this case, it is important to use the R^2 (adj) values for making comparisons.

The results (Table 8.8) show that when TN was included in the model (Model J), the value for R^2 (adj) was greater, SE of the prediction of maturity was smaller and F-ratio (used to test the significance of the model) was greater than when TN was not included. TN was therefore an important variable to include.

The regression analysis (SMS v all RP-HPLC peaks plus TN) provided a complex equation, involving 55 variables. The equation for Model J is:

$$\text{Predicted cheese maturity} = 15.188316 - 2.966638\,TN - 0.000064\,P1 + 0.000065\,P2 + 0.000251\,P3 + 0.000093\,P4 - 0.000197\,P5 - 0.000362\,P6 - 0.000311\,P7 + 0.000097\,P8 + 0.000053\,P9 + 0.000044\,P10 - 0.000102\,P11 + 0.000009\,P12 + 0.000046\,P13 - 0.000077\,P14 - 0.000247\,P15 + 0.000006\,P16 - 0.000018\,P17 + 0.000014\,P18 + 0.000169\,P19 - 0.000105\,P20 + 0.000057\,P21 + 0.000008\,P22 + 0.000022\,P23 + 0.000016\,P24 + 0.000003\,P25 + 0.000042\,P26 + 0.000199\,P27 + 0.000143\,P28 + 0.000210\,P29 + 0.000128\,P30 + 0.000296\,P31 + 0.000163\,P32 - 0.000256\,P33 + 0.000072\,P34 + 0.000131\,P35 + 0.000030\,P36 - 0.000391\,P37 - 0.000339\,P38 + 0.000177\,P39 - 0.000266\,P40 + 0.000229\,P41 - 0.000274\,P42 + 0.000151\,P43 - 0.000190\,P44 - 0.000051\,P45 - 0.000581\,P46 - 0.000027\,P47 - 0.000302\,P48 - 0.000005\,P49 - 0.000178\,P50 + 0.000140\,P51 + 0.000139\,P52 - 0.000055\,P53 - 0.000135\,P54$$

(Equation 10)

This equation was used to derive a maturity score for each cheese sample. Fig. 8.31 depicts a plot of the predicted cheese maturity score v SMS (graph 1) and a plot of the residuals (graph 2). There were no outliers, that is, no samples with predicted maturity scores with a standard residual of > 2 standard deviations.

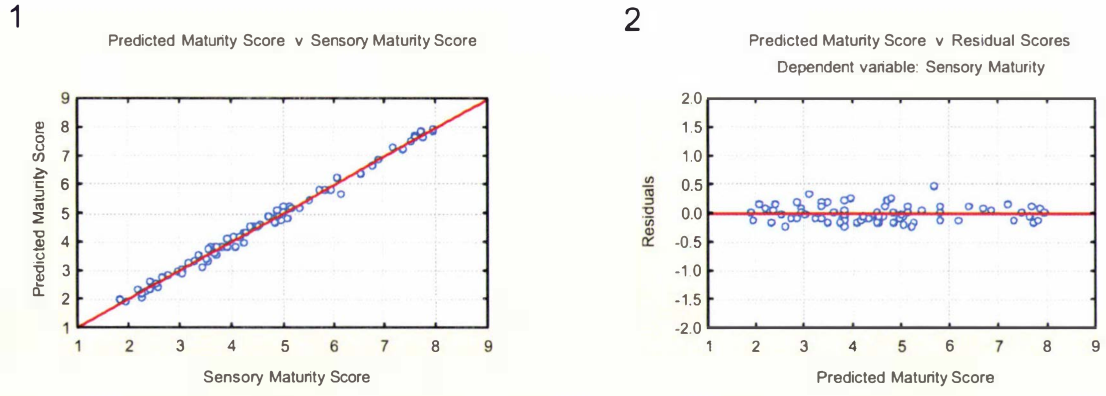


Fig. 8.31 *Predicted Cheddar cheese sensory maturity based on MLR analysis of SMS* v *TN and peaks 1 – 54.* Graph 1) Predicted maturity score v SMS and graph 2) predicted maturity score v the residual scores.

MLR analysis showed that the combination of 54 peak areas and TN (Model J) provided an excellent indication of cheese sensory maturity. In terms of the statistics, this model compared very favourably (R^2 (adj) = 0.9717, SE of prediction = 0.2714) with the best of the previous models (Model G) (R^2 (adj) = 0.8708, SE of prediction = 0.5802) that was based on TN and the scores of the first three principal components of the (RP-HPLC) covariance matrix (Table 8.8). However, obtaining the RP-HPLC data set was difficult and time-consuming, even with semi-automated analysis of the HPLC traces. The equation for the objective prediction of cheese maturity was also complex, incorporating 55 variables. This provided some concern as the number of variables (55) was essentially too large for the number of cases (77 samples). This meant that the d.f. and F-ratio were lower (d.f. = 21, F = 48.48) than when the principal components were used (Model G) (d.f. = 72, F = 129.04). As a result, the equation (Equation 10, Model I) is unlikely to be robust and problems could be expected if a new sample was different from the data set.

Different ways of reducing the data set, by removing peak data that was not particularly influential in the prediction of cheese maturity, were explored. These peaks were identified by their low Beta-coefficient values (this is the 'standardised regression coefficient that allows for a direct comparison between coefficients as to their relative explanatory power of the dependent variable') and high *p*-values (the level of significance and can be used to identify those variables not contributing significantly to the prediction of maturity).

The number of peaks could be reduced to 39 by removing 15 peaks in a 'one-step elimination' on the basis of the combination of very low Beta-coefficient values (< 0.1) and high *p*-values. Those peaks removed were peaks 9, 10, 11, 12, 16, 18, 22-25, 27, 30, 36, 45 and 49 (see Table 8.9).

A second approach was based on the recommendations of Hair et al (1998). It used a 'stepwise' method that involved removing the peak with the lowest Beta-coefficient value and highest *p*-value, re-applying the regression analysis and repeating the process until all the remaining peaks contributed significantly to the equation. Using this process, the number of peaks was reduced to 25. However the last seven peaks removed were actually contributing to the regression equation, and decreasing the number of peaks below 32 steadily increased the SE of prediction of maturity and decreased the R^2(adj) at the same F-ratio. It was therefore decided to use 32 peaks (Model K). The peaks removed were 8, 9, 12, 13, 16, 18, 20, 22-25, 27, 28, 30, 36, 42, 43, 45, 47, 49, 53 and 54 (Fig. 8.7, Fig. 8.8 and appendix, section 8.8.3.2). The results of these regression analyses are presented for comparison in Table 8.9.

Table 8.9 *A comparison of TN and all RP-HPLC peak areas (Model J), TN and 39 peaks, TN and 32 peaks (Model K) and TN and 25 peaks as predictors of cheese sensory maturity.* The table shows the statistics (R, R^2, R^2 (adj), *p*, SE of prediction, d.f. and F-ratio from MLR of SMS v TN and peaks 1-54, SMS v TN and 39 peaks, SMS v TN and 32 peaks and SMS v TN and 25 peaks.

Model	**Model J**		**Model K**	
Method	RP-HPLC	RP-HPLC	RP-HPLC	RP-HPLC
PCA	Corr. matrix	Corr. matrix	Corr. matrix	Corr. matrix
Peak selection	Factor scores	Factor scores and 'One-step elimination'	Factor scores and 'Stepwise elimination'	Factor scores and 'Stepwise elimination'
MLR	SMS v TN and peaks 1 - 54	SMS v TN and 39 peaks	SMS v TN and 32 peaks	SMS v TN and 25 peaks
R	0.9961	0.9950	0.9944	0.9918
R^2	0.9962	0.9899	0.9889	0.9837
R^2 (adj)	0.9717	0.9788	0.9803	0.9752
p-value	< 0.0001	< 0.0001	< 0.0001	< 0.0001
SE of prediction	0.2714	0.2350	0.2266	0.2542
d.f	55, 21	40, 36	33, 43	26,50
F	48.48	88.76	115.58	115.94
Outliers (Code No.)	-	4309	4309	4309, 4421

Both the 'one-step' and 'stepwise' approaches were essentially fine-tuning the model and both provided a very small improvement but did little to simplify the model. Using this approach the best of these models was based on 'stepwise elimination' with 32 peaks remaining (Model K), which provided a higher R^2 (adj), lower SE of prediction of maturity, greater degrees of freedom and correspondingly higher F-ratio. The following equation was derived from the regression analysis of the 32 peaks and TN with the SMS.

$$\text{Predicted cheese maturity} = 15.08415 - 2.82251\ TN - 0.00006\ P1 + 0.00015\ P2 + 0.00032\ P3 + 0.00007\ P4 - 0.00020\ P5 - 0.00037\ P6 - 0.00023\ P7 + 0.00005\ P10 - 0.00015\ P11 - 0.00013\ P14 - 0.00027\ P15 - 0.00004\ P17 + 0.00024\ P19 + 0.00007\ P21 + 0.00003\ P26 + 0.00020\ P29 + 0.00022\ P31 + 0.00011\ P32 - 0.00018\ P33 + 0.00009\ P34 + 0.00012\ P35 - 0.00043\ P37 - 0.00032\ P38 + 0.00015\ P39 - 0.00022\ P40 + 0.00025\ P41 - 0.00017\ P44 - 0.00061\ P46 - 0.00034\ P48 - 0.00013\ P50 + 0.00013\ P51 + 0.00009\ P52$$

(Equation 11)

This equation was used to calculate a maturity score for each of 77 cheese samples. Fig. 8.32 depicts the predicted cheese maturity score plotted against the SMS and provided an R^2 (adj) of 0.9803. There was one outlier, sample 4309 (Table 8.9).

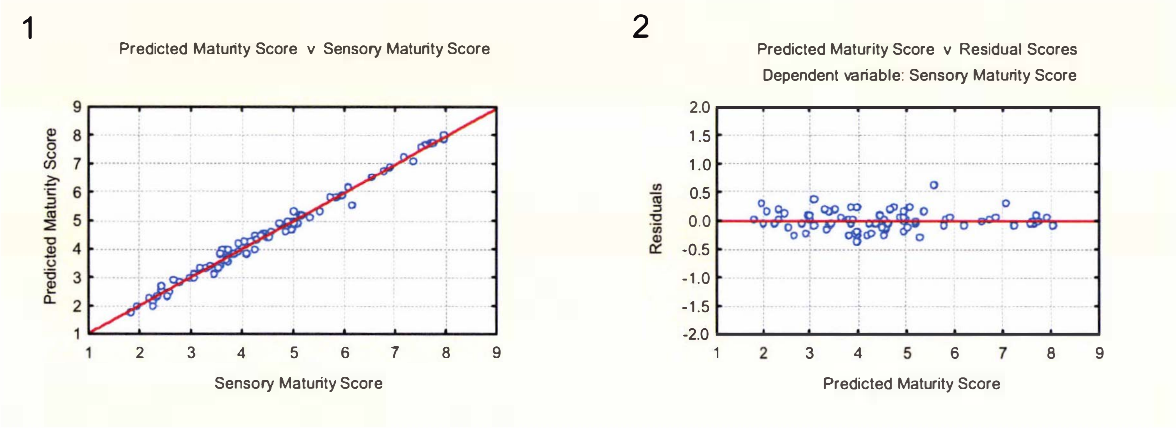


Fig. 8.32 *Predicted Cheddar cheese maturity based on MLR analysis of SMS* v *the combination of TN and the areas of 32 RP-HPLC peaks (Model K).* Graph 1) SMS v TN and 32 peaks (1 - 7, 10, 11, 14, 15, 17, 19, 21, 26, 29, 31 - 35, 37 - 41, 44, 46, 48, 50 - 52) and graph 2) predicted maturity score v the residual scores.

8.4.5.2 MLR of RP-HPLC peaks selected from PCA (covariance matrix) factor loading scores

Although the combination of 32 peaks and TN was highly correlated with the SMS, (R = 0.9944), a simpler model would be more desirable and more likely to be used. The plot of the factor loading scores for the covariance form of PCA (Fig. 8.15) had indicated that fewer peaks were closely associated with cheese maturity. The factor loading scores from the covariance matrix could therefore be useful to select those few peaks most closely associated with maturity in order to derive a simple model based on RP-HPLC. The factor loading scores for PC1, PC2 and PC3 were plotted (Fig. 8.33).

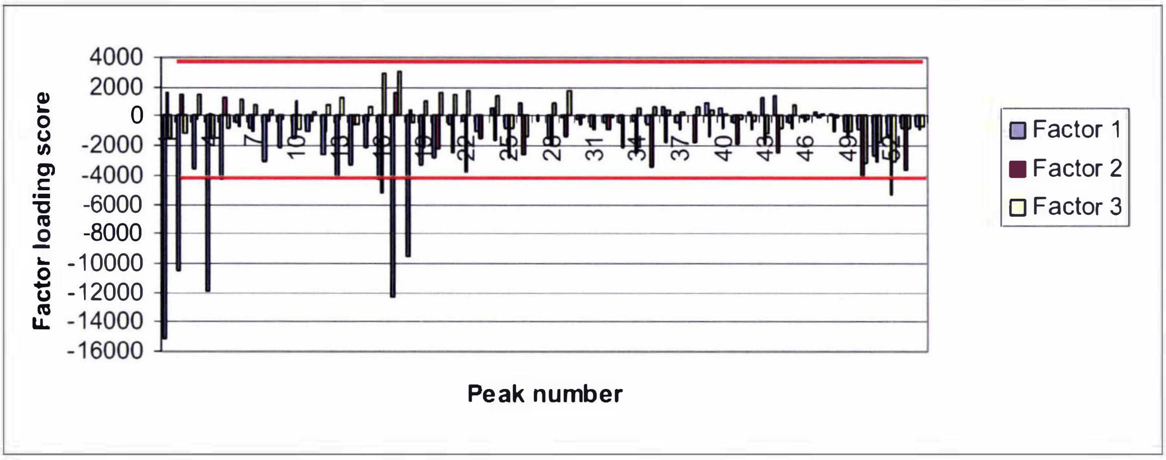


Fig. 8.33 *Factor loading scores for PC1, PC2 and PC3 of the RP-HPLC covariance matrix.* Peak number v factor loading score.

All peaks (Fig. 8.33) with factor loading scores > 4000 or < -4000 were considered likely to be important for the assessment of cheese maturity and were included in a model for predicting cheese maturity. Regression analysis of the principal component scores had shown that only PC1 was closely related to cheese maturity, so it was not surprising to see that the peaks having most influence on the prediction of maturity were those associated with the first factor or principal component (Fig. 8.15). The new data set contained peaks 1, 2, 4, 5, 13, 16, 17, 18, 50 and 52 (Fig. 8.7) and TN and was regressed against sensory maturity. Peaks 2, 5, and 13 were then removed (using 'stepwise elimination') because of very low Beta-coefficient values (< 0.1) and high *p*-values. The new data set, comprised of TN and peaks 1, 4, 16, 17, 18, 50 and 52, was regressed against sensory maturity (Model L) and the results were compared with those of Models J and K (Table 8.10).

Table 8.10 *A comparison of TN and all RP-HPLC peak areas (Model J), TN and 32 peaks (Model K), and TN and 7 peaks (Model L) as predictors of cheese sensory maturity.* Regression statistics (R, R^2, R^2 (adj), *p*, SE of prediction, d.f. and F-ratio from MLR of SMS v TN and peaks 1-54, SMS v TN and 32 peaks, and SMS v TN and 7 peaks.

Model	**Model J**	**Model K**	**Model L**
Method	RP-HPLC	RP-HPLC	RP-HPLC
PCA	Corr. matrix	Corr. matrix	Cov. matrix
Peak selection	Factor scores	Factor scores 'Stepwise elimination'	Factor scores 'Stepwise elimination'
MLR	SMS v TN and peaks 1 – 54	SMS v TN and 32 peaks	SMS v TN and 7 peaks
R	0.9961	0.9944	0.9617
R^2	0.9922	0.9889	0.9249
R^2 (adj)	0.9717	0.9803	0.9161
p-value	< 0.0001	< 0.0001	< 0.0001
SE of prediction	0.2714	0.2266	0.4676
d.f	55, 21	33, 43	8, 68
F	48.48	115.58	104.69
Outliers (Code No.)	-	4309	4323,4359

The regression equation is:

Predicted cheese maturity = 9.61716 - 1.99444 TN + 0.00003 P1 - 0.00006 P4 + 0.00002 P16 + 0.00006 P17 + 0.00008 P18 - 0.00008 P50 + 0.00007 P52*

(Equation 12)

* The very small fraction values are associated with peaks that may be very large at some point in maturation (see appendix, section 8.8.3.1). An examination of the values for peaks 50 and 52 showed that this pair of peaks contributed (between -0.01 and 0.29) to the maturity score (1.00 – 9.00).

The regression equation was used to predict cheese maturity and the observed SMSs were plotted against the predicted SMSs (Fig. 8.34). There were two outliers, samples 4323 and 4359 (Table 8.10).

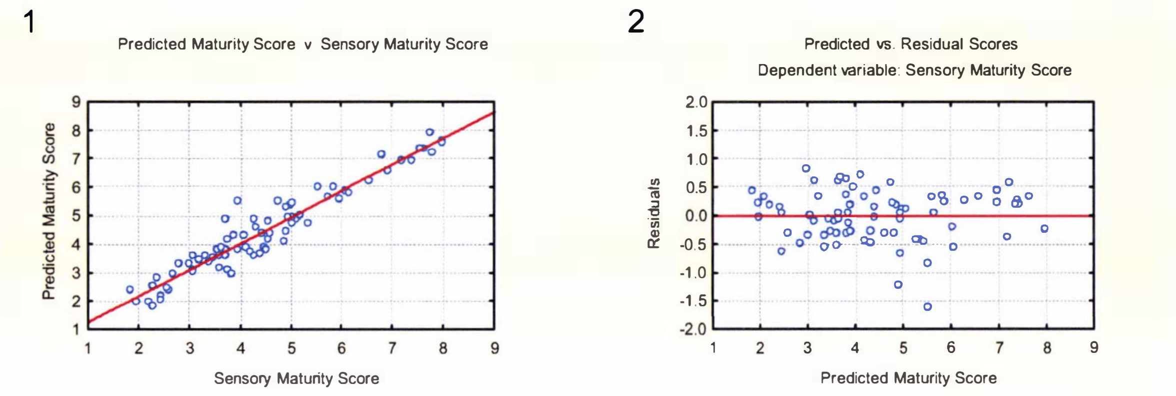


Fig. 8.34 *Predicted Cheddar cheese maturity, based on MLR analysis of SMS* v *TN and 7 RP-HPLC peaks (Model L).* Graph 1) SMS v TN and 7 peaks (1, 4, 16, 17, 18, 50 and 52) and graph 2) predicted maturity score v the residual scores.

TN and seven selected peaks correlated well with sensory maturity ($R = 0.9617$; $R^2(adj) = 0.9161$) (Model L). Although not as good as the analysis of the 32 peaks and TN (R^2 (adj) = 0.9803) (Model K), the R^2 (adj) of 0.9161 compared favourably with the R^2 (adj) of 0.8708 obtained using PCA of the (RP-HPLC) covariance matrix of the full data set (Model G). It also compared well with the R^2 values for the chemical analyses, in particular the combination of WSN and TN (Model C) (R^2 (adj) = 0.7980). Although this model had a greater SE of prediction of maturity (0.4676) than the model containing 32 peaks and TN (0.2266), it had the advantage of having just 7 peaks to measure and was much easier to apply.

8.4.5.3 MLR of SE-HPLC peaks selected from PCA (correlation matrix) factor loading scores

SE-HPLC is a simpler form of analysis than RP-HPLC, with 15 peaks forming the basis for cheese differentiation and the assessment of maturity.

PCA of both the correlation and covariance matrices had shown that the SE-HPLC 'peak' areas correlated well with sensory maturity. The factor loading scores and the close proximity of some 'peaks' to the maturity vector (Fig. 8.22) suggested the possibility that the method could be simplified, with fewer 'peaks' used for the prediction of maturity.

The factor loading scores were plotted for both the correlation (Fig. 8.35) and covariance (Fig. 8.38) matrices.

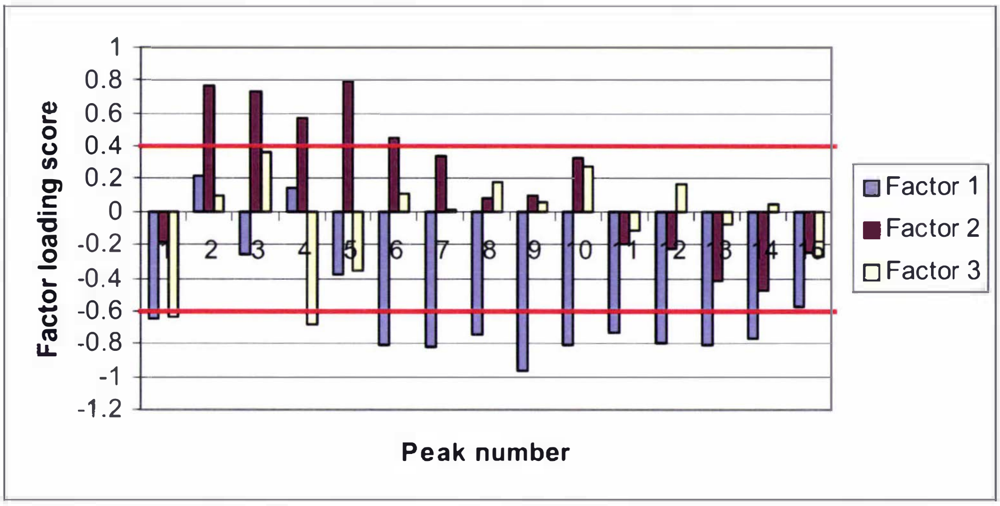


Fig. 8.35 *Factor loading scores for PC1, PC2 and PC3 of the (SE-HPLC) correlation matrix.* Peak number v factor loading score.

All 'peaks' (Fig. 8.35) with factor loading scores that were > 0.4 or < –0.4 were considered likely to be important for the assessment of cheese maturity. In the case of the correlation matrix all 'peaks' were considered important and were therefore included in the regression analysis.

The entire data set including TN was regressed against sensory maturity and the statistical results are included in Table 8.11.

Table 8.11 *Comparison of 4 models as predictors of sensory maturity.* Regression statistics (R, R^2, R^2 (adj), *p*, SE of prediction, d.f and F-ratio from MLR analysis of SMS v TN and RP-HPLC peaks 1-54 (Model J), SMS v TN and 32 RP-HPLC peaks Model K), SMS v TN and 7 RP-HPLC peaks (Model L), and SMS v TN and 15 SE-HPLC 'peaks' (Model M).

Model	**Model J**	**Model K**	**Model L**	**Model M**
Method	RP-HPLC	RP-HPLC	RP-HPLC	SE-HPLC
PCA	Corr. matrix	Corr. matrix	Cov. matrix	Corr. matrix
Peak selection	Factor scores	Factor scores 'Stepwise elimination'	Factor scores 'Stepwise elimination'	Factor scores
MLR	SMS v TN and peaks 1 – 54	SMS v TN and 32 peaks	SMS v TN and 7 peaks	SMS v TN and 'peaks' 1 – 15
R	0.9961	0.9944	0.9617	0.9585
R^2	0.9922	0.9889	0.9249	0.9187
R^2 (adj)	0.9717	0.9803	0.9161	0.8970
p-value	< 0.0001	< 0.0001	< 0.0001	< 0.0001
SE of prediction	0.2714	0.2266	0.4676	0.5180
d.f	55, 21	33, 43	8, 68	16, 60
F	48.48	115.58	104.69	42.37
Outliers (Code No.)	-	4309	4323, 4359	4079, 4361, 4367

The regression equation is:

$$\text{Predicted cheese maturity} = 9.53621 - 2.19866\ TN - 0.00503\ P1 - 0.00106\ P2 + 0.00052\ P3 - 0.00118\ P4 + 0.00024\ P5 - 0.00059\ P6 + 0.00257\ P7 + 0.00037\ P8 - 0.00104\ P9 - 0.00026\ P10 + 0.00047\ P11 + 0.00110\ P12 + 0.00020\ P13 + 0.00252\ P14 - 0.00104\ P15$$

(Equation 13)

This equation was used to determine the maturity score and this was plotted against the SMS (Fig. 8.30). There were three outliers, samples 4079, 4361 and 4367 (Table 8.11).

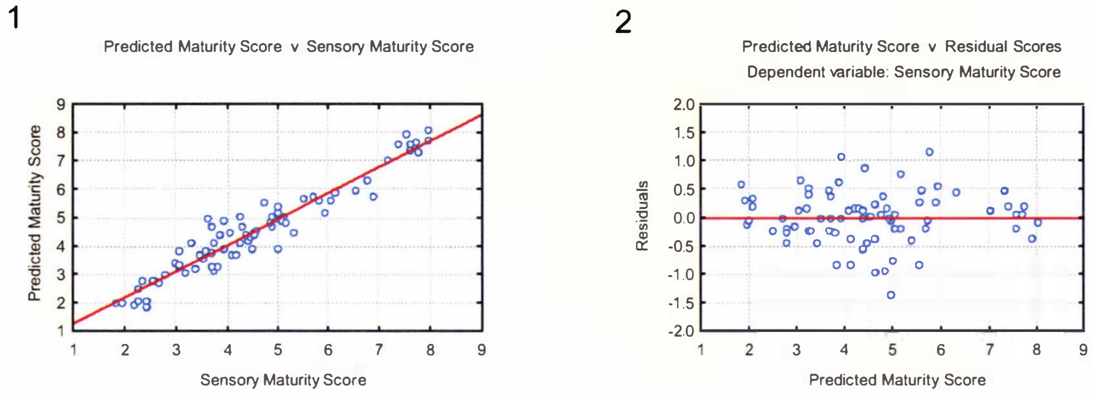


Fig. 8.36 *Predicted Cheddar cheese maturity based on MLR analysis of the SMS* v *the combination of TN and the areas of the 15 SE-HPLC 'peaks' (Model M).* Graph 1) SMS v TN and 15 SE-HPLC 'peaks', and graph 2) predicted maturity score v the residual scores.

'Peaks' 15, 8, 3, 10, 11, 2, 5 and 6 were successively eliminated ('stepwise elimination') from the analysis on the basis of their low Beta-coefficient values. The new data set (TN and 'peaks' 1, 4, 7, 9, 12, 13 and 14) was regressed against sensory maturity and the results were compared with those of the previous models (Table 8.12).

Table 8.12 *A comparison of five models as predictors of sensory maturity.* Regression statistics (R, R^2, R^2 (adj), *p*, SE of prediction, d.f. and F-ratio from MLR of SMS v TN and RP-HPLC peaks 1-54 (Model J), SMS v TN and 32 RP-HPLC peaks (Model K), SMS v TN and 7 RP-HPLC peaks (Model L), SMS v TN and 15 SE-HPLC 'peaks' (Model M), and SMS v TN and 7 SE-HPLC 'peaks' (Model N).

Model	**Model J**	**Model K**	**Model L**	**Model M**	**Model N**
Method	RP-HPLC	RP-HPLC	RP-HPLC	SE-HPLC	SE-HPLC
PCA	Corr. matrix	Corr. matrix	Cov. matrix	Corr. matrix	Corr. matrix
Peak selection	Factor scores	Factor scores 'Stepwise elimination'	Factor scores 'Stepwise elimination'	Factor scores	Factor scores 'Stepwise
MLR	SMS v TN and peaks 1 - 54	SMS v TN and 32 peaks	SMS v TN and 7 peaks	SMS v TN and 'peaks' 1 - 15	SMS v TN and 7 'peaks'
R	0.9961	0.9944	0.9617	0.9585	0.9533
R^2	0.9922	0.9889	0.9249	0.9187	0.9087
R^2 (adj)	0.9717	0.9803	0.9161	0.8970	0.8980
p-value	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
SE of prediction	0.2714	0.2266	0.4676	0.5180	0.5155
d.f	55, 21	33, 43	8, 68	16, 60	8, 68
F	48.48	115.58	104.69	42.37	84.63
Outliers (Code No.)	-	4309	4323, 4359	4079, 4361 4367	4079, 4323, 4361, 4367

The regression equation is:

$$\text{Cheddar cheese maturity} = 9.31131 - 2.07463\ TN - 0.00411\ P1 - 0.00108\ P4 + 0.00194\ P7 - 0.00103\ P9 + 0.00115\ P12 + 0.00075\ P13 + 0.00150\ P14$$

(Equation 14)

A graph of the sensory maturity v the predicted sensory maturity was derived from the regression equation (Fig. 8.31). There were four outliers, samples 4079, 4323, 4361 and 4367 (Table 8.12).

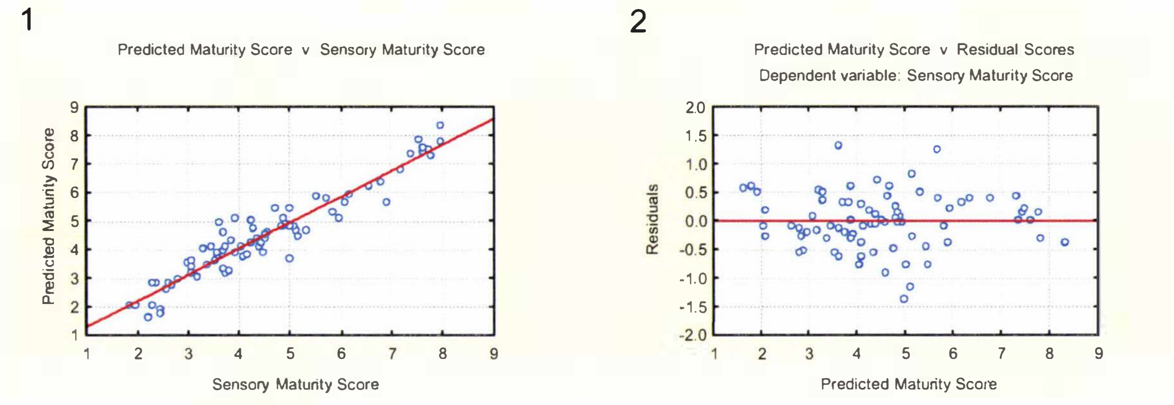


Fig. 8.37 *Predicted Cheddar cheese maturity, based on MLR analysis of SMS* v *the combination of TN and the areas of SE-HPLC 'peaks' 1, 4, 7, 9, 12, 13 and 14 (Model N).* Graph 1) SMS v TN and 7 SE-HPLC 'peaks', and graph 2) predicted maturity score v the residual scores.

'Stepwise elimination' of 'peaks' enabled the SE-HPLC model to be simplified and provided a simple means to predict cheese maturity. When TN and the 7 selected 'peaks' (1, 4, 7, 9, 12, 13 and 14) were included, regression analysis showed that there was a strong correlation ($R = 0.9533$, R^2 (adj) = 0.8980) between sensory maturity and the SE-HPLC 'peak' areas plus TN. This was essentially the same result as when all the 'peaks' were included ($R = 0.9585$, R^2 (adj) = 0.8970). The correlation with SMS ($R = 0.9533$, R^2 (adj) = 0.8980) was not quite as good as for 7 RP-HPLC peaks and TN ($R = 0.9617$, R^2 (adj) = 0.9161). The R^2 (adj) of 0.8980 also compared favourably with the values for the chemical analyses, and in particular the combination of WSN and TN (R^2 (adj) = 0.7980). The R^2 (adj) for the SE-HPLC method was less than for the RP-HPLC method (32 peaks and TN) which had an R^2 (adj) of 0.9717), however it provided the advantages of a simple and highly reproducible method, with easily identifiable peaks, and with only 7 'peaks' to measure.

8.4.5.4 MLR of SE-HPLC peaks selected from PCA (covariance matrix) factor loading scores

Although it is relatively simple to analyse and obtain peak areas from SE-HPLC traces, it may be possible to simplify the method by further reducing the number of 'peaks' necessary for the prediction of cheese maturity. The PCA loading plots (covariance matrix) indicated that very few 'peaks' were closely associated with cheese maturity. The factor loading scores for PC1, PC2 and PC3 were plotted (Fig. 8.38). 'Peaks' with factor loading scores that were greater than +400 or less than –400 were deemed important for the assessment of cheese maturity.

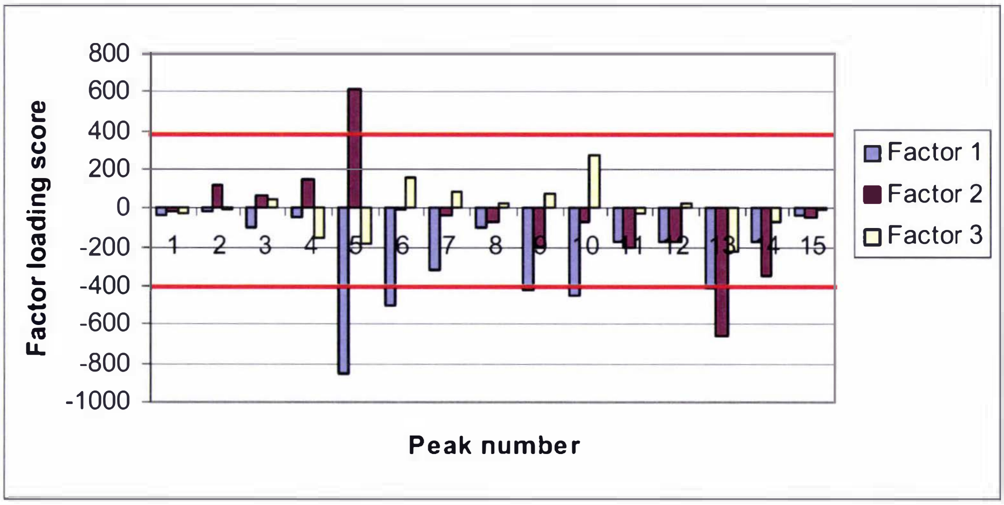


Fig. 8.38 *Factor loading scores for PC1, PC2 and PC3 of the (SE-HPLC) covariance matrix.* Peak number v factor loading score.

Five 'peaks' ('peaks' 5, 6, 9, 10, 13) plus TN were incorporated in a new data set that was regressed against sensory maturity. The results are included in Table 8.13.

Table 8.13 *Comparison of six models as predictors of sensory maturity.* Regression statistics (R, R^2, R^2 (adj), p, SE of prediction, d.f. and F-ratio from MLR of SMS v TN and RP-HPLC peaks 1-54 (Model J), SMS v TN and 32 RP-HPLC peaks (Model K), SMS v TN and 7 RP-HPLC peaks (Model L), SMS v TN and 15 SE-HPLC 'peaks' (Model M), SMS v TN and 7 SE-HPLC 'peaks' (Model N) and SMS v TN and 5 'peaks' (Model O).

Model	**Model J**	**Model K**	**Model L**	**Model M**	**Model N**	**Model O**
Method	RP-HPLC	RP-HPLC	RP-HPLC	SE-HPLC	SE-HPLC	SE-HPLC
PCA	Corr. matrix	Corr. matrix	Cov. matrix	Corr. matrix	Corr. matrix	Cov. matrix
Peak selection	Factor scores	Factor scores 'Stepwise elimination'	Factor scores 'Stepwise elimination'	Factor scores	Factor sores 'Stepwise elimination'	Factor score
MLR	SMS v TN, peaks 1 - 54	SMS v TN, 32 peaks	SMS v TN, 7 peaks	SMS v TN, 'peaks' 1 - 15	SMS v TN, 7 'peaks'	SMS v TN, 5 'peaks'
R	0.9961	0.9944	0.9617	0.9585	0.9533	0.9285
R^2	0.9922	0.9889	0.9249	0.9187	0.9087	0.8621
R^2 (adj)	0.9717	0.9803	0.9161	0.8970	0.8980	0.8503
p-value	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
SE of prediction	0.2714	0.2266	0.4676	0.5180	0.5155	0.6244
d.f	55, 21	33, 43	8, 68	16, 60	8, 68	6, 70
F	48.48	115.58	104.69	42.37	84.63	72.97
Outliers (Code No.)	-	4309	4323, 4359	4079, 4361 4367	4079, 4323 4361, 4367	4361, 4421

Although the reduced data set correlated well with sensory maturity (R = 0.9285), with an R^2 (adj) of 0.8503, there was little to be gained from using this approach as it did not improve the regression analysis beyond what was achieved by 'stepwise elimination' of the SE-HPLC 'peaks' (R^2 (adj) = 0.8980).

8.4.6 Models for the prediction of cheese maturity – a summary of all models and validation of selected models

The results related to each of the models investigated for their ability to predict the sensory maturity of the cheese are presented in Table 8.14.

Table 8.14 *Comparison of MI and Models A – O as predictors of cheese sensory maturity.*

Model		**Model A**	**Model B**	**Model C**	**Model D**	**Model E**	**Model F**	**Model G**	**Model H**	**Model I**
Method	MI	TN and Maturity Index	TN and NPN	TN and WSN	RP-HPLC	RP-HPLC	RP-HPLC	RP-HPLC	SE-HPLC	SE-HPLC
PCA	-			-	-	-	Corr. matrix	Cov. matrix	Corr. matrix	Cov. matrix
LR or MLR	SMS v MI	SMS v TN and MI	SMS v TN and NPN	SMS v TN, WSN	SMS v TN, ‘Peak' 1	SMS v TN, 'Peak' 17	SMS v TN, PC1, PC2, PC3	SMS v TN, PC1, PC2, PC3	SMS v TN, PC1, PC2, PC3	SMS v TN, PC1, PC2, PC3
R	0.8603	0.9006	0.8814	0.8963	0.9130	0.9278	0.9341	0.9368	0.9098	0.9224
R^2	0.7401	0.8111	0.7768	0.8033	0.8336	0.8609	0.8726	0.8776	0.8277	0.8508
R^2 (adj)	0.7366	0.8060	0.7708	0.7980	0.8291	0.8571	0.8655	0.8708	0.8181	0.8425
p-level	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
SE of prediction	0.8284	0.7109	0.7728	0.7255	0.6673	0.6102	0.5920	0.5802	0.6883	0.6406
d.f.	1, 75	2, 74	2, 74	2, 74	2, 74	2, 74	4, 72	4, 72	4, 72	4, 72
F	213.53	158.90	128.79	151.09	185.32	228.94	123.25	129.04	86.48	102.63
Outliers (Code No.)	4345, 4367	4314, 4374, 4420, 4339, 4074	4325, 4361	4325, 4421	4310, 4325	4328, 4329, 4361	4317, 4325, 4359	4317, 4323, 4325, 4359, 4361	4361, 4367	4361, 4362

Model	**Model J**	**Model K**	**Model L**	**Model M**	**Model N**	**Model O**
Method	RP-HPLC	RP-HPLC	RP-HPLC	SE-HPLC	SE-HPLC	SE-HPLC
PCA	Corr. matrix	Corr. matrix	Cov. matrix	Corr. matrix	Corr. matrix	Cov. matrix
Peak selection	Factor scores	Factor scores 'Stepwise elimination'	Factor scores 'Stepwise elimination'	Factor scores	Factor scores 'Stepwise elimination'	Factor scores
MLR	SMS v TN, peaks 1 - 54	SMS v TN, 32 peaks	SMS v TN, 7 peaks	SMS v TN, 'peaks' 1 - 15	SMS v TN, 7 'peaks'	SMS v TN, 5 'peaks'
R	0.9961	0.9944	0.9617	0.9585	0.9533	0.9285
R^2	0.9922	0.9889	0.9249	0.9187	0.9087	0.8621
R^2 (adj)	0.9717	0.9803	0.9161	0.8970	0.8980	0.8503
p-value	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
SE of prediction	0.2714	0.2266	0.4676	0.5180	0.5155	0.6244
d.f	55, 21	33, 43	8, 68	16, 60	8, 68	6, 70
F	48.48	115.58	104.69	42.37	84.63	72.97
Outliers (Code No.)	-	4309	4323, 4359	4079, 4361 4367	4079, 4323 4361, 4367	4361, 4421

It is clear that each of the models (Table 8.14) provided a marked improvement on the use of MI for predicting SMS. Although the combination of MI and TN (Model A) provided a reasonable model for the prediction of cheese maturity, this model would only be useful if a single storage protocol was used, and this is not always the case for NZ Cheddar cheese, where the storage temperature is sometimes changed during ripening. Models B-O, rather than being based on a measurement of the storage protocol, reflect the effect of the storage protocol as well as other influential variables on cheese proteolysis. Some of these models are better at capturing these effects than others and are therefore more likely to provide a reliable indication of cheese maturity.

The five models with equations that provided the best prediction of the sensory maturity of the cheese were selected for validation. The non-selection of some models does not mean they are unsuitable for predicting sensory maturity.

8.4.6.1 Five models for the prediction of cheese maturity

Five models were selected for validation. Those with equations correlated best with cheese sensory maturity were provided by RP-HPLC (all 54 peaks) plus TN (Model J), RP-HPLC (32 peaks) plus TN (Model K), RP-HPLC (peaks 1, 4, 16, 17, 18, 50 and 52) plus TN (Model L), SE-HPLC (all 15 'peaks') plus TN (Model M), and SE-HPLC ('peaks' 1, 4, 7, 9, 12, 13 and 14) plus TN (Model N). Each of these models was based on the measurement of peptides entering the aqueous phase, peptide intermediates and the end-products of the proteolytic reaction (see Table 8.14).

The distance between the SE of prediction of cheese maturity and the error associated with the assessment of sensory maturity (0.3464) was similar for each of the equations (Table 8.14). It was therefore not possible to tell on this basis whether SE-HPLC was better than RP-HPLC or vice versa.

The number of degrees of freedom indicates the generality of the models. The smaller the error (or residual) degrees of freedom, the less generalisable or robust the model, because most or all of the information is incorporated in the model. RP-HPLC with peaks 1-54 plus TN or 32 peaks plus TN resulted in the highest correlation with sensory maturity, at the lowest error (Table 8.14). However, in both cases, the ratio of peaks to samples was comparatively high and the residual degrees of freedom was relatively small when all the peaks were used (residual d.f. = 21) and larger when fewer peaks were used (residual d.f. = 43). Conversely the SE-HPLC-based models with 8 to 16 variables had the greatest degrees of freedom (residual d.f. = 68 and 60, respectively) and could be expected to be more robust.

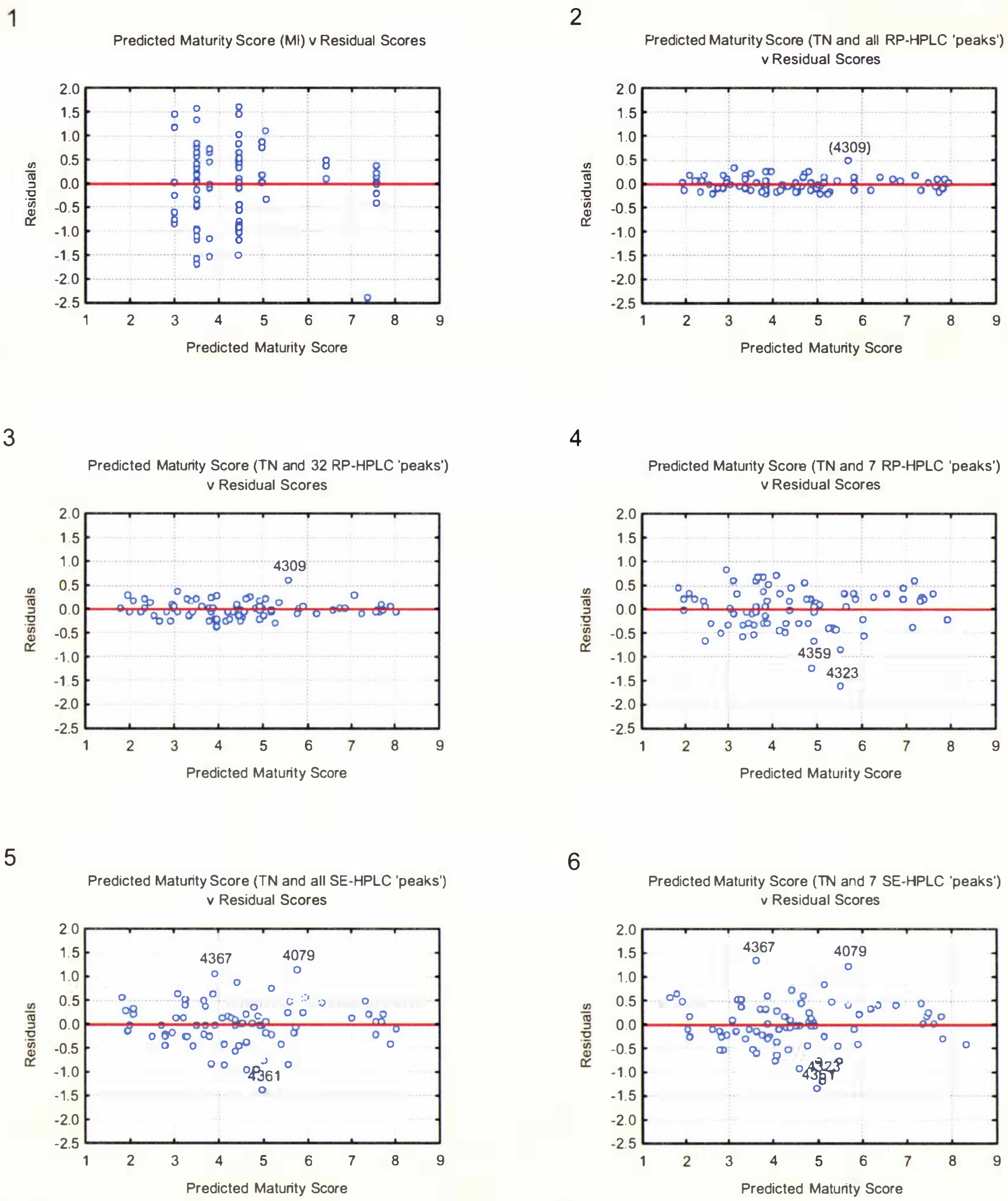


Fig. 8.39 *A comparison of the residual scores associated with SMS* v *MI and Models J - N.* Predicted maturity score v the residuals from the regression of (graph 1) SMS v MI, (graph 2) SMS v TN and all RP-HPLC peaks (Model J), (graph 3) SMS v TN and 32 RP-HPLC peaks (Model K), (graph 4) SMS v TN and 7 RP-HPLC peaks (Model L), (graph 5) SMS v TN and all SE-HPLC 'peaks' (Model M) and (graph 6) SMS v TN and 7 SE-HPLC 'peaks' (Model N).

Each of the residual plots (Fig. 8.39) was a "null" plot, indicating that there was no particular pattern associated with the residual scores.

The graphs of the residuals (Fig. 8.39) showed that in most cases the samples were within 2 standard deviations of the residual mean. There were six 'outliers' identified in total (Table 8.14 and Fig 8.39). All were within the mid-range of maturities (Fig. 8.39) and there seemed to be no particular association between any of them. These 'outliers' were identified as cheese samples 4309 from Southland, 4323 from Stirling, 4359 from Kiwi, 4079 from Southland, 4361 from Lichfield and 4367 from Waitoa. One of these, sample 4323 was identified as an outlier by the equations containing the reduced (7 peak) RP-HPLC and SE-HPLC data sets (Models L and N) as well as by the model based on TN and NPN (Model B). The peaks within these data sets were mostly comprised of small peptides and amino acids that could be expected to be contained within the NPN and, providing the SMS was accurate, this link may indicate a problem with using too few HPLC peaks in the model.

8.4.6.2 Validation of five models

The data in Table 8.14 and Fig. 8.39 show the high internal consistency of the methods. The question is how well these specific models using particular HPLC peaks would apply to a different group of samples. Ideally each of the five models should be tested with a new set of samples to make sure it is robust, and remains valid. Where no new samples are available (as in the present situation), other means of validation are available. A commonly used method for testing the founding principles of a model was used to validate the five models. The random function (Statistica 6.0) was used to select 10% of the samples from the full sample set. The remaining 90% of samples formed the training data set that was used to generate a new regression equation. This equation was used to calculate a maturity score for the remaining 10% of the samples and these scores were then regressed against the SMS. This process was repeated five times. The mean of the values obtained for each statistical parameter was determined and used to compare the models (Table 8.15). The full data set is included in the appendix (section 8.8.5).

Table 8.15 *Validation of Models J - N.* 90% of the samples were used to derive the new equation used to predict the maturity of the remaining 10% of samples. Regression analysis was then used to compare the predicted maturity score with the SMS. This was repeated 5 times for each. The means of the values obtained from the five replicates are presented in the table.

Model	**Model 'J'**	**Model 'K'**	**Model 'L'**	**Model 'M'**	**Model 'N'**
Method	RP-HPLC	RP-HPLC	RP-HPLC	SE-HPLC	SE-HPLC
PCA	Corr. matrix	Corr. matrix	Cov. matrix	Corr. matrix	Corr. matrix
Peak selection	Factor scores	Factor scores	Factor scores	Factor scores	Factor scores
		'Stepwise elimination'	'Stepwise elimination'		'Stepwise elimination'
MLR	SMS v TN and	SMS v TN and	SMS v TN and	SMS v TN and	SMS v TN,
	peaks 1 - 54	32 peaks	7 peaks	15 'peaks'	7 'peaks'
R	0.9583	0.9894	0.9652	0.9656	0.9644
R^2	0.9186	0.9790	0.9316	0.9326	0.9304
R^2 (adj)	0.9045	0.9756	0.9198	0.9209	0.9194
p-value	0.0005	0.0000	0.0003	0.0004	0.0005
SE of prediction	0.4921	0.2295	0.4553	0.4527	0.4534
d.f.	1, 6	1, 6	1, 6	1, 6	1, 6
F	84.03	473.57	89.70	99.89	99.26
Outliers (Code No.)	4347	4309, 4362	4323, 4359	4079, 4361, 4358, 4364, 4367	4079, 4319, 4323, 4361, 4367

The model based on TN and all of the RP-HPLC peaks was the only one to show substantial differences between the original statistical values (Table 14) and the values (the mean of five replicates) predicted by the validation data sets. The R^2 (adj) decreased from 0.9717 to 0.9045 (range 0.8456 - 0.9675) for the validation data sets, indicating that this method was not as robust as the other methods. The shortcomings of the model were probably due to the large number of peaks in relation to the number of samples.

The remaining four models were validated, indicating that the equations could be used to determine the maturity of unknown Cheddar cheese samples, provided they are within the compositional range of the samples used to formulate the equation and that the sensory maturity standard does not alter.

Those four validated models were:

A) Model K, based on TN and 32 RP-HPLC peaks. The equation used to derive the maturity score was:

$$\text{Cheddar cheese maturity score} = 15.08415 - 2.82251\,\text{TN} - 0.00006\,\text{P1} + 0.00015\,\text{P2} + 0.00032\,\text{P3} - 0.00007\,\text{P4} - 0.00020\,\text{P5} - 0.00037\,\text{P6} - 0.00023\,\text{P7} + 0.00005\,\text{P10} - 0.00015\,\text{P11} - 0.00013\,\text{P14} - 0.00027\,\text{P15} - 0.00004\,\text{P17} + 0.00024\,\text{P19} + 0.00007\,\text{P21} + 0.00003\,\text{P26} - 0.00020\,\text{P29} + 0.00022\,\text{P31} + 0.00011\,\text{P32} - 0.00018\,\text{P33} - 0.00009\,\text{P34} - 0.00012\,\text{P35} - 0.00043\,\text{P37} - 0.00032\,\text{P38} + 0.00015\,\text{P39} - 0.00022\,\text{P40} + 0.00025\,\text{P41} - 0.00017\,\text{P44} - 0.00061\,\text{P46} - 0.00034\,\text{P48} - 0.00013\,\text{P50} - 0.00013\,\text{P51} + 0.00009\,\text{P52}$$

B) Model L, based on TN and 7 RP-HPLC peaks. The equation used to derive the maturity score was:

Cheddar cheese maturity score = 9.61716 - 1.99444 TN + 0.00003 P1 - 0.00006 P4 + 0.00002 P16 + 0.00006 P17 + 0.00008 P18 - 0.00008 P50 + 0.00007 P52

C) Model M, based on TN and 15 SE-HPLC 'peaks'. The equation used to derive the maturity score was:

Cheddar cheese maturity score = 9.53621 - 2.19866 TN - 0.00503 P1 - 0.00106 P2 + 0.00052 P3 - 0.00118 P4 + 0.00024 P5 - 0.00059 P6 + 0.00257 P7 + 0.00037 P8 - 0.00104 P9 - 0.00026 P10 + 0.00047 P11 + 0.00110 P12 + 0.00020 P13 + 0.00252 P14 - 0.00104 P15

D) Model N, based on TN and 7 SE-HPLC 'peaks'. The equation used to derive the maturity score was:

Cheddar cheese maturity score = 9.31131 - 2.07463 TN - 0.00411 P1 - 0.00108 P4 + 0.00194 P7 - 0.00103 P9 + 0.00115 P12 + 0.00075 P13 + 0.00150 P14

A table that demonstrates the similarity of the maturity scores predicted by each of these equations is included in the appendix (section 8.8.6). The scores predicted by MI and the equations of the earlier models are included for comparison.

A generalised equation was written that describes the relationship between the sensory maturity of Cheddar cheese and instrumental data:

$$\textit{Predicted cheese maturity score} = a + \sum(b_i * X_i)$$

where a is a constant (the intercept),
where b is the regression coefficient (B coefficient) for a particular variable or predictor (i)
where i identifies the particular variable or predictor,
and where X is the measured value for the particular variable or predictor (i)

8.5 DISCUSSION

The maturation of Cheddar cheese is a slow process, but over a period, the cheese gradually develops the desired texture, flavour and aroma. The development of cheese maturity is influenced by a range of factors that include seasonal and regional differences in milk composition, differences in coagulant type and in starter and NSLAB strains and ripening temperature. It is therefore not appropriate to use cheese age as an indicator of the maturity. The development of accurate and objective methods for determining maturity is of interest to researchers, cheese manufacturers and the marketers of New Zealand Cheddar cheese.

In the current work, the relationship between sensory maturity and MI, NPN, WSN, or the area of SE-HPLC or RP-HPLC peaks containing water-soluble cheese peptides and amino acids was explored, and where appropriate the relationship was modelled and an equation developed so that cheese maturity could be predicted. In this way, a maturity score in the range of 1.00 – 9.00 could be assigned to each cheese. This is a significant advance on any preceding work, where the cheese has been ranked according to age (e.g. Pham and Nakai, 1984; Amantea et al, 1986), or broadly grouped on the basis of maturity (e. g. Santa-Maria et al, 1986; Bütikofer et al, 1998; García Ruiz et al, 1998).

8.5.1 Cheese age and ripening temperature as predictors of cheese sensory maturity

Neither age nor ripening temperature (Fig. 8.2) provided an adequate measure of the maturity of New Zealand Cheddar cheese. However, the combination of cheese age and ripening temperature or MI, which is sometimes used to predict the maturity of cheese used as an ingredient in processed cheese, provided a reasonable assessment of sensory maturity. The correlation (R) between SMS and MI was improved from 0.8603 to 0.9006 (Table 8.14) by using the combination of MI and TN to develop an equation (Equation 1) to predict cheese sensory maturity. An improvement in the correlation occurred whether the data from the two Cheddar specifications (each with a different amount of TN) was analysed together or separately (not reported).

It could be expected that cheese with a higher TN would provide more substrate for proteinases, but less water to facilitate enzyme activity. It could also be expected that cheese with a higher TN would require more rennet action to achieve the same texture and flavour release as cheese with a lower TN. Including TN as a variable in the regression analysis seems to capture the effect of TN on sensory maturity. In this way, the negative effect of a higher TN on the sensory panel's perception of maturity (the SMS) is balanced by decreasing the (calculated (MI) or instrumental) predicted maturity score more for cheese with a higher TN.

8.5.2 Traditional methods as predictors of cheese sensory maturity

Models B and C were based on traditional chemical methods for determining cheese maturity and involved the measurement of NPN or WSN, and TN.

Linear regression and MLR analysis was used to model the relationship between SMS and NPN, SMS and NPN/TN or SMS and the combination of TN and NPN, and equations were developed for predicting SMS. The correlation between SMS and maturity scores predicted by these equations was reasonable (Table 8.1), with the best correlation provided by the combination of NPN and TN ($R = 0.8814$, $R^2(\text{adj}) = 0.7708$) (Equation 2, Model B) (Table 8.14). The need to use TN and NPN as separate variables rather than expressed as NPN/TN may in part be related to the effect of TN on cheese texture and flavour release, and the effect of these on the assessment of cheese sensory maturity, as discussed earlier. The poorer correlation with SMS indicated that this model was not as useful as Model A for determining cheese maturity. NPN is more useful for conventional laboratory studies in which time-related changes in the maturity of cheese stored at the same temperature are monitored.

Aston and Creamer (1986) had demonstrated that much of the Cheddar flavour resides in the water-soluble fraction. This fraction is comprised of the peptides and amino acids as well as organic acids and their salts that are produced as the cheese ripens. The relationship between SMS and WSN, SMS and WSN/TN, or SMS and the combination of WSN and TN (Model C) was modelled and the resultant equations were used to provide a maturity score for each cheese sample. The maturity scores derived from the equation based on the combination of TN and WSN (Equation 3, Model C) provided the best correlation with the SMS ($R = 0.8963$, $R^2(\text{adj}) = 0.7980$) (Table 8.14). Although this was an improvement on the use of NPN and TN (Model B), and provided a reasonable estimation of maturity, the correlation was only approximately equivalent to the correlation provided by the MI and TN (Model A). It is therefore likely that ripening time and temperature had the major influence on proteolysis and the formation of WSN in the cheese, and that the other parameters that influenced cheese maturity resulted in more subtle changes that were recognised by the panel as a part of the assessment of sensory maturity but were not captured in the gross analysis of the amount of WSN.

8.5.3 RP-HPLC peaks as predictors of sensory maturity

RP-HPLC separation of the WSF highlighted 54 peaks, although perhaps undue emphasis was placed on those containing aromatic amino acids because of their comparatively high absorbance, although this effect was minimised by the careful selection of wavelength (Ch. 5). Some peaks were highly correlated with the SMS. Of these, 'peak' 1 comprised of many of the amino acids, and 'peak' 17, a very small peptide, were the most highly correlated with the SMS and were used in conjunction with TN to develop equations (Equations 4 and 5) for predicting cheese sensory maturity (Models D and E). The correlation between the predicted maturity scores and the SMS provided an R of 0.9130 (R^2 (adj) = 0.8291) and 0.9278 (R^2 (adj) = 0.8571) for models D and E, respectively.

MLR analysis of TN and the first three principal components derived from the RP-HPLC peak area data using PCA, enabled the relationship between the area of all of the peaks and sensory maturity to be determined (Table 8.14). While both the correlation and the covariance forms of PCA provided good differentiation of cheese maturity, PCA of the covariance matrix provided the best correlation with the SMS (in agreement with the findings of Ch. 7), with an R of 0.9368 (R^2 (adj) = 0.8708) for the maturity scores predicted by the RP-HPLC-based equation incorporating TN and the first three principal components (Equation 7, Model G). This model was based on the first three principal components that collectively accounted for 88% of the variation in the data. It was not possible to provide any useful improvement in the predictive ability of the model by incorporating more than the first three principal components. The use of only three principal components provided a significant improvement on the work of O'Shea et al (1996) who used 19 - 20 principal components to explain 82 - 84% of the RP-HPLC peak data and to group Cheddar cheese (not particularly effectively) into the mild, mature and extra-mature cheese categories determined by cheese graders.

The PCA factor loading scores were also useful for reducing the very large RP-HPLC data set by eliminating data from peaks that did not contribute (statistically) to mature flavour and texture. In this way, only the key peaks, related to sensory maturity, were used to determine the relationship between the peak area data and the SMS. This approach provided the means to simplify the peak integration and the analysis of the peak data. The relationship between the selected peaks and the SMS was modelled using MLR, and the equation was further simplified by using the process of 'stepwise elimination' to remove peaks with very low Beta-coefficient values (< 0.1) and high *p*-values that were not contributing significantly to the regression equation that related them to mature flavour and texture.

The first of these equations incorporated TN and all of the RP-HPLC peaks (Equation 10, Model J). However, the low ratio of samples (77) to peaks (54) meant that the MLR equation was not robust and could not handle new and different samples well, despite the high correlation between the predicted maturity scores and the SMSs (Table 8.14). Two apparently robust equations based on RP-HPLC were formed using a combination of PCA and MLR. The first of these equations (Equation 11) incorporated TN and 32 peaks selected on the basis of their contribution to the regression equation that related them to sensory maturity (Model K). The predicted maturity scores were highly correlated with the SMS ($R = 0.9944$, $R^2\,(adj) = 0.9803$) (Table 8.14). The graphs of the SMS versus the area of each of the peaks included in the equation (see appendix, section 8.8.3.2) indicated that while most peaks increased during maturation (as the SMS increased) several decreased and two showed no particular relationship with the SMS. The black-numbered peaks on the RP-HPLC trace (Fig. 8.40) indicate those peaks included in the equation and the direction of the arrows indicates either an overall increase or decrease in peak area with cheese maturation. The second of the two equations (Equation 12) predicted maturity scores that were also highly correlated with the SMS ($R = 0.9617$, $R^2\,(adj) = 0.9161$). This equation modelled the relationship between TN and 7 RP-HPLC peaks (Model L) that were selected on the basis of their PCA factor loading scores (which highlighted their close association with SMS), with the subsequent elimination of peaks not contributing significantly to the regression equation relating them to sensory maturity. With the exception of peak 50, all the peaks incorporated in the equation increased in area as the SMS increased, although the slope of the trend line and the distribution of points were different for each peak. The peaks used in this model are highlighted in Fig. 8.40.

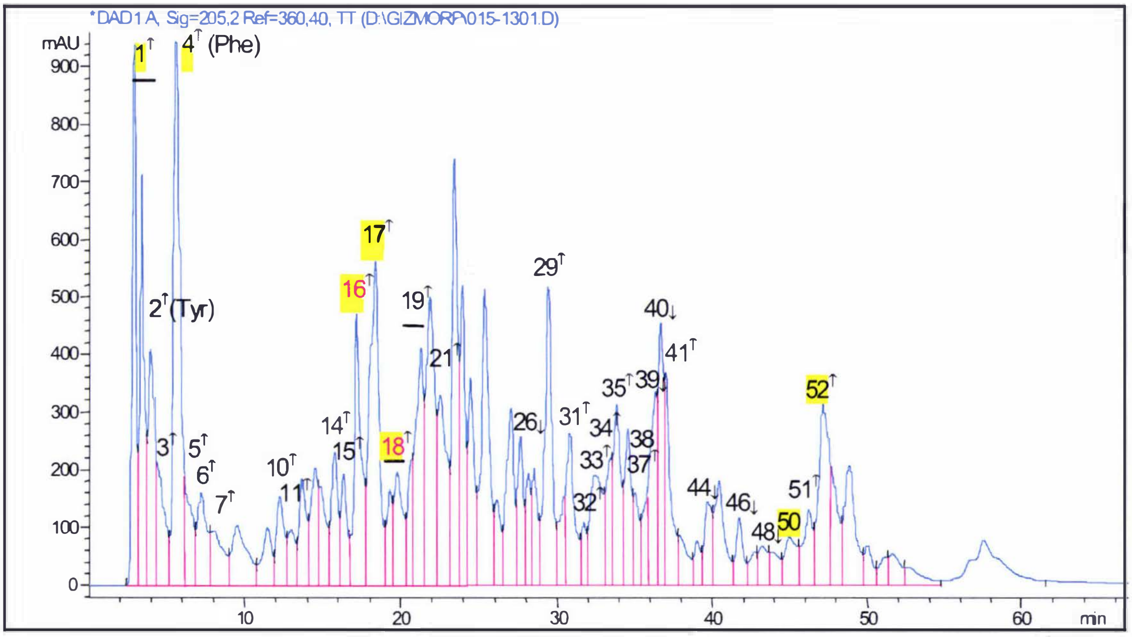


Fig. 8.40 RP-HPLC peaks of the WSF of Cheddar cheese after 3 m at 10°C. Model K (32 peaks and TN) includes the black-numbered peaks. Model L (7 peaks and TN) includes the yellow highlighted peaks. The arrows indicate whether the peaks increased or decreased in area during maturation.

The method of peak selection meant it was mainly the peaks that increased or decreased during maturation that were selected and that most of the peaks that were obvious intermediates in the process or showed no consistent change during maturation were excluded. An examination of the slopes of the trend lines of the peaks (SMS v peak area) (see appendix, section 8.8.3.2) showed that the peaks could be divided into 3 broad groups. The first group was comprised of the later-eluting peaks (peaks 49 – 54), most of which showed only a small increase in area as the cheese matured. It is likely that these were comprised of the first large water-soluble peptides that became the substrates for cheese enzymes, but were produced at a greater rate than they were hydrolysed. The second group (peaks 21 – 48) encompassed all of the peaks that decreased in concentration as the cheese matured, as well as many that were weakly or poorly associated with cheese maturity. It is probable that the peptides within these peaks increased and decreased in concentration and were transient peptide intermediates. The third grouping (peaks 1 – 20) was comprised of peaks that increased in area markedly throughout maturation. These peaks were likely to be comprised of the small peptides and amino acids. The amino acids (peaks 1, 2, 3 and 4 and 9) would inevitably have been depleted, but at a much slower rate than they were produced, as they were catabolised in the more mature cheese. However, the slope of the trend lines and the distribution of sample points (see appendix, section 8.8.3.2) indicated that, over the maturity range investigated, the rate of gain was likely to be considerably greater than the rate of loss, and this did not appear to affect the predictive ability of the equation. This appeared to be the case for even the maturest of the cheese examined in this study.

8.5.4 SE-HPLC peaks as predictors of sensory maturity

In order to simplify the sample and data analysis, a SE-HPLC method that was developed for the analysis of cheese (Chs. 5 – 7), was used to provide the data for the development of three further equations. Using this method, the WSF can be divided into approximately 15 fractions on the basis of the molecular weight of the peptides present (Ch. 5), providing a distinctive molecular weight profile for different cheese types (Ch. 7). The peaks are very reproducible (Ch. 5), and the peak data is obtained easily, providing a major advantage over RP-HPLC, which is subject to shifts in retention time and changes in peak height and width as the column deteriorates.

PCA of both the correlation and covariance matrices was used to analyse the SE-HPLC 'peak' data. The relationship between the SMS and the first three principal components and TN was modelled using MLR and equations were developed to predict cheese maturity. While both forms of PCA provided the means to achieve good differentiation of cheese maturity, the maturity scores predicted by the equation based on PCA of the covariance matrix (Equation 9, Model I) provided the best correlation with the SMS ($R = 0.9224$, R^2 (adj) = 0.8425). This model compared well with

Model G based on PCA (covariance matrix) of the RP-HPLC peak data ($R = 0.9368$, R^2 (adj) = 0.8708).

MLR was also used to model the relationship between the SMS, TN and the area of each of the SE-HPLC 'peaks' (Model M) and an equation was developed to predict cheese maturity (Equation 13). The maturity scores predicted by this equation correlated well with the SMS ($R = 0.9585$, R^2 (adj) = 0.8970). The predictive ability of this equation was similar to that of Equation 9 (Model I), and while not as good as Equation 11 based on TN and 32 RP-HPLC peaks, was similar to that of Equation 12 based on TN and 7 RP-HPLC peaks (Table 8.14). Peaks with very low Beta-coefficient values (< 0.1) that did not contribute significantly to the regression equation were eliminated and a second equation (Equation 14) was developed that modelled the relationship between SMS, TN and 7 SE-HPLC 'peaks' (1, 4, 9, 12, 13 and 14) (Model N). The maturity scores predicted by this equation were also well correlated with the SMS ($R = 0.9533$, R^2 (adj) = 0.8980). The 'peaks' included in this equation are highlighted in Fig. 8.41. The arrows indicate whether the peak increased or decreased in area as the SMS increased (Fig. 8.41). A double-headed arrow indicates 'peaks' with no consistent relationship with SMS.

The small number of SE-HPLC peaks meant that the selection of key 'peaks' using the PCA (covariance matrix) factor loading scores was less useful than it was for the RP-HPLC-based models (Models K and L). The model based on this approach (Model O) was discarded because the correlation between the predicted maturity scores and the SMS ($R = 0.9285$, R^2 (adj) = 0.8621), while reasonable, was not as good as it was for the other SE-HPLC-based models (Models M and N).

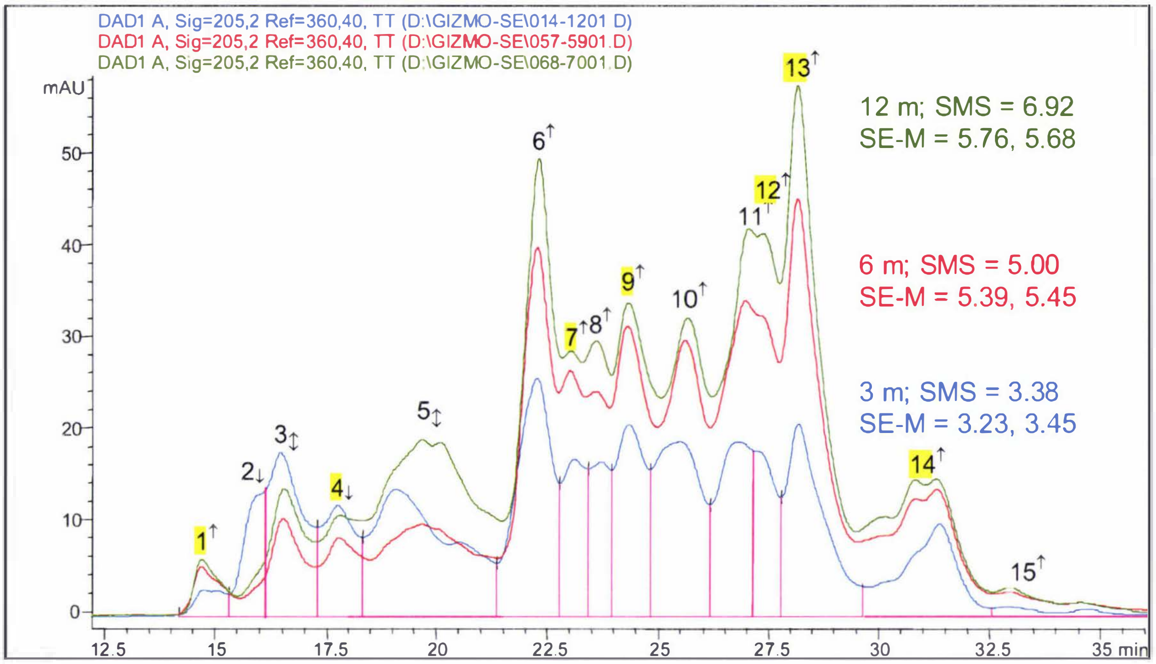


Fig. 8.41 Cheddar cheese manufactured at different times of the year at the same factory, and ripened at 10°C for 3, 6 or 12 months. For each cheese, the SMS and the SE-HPLC maturity scores (SE-M) for Models M and N respectively are recorded alongside the corresponding chromatograph. Model M (TN and 15 'peaks') includes all of the numbered 'peaks' and Model N) (TN and 7 'peaks') includes those 'peaks' that are highlighted. Arrows indicate whether a peak increased or decreased in area.

A comparison of the 'peaks' included in the two SE-HPLC-based equations and the graphs of the SMS versus the area of each peak indicated that the high molecular weight peptides ('peaks' 1 – 5) were likely to be comprised of the first large water-soluble peptide intermediates that were hydrolysed at approximately the same rate as they were produced, or in the case of peak 4, at a greater rate than they were produced. 'Peaks' 6 – 15 each increased in area with increasing maturity, with 'peaks' 11, 12 and 13, comprised of the smallest peptides and amino acids, increasing the most as could be expected.

In summary, each of the four validated equations (Equations 11 and 12 based on RP-HPLC, and Equations 13 and 14 based on SE-HPLC) provided maturity scores that were highly correlated with the SMSs (R = 0.9944, 0.9617, 0.9585 and 0.9533), and each could be used to determine the maturity of Cheddar cheese. The R^2(adj) values (0.9803, 0.9161, 0.8970 and 0.8980) indicated that Equation 11 based on RP-HPLC and 32 peaks was superior to the other equations that provided very similar results. However, the values for the SE of prediction of maturity (0.2266, 0.4676, 0.5180 and 0.5155) were approximately the same distance from the error associated with the determination of sensory maturity of ± 0.3464 and therefore it was not possible to determine which of the methods was the best on this basis.

As a cautionary note, it should be remembered that none of these equations identify flavour defects and therefore they should be used in conjunction with a simple sensory grading to ascertain whether or not there were defects present. To overcome this, it would be necessary to locate defective cheese (there were none present in the sample set used to develop the models), ascertain the peak areas and add this information to the current data set. PCA could then be used to determine which peaks were most closely associated with the defect and predictive models could be developed to identify defective cheese. This approach could also be taken to form equations to predict each of the sensory attributes, which included both flavour and textural properties. In this way, it could be possible to identify peaks associated with a particular attribute. The interrelationship could be explored further to determine whether it was merely a correlation or whether there was a link, perhaps with the peptides or the amino acids within them, that might help characterise the ripening of the cheese. The potential of this approach has been explored by Noël et al (1998) in order to relate the sensory textural properties of Appenzeller and Parmigiano Reggiano to instrumental data that included RP-HPLC peaks, but no predictive equations were developed.

Two observations secondary to the objectives of the current work warrant discussion because they highlight potential problems with the (mis)use of methodologies such as those used in the current work.

In Ch. 7, it was demonstrated that PCA (correlation matrix) of the RP-HPLC or SE-HPLC peak data could differentiate various cheese types. In the present chapter, it was demonstrated that PCA (correlation or covariance matrices) of the RP-HPLC or SE-HPLC peak area data enabled cheese from one particular factory to be differentiated from other cheese. This cheese, from the Southland factory, was differentiated from the other Cheddar cheese, despite being made to the same specification (and the sub-specification that was used at seven of the eight factories). As well, an examination of the RP-HPLC data showed that most cheese made at the Southland factory contained greater quantities of amino acids including Tyr and Phe, than the Waitoa cheese, indicating greater chymosin and possibly microbial enzyme activity in the Southland cheese, despite being stored at the same temperature for the same period of time. The SMS of the maturest Southland cheese did not reflect this and it seems likely that the sensory panel was reluctant to allocate scores between 8.00 and 9.00. This is apparently typical for a sensory panel (Dr R Crawford, Statistician, NZDRI, 2002).

Cheese made at the Southland and Waitoa factories is sold as mature Cheddar and is therefore stored for longer than cheese manufactured at the other factories. The differentiation of the Southland cheese was likely to be influenced by the different NSLAB species known to exist at this factory and the effect of possible differences in enzyme complement on peptides and amino acids present in the cheese. Southland was the only New Zealand factory to possess predominantly *Lactobacillus rhamnosus* strains at the time the cheese was made, with the species identified in the other factories being comprised predominantly of *Lactobacillus paracasei* subsp. *paracasei* or a mixture of the two species (Dr V Crow and B Curry, personal communications, 2002). The differences in the maturity of the cheese made at the two factories may have been due to differences in the rate of cooling of the cheese post-manufacture. Cheese at both factories was cooled to 18°C prior to storage. However, the 'rapid cooling' equipment at the Southland factory was not as efficient at reducing the temperature of the cheese as that at the Waitoa factory and the cheese temperature was likely to have been approximately 3°C higher when it was stored (P Elston, personal communication). The higher temperature would have enabled greater initial chymosin and starter enzyme action and may also have allowed NSLAB numbers to increase in the young cheese. This could account for differences in the cheese maturity. The rate of cooling of cheese has been shown to affect flavour development (Fryer, 1982), and may account for the higher SMS assigned by the sensory panel. Aston et al (1983) found that storing cheese at 20°C prior to storage at lower temperature accelerated Cheddar cheese ripening without the development of off-flavours.

Several European countries are interested in protecting the regional Appellation of a range of cheeses. These cheeses are clearly labelled to set them apart from copies produced in other regions or countries. However, there are apparently large quantities of cheese being produced outside the particular region and marketed with the Appellation. An International Dairy Federation Action Team is currently investigating suitable methods for determining the origins of cheese within a single type. The current work suggests that methods such as PCA of the RP-HPLC or SE-HPLC peak area data that differentiate mainly on the basis of secondary proteolysis may be useful if the cheese is sufficiently different. In fact, others have used a similar approach to differentiate Emmental cheese made with or without the addition of NSLAB (Bütikofer et al (1998), using PCA of RP-HPLC peak height data), or to differentiate the effects of different NSLAB species or strains used in the accelerated ripening of cheese (Lynch et al, 1999). This approach may lead to incorrect assignment of cheese if NSLAB strains are implicated in the differentiation, because the NSLAB population within a factory is subject to change, both within a manufacturing season and between seasons (Crow et al, 2001). As well, it would be possible to determine the NSLAB species or strains present in Appellation cheese and to add them as adjuncts.

8.6 CONCLUSIONS

The relationship between the sensory maturity of a diverse range of New Zealand Cheddar cheese of differing maturities and various instrumental measurements can be modelled and equations developed to predict cheese maturity.

The predictive ability of the cheese MI (used as a tool for designing processed cheese formulations) can be improved by incorporating TN in the equation.

Equations that use a combination of TN and WSN or TN and NPN have a similar predictive ability to the improved MI equation and have a better predictive ability than equations using WSN and NPN alone.

RP-HPLC peak areas of water-soluble peptides and amino acids can be used to develop equations that provide a very good prediction of cheese maturity.

- Equations that incorporate TN and 'peak' 1 or TN and 'peak' 17 have a better predictive ability than equations based on TN and MI, TN and NPN, or TN and WSN.
- Equations that incorporate TN and the scores of the first three principal components (from PCA (covariance matrix) of the RP-HPLC data) have a very good predictive ability that is better than that of equations based on the MI and TN, WSN and TN, NPN and TN, or TN and the area of 'peak' 1 or 'peak' 17.
- An equation that includes TN and 32 RP-HPLC peaks has an excellent predictive ability.

SE-HPLC provides a simpler means of sample and data analysis than RP-HPLC and the peak areas of water-soluble peptides and amino acids can be used to develop equations that provide a reasonable prediction of cheese maturity.

- Equations that incorporate TN and the scores of the first three principal components (from PCA (covariance matrix) of the SE-HPLC data) have a very good predictive ability that is better than that of equations based on the combinations of MI and TN, WSN and TN, NPN and TN, or TN and the area of RP-HPLC 'peak' 1 or 'peak' 17.
- An equation that includes TN and the area of the 15 SE-HPLC 'peaks' provided a very good prediction of maturity that was similar to the best of the RP-HPLC-based equations.

Predictive models for cheese maturity will only be successful if applied to cheeses that lie within the composition and maturity ranges of the standards used to define the parameters in the equations.

The combination of either RP-HPLC or SE-HPLC with the covariance form of PCA, as well as providing very good differentiation of cheese of differing maturities, enabled the HPLC 'peaks' most closely associated with sensory maturity to be identified and this could be useful for characterising cheese ripening.

The combination of either RP-HPLC or SE-HPLC with the correlation form of PCA highlighted some differences between the cheese from different factories. While it may be suggested that this could provide the means to authenticate cheese of a particular origin, the influence of NSLAB strain on the differentiation is of concern because it changes periodically within a factory.

8.7 RECOMMENDATIONS

It is recommended that one model, developed in the current study, is selected and developed for transfer to the New Zealand cheese industry for determining the maturity of Cheddar cheese.

The model selected for further development should be based on HPLC analysis of the WSF of cheese. SE-HPLC is recommended as the most practical instrumental method for development and transfer. It provided the simplest form of sample analysis, repeatable peak elution, relatively simple integration of the peaks, repeatable peak area data (Ch. 5, Fig. 5.14 and Table 5.5), peak area data that were highly correlated with sensory data ($R = 0.9585$) and low and acceptable residuals (SE of prediction = 0.5180).

Instrumental models do not identify flavour defects, and it is therefore recommended that each cheese analysed for maturity be subjected to simple sensory grading.

The relationship between RP-HPLC peak data and data pertaining to particular sensory attributes associated with cheese flavour (e.g. savoury, sweet, fermented, acidic, salty, bitter, creamy, yoghurt, cooked) or texture (e.g. firm, smooth, crumbly, creamy) should be explored using PCA. This approach may be useful for understanding and characterising cheese maturity and as well, it may be possible to develop instrumental models to predict some sensory attributes.

PCA of RP-HPLC or SE-HPLC peak data is not recommended for distinguishing cheese from a particular country or region, because much of the differentiation appears to be attributable to NSLAB species and strains, and these can change with time.

The approach taken for estimating the sensory maturity of Cheddar cheese should be extended to other cheese types and a predictive equation developed for each cheese type.

9.0 GENERAL DISCUSSION, CONCLUSIONS AND SCOPE FOR FUTURE WORK

9.1 GENERAL DISCUSSION

Successful product development and marketing relies on an excellent understanding of the product and the effect of manufacturing processes and storage on product characteristics. In cheese, proteolysis is considered one of the most important biochemical events to be influenced by these processes and plays an important role in the development of cheese texture (e.g. de Jong 1976, 1977, 1978; Creamer and Olson, 1982; Johnston et al, 1994; Watkinson et al, 2001), physical properties (e.g. Watkinson and Johnston, 1993; Ramkumar et al, 1998; Watkinson et al, 1997; Watkinson and Jackson, 1999) and flavour (e.g. McGugan et al, 1979; Aston and Creamer, 1986; Cliffe et al, 1993; Engels and Visser, 1994; Singh et al, 1994; Molina et al, 1999). Recent reviews in the area include those of Christensen et al (1999), McSweeney and Sousa (2000), Kelly and McSweeney (2001) and Sousa et al (2001).

The main proteolytic enzymes in cheese are rennet, plasmin, Cathepsin D, and the peptidases of the cheese microflora, and their action in cheese has been widely studied using a range of methods that have been the subject of several reviews (Fox et al, 1995; McSweeney and Fox, 1997; Wallace and Fox, 1998; Ardö, 1999, Bütikofer and Ardö, 1999; Otte et al, 1999; Singh et al, 1999).

Traditionally, proteolysis has been monitored by measuring the (Kjeldahl) nitrogen present in some fraction of the cheese. Most commonly, non-casein nitrogen (NCN) comprised of pH 4.6-soluble peptides, and non-protein nitrogen (NPN) comprised of small 12% TCA-soluble peptides of less than ~20 amino acids, have been used to provide information about the rate and amount of protease and peptidase activity in cheese. In the present study, these methods were used to demonstrate differences in the amount of protease and peptidase action in seven types of cheese (Ch. 6), to demonstrate differences in the rates of protease and peptidase activity in each of five cheese types ripened over a period of six months (Ch. 7), and to demonstrate the potential of NPN for determining cheese maturity (Ch. 8).

Two methods commonly used to analyse proteolysis are urea-PAGE and RP-HPLC. Urea-PAGE separates the proteins and peptides in cheese on the basis of their size and charge and is widely used to monitor primary proteolysis, i.e. the hydrolysis of the caseins to large peptides (e.g. Creamer and Richardson, 1974; McSweeney et al, 1994b; Johnston et al, 1994; Watkinson et al, 1997). In New Zealand, the advent of the Mini-Protean gel electrophoresis system meant that urea-PAGE became a simple and relatively fast means of assessing proteolysis in a number of cheese samples in a single analysis (Creamer, 1991). The first application of urea-PAGE to systematic cheese product development in New Zealand, was in the extensive study of the effect of the level of chymosin or Rennilase™ addition on proteolysis and the development of cheese sensory properties (Johnston et al, 1994). To achieve semi-quantitative results it was necessary to standardise cheese sample storage, protein and peptide extraction, gel preparation and gel analysis (Coker, 1994). Since that time, urea-PAGE has been used to routinely monitor the effect of manufacturing processes on proteolysis during ripening in order to guide the development of many cheese types including a range of styles of Mozzarella for different markets (K A Johnston and C J Coker, unpublished). RP-HPLC, which separates peptides in cheese on the basis of their hydrophobicity, has been widely used to isolate cheese peptides prior to their identification using mass spectrometry (eg, Singh et al, 1994, 1995, 1997, 1999; Fernández et al, 1998; Alli et al, 1998).

The main objectives of the work in the present study were to determine whether methods for assessing proteolysis in cheese could be used to differentiate cheese according to type at all stages of maturity (limited to 6 months) and to differentiate cheese of a single type according to maturity. This type of work has particular significance for international trade because, in 1993, European legislation (Council Regulation 2081/92) "providing for the protection of food names on a geographical or traditional recipe basis within the European Union (EU)" came into force. Since then, the EU has granted Protected Designation of Origin (PDO) (Appellation of Origin) status to approximately 150 cheese types. Generic food types are not eligible for PDO status unless they include a regional appellation e.g. Brie de Mehun, or Camembert de Normandie (Anon, Dairy Reporter, 2003). Legal challenges have arisen when PDO status has been granted to cheese types commonly manufactured in many regions or countries. For example, the decision to grant PDO status to Feta in October 2002, is to be challenged in the European Court of Justice. Similarly, the debate on whether Parmesan is a generic name, or a direct translation of Parmigiano Reggiano, which already has PDO status, is unresolved and may involve a court case (Dairy Reporter, 2003). With PDO in mind, the statement of intent of the Action Team on Cheese Ripening of the IDF Standing Committee on Dairy Technology (2002) reads, "It is the intention of the team to work on methods that can be applied as standards for indices of cheese maturation, for indices of cheese quality, for authentication and for Appellation issues as each of these areas affect International trade".

Traditionally cheese types and maturity are identified by sensory evaluation by experts. There have been several attempts to identify methods suitable for differentiating cheese types objectively. Subjective analysis of the results of urea-PAGE and RP-HPLC analysis of the water-insoluble fraction (WISF) or water-soluble fraction (WSF) of different cheese types has led to the suggestion that these methods be used for the classification of cheese (e.g. Marcos et al, 1979; Haasnoot et al, 1989; Fox, 1993). In the present study, a preliminary investigation (Ch. 6) showed that proteolysis was sufficiently different in Swiss, Elsberg, Cheddar, Mozzarella, Gouda, Feta and Blue cheese at optimum ripeness, for each cheese type to have a different peptide profile when examined by RP-HPLC of the WSF, but that the traces were complex with a large number of peaks. Urea-PAGE of the urea-soluble fraction (USF) (which contains all the proteins, peptides and amino acids in cheese) or WSF was used to analyse the same cheeses, and although there were marked differences in proteolysis between the Blue cheese and the other cheese types, differences between the remaining cheese types were less obvious than when RP-HPLC of the WSF was used. These findings were similar to those of McGoldrick and Fox (1999) who suggested (following subjective examination) that RP-HPLC of extracts of the WSF had more potential than urea-PAGE of the water-insoluble fraction (WISF) of cheese for differentiating Cheddar, and a range of British, Dutch, Swiss and Italian cheese types. Both studies identified stage of maturation as a potential problem for differentiating cheese. The type of rennet, starter culture and ripening temperature were also suggested by McGoldrick and Fox (1999) as potential problems for differentiating cheese type using urea-PAGE of the WISF or RP-HPLC of extracts of the WSF.

The complexity of the RP-HPLC traces made it desirable to develop simpler methods for analysing proteolysis in cheese. A SE-HPLC method designed to fractionate protein and peptide mixtures (Swergold and Rubin, 1982) that was modified and used to monitor whey protein hydrolysis (Motion, 1992) seemed to have potential for analysing proteolysis in cheese.

The SE-HPLC method, which employs a TSK G-2000 SWXL column (instead of the TSK G-3000 XL column reported by Motion (1992)) and a solvent comprising 36% acetonitrile and 0.1% TFA in water, was shown to provide useful separation of the components of both the USF and WSF of Cheddar cheese. Excellent repeatability of both the extraction of the WSF and SE-HPLC analysis was demonstrated. The traces could be divided into about 16 peaks and the relationship between peak (peptide) elution time and molecular weight could be determined from a standard curve prepared from the elution times of molecular weight standards. While proteins and most peptides eluted according to their molecular weight within the particular solvent conditions, there were some anomalies in the order of elution of the smallest peptides and amino acids. A large ratio of positive charge to size influenced the order of elution of some of the smallest peptides and amino acids, despite the use of an ion-pairing agent (TFA, 0.1%) and low solvent pH to minimise charge-related interactions between the peptides and column matrix (Ch. 5, Table 5.4). The large ratio of hydrophobicity to size of some of the smallest peptides and amino acids had only a minor effect on elution time, due to the high solvent concentration (Ch. 5, Tables 5.2 and 5.3). A wavelength of 205 nm was chosen for monitoring proteolysis and for determining peak area because it was close to the absorbance maxima for most non-aromatic amino acids, minimised the disproportionate contribution of the aromatic amino acids, was above the background absorbance noise (< 200 nm), and was close to the absorbance maxima demonstrated for peptide bonds (~205 nm).

The new SE-HPLC method compared favourably with urea-PAGE and RP-HPLC. The molecular weight distribution of the WSF typically ranged from 20000 to 165 Da, and the molecular weight distribution profiles of the WSF were different for each of the seven cheese types (Ch. 6, Fig. 6.3) and simple in comparison with the RP-HPLC profiles (Ch. 6, Fig. 6.7). These findings suggested that SE-HPLC had potential for differentiating cheese types. The molecular weight distribution of the USF proved less useful for the analysis of proteolysis in cheese because large quantities of high molecular weight peptides of > 15000 Da dominated the trace, causing the profiles of the lower molecular weight peptide peaks to be flatter than the profiles of the same peaks in the WSF and making it more difficult to differentiate the cheese from one another (c.f. Figs. 6.1 and 6.3, Ch. 6). Nevertheless, SE-HPLC analysis of the USF of extensively hydrolysed cheese products, such as enzyme-modified cheese flavour ingredients, has proven useful for comparing products and for monitoring the hydrolysis process (Wilkinson et al, 1992; Coker et al, 2000).

The objectives of the second phase of this study (Ch. 7) were to examine the maturation of several cheese types, to determine the effect of maturation on cheese differentiation and to develop the means to differentiate cheese at all stages of maturity. The differentiation of cheese type or the broad grouping according to maturity had been attempted by several authors using different analytical methods and using multivariate statistical methods to analyse the data. The limited success of these studies, was due perhaps to the poor columns available at the time (Pham and Nakai, 1984; Smith and Nakai, 1990), the choice of method of sample analysis (SDS-PAGE; Dewettinck et al, 1997) or the difficulty in obtaining a good RP-HPLC data set (O'Shea et al, 1996).

In the present study, five cheese types, manufactured at either NZDRI (Cheddar and Mozzarella) or commercially (Swiss, Elsberg and Gouda), were ripened at NZDRI for six months under conditions normal for the variety and were sampled regularly. Gross proteolysis in the cheese was examined using NCN and NPN, and for most cheese types, NCN/TN, NPN/TN and NPN/NCN increased in the early stages of maturation and levelled off as proteolysis progressed (Ch. 7, Fig. 7.1A, B). The exception was Mozzarella in which NPN increased very slowly throughout maturation, reflecting the effect of the high stretch temperature on microbial survival. Urea-PAGE of the USF provided the means to identify the enzymes responsible for primary proteolysis in each of the cheese types. The order of greatest chymosin activity, as determined by the hydrolysis of α_{s1}-casein and formation of α_{s1}-I-casein (Ch. 7, Fig. 7.3A), was Elsberg > Cheddar > Gouda ≈ Swiss > Mozzarella, with the amount dependent mainly on the amount of rennet added, whether or not the curd was washed, the cook temperature, drain pH, stretch temperature (Mozzarella), cheese pH and the storage temperature. The order of greatest plasmin activity, as determined by the hydrolysis of β-casein and formation of γ-casein (Ch. 7, Fig. 7.3A), was Elsberg ≈ Swiss > Gouda ≈ Mozzarella > Cheddar, with the amount dependent mainly on cook temperature, stretch temperature (Mozzarella), cheese pH and storage temperature. The effect of these variables on plasmin activity is complex because plasminogen, plasmin and the plasminogen activators (associated with the casein) as well as any plasminogen activator inhibitors and plasmin inhibitors contained in the cheese serum, are affected differently (see Ch. 2, Fig. 2.3). The ratio of α_{s1}-I-:α_{s1}-casein reflects chymosin activity and provided a suitable index of the maturity of very young cheese (Ch. 7, Fig. 7.3C), as suggested by Haasnoot et al (1989). The ratio of γ-:β-casein reflects plasmin activity and was suitable for determining the maturity of Gouda (Haasnoot et al, 1989), Cheddar and Mozzarella cheese, although the trends for β- and γ-casein suggested that this ratio would cease to be useful as proteolysis progressed, as was the case for Elsberg and Swiss cheese after 120 d (Ch. 7, Fig. 7.3C).

Analysis of the USF by urea-PAGE and the WSF by urea-PAGE, RP-HPLC and SE-HPLC provided patterns of the changes in concentration of the proteins and peptides during the ripening period. Principal component analysis (PCA) with either the correlation or covariance matrix provided an objective interpretation of the peak (PAGE bands were converted to peaks) area data, with a large proportion of the variation in the data (72 – 97%) explained by the first three principal components (PC1 = 47 – 86%) that could be plotted to demonstrate cheese differentiation. This was a marked improvement on the results of Smith and Nakai (1990), who used PCA to analyse the peak area data from RP-HPLC (C8 column) analysis of the WSF of a range of Cheddar, Edam, Gouda, Swiss and Parmesan cheeses. They explained 74% of the variation in their RP-HPLC peak area data (55 peaks from 106 cheeses) using 17 principal components (c.f. 83 – 85% with 3 principal components), with no principal component accounting for more than 13% of the variation (c.f. 47 – 54% for PC1). In this instance, it was necessary to use discriminant analysis of the 17 principal components to achieve differentiation (64% correctly classified).

There were no 'hard and fast' rules regarding the best form of PCA to analyse the data, and the importance or relevance of giving greater weighting to peaks that differed the most between samples (covariance matrix) or giving the same weighting to all peaks across the sample range (correlation matrix) needed to be carefully considered for each new data set. When PCA with the correlation matrix was used, the data for each peak was standardised to a zero mean and a standard deviation of one across the entire sample range, and the relative (between sample) variance in the area of a particular peak was captured, which meant that minor and major peaks could be equally important. There was no standardisation of the data when PCA with the covariance matrix was used, although the data for each peak was centred around the mean for that peak across the sample range. Therefore the peaks maintained their original variance, which meant that the differentiation of samples was influenced more by peaks that changed the most in size between samples, thereby minimising minor effects or differences between cheeses. For urea-PAGE of the USF, this meant that the covariance form of PCA, which accounted for 97% of the variation in the peak area data within three principal components (Ch. 7, Fig. 7.19), was best for differentiating maturity. Urea-PAGE captures primary proteolysis; the hydrolysis of α_{s1}-casein to α_{s1}-I-casein and its subsequent hydrolysis, the hydrolysis of α_{s2}-casein, and the progressive hydrolysis of β-casein to β-I-, β-II- and β-III-casein by chymosin or to γ_1-, γ_2- and γ_3-casein by plasmin. These were particularly important markers of maturation in each cheese type, and when maturation reached a stage where there was little change in the amount of these markers, differentiation of maturity was no longer possible. Therefore, urea-PAGE was only suitable for monitoring maturation during the early stages of maturation or in cheese types in which maturation was slow. The correlation form of PCA, which captured 79% of the variation in the peak area data within three principal components (Ch. 7, Fig. 7.17), gave equal importance to the caseins and minor peptide intermediates and although this was unhelpful for the differentiation of maturity, it was useful for the differentiation of cheese type.

The WSF contains peptides and amino acids, and when analysed by urea-PAGE, provided no clear basis for differentiating cheese maturity because only large peptide intermediates were captured in the gel. Although the correlation form of PCA provided good differentiation of all cheese types urea-PAGE of the WSF could not be recommended because of its lack of sensitivity.

PCA with the correlation matrix captured 83% of the variance in the RP-HPLC peak area data within three principal components (Ch. 7, Fig. 7.36), and was useful for emphasising small differences between cheese types, because small peaks that changed in size across the sample range were given the same status as large peaks that changed in size across the sample range, including those containing the highly absorbing aromatic amino acids. However, it was less useful for determining maturity because some peaks were more important than others as markers of cheese maturity, the aromatic amino acids and peptides containing aromatic amino acids were important, some minor peaks were intermediates that increased and then decreased in concentration, and to further complicate the issue, different peaks were important to the assessment of the maturity of each cheese type. Some of these effects on the differentiation of maturity as well as any problems with differentiating cheese type could be overcome by examining the principal component plots and sequentially removing data associated with any cheese type that stood out as being very different, before using PCA to analyse the reduced data set. When PCA with the covariance matrix was used to analyse the RP-HPLC data, 85% of the variance was captured in three principal components. Differentiation was therefore influenced more by peaks that changed the most in size between samples and this allowed maturity trends to be established for each cheese type.

The SE-HPLC peaks were common to all cheese types (because each peak generally contained several peptides), most increased in size during maturation, and the relativity of the peaks within a sample provided a 'fingerprint' that was different for each cheese type (Ch. 7, Fig. 7.5 - 7.9). Therefore, PCA with the covariance matrix, which accounted for 90% of the variance in the peak area data within three principal components (Ch. 7, Fig. 7.31), was most useful for differentiating both cheese type and maturity. The Mozzarella cheese was clearly separated from the other cheese types and removal of the Mozzarella data followed by PCA of the remaining data (Ch. 7, Fig. 7.33) removed the influence of some peaks that were very large in the Mozzarella samples and improved differentiation of the other cheese types.

In summary, for the range of cheese analysed, several methods of sample analysis provided useful and objective differentiation of cheese type and maturity when the peak area data was analysed using the appropriate form of PCA. In most cases, it was useful to analyse the data from different perspectives using both forms of PCA. Urea-PAGE was more suited to analysing young cheese or cheese that matured slowly, and has potential for developing a maturity index for young cheese sold as high-value 'functional' cheese for processing. SE-HPLC and RP-HPLC captured secondary proteolysis and both have potential for differentiating cheese type and for determining the maturity of cheese within a wider maturity range.

The focus of the final part of this study (Ch. 8) was on Cheddar cheese, which constitutes 50% of all cheese manufactured in New Zealand. The variability in proteolysis within (77) Cheddar cheese, manufactured at eight New Zealand factories to one of two sub-specifications, over two seasons using different types of rennet and different starter strains was examined. Models were developed to explain the relationship between various indices of maturation, and equations were constructed to predict the sensory maturity score (SMS) on a scale of 1.00 – 9.00 (supplied by the Sensory Science Team, NZDRI). It was considered more appropriate to relate proteolysis to cheese maturity than age, because New Zealand's seasonal milk supply and distant markets make it necessary to manufacture and ripen Cheddar cheese under a range of conditions, to ensure a year-round supply at the maturity required by the customer.

A 'Maturity Index' (the product of time (months) and temperature (°C)), commonly used in New Zealand, did in fact provide a reasonable estimation ($R^2 = 0.74$) of the SMS. The inclusion of TN in the regression equation resulted in a marked improvement in the prediction of maturity (R^2 (adj) = 0.81), because it adjusted for the effect of a higher protein content on the structure of the cheese matrix and consequent texture and flavour release experienced by the sensory panel. For this reason, it was useful to include TN in all models. The predictive ability of the equation was similar to that of the mathematical (kinetic) expressions based on time and temperature that were developed by Bouzas et al (1993) to predict proteolysis. These authors cautioned that their equations could only be used to predict proteolysis in cheese from a single factory that had been made with high quality milk to a precise specification.

NPN has been widely used as an indicator of cheese maturity, and in Ch. 7, it was shown to increase steadily in Cheddar cheese during maturation at 13°C for 6 months. In Ch. 8, the relationship between NPN, TN and SMS was modelled using multiple linear regression (MLR). The predictive ability of the regression equation (R^2 (adj) = 0.77), although reasonable, was less useful than MI and TN. The residual scores were spread evenly across the range of SMSs, indicating that MLR was appropriate for modelling the relationship between TN, NPN and SMS, and that the rate of NPN formation was reasonably constant throughout the maturation period.

The focus of the remainder of this study (Ch. 8) was on modelling the relationship between the SMS and peak area data from RP-HPLC and SE-HPLC of the WSF of Cheddar cheese, in order to develop accurate equations for predicting cheese maturity. The WSF was demonstrated by Aston and Creamer (1986) to have typical Cheddar flavour and contains peptides, amino acids, organic acids and their salts, that are produced during maturation. It was therefore appropriate to model the relationship between WSN, TN and SMS. The predictive ability of the regression equation (R^2 (adj) = 0.80), was perhaps less useful than the equation based on MI and TN (R^2 (adj) = 0.81). An examination of the residual scores showed that they were evenly spread across the range of SMSs.

In the previous study (Ch. 7), RP-HPLC peaks containing Tyr, Phe or Trp and 'peak' 17 were identified as important markers of Cheddar cheese maturation. Phe and Tyr to a lesser extent, are located at the N-terminus (mainly) of peptides produced by chymosin action on casein and are exposed to the action of the amino- and/or carboxypeptidases released by the cheese microflora. Their presence is therefore a reflection of aspects of both primary and secondary proteolysis, making them likely indicators of cheese maturity. In the current study the relationship between TN and Tyr, Phe, Trp or 'peak' 17 and SMS was modelled, and in each case the regression equations provided a reasonable prediction of the SMS (R^2 (adj) = 0.82 for Tyr and TN; R^2 (adj) = 0.78 for Phe and TN; R^2 (adj) = 0.79 for Trp and TN; and R^2 (adj) = 0.86 for 'peak' 17 and TN).

The large multi-dimensional data sets resulting from RP-HPLC (54 peaks) and SE-HPLC (15 peaks) analysis of the WSF of 77 Cheddar cheeses were reduced to a small number of uncorrelated principal components using PCA with either the correlation or covariance matrix. PC1 - PC3 of the covariance matrix accounted for most of the variance in the RP-HPLC and SE-HPLC peak area data (88% and 93%, respectively) and provided a better interpretation of the data and assessment of cheese maturity than PC1 - PC3 of the correlation matrix, which was in agreement with the earlier findings. The relationship between SMS and TN and PC1 - PC3 of the RP-HPLC and SE-HPLC covariance matrices was modelled using principal component regression (PCR). The resultant equations predicted maturity scores that correlated well with the SMSs (RP-HPLC: $R = 0.94$ and R^2 (adj) = 0.87; SE-HPLC: $R = 0.92$ and R^2 (adj) = 0.84) and were an improvement on those provided by chemical indices and most individual RP-HPLC peaks (peak 17 being the exception). It was not possible to improve the predictive ability of the equations by including more principal components, without risking an increase in error. These results were a marked improvement on those of O'Shea et al (1996) who analysed the retentate and permeate of the WSF of Cheddar cheese and used 19 or 20 principal components to explain 82 or 84% of the variance in RP-HPLC peak area data (PC1 = 32.4% or 20.7%), and to broadly group the cheese into the four categories determined by cheese grader.

In an endeavour to improve the prediction of the SMS, MLR was used to model the relationship between SMS and the 54 RP-HPLC peaks and TN, or the 15 SE-HPLC peaks and TN. For the SE-HPLC model, the predicted maturity scores correlated well with the SMSs ($R = 0.96$, R^2 (adj) = 0.90). This was a marked improvement on the model based on principal component scores. The data pertaining to peaks not contributing significantly to the equation were removed and MLR was used to provide a new equation based on 7 peaks and TN ($R = 0.95$, R^2 (adj) = 0.90). There is a tendency to 'overfit' the data (that is, to make the data from dissimilar cheeses fit the model) when MLR is used, and it was therefore necessary to validate the models. Both SE-HPLC models could be validated and were considered robust. The first of the RP-HPLC models, based on TN and 54 peaks could not be validated. The data from peaks not contributing significantly to the equation were removed and two further models, based on TN and 32 RP-HPLC peaks or TN and 7 RP-HPLC peaks, were developed and subsequently validated. For both models, the predicted maturity scores were highly correlated with the SMSs (TN and 32 RP-HPLC peaks: $R = 0.99$, R^2 (adj) = 0.98; TN and 7 RP-HPLC peaks: $R = 0.96$, R^2 (adj) = 0.92). The equations developed in this study could be used to predict the maturity of New Zealand Cheddar cheese within the sample range used to develop the models. To encompass a wider range of Cheddar cheese from different factories, or made using different processes, data from these cheeses would be incorporated in an accumulating database that would be used for new models.

To summarise, both RP-HPLC and SE-HPLC provided useful separation of the peptides in the WSF of cheese. The relationship between peak area data and SMS could be modelled and the resultant equations used to provide objectivity to the assessment of the maturity of Cheddar cheese.

Models developed using MLR of the peak area data provided a better prediction of the SMS than those based on PCR. The equations from each of the validated models were used to assign a maturity score (on a scale of 1.00 - 9.00) to each cheese. Despite the higher resolution of RP-HPLC and better predictive ability, particularly of the 32 peak RP-HPLC-based model, SE-HPLC-based models are considered more practical. The SE-HPLC method is comparatively simple, with a single mode of separation that groups water-soluble peptides according to molecular weight. The columns are robust, the elution is isocratic, the analysis time is short, the elution time of each peak is consistent, the results are repeatable, and most importantly, peak recognition is straightforward.

It was clear from the (PCA) results of this study, that differences in the manufacturing processes of different cheese types influence the pathways and rates of proteolysis in cheese during ripening. Most of these manufacturing processes are under tight control in New Zealand factories and it could be expected that cheese of the same type that is made to a tight specification and stored in the same way would be similar. However, this was not the case and there were often differences in the maturation of cheese produced at different factories, or even at the same factory. These differences were greater than could be expected from minor differences in cheese composition, and in the current study (Ch. 8) were reflected in differences in proteolysis that were captured by high-resolution chromatographic methods. Prediction of maturity on the basis of proteolysis patterns was very effective because it captured the influence of factors such as regional milk source, seasonal milk supply, milk composition, factory differences, cheese chemical composition, type of rennet, starter strains, NSLAB species and strains, and cheese storage conditions on the development of Cheddar cheese maturity.

9.2 CONCLUSIONS

SE-HPLC and RP-HPLC of the WSF of cheese, urea-PAGE of the USF of cheese and NPN/TN can be used to follow increasing maturation.

SE-HPLC and RP-HPLC of the WSF are more suitable than urea-PAGE of the USF of cheese or NPN/TN for monitoring proteolysis in the later stages of maturation of most cheese types.

SE-HPLC is a reliable method of sample analysis that gives repeatable results.

Application of multivariate statistical methods to SE-HPLC (WSF) or RP-HPLC (WSF) data from a range of cheese types can allocate each cheese to one of the set of types and provide a maturity trend for each type.

Multivariate statistical methods can be used to model the relationship between SE-HPLC (WSF) or RP-HPLC (WSF) data from a large range of cheese of one type and a sensory maturity score.

An equation that describes the relationship between SE-HPLC (WSF) or RP-HPLC (WSF) data and sensory maturity score can be used to provide a reasonably accurate prediction of the sensory maturity score of a cheese of the type used to develop the equation.

RP-HPLC (WSF) provides greater accuracy than SE-HPLC (WSF) but it is less practical because column aging alters the elution pattern and peak matching between samples is laborious.

SE-HPLC (WSF) is more suitable than other methods because sample analysis time is comparatively short, peak area and elution time are repeatable, peak selection is unambiguous, automation of peak selection on the basis of molecular weight (using a calibration curve) is possible, and a data set is comparatively quick to obtain.

9.3 SCOPE FOR FUTURE WORK

The present study shows the potential of SE-HPLC as a method for examining cheese maturity and type (Chapters 5, 6, 7, 8), as well as variations within a cheese type (Chapter 8).

In the future, it would be useful to develop SE-HPLC for routine use. To achieve this it will be necessary to automate SE-HPLC peak selection. ‘Peak’ areas will be determined within designated molecular weight cut-off points, derived from a standard curve formed from the elution times of molecular weight standards analysed at the same time as any samples.

It is intended that a database will be developed, containing the SE-HPLC (WSF) ‘peak’ areas of samples of large numbers of cheese types, made using different manufacturing processes and sampled at different stages of maturity. The database will be used to determine the type and stage of maturity of unknown cheese samples, and will be most useful for identifying particular processes used in the manufacture of competitor products.

The intention is also to develop a maturity index for the very young Cheddar cheese used in processed cheese formulations. This will involve correlation of SE-HPLC peak areas with the “processibility” of the cheese. It is intended that the maturity index will be used as a formulating tool for processed cheese manufacture.

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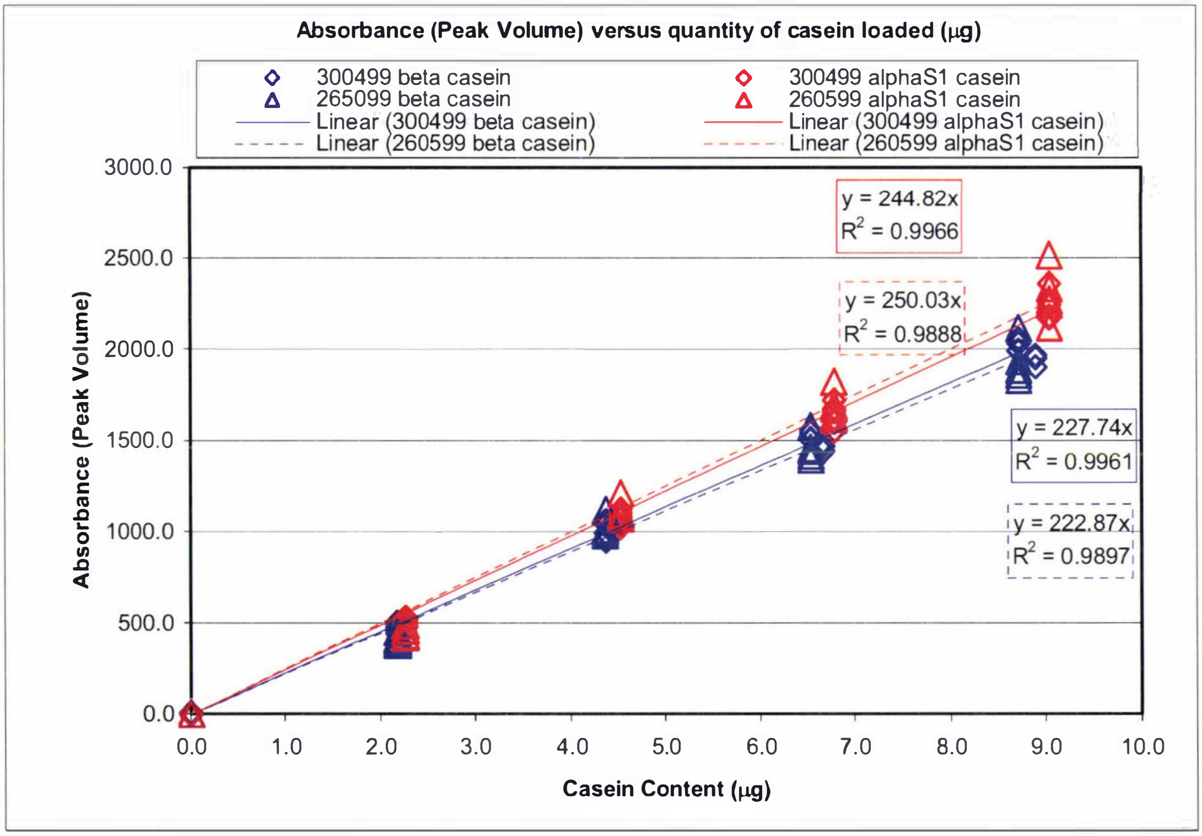
APPENDICES

4.3 APPENDIX – CHAPTER 4

4.3.1 Urea-PAGE – Linear range for casein quantity versus Coomassie dye bound

The graph represents the results obtained from thirteen gels, run as one batch of five gels (batch 300499) and a second batch of eight gels (batch 260599) (White N and C Coker, 1999, unpublished). Rennet casein standards were loaded on to each of the gels and the band absorbance values obtained for β- and α_{s1}-casein were used separately to bring the absorbance values of the bands on each gel set into alignment, thus allowing for differences in the gels. The concentrations of β-casein loaded on to each gel were 0, 2.2, 4.4, 6.5 and 8.7 μg. The concentrations of α_{s1}-casein loaded on to each gel were 0, 2.3, 5.0, 4.5, 6.8 and 9.1 μg. The absorbance for each band was plotted against the casein concentration.



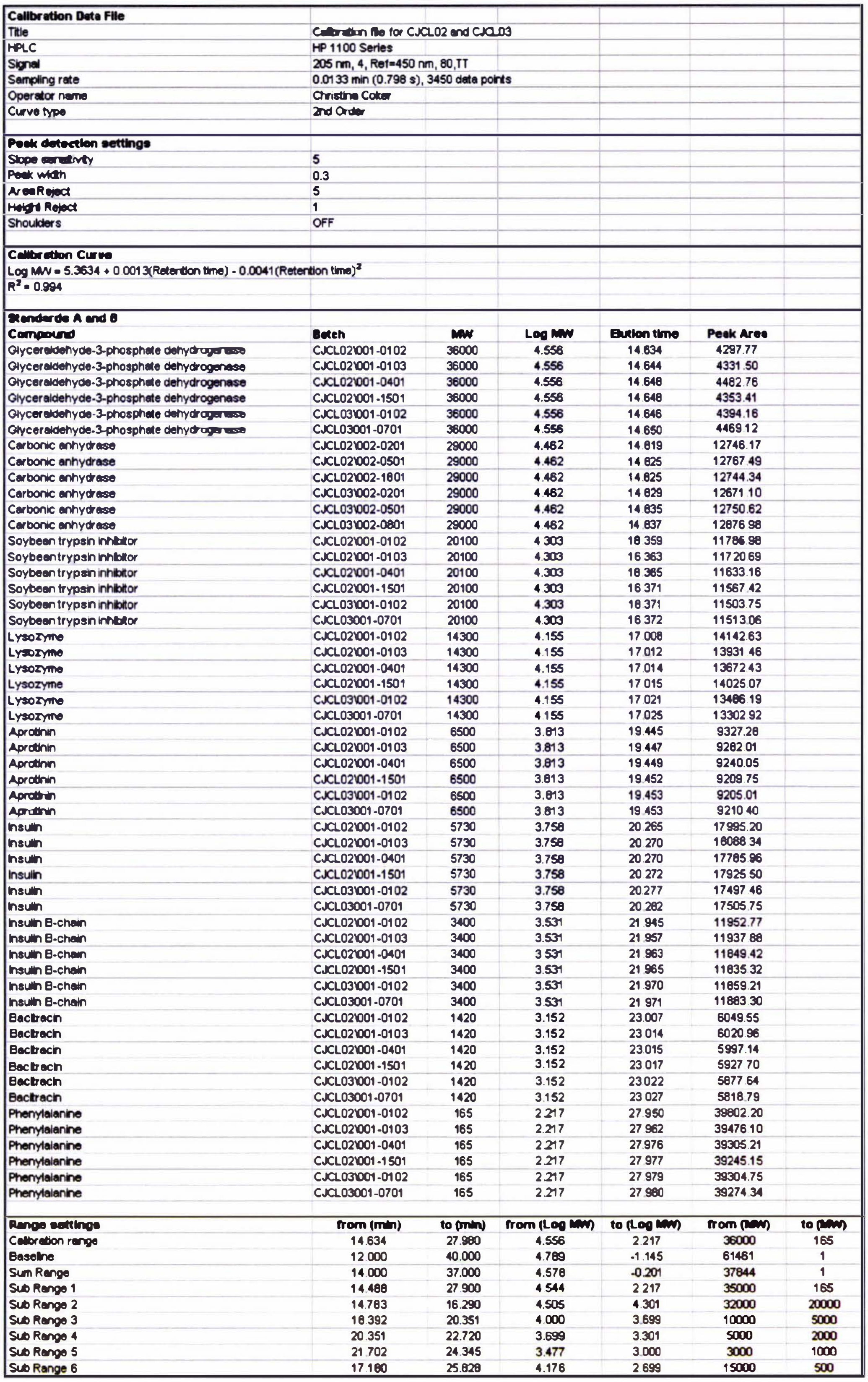
The graph demonstrates:

- very good reproducibility within a gel set
- very good reproduciblity between gels, and
- the linear relationship between the amount of casein loaded on to the gel and the absorbance or amount of Coomassie (R250) dye bound.

5.8 APPENDIX – CHAPTER 5

5.8.1 SE-HPLC - Calibration table for estimates of molecular weight distribution (Fig. 5.5)

The table includes details of sample analysis, peak detection, and the molecular weights and retention times of each of the protein standards used to calculate the standard curve in Fig. 5.5.

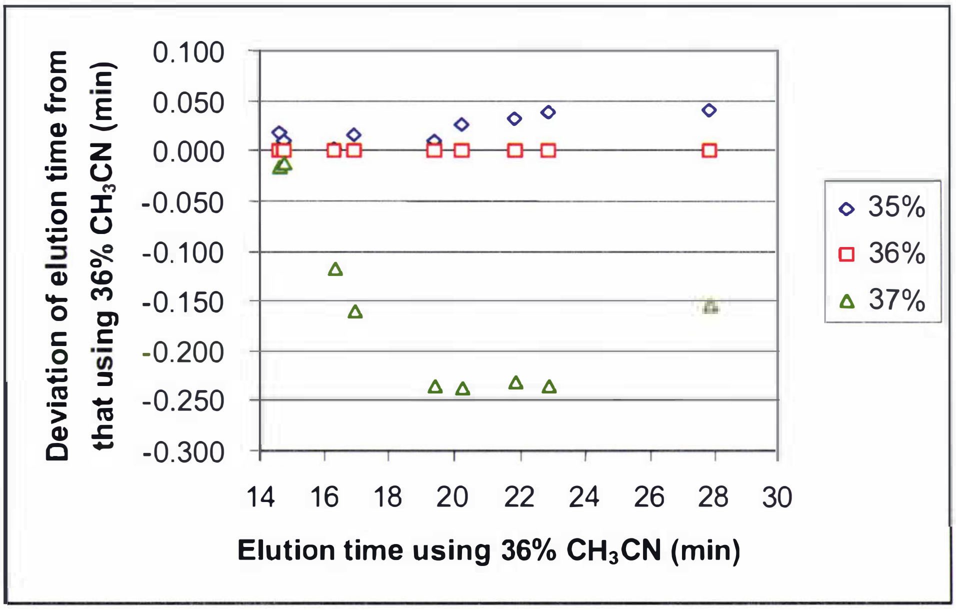


Calibration Data File						
Title	Calibration file for CJCL02 and CJCL03					
HPLC	HP 1100 Series					
Signal	205 nm, 4, Ref=450 nm, 80,TT					
Sampling rate	0.0133 min (0.798 s), 3450 data points					
Operator name	Christine Coker					
Curve type	2nd Order					
Peak detection settings						
Slope sensitivity	5					
Peak width	0.3					
AreaReject	5					
Height Reject	1					
Shoulders	OFF					
Calibration Curve						
Log MW = 5.3634 + 0.0013(Retention time) - 0.0041(Retention time)^2						
R^2 = 0.994						
Standards A and B						
Compound	**Batch**	**MW**	**Log MW**	**Elution time**	**Peak Area**	
Glyceraldehyde-3-phosphate dehydrogenase	CJCL02\001-0102	36000	4.556	14.634	4297.77	
Glyceraldehyde-3-phosphate dehydrogenase	CJCL02\001-0103	36000	4.556	14.644	4331.50	
Glyceraldehyde-3-phosphate dehydrogenase	CJCL02\001-0401	36000	4.556	14.648	4482.76	
Glyceraldehyde-3-phosphate dehydrogenase	CJCL02\001-1501	36000	4.556	14.648	4353.41	
Glyceraldehyde-3-phosphate dehydrogenase	CJCL03\001-0102	36000	4.556	14.646	4394.16	
Glyceraldehyde-3-phosphate dehydrogenase	CJCL03001-0701	36000	4.556	14.650	4469.12	
Carbonic anhydrase	CJCL02\002-0201	29000	4.462	14.619	12746.17	
Carbonic anhydrase	CJCL02\002-0501	29000	4.462	14.625	12767.49	
Carbonic anhydrase	CJCL02\002-1601	29000	4.462	14.625	12744.34	
Carbonic anhydrase	CJCL03\002-0201	29000	4.462	14.629	12671.10	
Carbonic anhydrase	CJCL03\002-0501	29000	4.462	14.635	12750.62	
Carbonic anhydrase	CJCL03\002-0601	29000	4.462	14.637	12676.98	
Soybean trypsin inhibitor	CJCL02\001-0102	20100	4.303	16.359	11786.98	
Soybean trypsin inhibitor	CJCL02\001-0103	20100	4.303	16.363	11720.69	
Soybean trypsin inhibitor	CJCL02\001-0401	20100	4.303	16.365	11633.16	
Soybean trypsin inhibitor	CJCL02\001-1501	20100	4.303	16.371	11567.42	
Soybean trypsin inhibitor	CJCL03\001-0102	20100	4.303	16.371	11503.75	
Soybean trypsin inhibitor	CJCL03001-0701	20100	4.303	16.372	11513.06	
Lysozyme	CJCL02\001-0102	14300	4.155	17.008	14142.63	
Lysozyme	CJCL02\001-0103	14300	4.155	17.012	13931.46	
Lysozyme	CJCL02\001-0401	14300	4.155	17.014	13672.43	
Lysozyme	CJCL02\001-1501	14300	4.155	17.015	14025.07	
Lysozyme	CJCL03\001-0102	14300	4.155	17.021	13486.19	
Lysozyme	CJCL03001-0701	14300	4.155	17.025	13302.92	
Aprotinin	CJCL02\001-0102	6500	3.813	19.445	9327.28	
Aprotinin	CJCL02\001-0103	6500	3.813	19.447	9282.01	
Aprotinin	CJCL02\001-0401	6500	3.813	19.449	9240.05	
Aprotinin	CJCL02\001-1501	6500	3.813	19.452	9209.75	
Aprotinin	CJCL03\001-0102	6500	3.813	19.453	9205.01	
Aprotinin	CJCL03001-0701	6500	3.813	19.453	9210.40	
Insulin	CJCL02\001-0102	5730	3.758	20.265	17995.20	
Insulin	CJCL02\001-0103	5730	3.758	20.270	18088.34	
Insulin	CJCL02\001-0401	5730	3.758	20.270	17785.96	
Insulin	CJCL02\001-1501	5730	3.758	20.272	17925.50	
Insulin	CJCL03\001-0102	5730	3.758	20.277	17497.46	
Insulin	CJCL03001-0701	5730	3.758	20.282	17505.75	
Insulin B-chain	CJCL02\001-0102	3400	3.531	21.945	11952.77	
Insulin B-chain	CJCL02\001-0103	3400	3.531	21.957	11937.88	
Insulin B-chain	CJCL02\001-0401	3400	3.531	21.963	11849.42	
Insulin B-chain	CJCL02\001-1501	3400	3.531	21.965	11835.32	
Insulin B-chain	CJCL03\001-0102	3400	3.531	21.970	11859.21	
Insulin B-chain	CJCL03001-0701	3400	3.531	21.971	11883.30	
Bacitracin	CJCL02\001-0102	1420	3.152	23.007	6049.55	
Bacitracin	CJCL02\001-0103	1420	3.152	23.014	6020.96	
Bacitracin	CJCL02\001-0401	1420	3.152	23.015	5997.14	
Bacitracin	CJCL02\001-1501	1420	3.152	23.017	5927.70	
Bacitracin	CJCL03\001-0102	1420	3.152	23.022	5877.64	
Bacitracin	CJCL03001-0701	1420	3.152	23.027	5818.79	
Phenylalanine	CJCL02\001-0102	165	2.217	27.950	39802.20	
Phenylalanine	CJCL02\001-0103	165	2.217	27.962	39476.10	
Phenylalanine	CJCL02\001-0401	165	2.217	27.976	39305.21	
Phenylalanine	CJCL02\001-1501	165	2.217	27.977	39245.15	
Phenylalanine	CJCL03\001-0102	165	2.217	27.979	39304.75	
Phenylalanine	CJCL03001-0701	165	2.217	27.980	39274.34	
Range settings	**from (min)**	**to (min)**	**from (Log MW)**	**to (Log MW)**	**from (MW)**	**to (MW)**
Calibration range	14.634	27.980	4.556	2.217	36000	165
Baseline	12.000	40.000	4.789	-1.145	61461	1
Sum Range	14.000	37.000	4.578	-0.201	37844	1
Sub Range 1	14.488	27.900	4.544	2.217	35000	165
Sub Range 2	14.763	16.290	4.505	4.301	32000	20000
Sub Range 3	16.392	20.351	4.000	3.699	10000	5000
Sub Range 4	20.351	22.720	3.699	3.301	5000	2000
Sub Range 5	21.702	24.345	3.477	3.000	3000	1000
Sub Range 6	17.160	25.828	4.176	2.699	15000	500

5.8.2 SE-HPLC – Elution times of molecular weight standards using 35, 36 or 37% acetonitrile

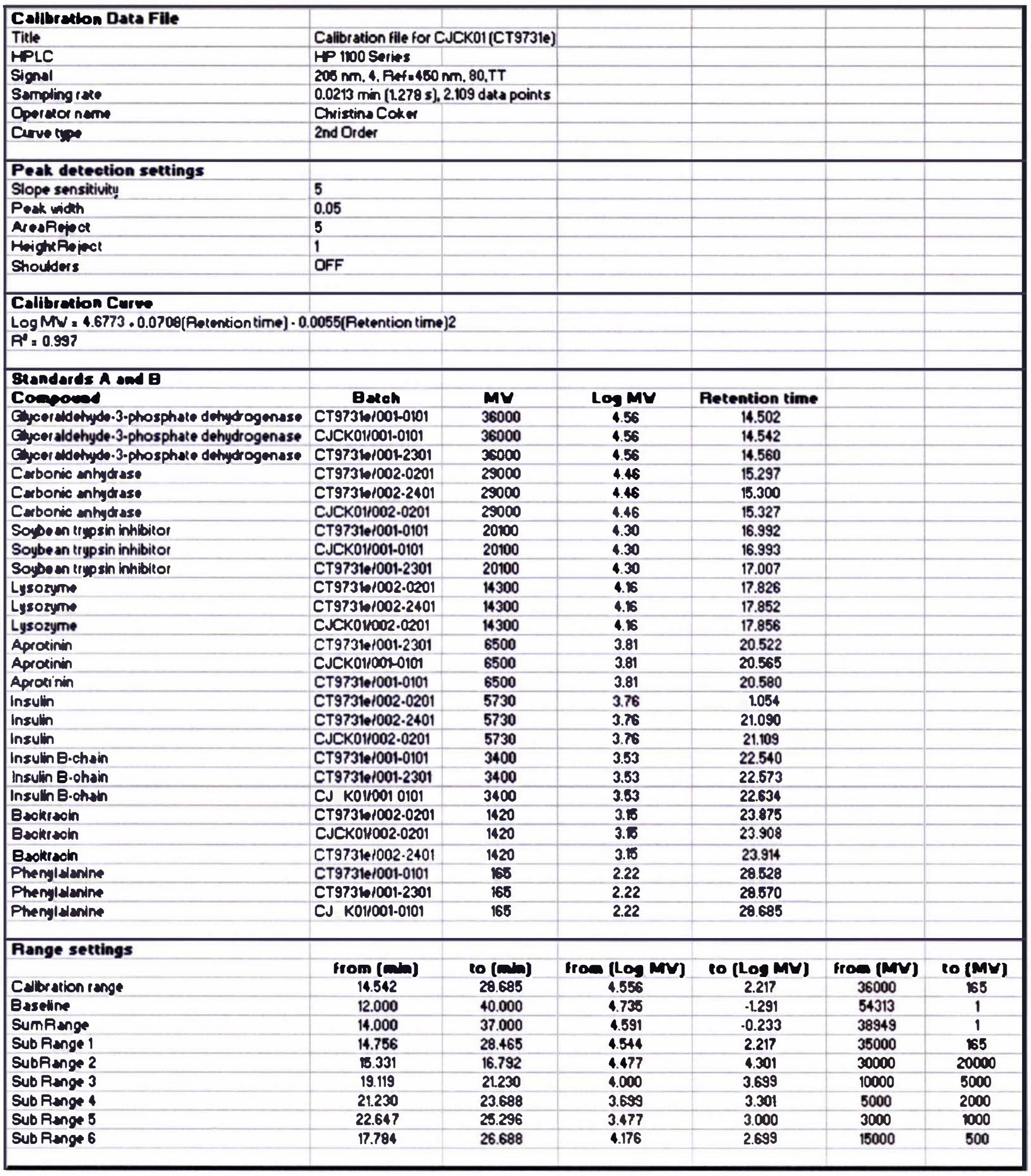
Effect of acetonitrile concentration on elution time (min)

Peak identity	35%	36%	37%
G	14.622	14.600	14.585
G	14.625	14.610	14.594
C	14.790	14.779	14.770
C	14.804	14.792	14.778
S	16.341	16.346	16.228
S	16.367	16.356	16.238
L	16.989	16.970	16.816
L	17.003	16.990	16.824
A	19.434	19.428	19.186
A	19.453	19.437	19.210
I	20.252	20.222	19.991
I	20.266	20.242	20.000
I-B	21.938	21.904	21.663
I-B	21.946	21.913	21.691
B	22.992	22.952	22.721
B	23.006	22.969	22.732
P	27.914	27.879	27.723
P	27.936	27.888	27.737



5.8.3 SE-HPLC - Calibration table for estimates of molecular weight distribution (Fig. 5.13)

The table includes details of sample analysis, peak detection, and the molecular weights and retention times of each of the protein standards used to calculate the standard curve in Fig. 5.13.

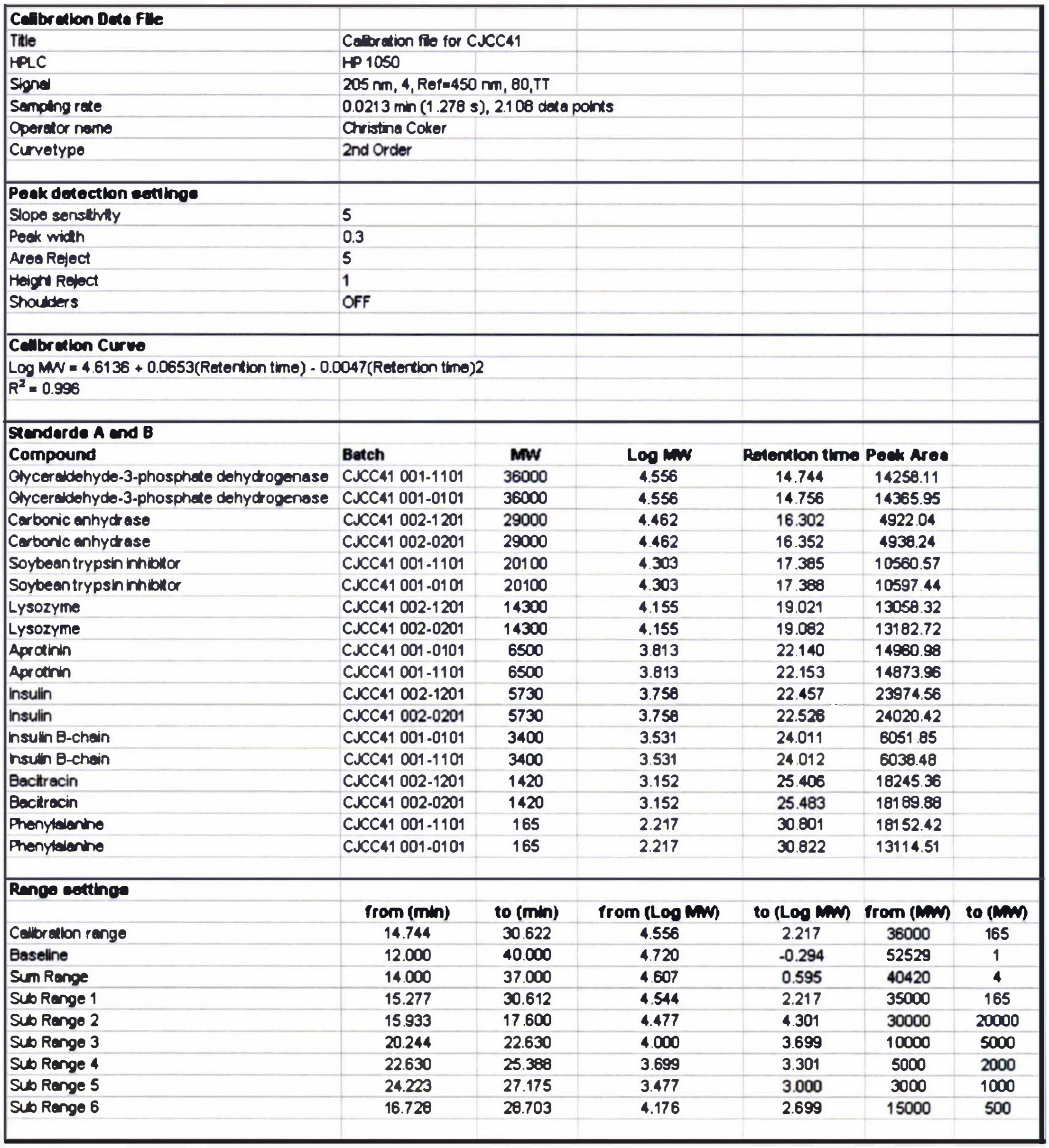


Calibration Data File						
Title	Calibration file for CJCK01 (CT9731e)					
HPLC	HP 1100 Series					
Signal	205 nm, 4, Ref=450 nm, 80,TT					
Sampling rate	0.0213 min (1.278 s), 2.109 data points					
Operator name	Christina Coker					
Curve type	2nd Order					
Peak detection settings						
Slope sensitivity	5					
Peak width	0.05					
AreaReject	5					
HeightReject	1					
Shoulders	OFF					
Calibration Curve						
Log MV = 4.6773 + 0.0708(Retention time) - 0.0055(Retention time)2						
R^2 = 0.997						
Standards A and B						
Compound	**Batch**	**MV**	**Log MV**	**Retention time**		
Glyceraldehyde-3-phosphate dehydrogenase	CT9731e/001-0101	36000	4.56	14.502		
Glyceraldehyde-3-phosphate dehydrogenase	CJCK01/001-0101	36000	4.56	14.542		
Glyceraldehyde-3-phosphate dehydrogenase	CT9731e/001-2301	36000	4.56	14.560		
Carbonic anhydrase	CT9731e/002-0201	29000	4.46	15.297		
Carbonic anhydrase	CT9731e/002-2401	29000	4.46	15.300		
Carbonic anhydrase	CJCK01/002-0201	29000	4.46	15.327		
Soybean trypsin inhibitor	CT9731e/001-0101	20100	4.30	16.992		
Soybean trypsin inhibitor	CJCK01/001-0101	20100	4.30	16.993		
Soybean trypsin inhibitor	CT9731e/001-2301	20100	4.30	17.007		
Lysozyme	CT9731e/002-0201	14300	4.16	17.826		
Lysozyme	CT9731e/002-2401	14300	4.16	17.852		
Lysozyme	CJCK01/002-0201	14300	4.16	17.856		
Aprotinin	CT9731e/001-2301	6500	3.81	20.522		
Aprotinin	CJCK01/001-0101	6500	3.81	20.565		
Aprotinin	CT9731e/001-0101	6500	3.81	20.580		
Insulin	CT9731e/002-0201	5730	3.76	1.054		
Insulin	CT9731e/002-2401	5730	3.76	21.090		
Insulin	CJCK01/002-0201	5730	3.76	21.109		
Insulin B-chain	CT9731e/001-0101	3400	3.53	22.540		
Insulin B-chain	CT9731e/001-2301	3400	3.53	22.573		
Insulin B-chain	CJ K01/001 0101	3400	3.53	22.634		
Bacitracin	CT9731e/002-0201	1420	3.15	23.875		
Bacitracin	CJCK01/002-0201	1420	3.15	23.908		
Bacitracin	CT9731e/002-2401	1420	3.15	23.914		
Phenylalanine	CT9731e/001-0101	165	2.22	28.528		
Phenylalanine	CT9731e/001-2301	165	2.22	28.570		
Phenylalanine	CJ K01/001-0101	165	2.22	28.685		
Range settings						
	from (min)	**to (min)**	**from (Log MV)**	**to (Log MV)**	**from (MV)**	**to (MV)**
Calibration range	14.542	28.685	4.556	2.217	36000	165
Baseline	12.000	40.000	4.735	-1.291	54313	1
SumRange	14.000	37.000	4.591	-0.233	38949	1
Sub Range 1	14.756	28.465	4.544	2.217	35000	165
SubRange 2	15.331	16.792	4.477	4.301	30000	20000
Sub Range 3	19.119	21.230	4.000	3.699	10000	5000
Sub Range 4	21.230	23.688	3.699	3.301	5000	2000
Sub Range 5	22.647	25.296	3.477	3.000	3000	1000
Sub Range 6	17.784	26.688	4.176	2.699	15000	500

6.8 APPENDIX – CHAPTER 6

6.8.1 SE-HPLC - Calibration table for molecular weight standard sets A and B (Fig. 6.2)

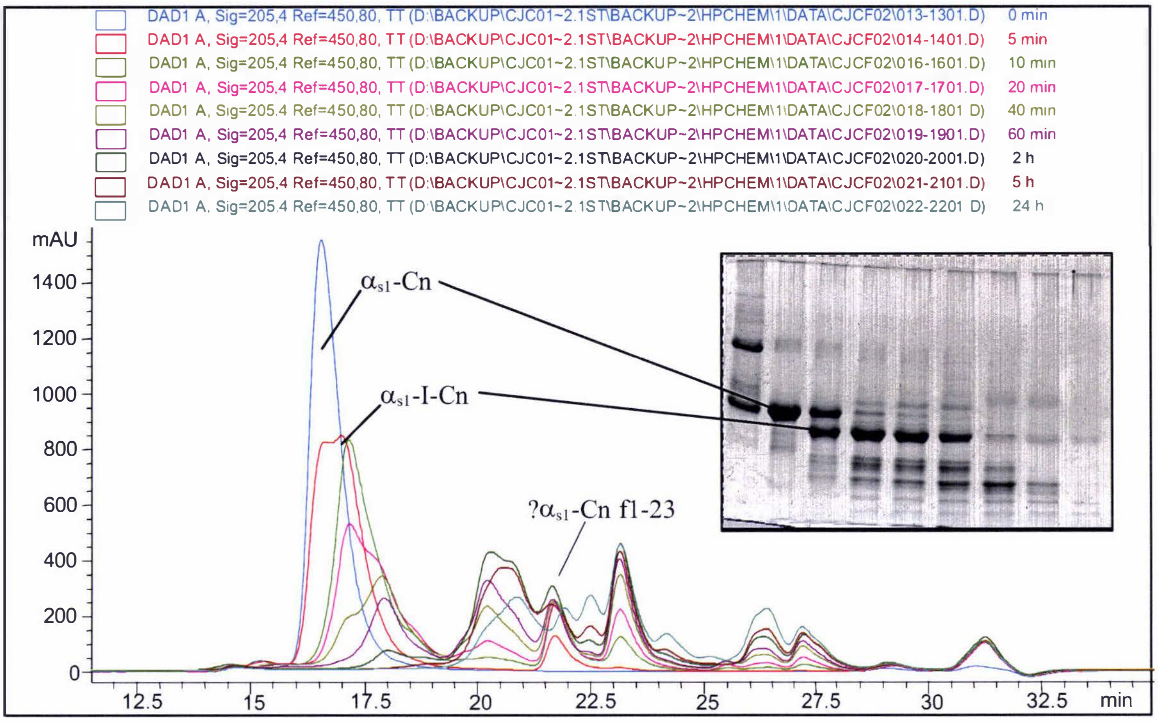
The table includes details of SE-HPLC analysis, peak detection, and the molecular weight and retention time of each protein standard used to calculate the standard curve (Fig. 6.2), as well as retention times calculated from the standard curve and used to determine the position of the molecular weight markers (Fig. 6.1).



Calibration Data File						
Title	Calibration file for CJCC41					
HPLC	HP 1050					
Signal	205 nm, 4, Ref=450 nm, 80,TT					
Sampling rate	0.0213 min (1.278 s), 2108 data points					
Operator name	Christina Coker					
Curvetype	2nd Order					
Peak detection settings						
Slope sensitivity	5					
Peak width	0.3					
Area Reject	5					
Height Reject	1					
Shoulders	OFF					
Calibration Curve						
Log MW = 4.6136 + 0.0653(Retention time) - 0.0047(Retention time)2						
R^2 = 0.996						
Standards A and B						
Compound	**Batch**	**MW**	**Log MW**	**Retention time**	**Peak Area**	
Glyceraldehyde-3-phosphate dehydrogenase	CJCC41 001-1101	36000	4.556	14.744	14258.11	
Glyceraldehyde-3-phosphate dehydrogenase	CJCC41 001-0101	36000	4.556	14.756	14365.95	
Carbonic anhydrase	CJCC41 002-1201	29000	4.462	16.302	4922.04	
Carbonic anhydrase	CJCC41 002-0201	29000	4.462	16.352	4938.24	
Soybean trypsin inhibitor	CJCC41 001-1101	20100	4.303	17.385	10560.57	
Soybean trypsin inhibitor	CJCC41 001-0101	20100	4.303	17.388	10597.44	
Lysozyme	CJCC41 002-1201	14300	4.155	19.021	13058.32	
Lysozyme	CJCC41 002-0201	14300	4.155	19.082	13182.72	
Aprotinin	CJCC41 001-0101	6500	3.813	22.140	14980.98	
Aprotinin	CJCC41 001-1101	6500	3.813	22.153	14873.96	
Insulin	CJCC41 002-1201	5730	3.758	22.457	23974.56	
Insulin	CJCC41 002-0201	5730	3.758	22.528	24020.42	
Insulin B-chain	CJCC41 001-0101	3400	3.531	24.011	6051.85	
Insulin B-chain	CJCC41 001-1101	3400	3.531	24.012	6038.48	
Bacitracin	CJCC41 002-1201	1420	3.152	25.406	18245.36	
Bacitracin	CJCC41 002-0201	1420	3.152	25.483	18189.88	
Phenylalanine	CJCC41 001-1101	165	2.217	30.801	18152.42	
Phenylalanine	CJCC41 001-0101	165	2.217	30.822	13114.51	
Range settings						
	from (min)	**to (min)**	**from (Log MW)**	**to (Log MW)**	**from (MW)**	**to (MW)**
Calibration range	14.744	30.622	4.556	2.217	36000	165
Baseline	12.000	40.000	4.720	-0.294	52529	1
Sum Range	14.000	37.000	4.607	0.595	40420	4
Sub Range 1	15.277	30.612	4.544	2.217	35000	165
Sub Range 2	15.933	17.600	4.477	4.301	30000	20000
Sub Range 3	20.244	22.630	4.000	3.699	10000	5000
Sub Range 4	22.630	25.388	3.699	3.301	5000	2000
Sub Range 5	24.223	27.175	3.477	3.000	3000	1000
Sub Range 6	16.728	28.703	4.176	2.699	15000	500

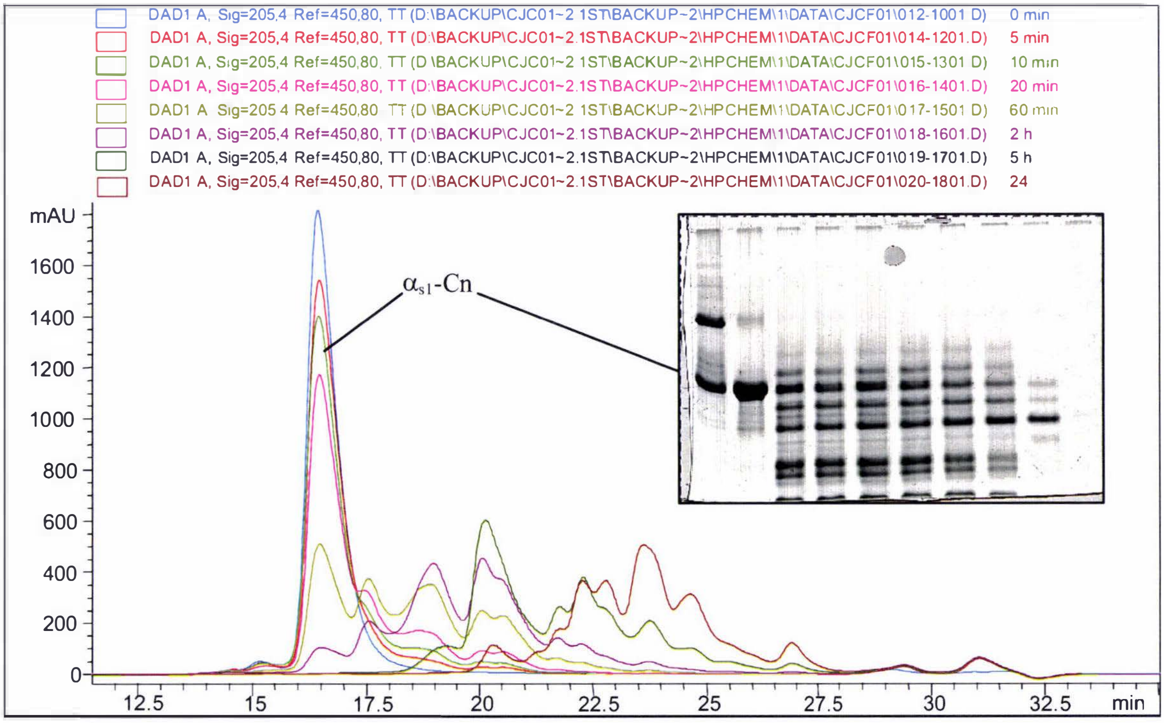
6.8.2 Chymosin hydrolysate of α_{s1}-casein

The casein was purified and hydrolysed using the methods described in Ch. 4, sections 4.2.4 and 4.2.5, respectively. Samples were taken at 0, 5, 10, 20, 40 and 60 min, and 2, 5 and 24 h and analysed by urea-PAGE and SE-HPLC using the methods described in Ch. 4, sections 4.2.3.1 and 4.2.3.3.



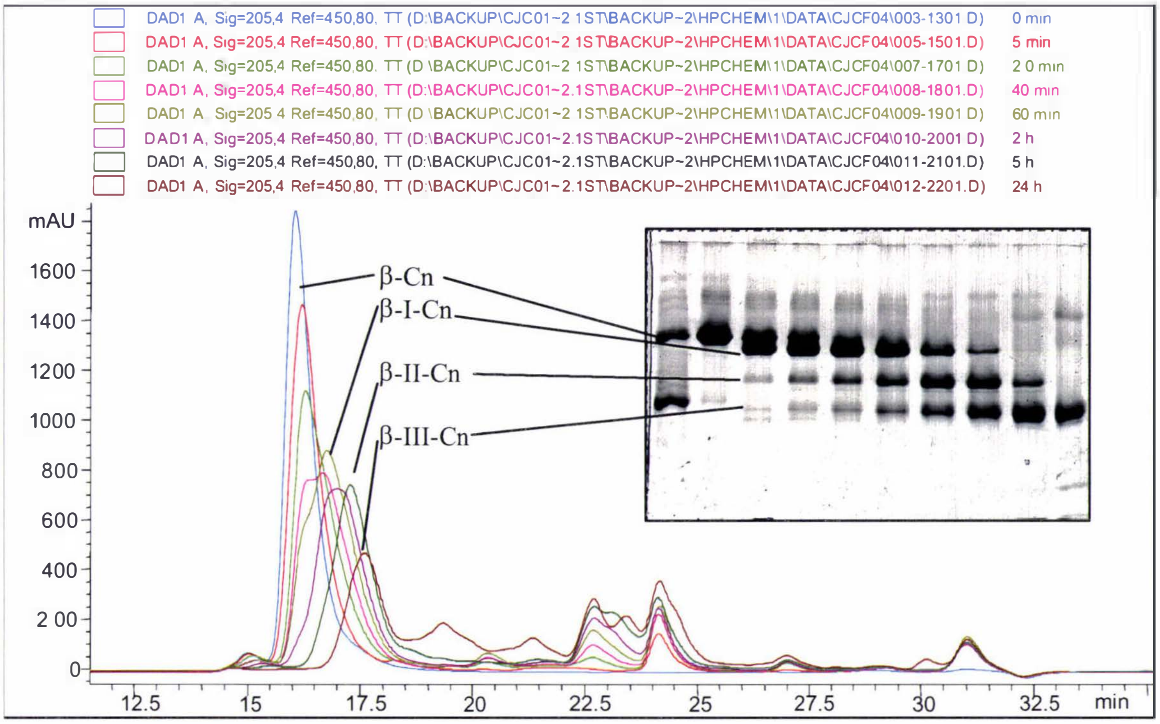
6.8.3 Plasmin hydrolysate of α_{s1}-casein

See Ch. 4, sections 4.2.4 and 4.2.5 for details of casein purification and hydrolysis. Samples were taken at 0, 5, 10, 20 and 60 min, and 2, 5 and 24 h and analysed by urea-PAGE and SE-HPLC.



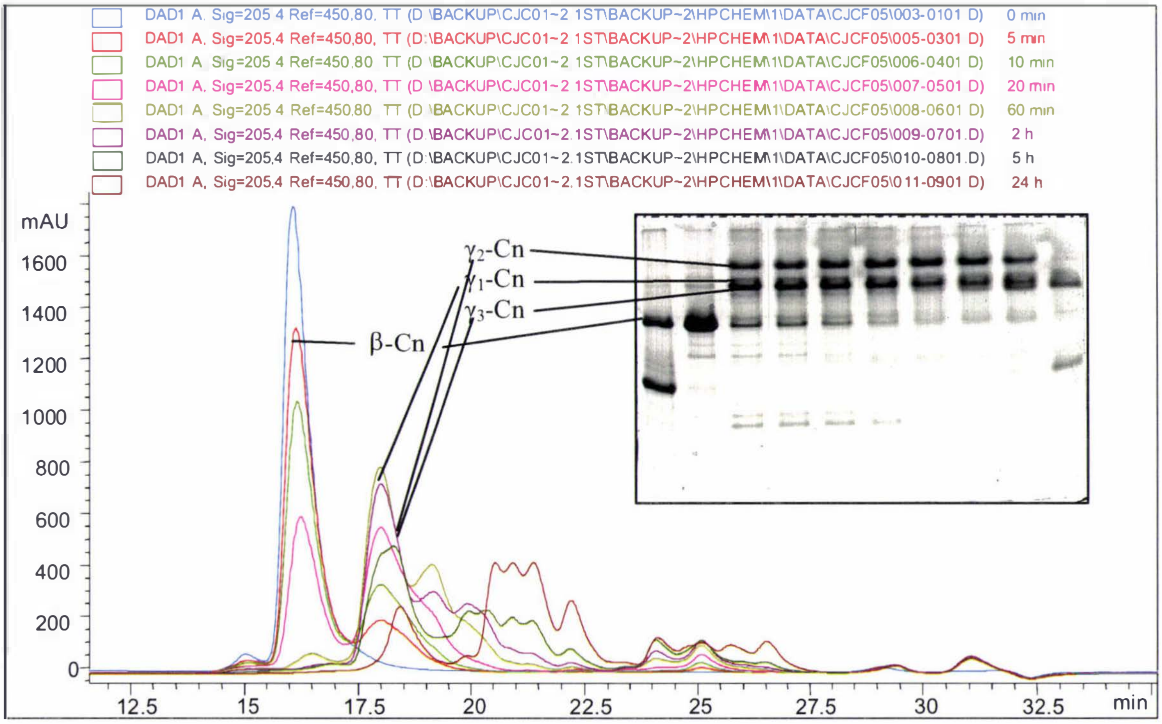
6.8.4 Chymosin hydrolysate of β-casein

See Ch. 4, sections 4.2.4 and 4.2.5 for details of casein purification and hydrolysis. Samples were taken at 0, 5, 20, 40 and 60 min, and 2, 5 and 24 h and analysed by urea-PAGE and SE-HPLC.



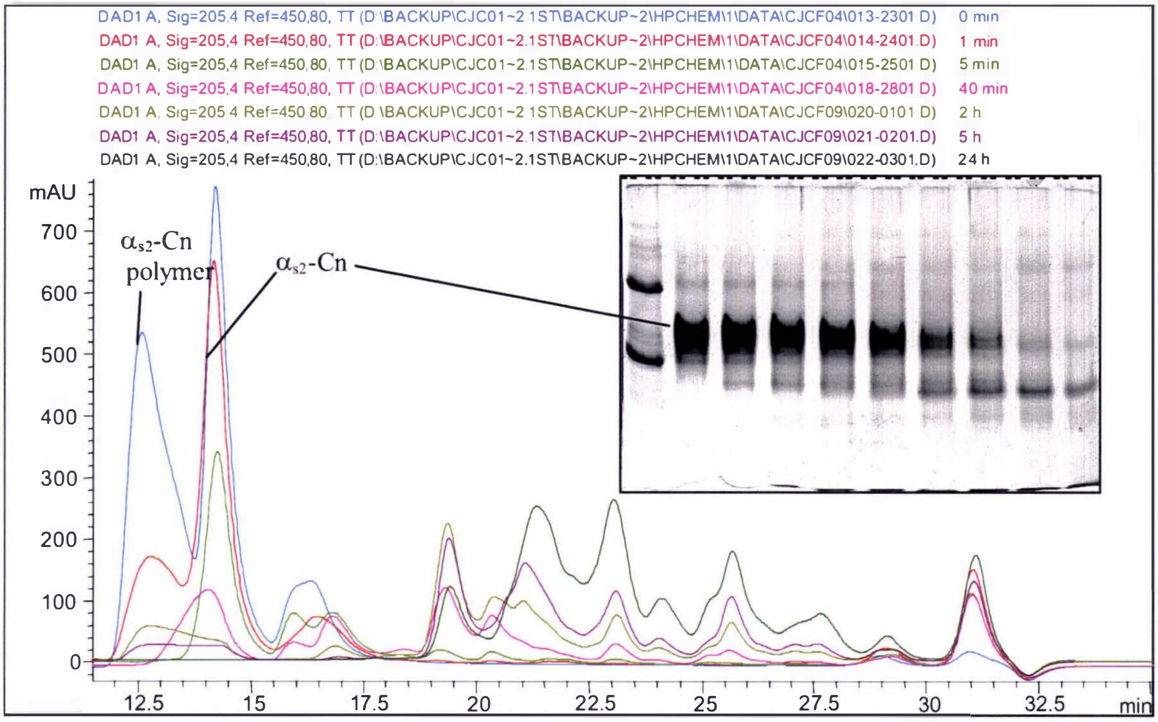
6.8.5 Plasmin hydrolysate of β-casein

See Ch. 4, sections 4.2.4 and 4.2.5 for details of casein purification and hydrolysis. Samples were taken at 0, 5, 10, 20 and 60 min and 2, 5 and 24 h and analysed by urea-PAGE and SE-HPLC.



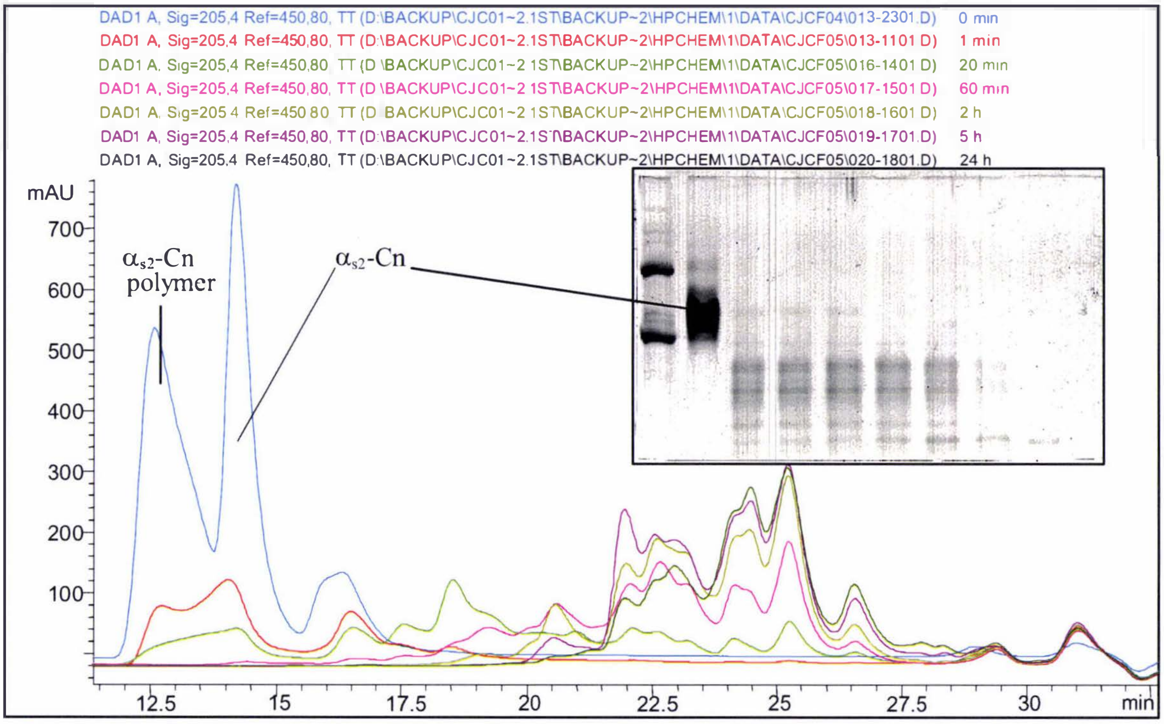
6.8.6 Chymosin hydrolysate of α_{s2}-casein

See Ch. 4, sections 4.2.4 and 4.2.5 for details of casein purification and hydrolysis. Samples were taken at 0, 1, 5 and 40 min, and 2, 5 and 24 h and analysed by urea-PAGE and SE-HPLC.



6.8.7 Plasmin hydrolysate of α_{s2}-casein

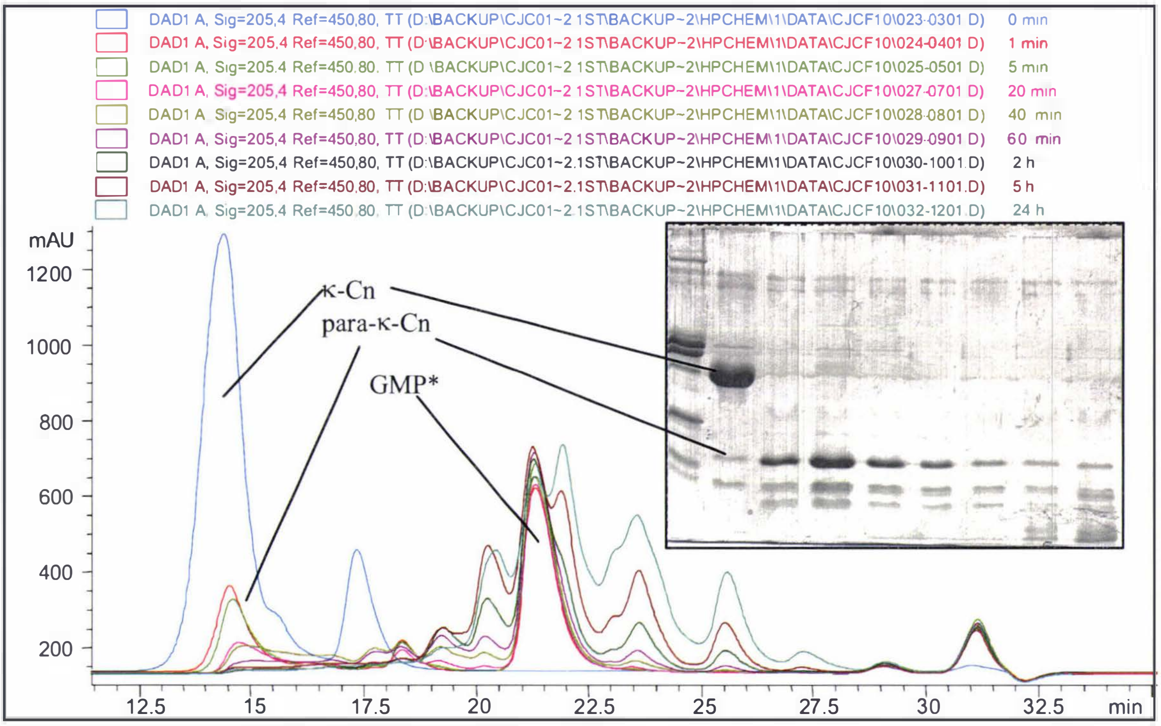
See Ch. 4, sections 4.2.4 and 4.2.5 for details of casein purification and hydrolysis. Samples were taken at 0, 1, 20 and 60 min and 2, 5 and 24 h and analysed by urea-PAGE and SE-HPLC.



6.8.8 Chymosin hydrolysate of κ-casein

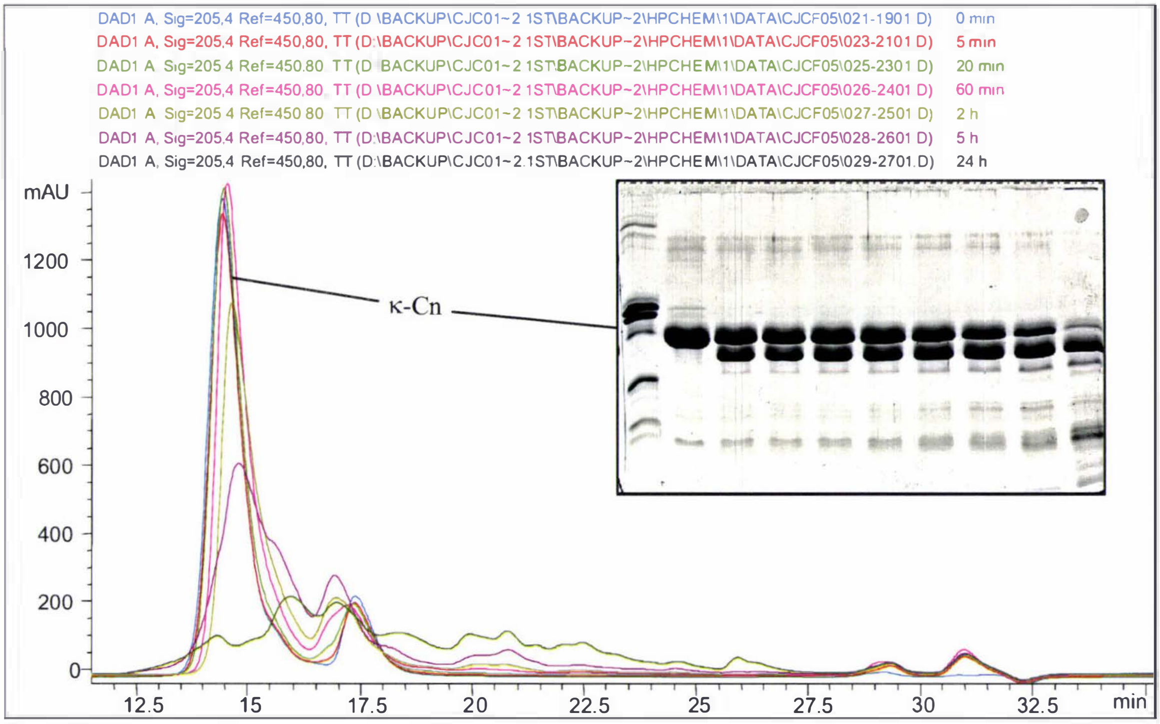
See Ch. 4, sections 4.2.4 and 4.2.5 for details of casein purification and hydrolysis. Samples were taken at 0, 1, 5, 20, 40 and 60 min and 2, 5 and 24 h and analysed by SDS-PAGE and SE-HPLC using the methods described in Ch. 4, sections 4.2.3.2 and 4.2.3.1.

*GMP position confirmed using standards



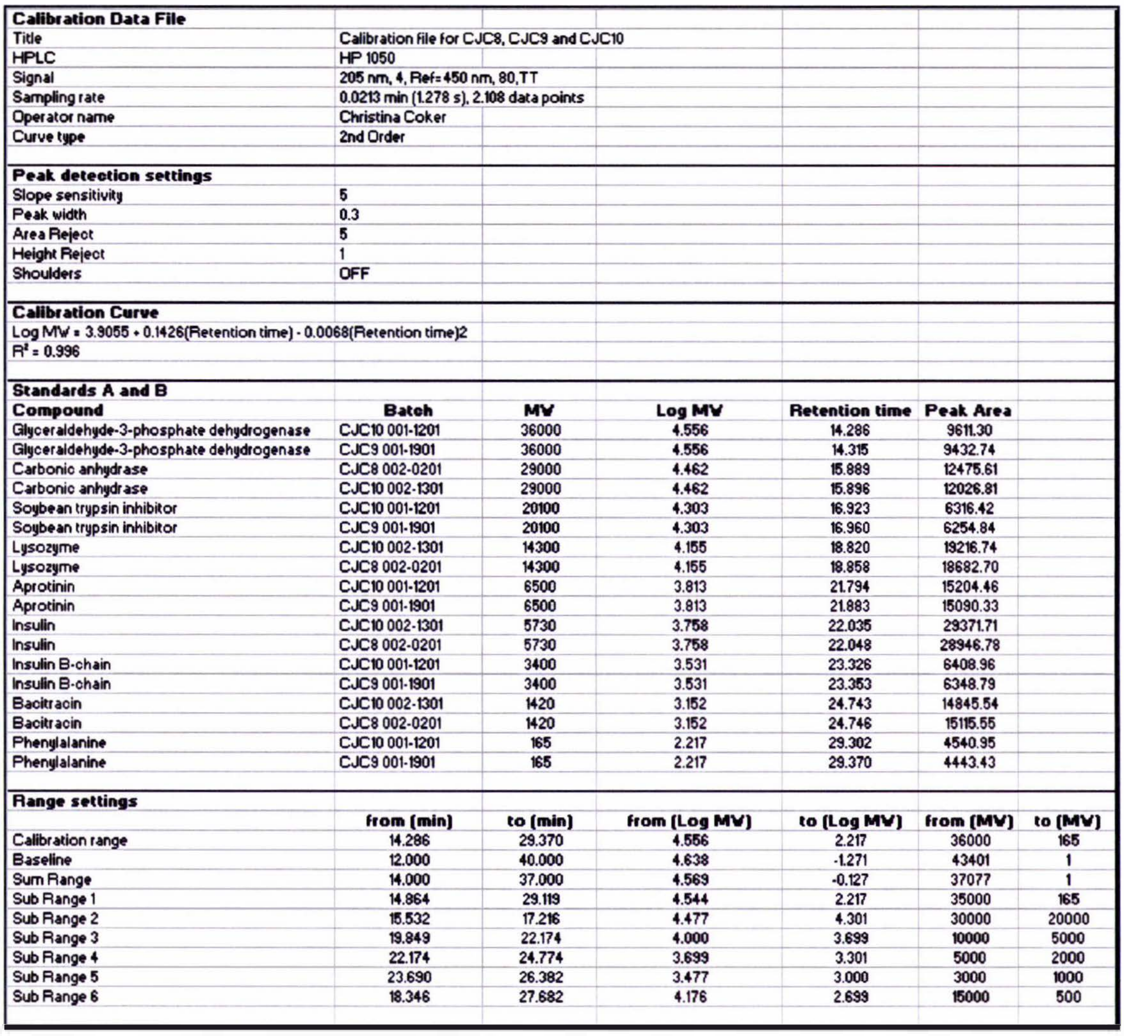
6.8.9 Plasmin hydrolysate of κ-casein

See Ch. 4, sections 4.2.4 and 4.2.5 for details of casein purification and hydrolysis. Samples were taken at 0, 5, 20 and 60 min and 2, 5 and 24 h and analysed by SDS-PAGE and SE-HPLC using the methods described in Ch. 4, sections 4.2.3.2 and 4.2.3.1.



6.8.10 SE-HPLC - Calibration table for molecular weight standard sets A and B (Fig. 6.4)

The table includes details of sample analysis, peak detection, and the molecular weight and retention time of each protein standard used to calculate the standard curve (Fig. 6.4), as well as retention times calculated from the standard curve and used to create the molecular weight markers (Fig. 6.3).



Calibration Data File						
Title	Calibration file for CJC8, CJC9 and CJC10					
HPLC	HP 1050					
Signal	205 nm, 4, Ref=450 nm, 80,TT					
Sampling rate	0.0213 min (1.278 s), 2.108 data points					
Operator name	Christina Coker					
Curve type	2nd Order					
Peak detection settings						
Slope sensitivity	5					
Peak width	0.3					
Area Reject	5					
Height Reject	1					
Shoulders	OFF					
Calibration Curve						
Log MW = 3.9055 + 0.1426(Retention time) - 0.0068(Retention time)2						
R^2 = 0.996						
Standards A and B						
Compound	**Batch**	**MW**	**Log MW**	**Retention time**	**Peak Area**	
Glyceraldehyde-3-phosphate dehydrogenase	CJC10 001-1201	36000	4.556	14.286	9611.30	
Glyceraldehyde-3-phosphate dehydrogenase	CJC9 001-1901	36000	4.556	14.315	9432.74	
Carbonic anhydrase	CJC8 002-0201	29000	4.462	15.889	12475.61	
Carbonic anhydrase	CJC10 002-1301	29000	4.462	15.896	12026.81	
Soybean trypsin inhibitor	CJC10 001-1201	20100	4.303	16.923	6316.42	
Soybean trypsin inhibitor	CJC9 001-1901	20100	4.303	16.960	6254.84	
Lysozyme	CJC10 002-1301	14300	4.155	18.820	19216.74	
Lysozyme	CJC8 002-0201	14300	4.155	18.858	18682.70	
Aprotinin	CJC10 001-1201	6500	3.813	21.794	15204.46	
Aprotinin	CJC9 001-1901	6500	3.813	21.883	15090.33	
Insulin	CJC10 002-1301	5730	3.758	22.035	29371.71	
Insulin	CJC8 002-0201	5730	3.758	22.048	28946.78	
Insulin B-chain	CJC10 001-1201	3400	3.531	23.326	6408.96	
Insulin B-chain	CJC9 001-1901	3400	3.531	23.353	6348.79	
Bacitracin	CJC10 002-1301	1420	3.152	24.743	14845.54	
Bacitracin	CJC8 002-0201	1420	3.152	24.746	15115.55	
Phenylalanine	CJC10 001-1201	165	2.217	29.302	4540.95	
Phenylalanine	CJC9 001-1901	165	2.217	29.370	4443.43	
Range settings						
	from (min)	**to (min)**	**from (Log MW)**	**to (Log MW)**	**from (MW)**	**to (MW)**
Calibration range	14.286	29.370	4.556	2.217	36000	165
Baseline	12.000	40.000	4.638	-1.271	43401	1
Sum Range	14.000	37.000	4.569	-0.127	37077	1
Sub Range 1	14.864	29.119	4.544	2.217	35000	165
Sub Range 2	15.532	17.216	4.477	4.301	30000	20000
Sub Range 3	19.849	22.174	4.000	3.699	10000	5000
Sub Range 4	22.174	24.774	3.699	3.301	5000	2000
Sub Range 5	23.690	26.382	3.477	3.000	3000	1000
Sub Range 6	18.346	27.682	4.176	2.699	15000	500

7.8 APPENDIX – CHAPTER 7

7.8.1 Alkaline urea-PAGE of the USF

Peak data (average of two values) for PCA

	Cheese	Ripening time	1	2	3	4	5	6	7	8	9	10	11	12
24	Cheddar	1 d	0.206	0.311	0.453	0.490	14.980	0.139	0.971	1.686	1.748	15.589	1.404	0.232
25	Cheddar	14 d	0.552	0.201	0.865	0.369	14.996	0.336	1.115	1.607	1.683	12.019	3.467	0.460
26	Cheddar	30 d	0.640	0.233	1.079	0.436	13.878	0.413	1.128	1.125	1.954	6.591	5.123	0.794
27	Cheddar	63 d	0.709	0.341	1.695	0.755	12.936	0.527	0.912	1.060	2.989	2.911	4.875	1.224
28	Cheddar	91 d	0.764	0.363	2.242	1.105	12.663	0.564	1.013	0.980	3.586	2.502	4.255	1.476
29	Cheddar	133 d	0.796	0.278	2.466	1.285	10.516	0.535	0.853	0.691	3.394	2.080	2.833	1.338
30	Cheddar	182 d	1.006	0.423	3.123	1.748	9.847	0.545	0.934	0.774	4.009	2.122	2.059	1.458
8	Elsberg	2 d	1.596	0.614	2.238	1.386	18.043	1.630	1.043	2.695	3.150	17.804	3.811	1.526
9	Elsberg	24 d	1.558	0.309	4.095	1.748	10.506	1.567	0.680	0.891	2.436	4.542	8.794	1.263
10	Elsberg	42 d	1.530	0.331	4.580	1.672	9.100	1.273	0.603	0.896	2.681	2.709	9.017	1.473
11	Elsberg	63 d	1.601	0.449	4.601	1.648	7.960	1.124	0.581	0.875	2.646	2.225	8.706	1.558
12	Elsberg	91 d	1.738	0.441	4.938	1.797	6.974	1.123	0.635	0.680	2.859	1.647	8.112	1.723
13	Elsberg	120 d	1.791	0.449	5.220	1.863	6.547	0.959	0.669	0.596	3.152	1.396	7.594	1.857
14	Elsberg	150 d	1.989	0.463	5.652	1.952	5.787	0.897	0.570	0.613	3.393	1.307	7.014	2.051
15	Elsberg	182 d	1.848	0.460	5.598	1.949	5.588	0.876	0.577	0.704	3.466	1.299	5.793	1.776
16	Gouda	1 d	0.700	0.380	0.946	0.372	15.346	0.564	0.629	1.962	1.938	15.895	1.297	0.276
17	Gouda	17 d	0.812	0.423	1.462	0.472	13.837	0.785	0.720	2.032	1.894	13.135	3.425	0.286
18	Gouda	31 d	0.865	0.752	1.888	0.685	13.174	0.770	1.026	2.315	1.744	11.588	4.774	0.273
19	Gouda	50 d	1.083	0.738	2.570	0.967	12.178	0.787	1.186	1.678	2.092	8.771	6.036	0.512
20	Gouda	91 d	0.984	0.658	2.505	0.739	11.231	0.723	0.874	1.118	1.948	7.838	5.767	0.377
21	Gouda	120 d	1.197	0.835	3.227	0.841	9.866	0.718	0.871	1.014	2.016	4.871	7.119	0.482
22	Gouda	150 d	1.351	0.989	3.840	1.041	8.992	0.675	0.944	0.915	2.172	3.638	7.143	0.462
23	Gouda	182 d	1.617	1.127	4.386	1.100	6.218	0.532	0.651	0.722	1.933	2.035	6.269	0.570
31	Mozzarella	1 d	0.647	0.215	0.815	0.364	16.203	0.241	1.144	2.180	2.186	18.490	0.902	0.988
32	Mozzarella	14 d	0.610	0.211	1.121	0.464	15.498	0.256	0.492	1.695	2.683	18.639	1.204	0.972
33	Mozzarella	30 d	0.707	0.231	1.673	0.712	14.401	0.436	0.581	1.528	2.302	17.446	1.830	0.925
34	Mozzarella	63 d	0.922	0.302	2.743	1.205	11.963	0.383	0.601	1.146	2.024	13.802	3.620	1.172
35	Mozzarella	91 d	1.059	0.284	3.559	1.592	11.099	0.391	0.700	1.000	1.967	11.537	4.976	1.373
36	Mozzarella	133 d	1.181	0.289	3.897	1.843	9.578	0.469	0.594	0.742	1.774	9.010	5.913	1.556
37	Mozzarella	182 d	1.316	0.300	3.969	1.882	7.027	0.366	0.455	0.524	1.757	5.396	5.631	1.984
1	Swiss	1 d	1.343	0.632	1.283	0.580	17.087	0.498	0.897	2.607	2.702	19.353	0.785	0.356
2	Swiss	14 d	1.309	0.799	1.985	0.728	13.803	0.477	0.819	1.818	1.918	16.345	1.737	0.503
3	Swiss	35 d	1.987	1.043	3.967	1.219	9.842	0.396	0.467	0.684	2.051	10.601	4.906	1.143
4	Swiss	91 d	2.595	1.330	4.519	1.446	7.458	0.378	0.499	0.442	2.214	7.710	5.657	1.373
5	Swiss	120 d	2.888	1.615	4.626	1.585	6.583	0.466	0.597	0.460	2.534	6.562	6.378	1.744
6	Swiss	150 d	2.977	2.144	4.817	1.852	6.617	0.470	0.797	0.571	2.656	6.638	6.571	1.902
7	Swiss	182 d	3.075	1.940	4.919	2.069	6.458	0.574	0.939	0.611	2.948	5.979	6.601	2.132

7.8.1.1 PCA of the correlation matrix (PAGE/USF)

PCA: Peaks 1 - 12

Unrotated Factor Loadings (Factor-variable correlations) and Communalities

Peak	Factor 1	Factor 2	Factor 3	Communality
1	-0.792	0.380	-0.385	0.920
2	-0.448	0.662	-0.557	0.949
3	-0.972	0.047	-0.010	0.946
4	-0.920	-0.127	0.015	0.863
5	0.934	-0.180	-0.145	0.927
6	-0.375	-0.639	-0.430	0.734
7	0.483	-0.208	-0.566	0.596
8	0.841	-0.148	-0.370	0.867
9	-0.437	-0.565	-0.130	0.527
10	0.787	0.271	-0.104	0.704
11	-0.799	-0.107	-0.137	0.669
12	-0.799	-0.168	0.096	0.676
Variance	6.662	1.539	1.177	9.38
% Variance	55.52	12.83	9.81	78.15

Factor Score Coefficients

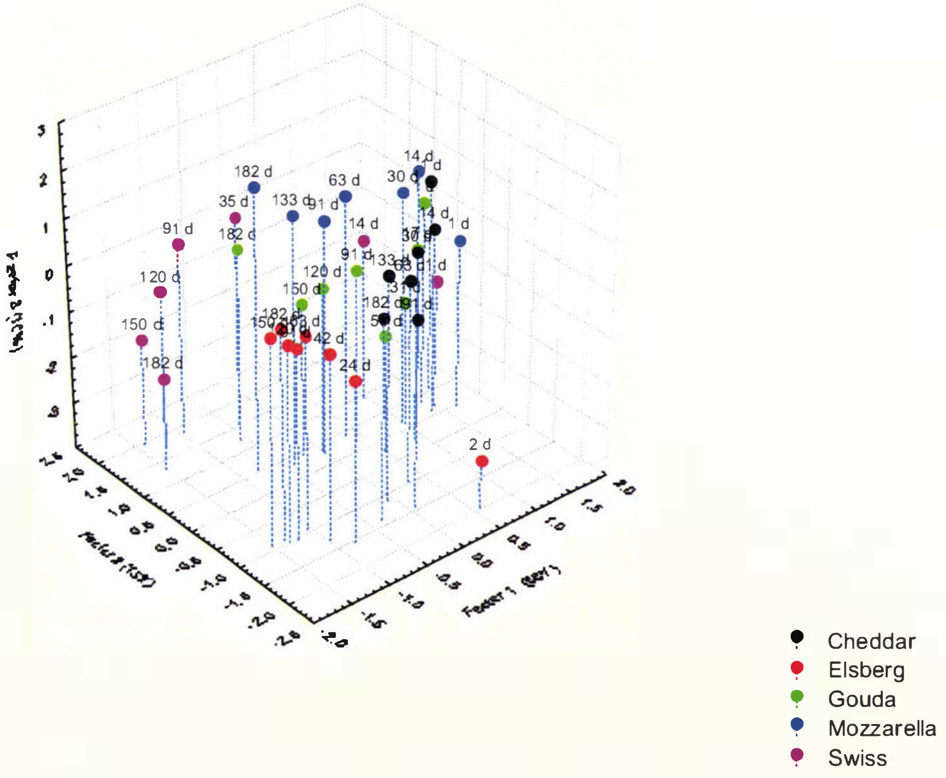
Peak	Factor 1	Factor 2	Factor 3
1	-0.119	0.247	-0.327
2	-0.067	0.430	-0.473
3	-0.146	0.030	-0.009
4	-0.138	-0.082	0.012
5	0.140	-0.117	-0.123
6	-0.056	-0.415	-0.365
7	0.072	-0.135	-0.480
8	0.126	-0.096	-0.315
9	-0.066	-0.367	-0.111
10	0.118	0.176	-0.088
11	-0.120	-0.070	-0.116
12	-0.120	-0.109	0.081

Factor Scores

	Cheese	Ripening time	Factor 1	Factor 2	Factor 3
24	Cheddar	1 d	1.634	0.548	0.608
25	Cheddar	14 d	1.369	0.112	0.048
26	Cheddar	30 d	0.878	-0.257	0.142
27	Cheddar	63 d	0.315	-0.926	0.377
28	Cheddar	91 d	0.076	-1.409	0.082
29	Cheddar	133 d	-0.063	-1.079	0.853
30	Cheddar	182 d	-0.328	-1.347	0.354
8	Elsberg	2 d	0.583	-1.962	-2.905
9	Elsberg	24 d	-0.786	-1.501	-0.501
10	Elsberg	42 d	-0.959	-1.267	0.020
11	Elsberg	63 d	-1.024	-0.898	0.168
12	Elsberg	91 d	-1.211	-0.999	0.147
13	Elsberg	120 d	-1.296	-0.967	0.260
14	Elsberg	150 d	-1.482	-0.895	0.475
15	Elsberg	182 d	-1.326	-0.891	0.522
16	Gouda	1 d	1.389	0.352	0.446
17	Gouda	17 d	1.082	0.020	-0.125
18	Gouda	31 d	0.991	0.120	-1.283

19	Gouda	50 d	0.503	-0.206	-1.442
20	Gouda	91 d	0.386	0.202	-0.206
21	Gouda	120 d	0.003	0.317	-0.389
22	Gouda	150 d	-0.212	0.414	-0.667
23	Gouda	182 d	-0.551	1.212	0.202
31	Mozzarella	1 d	1.642	-0.006	-0.290
32	Mozzarella	14 d	1.184	0.166	1.345
33	Mozzarella	30 d	0.971	0.161	1.062
34	Mozzarella	63 d	0.364	0.399	1.232
35	Mozzarella	91 d	-0.007	0.259	1.067
36	Mozzarella	133 d	-0.396	0.318	1.396
37	Mozzarella	182 d	-0.753	0.532	2.070
1	Swiss	1 d	1.459	0.181	-1.169
2	Swiss	14 d	0.974	0.937	-0.454
3	Swiss	35 d	-0.388	1.526	0.566
4	Swiss	91 d	-0.925	1.843	0.161
5	Swiss	120 d	-1.239	1.728	-0.570
6	Swiss	150 d	-1.374	1.944	-1.652
7	Swiss	182 d	-1.484	1.315	-1.949

PC1 (56%) v PC2 (13%) v PC3 (10%)



7.8.1.2 PCA of the covariance matrix (PAGE/USF)

PCA: Peaks 1 - 12

Unrotated Factor Loadings (Factor-variable correlations) and Communalities

Peak	Factor 1	Factor 2	Factor 3	Communality
1	-0.358	-0.473	0.002	0.352
2	-0.125	-0.263	-0.031	0.086
3	-1.282	-0.781	0.043	2.254
4	-0.411	-0.231	-0.025	0.223
5	3.220	1.437	0.537	12.723
6	-0.117	0.022	0.215	0.060
7	0.057	0.119	0.035	0.019
8	0.539	0.128	0.135	0.325
9	-0.246	0.164	-0.203	0.129
10	5.850	-1.197	0.110	35.665
11	-1.961	-0.500	1.255	5.672
12	-0.343	-0.187	-0.091	0.161
Variance	50.88	4.80	1.99	57.67
% Variance	86.43	8.15	3.39	97.96

Factor Score Coefficients

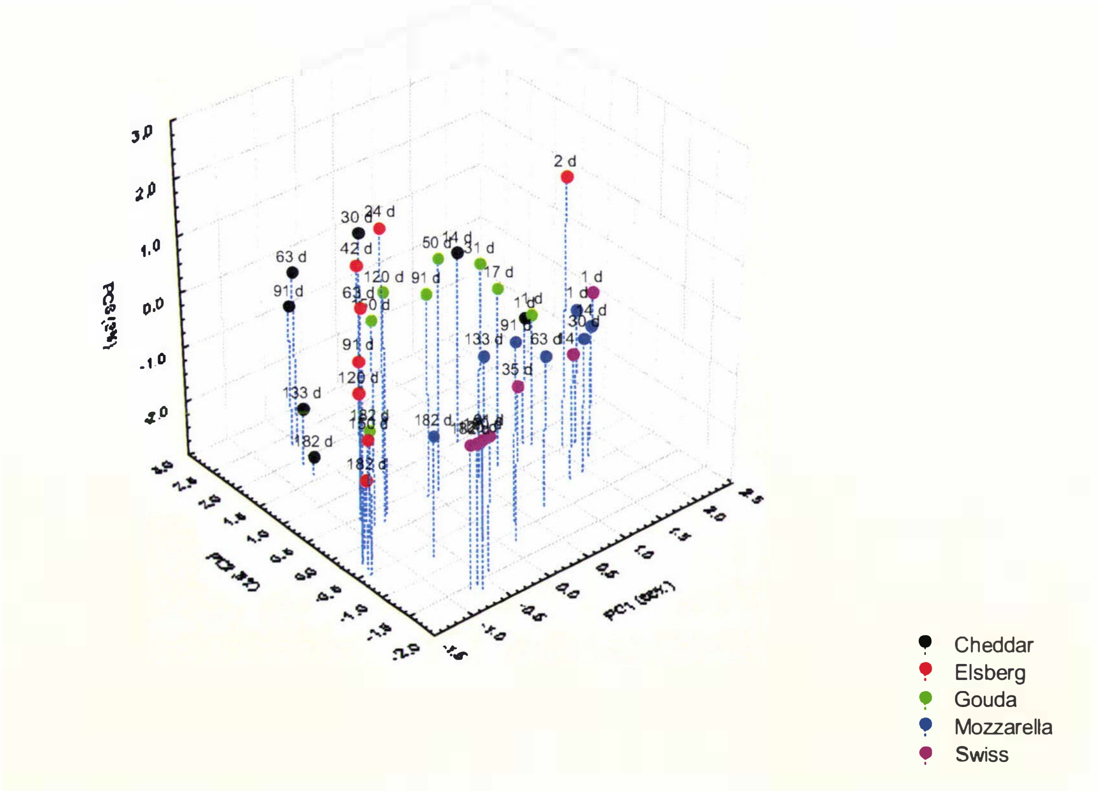
Peak	Factor 1	Factor 2	Factor 3
1	-0.007	-0.099	0.001
2	-0.002	-0.055	-0.015
3	-0.025	-0.163	0.022
4	-0.008	-0.048	-0.013
5	0.063	0.300	0.269
6	-0.002	0.005	0.108
7	0.001	0.025	0.018
8	0.011	0.027	0.068
9	-0.005	0.034	-0.102
10	0.115	-0.250	0.055
11	-0.039	-0.104	0.629
12	-0.007	-0.039	-0.046

Factor Scores

	Cheese	Ripening time	Factor 1	Factor 2	Factor 3
24	Cheddar	1 d	1.310	0.404	-0.669
25	Cheddar	14 d	0.807	0.986	0.464
26	Cheddar	30 d	0.033	1.769	0.842
27	Cheddar	63 d	-0.468	2.315	0.116
28	Cheddar	91 d	-0.531	2.299	-0.435
29	Cheddar	133 d	-0.669	1.853	-1.927
30	Cheddar	182 d	-0.702	1.585	-2.645
8	Elsberg	2 d	1.595	0.063	1.846
9	Elsberg	24 d	-0.662	0.225	2.211
10	Elsberg	42 d	-0.984	0.162	1.815
11	Elsberg	63 d	-1.101	-0.049	1.267
12	Elsberg	91 d	-1.222	-0.216	0.560
13	Elsberg	120 d	-1.269	-0.280	0.052
14	Elsberg	150 d	-1.320	-0.521	-0.555
15	Elsberg	182 d	-1.282	-0.413	-1.369
16	Gouda	1 d	1.358	0.327	-0.572
17	Gouda	17 d	0.848	0.243	0.252

18	Gouda	31 d	0.567	0.198	0.878
19	Gouda	50 d	0.101	0.315	1.175
20	Gouda	91 d	-0.056	0.305	0.673
21	Gouda	120 d	-0.559	0.338	0.985
22	Gouda	150 d	-0.777	0.253	0.681
23	Gouda	182 d	-1.122	-0.240	-0.712
31	Mozzarella	1 d	1.727	0.011	-0.515
32	Mozzarella	14 d	1.672	-0.331	-0.594
33	Mozzarella	30 d	1.424	-0.552	-0.502
34	Mozzarella	63 d	0.745	-0.812	-0.231
35	Mozzarella	91 d	0.351	-0.822	0.268
36	Mozzarella	133 d	-0.087	-0.847	0.313
37	Mozzarella	182 d	-0.661	-0.734	-0.795
1	Swiss	1 d	1.873	-0.056	-0.273
2	Swiss	14 d	1.258	-0.572	-0.697
3	Swiss	35 d	0.148	-1.141	-0.186
4	Swiss	91 d	-0.390	-1.398	-0.554
5	Swiss	120 d	-0.616	-1.517	-0.440
6	Swiss	150 d	-0.621	-1.622	-0.320
7	Swiss	182 d	-0.715	-1.528	-0.402

PC1 (86%) v PC2 (8%) v PC3 (3%)



7.8.2 Alkaline urea-PAGE of the WSF

Peak data for PCA

	Cheese	Ripening time	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
24	Cheddar	1 d	0.136	0.174	0.173	0.569	1.019	0.391	2.953	0	0.827	0.142	0	0.161	0.094	0.169	0
25	Cheddar	14 d	0.101	0.150	1.046	1.270	3.208	0.879	2.663	0	1.022	0.128	0	0.118	0.083	0.228	0
26	Cheddar	30 d	0.118	0.185	1.353	1.514	2.494	0.591	1.615	0	0.899	0.166	0	0.153	0.102	0.269	0
27	Cheddar	63 d	0.143	0.251	1.693	2.196	1.675	0.425	1.449	0	0.782	0.253	0	0.157	0.076	0.351	0
28	Cheddar	91 d	0.170	0.195	0.591	1.433	1.322	0.382	1.240	0	0.582	0.478	0	0.175	0.101	0.492	0
29	Cheddar	133 d	0.172	0.196	0.286	1.119	1.355	0.408	1.500	0	0.589	0.506	0	0.126	0.121	0.525	0
30	Cheddar	182 d	0.114	0.141	0.250	0.480	0.653	0.177	0.720	0	0.381	0.456	0	0.125	0.071	0.387	0
31	Elsberg	2 d	0.112	0.262	0.367	1.236	0.424	0.370	0.864	0	0.419	0.379	0.113	0	0	0.411	0
32	Elsberg	24 d	0.203	0.427	0.581	1.313	0.412	0.302	1.017	0	0.416	0.318	0.108	0	0	0.381	0
33	Elsberg	63 d	0.265	0.495	0.521	1.261	0.423	0.297	0.940	0	0.379	0.278	0.113	0	0	0.299	0
34	Elsberg	91 d	0.060	0.155	0.150	0.573	0.326	0.266	0.802	0	0.234	0.281	0.145	0	0	0.242	0
35	Elsberg	120 d	0.103	0.209	0.127	0.487	0.221	0.248	0.546	0	0.252	0.210	0.120	0	0	0.199	0
36	Elsberg	150 d	0.109	0.244	0.132	0.643	0.187	0.337	0.676	0	0.258	0.265	0.149	0	0	0.202	0
37	Elsberg	182 d	0.132	0.231	0.274	0.924	0.356	0.367	0.648	0	0.316	0.307	0.156	0	0	0.324	0
1	Gouda	1 d	0.026	0.037	0.240	0.702	0.653	0.439	0.444	0	0.292	0.197	0.093	0.196	0	0.154	0.035
2	Gouda	17 d	0.074	0.080	0.813	0.825	1.146	0.465	0.572	0	0.357	0.279	0.124	0.168	0	0.191	0.044
3	Gouda	31 d	0.062	0.114	0.738	0.901	1.077	0.454	0.668	0	0.423	0.351	0.108	0.237	0	0.279	0.071
4	Gouda	50 d	0.065	0.108	0.651	0.865	0.963	0.386	0.618	0	0.403	0.422	0.153	0.238	0	0.315	0.035
5	Gouda	63 d	0.055	0.100	0.366	0.744	0.774	0.395	0.481	0	0.334	0.429	0.159	0.250	0	0.298	0.057
6	Gouda	91 d	0.125	0.203	0.685	0.995	1.114	0.493	0.680	0	0.573	0.595	0.190	0.320	0	0.398	0.079
7	Gouda	120 d	0.099	0.157	0.422	0.852	1.016	0.478	0.571	0	0.464	0.597	0.202	0.267	0	0.429	0.070
8	Gouda	150 d	0.187	0.276	0.579	0.822	0.964	0.462	0.583	0	0.669	0.697	0.297	0.532	0	0.581	0.149
9	Gouda	182 d	0.141	0.269	0.354	0.658	0.806	0.374	0.506	0	0.655	0.500	0.262	0.479	0	0.454	0.100
17	Mozzarella	1 d	0.156	0.067	7.349	0.717	0	0.168	0.216	0.351	0.368	0.232	0.237	0.067	0.053	0.093	0
18	Mozzarella	14 d	0.165	0.062	4.383	0.800	0	0.272	0.366	0.351	0.665	0.622	0.499	0.098	0.070	0.166	0
19	Mozzarella	30 d	0.205	0.076	4.048	0.917	0	0.238	0.376	0.171	0.710	0.893	0.570	0.115	0.067	0.177	0
20	Mozzarella	63 d	0.193	0.102	1.910	0.936	0	0.278	0.414	0	1.085	1.599	0.992	0.184	0.103	0.283	0
21	Mozzarella	91 d	0.047	0.032	0.494	0.830	0	0.323	0.450	0	1.301	2.015	1.210	0.267	0.170	0.498	0
22	Mozzarella	133 d	0.256	0.135	1.285	1.050	0	0.386	0.532	0	1.470	2.341	1.558	0.323	0.182	0.684	0
23	Mozzarella	182 d	0.160	0.118	0.666	1.148	0	0.493	0.554	0	1.165	2.034	1.321	0.280	0.262	0.765	0
10	Swiss	1 d	0	0	0	0.608	0	0.238	0.159	0	0.270	0.237	0.247	0.132	0	0.060	0
11	Swiss	14 d	0	0	0	0.696	0	0.244	0.173	0	0.365	0.408	0.266	0.132	0	0.195	0
12	Swiss	35 d	0	0	0	0.815	0	0.311	0.203	0	0.340	0.578	0.350	0.164	0	0.225	0
13	Swiss	91 d	0	0	0	0.854	0	0.320	0.265	0	0.165	0.411	0.280	0.149	0	0.155	0
14	Swiss	120 d	0	0	0	0.734	0	0.293	0.191	0	0.184	0.386	0.259	0.147	0	0.118	0
15	Swiss	150 d	0	0	0	0.774	0	0.304	0.231	0	0.240	0.408	0.265	0.225	0	0.177	0
16	Swiss	182 d	0	0	0	0.930	0	0.337	0.255	0	0.228	0.382	0.224	0.144	0	0.186	0

7.8.2.1 PCA of the correlation matrix (PAGE/WSF)

PCA: Peaks 1, 2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15

Unrotated Factor Loadings (Factor-variable correlations) and Communalities

Peak	Factor 1	Factor 2	Factor 3	Communality
1	-0.622	0.137	0.368	0.541
2	-0.281	0.551	0.202	0.424
4	-0.479	0.476	0.308	0.551
5	-0.279	0.872	-0.112	0.850
6	-0.453	0.644	-0.283	0.700
7	-0.334	0.751	0.278	0.752
8	0.131	-0.308	0.386	0.261
9	-0.926	-0.109	0.093	0.878
10	-0.743	-0.641	-0.045	0.966
11	-0.621	-0.743	0.002	0.937
12	-0.460	-0.119	-0.809	0.881
13	-0.819	-0.238	0.314	0.827
14	-0.827	-0.030	-0.206	0.727
15	-0.099	0.191	-0.856	0.779
Variance	4.48	3.47	2.13	10.07
% Variance	31.99	24.75	15.21	71.95

Factor Score Coefficients

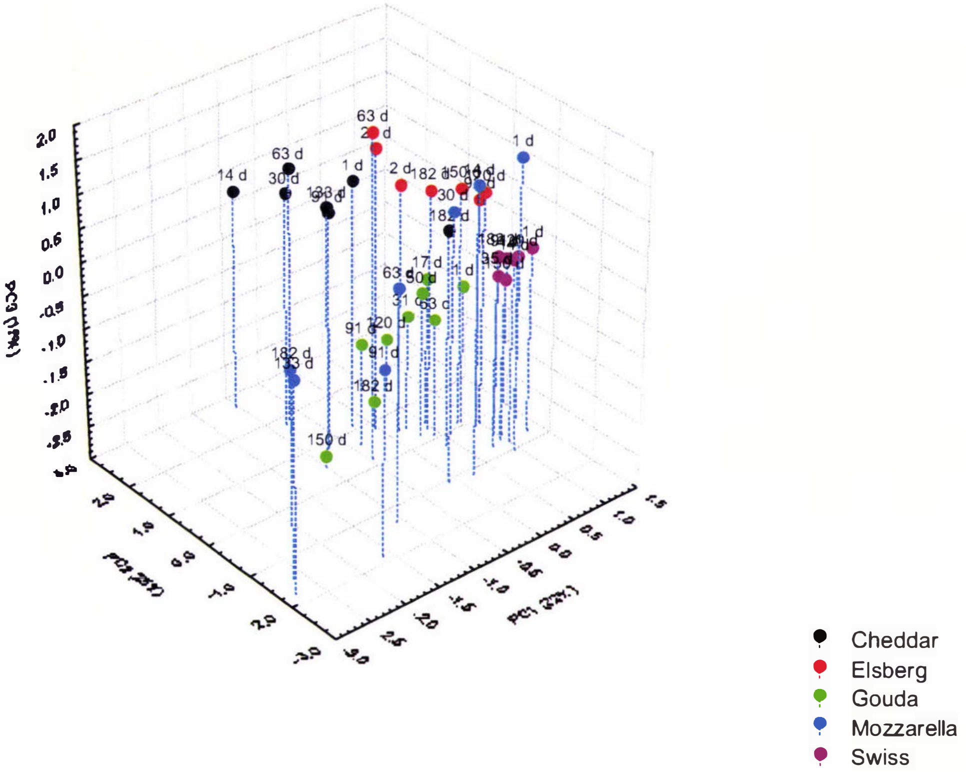
Peak	Factor 1	Factor 2	Factor 3
1	-0.139	0.040	0.173
2	-0.063	0.159	0.095
4	-0.107	0.137	0.145
5	-0.062	0.252	-0.052
6	-0.101	0.186	-0.133
7	-0.075	0.217	0.131
8	0.029	-0.089	0.181
9	-0.207	-0.031	0.044
10	-0.166	-0.185	-0.021
11	-0.139	-0.214	0.001
12	-0.103	-0.034	-0.380
13	-0.183	-0.069	0.148
14	-0.185	-0.009	-0.097
15	-0.022	0.055	-0.402

Factor Scores

	Cheese	Ripening time	Factor 1	Factor 2	Factor 3
24	Cheddar	1 d	-0.202	1.093	0.766
25	Cheddar	14 d	-1.001	2.689	0.342
26	Cheddar	30 d	-0.783	1.788	0.543
27	Cheddar	63 d	-0.839	1.596	1.007
28	Cheddar	91 d	-0.715	0.868	0.599
29	Cheddar	133 d	-0.739	0.862	0.671
30	Cheddar	182 d	0.385	-0.255	0.284
31	Elsberg	2 d	0.165	0.539	0.767
32	Elsberg	24 d	-0.025	0.821	1.269
33	Elsberg	63 d	-0.021	0.911	1.478
34	Elsberg	91 d	0.954	-0.071	0.456

35	Elsberg	120 d	0.997	-0.122	0.565
36	Elsberg	150 d	0.798	0.120	0.610
37	Elsberg	182 d	0.447	0.291	0.658
1	Gouda	1 d	0.842	0.101	-0.875
2	Gouda	17 d	0.513	0.452	-0.752
3	Gouda	31 d	0.263	0.515	-1.241
4	Gouda	50 d	0.297	0.231	-0.806
5	Gouda	63 d	0.418	0.099	-1.198
6	Gouda	91 d	-0.375	0.619	-1.431
7	Gouda	120 d	-0.143	0.392	-1.351
8	Gouda	150 d	-0.982	0.486	-2.824
9	Gouda	182 d	-0.448	0.210	-2.118
17	Mozzarella	1 d	0.934	-1.024	1.449
18	Mozzarella	14 d	0.233	-1.131	1.354
19	Mozzarella	30 d	-0.079	-1.038	1.085
20	Mozzarella	63 d	-1.057	-1.329	0.517
21	Mozzarella	91 d	-1.602	-1.864	-0.177
22	Mozzarella	133 d	-2.801	-1.766	0.193
23	Mozzarella	182 d	-2.642	-1.444	0.129
10	Swiss	1 d	1.314	-0.749	-0.166
11	Swiss	14 d	1.008	-0.784	-0.205
12	Swiss	35 d	0.786	-0.741	-0.343
13	Swiss	91 d	1.036	-0.570	-0.253
14	Swiss	120 d	1.153	-0.661	-0.258
15	Swiss	150 d	0.950	-0.656	-0.512
16	Swiss	182 d	0.960	-0.481	-0.232

PC1 (32%) v PC2 (25%) v PC3 (15%)



7.8.2.2 PCA of the covariance matrix (PAGE/WSF)

PCA: Peaks 1, 2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15

Unrotated Factor Loadings (Factor-variable correlations) and Communalities

Peak	Factor 1	Factor 2	Factor 3	Communality
1	-0.144	-0.491	0.149	0.284
2	-0.392	-0.074	0.111	0.172
4	-0.470	-0.372	-0.314	0.458
5	-0.950	-0.145	-0.250	0.986
6	-0.712	-0.373	-0.285	0.728
7	-0.868	-0.251	0.427	0.998
8	0.216	0.099	0.074	0.062
9	-0.103	-0.967	0.053	0.948
10	0.516	-0.849	-0.035	0.989
11	0.624	-0.763	0.011	0.971
12	0.061	-0.411	-0.411	0.342
13	0.002	-0.863	0.166	0.773
14	0.011	-0.716	-0.185	0.546
15	-0.096	0.038	-0.456	0.218
Variance	0.955	0.467	0.122	1.545
% Variance	56.95	27.87	7.28	92.11

Factor Score Coefficients

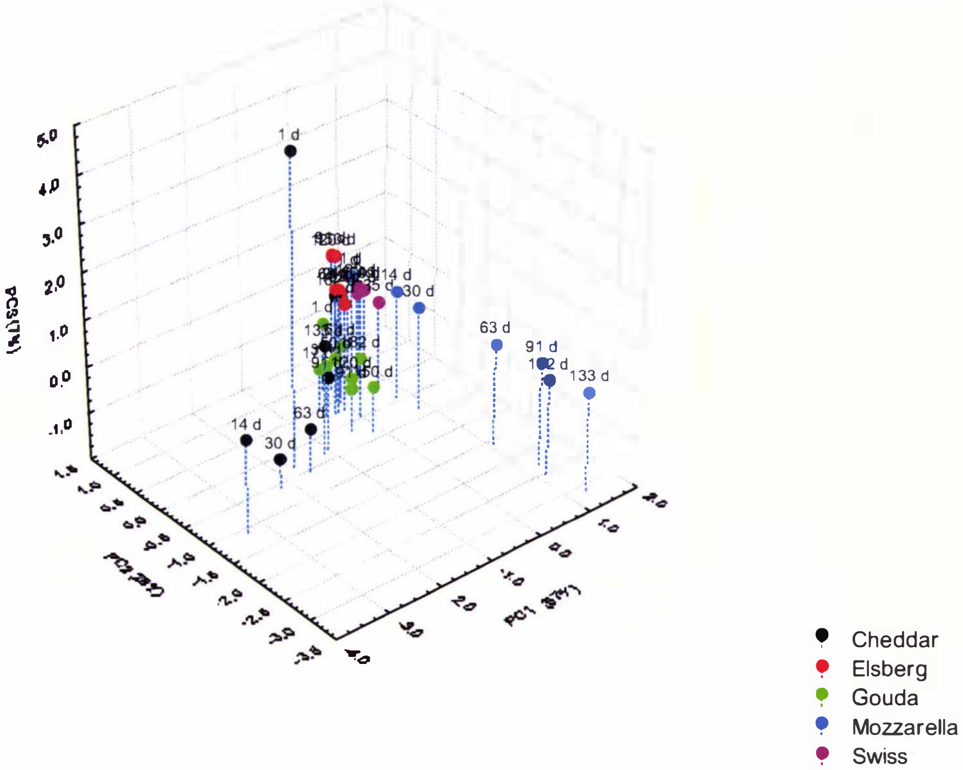
Peak	Factor 1	Factor 2	Factor 3
1	-0.011	-0.078	0.091
2	-0.048	-0.018	0.106
4	-0.164	-0.266	-0.858
5	-0.736	-0.229	-1.514
6	-0.095	-0.102	-0.299
7	-0.566	-0.334	2.179
8	0.019	0.018	0.051
9	-0.035	-0.681	0.142
10	0.292	-0.980	-0.155
11	0.245	-0.612	0.034
12	0.008	-0.109	-0.418
13	0.000	-0.119	0.088
14	0.002	-0.250	-0.248
15	-0.004	0.003	-0.132

Factor Scores

	Cheese	Ripening time	Factor 1	Factor 2	Factor 3
24	Cheddar	1 d	-1.707	-0.321	4.657
25	Cheddar	14 d	-3.325	-1.087	-0.011
26	Cheddar	30 d	-2.205	-0.581	-1.375
27	Cheddar	63 d	-1.578	-0.529	-1.079
28	Cheddar	91 d	-0.996	-0.296	-0.437
29	Cheddar	133 d	-1.110	-0.347	0.351
30	Cheddar	182 d	-0.030	0.504	0.328
31	Elsberg	2 d	-0.088	0.282	0.356
32	Elsberg	24 d	-0.200	0.283	0.704
33	Elsberg	63 d	-0.168	0.396	0.599
34	Elsberg	91 d	0.130	0.762	0.988
35	Elsberg	120 d	0.337	0.976	0.700
36	Elsberg	150 d	0.275	0.813	0.872

37	Elsberg	182 d	0.130	0.588	0.276
1	Gouda	1 d	0.022	0.836	-0.513
2	Gouda	17 d	-0.410	0.491	-1.086
3	Gouda	31 d	-0.411	0.321	-0.888
4	Gouda	50 d	-0.253	0.287	-0.789
5	Gouda	63 d	-0.011	0.447	-0.720
6	Gouda	91 d	-0.383	-0.159	-1.073
7	Gouda	120 d	-0.214	0.007	-1.043
8	Gouda	150 d	-0.135	-0.348	-1.030
9	Gouda	182 d	-0.006	0.032	-0.708
17	Mozzarella	1 d	0.700	0.929	0.169
18	Mozzarella	14 d	0.759	0.077	0.356
19	Mozzarella	30 d	0.828	-0.304	0.239
20	Mozzarella	63 d	1.092	-1.572	0.195
21	Mozzarella	91 d	1.259	-2.307	0.221
22	Mozzarella	133 d	1.338	-3.119	0.140
23	Mozzarella	182 d	1.163	-2.527	0.063
10	Swiss	1 d	0.750	1.041	0.040
11	Swiss	14 d	0.778	0.734	-0.051
12	Swiss	35 d	0.807	0.475	-0.157
13	Swiss	91 d	0.705	0.786	-0.036
14	Swiss	120 d	0.755	0.881	-0.072
15	Swiss	150 d	0.731	0.768	-0.062
16	Swiss	182 d	0.671	0.781	-0.122

PC1 (57%) v PC2 (28%) v PC3 (7%)



7.8.3 SE-HPLC of the WSF

Peak data for PCA

	Cheese	Ripening time	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	Cheddar	1 d	230.67	1640.33	305.87	247.56	520.38	240.29	264.72	311.77	411.45	440.04	398.31	109.65	663.45	614.39
2	Cheddar	14 d	208.56	2659.94	365.35	260.04	791.90	557.17	508.83	426.81	531.94	652.36	607.93	222.92	898.68	826.26
3	Cheddar	30 d	200.38	2615.13	422.25	409.52	1084.81	1055.42	699.66	517.58	673.12	916.53	896.85	222.75	1203.19	717.29
4	Cheddar	63 d	222.55	2412.29	612.84	589.16	1800.52	1659.07	982.31	799.61	955.24	1394.16	1237.35	510.92	1730.11	932.84
5	Cheddar	91 d	256.18	2356.94	774.33	808.43	2096.44	1854.73	1238.59	878.87	1006.60	1863.76	1365.25	696.15	2116.80	824.20
6	Cheddar	133 d	239.63	1482.37	862.03	734.16	2201.47	2110.69	1185.35	828.10	1368.03	1839.52	1730.23	701.62	2688.09	955.91
7	Cheddar	182 d	335.22	1116.09	867.08	625.27	2684.61	2315.38	1267.94	1015.43	1516.81	2466.16	1771.98	711.32	3718.14	1056.39
8	Elsberg	2 d	240.93	916.38	441.79	373.50	429.50	418.44	259.14	210.06	302.40	315.05	141.08	117.02	570.65	302.69
9	Elsberg	24 d	490.19	1593.33	1257.15	1219.11	1908.32	1763.37	1022.56	499.06	729.00	847.70	378.85	252.45	2492.22	993.23
10	Elsberg	42 d	598.76	2387.62	1487.64	1313.50	2419.37	1923.44	1591.64	505.52	832.32	1123.97	469.81	352.02	3157.14	1140.25
11	Elsberg	63 d	591.08	1494.53	1182.84	1179.18	2444.38	1917.75	1645.26	491.66	850.67	1190.97	557.34	359.63	3458.18	1255.84
12	Elsberg	91 d	623.95	2003.19	1419.37	1399.31	2446.84	2038.34	1721.67	496.65	852.51	1266.53	620.07	303.86	3637.06	1387.82
13	Elsberg	120 d	727.78	1686.84	1530.77	1177.93	2919.00	2324.00	1933.38	469.65	975.40	1302.57	725.88	368.87	4510.08	1459.17
14	Elsberg	150 d	864.76	1645.86	1652.95	1023.75	3240.82	2322.10	1886.00	509.03	860.30	1274.68	766.59	359.35	5002.12	1659.01
15	Elsberg	182 d	894.27	1686.65	1609.31	1065.56	3469.10	2744.48	2149.27	495.18	948.54	1494.72	858.36	433.71	5481.40	1873.46
16	Gouda	1 d	241.32	454.15	291.69	187.77	326.42	208.51	191.42	125.58	264.93	257.28	122.50	115.98	755.29	458.44
17	Gouda	17 d	265.11	760.92	551.58	304.60	629.60	481.35	464.30	240.55	470.30	496.50	307.31	217.62	1011.44	650.42
18	Gouda	31 d	280.61	928.74	739.36	463.20	833.30	675.59	609.13	352.38	525.40	625.65	354.93	288.85	1219.41	788.90
19	Gouda	50 d	334.43	1580.07	1035.20	872.70	1236.54	976.78	782.53	424.44	632.76	723.47	429.57	307.90	1368.16	658.29
20	Gouda	63 d	306.01	1003.49	903.12	705.20	1056.20	910.02	818.56	405.71	698.24	695.34	488.90	283.82	1460.25	732.59
21	Gouda	91 d	414.19	1242.90	1196.24	1048.33	1569.20	1340.57	1038.96	492.55	917.40	901.82	640.00	319.57	1924.07	747.75
22	Gouda	120 d	391.18	1266.73	1361.38	1135.74	1843.73	1384.77	1251.08	599.62	1091.89	972.57	614.82	463.23	2195.82	798.64
23	Gouda	150 d	688.67	1138.59	1343.07	757.60	2423.00	1573.48	1313.64	653.52	1185.68	1009.31	726.14	359.95	2582.50	1019.72
24	Gouda	182 d	546.00	1138.45	1426.20	1144.13	2387.38	1704.08	1512.66	625.92	1386.21	1094.16	907.06	350.86	2640.71	841.51
25	Mozzarella	1 d	104.33	3697.22	707.18	506.15	292.51	168.13	139.49	120.77	124.94	109.96	85.21	74.21	605.77	1663.87
26	Mozzarella	14 d	33.55	2195.34	1367.35	711.42	437.28	177.47	191.78	152.01	118.47	251.08	104.24	65.17	563.25	1531.94
27	Mozzarella	30 d	38.27	2279.59	1888.73	1130.77	700.90	205.25	321.68	178.33	248.13	301.45	185.06	121.22	715.88	1949.76
28	Mozzarella	63 d	33.35	1499.97	2903.21	2251.21	1363.53	369.12	626.85	239.23	419.46	617.17	289.47	124.72	612.74	1367.11
29	Mozzarella	91 d	22.54	639.58	3239.34	2808.69	1667.77	546.61	913.91	294.90	493.60	1075.25	475.61	204.61	759.40	1654.68
30	Mozzarella	133 d	32.06	1669.12	3161.75	3716.71	3073.55	695.67	1020.09	425.54	717.63	921.57	593.19	173.04	741.69	1216.83
31	Mozzarella	182 d	62.67	1427.61	4152.00	3732.20	4196.74	1568.97	1174.59	523.14	1507.88	2166.27	1008.01	447.99	953.01	1119.99
32	Swiss	1 d	224.95	208.02	189.86	309.39	309.06	84.86	56.99	101.23	277.33	251.41	86.56	87.39	483.84	513.07
33	Swiss	14 d	381.82	299.53	791.81	399.80	1260.41	265.47	193.52	257.52	662.34	666.20	320.08	209.43	657.45	671.77
34	Swiss	35 d	219.93	321.98	1145.78	895.19	2321.51	458.21	582.41	378.92	1334.50	1237.08	716.41	309.18	1207.01	639.91
35	Swiss	91 d	328.47	531.37	1586.61	689.58	3786.45	840.99	1081.74	789.41	1467.70	1737.51	510.50	311.80	1893.46	780.20
36	Swiss	120 d	371.41	474.99	1789.96	686.74	3946.76	943.31	1215.38	887.84	1794.88	1719.66	343.22	911.34	2181.52	779.41
37	Swiss	150 d	365.21	467.19	1798.85	609.02	4215.62	1128.80	1154.42	710.31	2164.38	1861.47	590.79	419.86	2446.93	602.21
38	Swiss	182 d	440.61	521.52	1571.13	814.49	4377.03	1139.69	1510.27	817.14	1980.30	2067.39	695.12	375.38	2704.30	672.60

7.8.3.1 PCA of the correlation matrix (SE-HPLC/WSF)

PCA: Peaks 1 - 14

Unrotated Factor Loadings (Factor-variable correlations) and Communalities

Peak	Factor 1	Factor 2	Factor 3	Communality
1	-0.590	-0.185	0.591	0.731
2	0.073	0.317	0.567	0.427
3	-0.314	0.837	-0.406	0.965
4	-0.221	0.860	-0.336	0.901
5	-0.870	0.182	-0.275	0.866
6	-0.876	0.031	0.402	0.931
7	-0.904	0.182	0.279	0.928
8	-0.865	-0.284	-0.195	0.868
9	-0.825	-0.202	-0.424	0.901
10	-0.915	-0.041	-0.306	0.933
11	-0.741	-0.148	-0.044	0.573
12	-0.817	-0.267	-0.165	0.766
13	-0.815	-0.019	0.517	0.931
14	-0.162	0.760	0.462	0.817
Variance	7.01	2.44	2.09	11.54
% Variance	50.10	17.41	14.89	82.40

Factor Score Coefficients

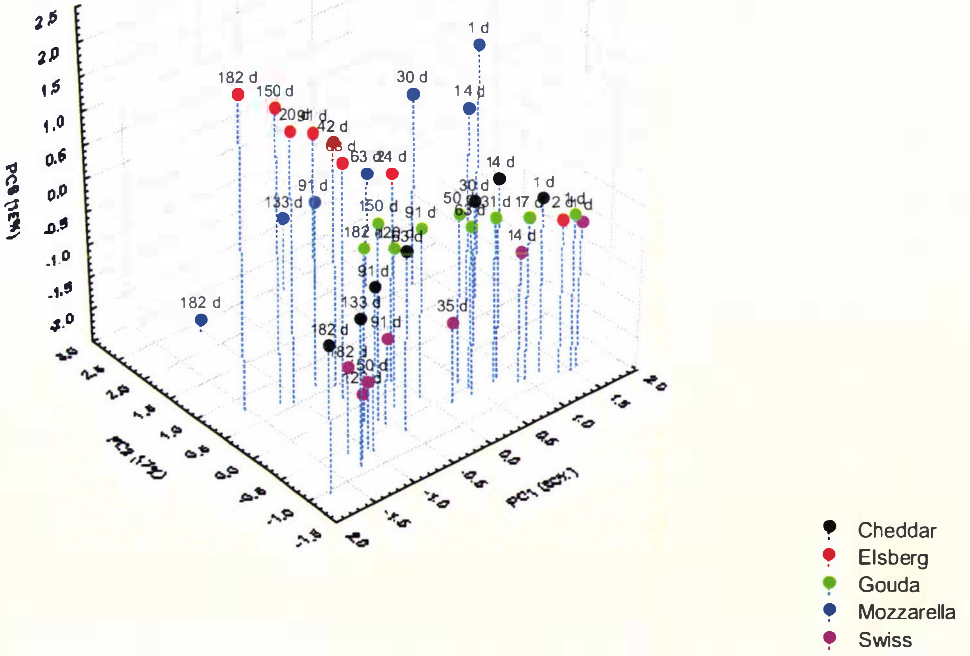
Peak	Factor 1	Factor 2	Factor 3
1	-0.084	-0.076	0.283
2	0.010	0.130	0.272
3	-0.045	0.344	-0.195
4	-0.032	0.353	-0.161
5	-0.124	0.075	-0.132
6	-0.125	0.013	0.193
7	-0.129	0.075	0.134
8	-0.123	-0.117	-0.094
9	-0.118	-0.083	-0.203
10	-0.130	-0.017	-0.147
11	-0.106	-0.061	-0.021
12	-0.116	-0.109	-0.079
13	-0.116	-0.008	0.248
14	-0.023	0.312	0.222

Factor scores

	Cheese	Ripening time	Factor 1	Factor 2	Factor 3
1	Cheddar	1 d	1.223	-0.798	0.142
2	Cheddar	14 d	0.816	-0.559	0.513
3	Cheddar	30 d	0.421	-0.628	0.413
4	Cheddar	63 d	-0.481	-0.678	0.175
5	Cheddar	91 d	-0.961	-0.754	-0.037
6	Cheddar	133 d	-1.216	-0.881	-0.284
7	Cheddar	182 d	-1.735	-1.048	-0.295
31	Elsberg	2 d	1.373	-0.946	-0.155
32	Elsberg	24 d	-0.049	0.151	0.656
33	Elsberg	42 d	-0.542	0.496	1.163
34	Elsberg	63 d	-0.616	0.253	1.043
35	Elsberg	91 d	-0.705	0.641	1.314
36	Elsberg	120 d	-1.059	0.555	1.548
37	Elsberg	150 d	-1.179	0.646	1.900
38	Elsberg	182 d	-1.527	0.789	2.185
15	Gouda	1 d	1.487	-1.010	-0.119

16	Gouda	17 d	1.024	-0.794	-0.049
17	Gouda	31 d	0.738	-0.606	0.001
18	Gouda	50 d	0.445	-0.350	0.071
19	Gouda	63 d	0.470	-0.506	-0.037
20	Gouda	91 d	-0.012	-0.292	0.055
21	Gouda	120 d	-0.318	-0.255	-0.096
22	Gouda	150 d	-0.589	-0.333	0.471
23	Gouda	182 d	-0.706	-0.240	0.103
8	Mozzarella	1 d	1.617	0.829	1.380
9	Mozzarella	14 d	1.520	0.856	0.467
10	Mozzarella	30 d	1.269	1.523	0.464
11	Mozzarella	63 d	0.873	1.835	-0.720
12	Mozzarella	91 d	0.429	2.218	-1.156
13	Mozzarella	133 d	0.146	2.415	-1.373
14	Mozzarella	182 d	-1.006	2.364	-2.312
24	Swiss	1 d	1.603	-0.981	-0.288
25	Swiss	14 d	0.934	-0.798	-0.499
26	Swiss	35 d	0.175	-0.597	-1.274
27	Swiss	91 d	-0.573	-0.453	-1.190
28	Swiss	120 d	-1.131	-0.788	-1.510
29	Swiss	150 d	-0.975	-0.675	-1.473
30	Swiss	182 d	-1.183	-0.603	-1.197

PC1 (50%) v PC2 (17%) v PC3 (15%)



7.8.3.2 PCA of the correlation matrix – without Mozzarella cheese data (SE-HPLC/WSF)

PCA: Peaks 1 - 14

Unrotated Factor Loadings (Factor-variable correlations) and Communalities

Peak	Factor 1	Factor 2	Factor 3	Communality
1	-0.686	0.631	0.256	0.934
2	-0.256	0.350	-0.772	0.785
3	-0.813	0.045	0.528	0.942
4	-0.739	0.398	0.128	0.722
5	-0.857	-0.244	0.384	0.942
6	-0.897	0.263	-0.295	0.960
7	-0.950	0.259	0.027	0.971
8	-0.750	-0.582	-0.199	0.941
9	-0.689	-0.607	0.327	0.950
10	-0.826	-0.510	-0.055	0.947
11	-0.596	-0.369	-0.625	0.881
12	-0.694	-0.517	-0.202	0.790
13	-0.905	0.347	0.005	0.940
14	-0.757	0.549	-0.153	0.897
Variance	8.14	2.67	1.80	12.60
% Variance	58.11	19.07	12.84	90.01

Factor Score Coefficients

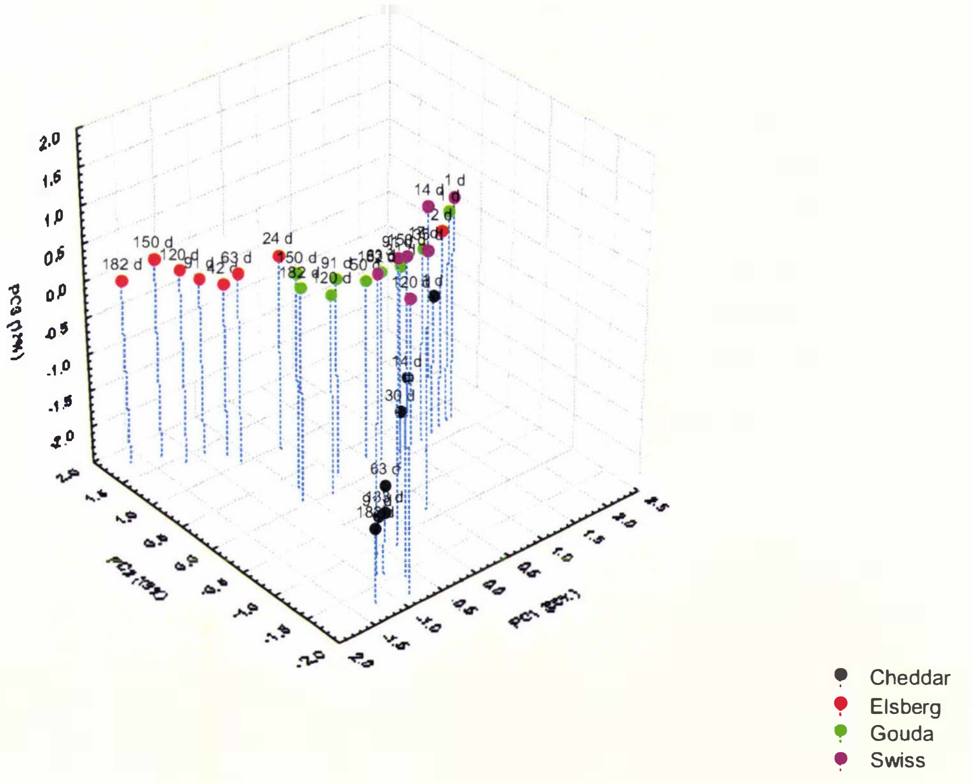
Peak	Factor 1	Factor 2	Factor 3
1	-0.084	0.236	0.143
2	-0.031	0.131	-0.430
3	-0.100	0.017	0.294
4	-0.091	0.149	0.071
5	-0.105	-0.092	0.214
6	-0.110	0.099	-0.164
7	-0.117	0.097	0.015
8	-0.092	-0.218	-0.110
9	-0.085	-0.227	0.182
10	-0.102	-0.191	-0.031
11	-0.073	-0.138	-0.348
12	-0.085	-0.194	-0.113
13	-0.111	0.130	0.003
14	-0.093	0.205	-0.085

Factor Scores

	Cheese	Ripening time	Factor 1	Factor 2	Factor 3
1	Cheddar	1 d	1.452	0.179	-0.588
2	Cheddar	14 d	1.010	0.133	-1.506
3	Cheddar	30 d	0.669	-0.115	-1.695
4	Cheddar	63 d	-0.169	-0.759	-1.969
5	Cheddar	91 d	-0.615	-1.099	-1.988
6	Cheddar	133 d	-0.794	-1.398	-1.663
7	Cheddar	182 d	-1.222	-1.713	-1.480
24	Elsberg	2 d	1.618	0.222	0.208
25	Elsberg	24 d	-0.130	0.955	0.185
26	Elsberg	42 d	-0.698	1.230	-0.130
27	Elsberg	63 d	-0.656	1.040	0.097
28	Elsberg	91 d	-0.884	1.404	-0.078
29	Elsberg	120 d	-1.167	1.399	0.149
30	Elsberg	150 d	-1.316	1.651	0.235
31	Elsberg	182 d	-1.668	1.758	0.024

8	Gouda	1 d	1.768	0.257	0.390
9	Gouda	17 d	1.261	0.147	0.151
10	Gouda	31 d	0.917	0.143	0.049
11	Gouda	50 d	0.518	0.291	-0.069
12	Gouda	63 d	0.618	0.142	0.105
13	Gouda	91 d	0.061	0.240	0.168
14	Gouda	120 d	-0.245	-0.007	0.234
15	Gouda	150 d	-0.467	0.286	0.424
16	Gouda	182 d	-0.606	0.088	0.399
17	Swiss	1 d	1.859	0.268	0.559
18	Swiss	14 d	1.144	-0.054	0.872
19	Swiss	35 d	0.385	-0.834	0.991
20	Swiss	91 d	-0.335	-1.114	1.330
21	Swiss	120 d	-0.807	-1.813	1.389
22	Swiss	150 d	-0.641	-1.574	1.727
23	Swiss	182 d	-0.859	-1.350	1.481

PC1 (58%) v PC2 (19%) v PC3 (13%)



7.8.3.3 PCA of the covariance matrix (SE-HPLC/WSF)

PCA: Peaks 1 - 14

Unrotated Factor Loadings (Factor-variable correlations) and Communalities

Peak	Factor 1	Factor 2	Factor 3	Communality
1	-0.659	-0.546	0.002	0.733
2	0.070	-0.211	0.837	0.749
3	-0.431	0.850	0.234	0.963
4	-0.326	0.822	0.384	0.930
5	-0.904	0.282	-0.236	0.953
6	-0.867	-0.332	0.228	0.914
7	-0.950	-0.124	0.153	0.941
8	-0.724	-0.121	-0.288	0.621
9	-0.753	0.121	-0.524	0.857
10	-0.839	0.110	-0.269	0.789
11	-0.594	-0.162	0.005	0.379
12	-0.672	-0.135	-0.253	0.533
13	-0.865	-0.443	0.130	0.961
14	-0.305	0.134	0.758	0.686
Variance	4000489	1598624	934670	6533783
% Variance	54.85	21.92	12.81	89.58

Factor Score Coefficients

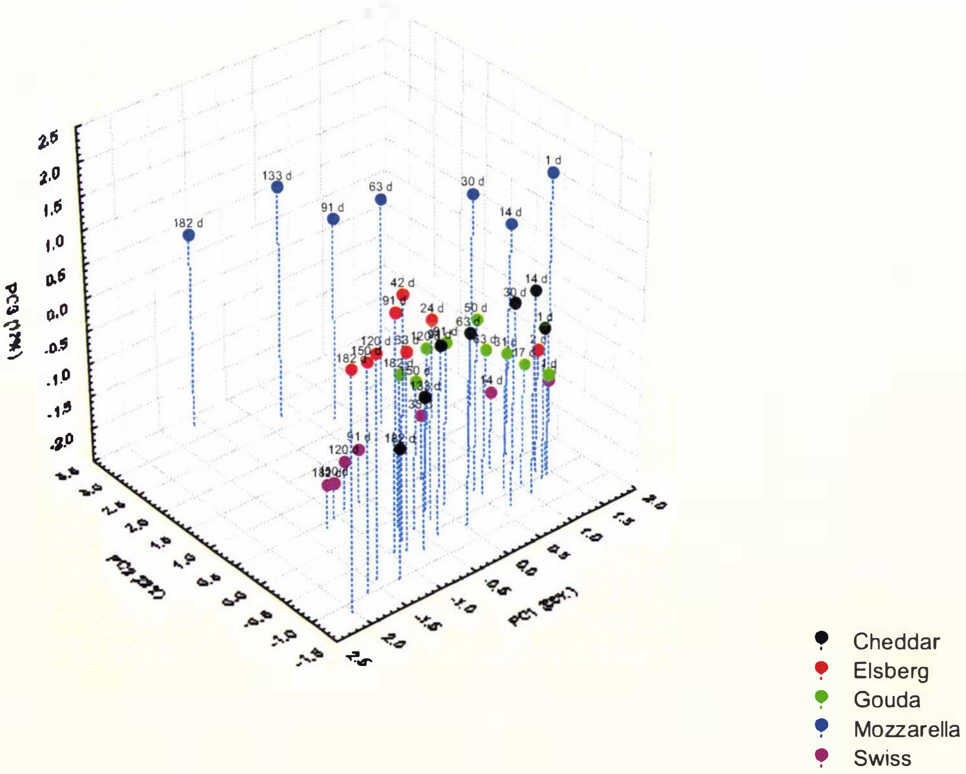
Peak	Factor 1	Factor 2	Factor 3
1	-0.000037	-0.000077	0.000001
2	0.000014	-0.000104	0.000710
3	-0.000093	0.000461	0.000216
4	-0.000068	0.000427	0.000341
5	-0.000276	0.000216	-0.000309
6	-0.000164	-0.000157	0.000185
7	-0.000132	-0.000043	0.000091
8	-0.000043	-0.000018	-0.000073
9	-0.000096	0.000038	-0.000285
10	-0.000126	0.000041	-0.000172
11	-0.000060	-0.000041	0.000002
12	-0.000032	-0.000016	-0.000052
13	-0.000286	-0.000366	0.000183
14	-0.000031	0.000035	0.000335

Factor Scores

	Cheese	Ripening time	Factor 1	Factor 2	Factor 3
1	Cheddar	1 d	1.312	-0.528	-0.191
2	Cheddar	14 d	1.028	-0.679	0.576
3	Cheddar	30 d	0.672	-0.720	0.555
4	Cheddar	63 d	0.018	-0.667	0.401
5	Cheddar	91 d	-0.350	-0.608	0.363
6	Cheddar	133 d	-0.648	-0.728	-0.200
7	Cheddar	182 d	-1.229	-1.020	-0.541
31	Elsberg	2 d	1.359	-0.358	-0.634
32	Elsberg	24 d	-0.215	-0.295	0.516
33	Elsberg	42 d	-0.727	-0.407	1.174
34	Elsberg	63 d	-0.820	-0.613	0.503
35	Elsberg	91 d	-0.949	-0.547	1.085
36	Elsberg	120 d	-1.433	-0.834	0.877
37	Elsberg	150 d	-1.658	-0.959	0.910

38	Elsberg	182 d	-2.012	-1.157	1.070
15	Gouda	1 d	1.409	-0.508	-0.959
16	Gouda	17 d	1.067	-0.442	-0.665
17	Gouda	31 d	0.838	-0.368	-0.430
18	Gouda	50 d	0.536	-0.153	0.113
19	Gouda	63 d	0.576	-0.284	-0.304
20	Gouda	91 d	0.089	-0.163	-0.028
21	Gouda	120 d	-0.155	-0.099	-0.014
22	Gouda	150 d	-0.477	-0.318	-0.262
23	Gouda	182 d	-0.594	-0.164	-0.177
8	Mozzarella	1 d	1.462	-0.420	1.984
9	Mozzarella	14 d	1.317	0.177	1.016
10	Mozzarella	30 d	1.063	0.595	1.385
11	Mozzarella	63 d	0.614	1.759	0.965
12	Mozzarella	91 d	0.250	2.236	0.598
13	Mozzarella	133 d	-0.212	2.742	1.030
14	Mozzarella	182 d	-1.112	3.304	0.453
24	Swiss	1 d	1.530	-0.350	-1.175
25	Swiss	14 d	0.984	0.074	-1.310
26	Swiss	35 d	0.223	0.468	-1.518
27	Swiss	91 d	-0.621	0.567	-1.662
28	Swiss	120 d	-0.846	0.577	-1.753
29	Swiss	150 d	-1.053	0.498	-1.939
30	Swiss	182 d	-1.234	0.392	-1.813

PC1 (55%) v PC2 (22%) v PC3 (13%)



7.8.3.4 PCA of the covariance matrix - without Mozzarella cheese data (SE-HPLC/WSF)

PCA: Peaks 1 – 14

Unrotated Factor Loadings (Factor-variable correlations) and Communalities

Peak	Factor 1	Factor 2	Factor 3	Communality
1	-0.757	0.253	0.523	0.911
2	-0.210	0.782	-0.465	0.872
3	-0.832	-0.336	0.222	0.854
4	-0.729	0.208	0.193	0.611
5	-0.873	-0.461	-0.058	0.978
6	-0.883	0.409	-0.090	0.955
7	-0.962	0.168	0.061	0.956
8	-0.662	-0.257	-0.636	0.909
9	-0.655	-0.653	-0.310	0.952
10	-0.785	-0.324	-0.464	0.937
11	-0.509	0.189	-0.671	0.745
12	-0.601	-0.185	-0.479	0.625
13	-0.946	0.242	0.186	0.988
14	-0.796	0.474	0.216	0.906
Variance	4065159	1023560	411865	5500583
% Variance	69.54	17.51	7.05	94.10

Factor Score Coefficients

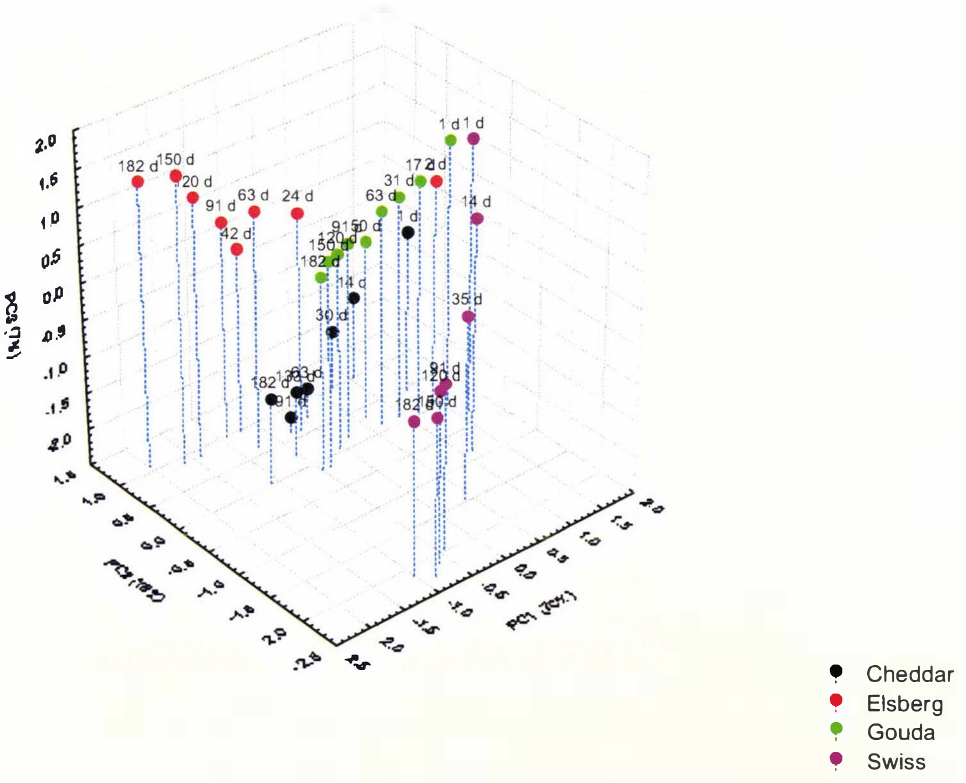
Peak	Factor 1	Factor 2	Factor 3
1	-0.000037	0.000049	0.000250
2	-0.000037	0.000550	-0.000814
3	-0.000100	-0.000161	0.000264
4	-0.000063	0.000071	0.000164
5	-0.000253	-0.000531	-0.000166
6	-0.000162	0.000299	-0.000163
7	-0.000133	0.000092	0.000083
8	-0.000038	-0.000058	-0.000359
9	-0.000078	-0.000309	-0.000366
10	-0.000109	-0.000179	-0.000637
11	-0.000051	0.000076	-0.000666
12	-0.000028	-0.000034	-0.000220
13	-0.000306	0.000311	0.000593
14	-0.000068	0.000161	0.000182

Factor scores

	Cheese	Ripening time	Factor 1	Factor 2	Factor 3
1	Cheddar	1 d	1.384	0.463	-0.273
2	Cheddar	14 d	1.051	1.024	-1.374
3	Cheddar	30 d	0.713	1.014	-1.698
4	Cheddar	63 d	0.087	0.738	-2.089
5	Cheddar	91 d	-0.264	0.625	-2.279
6	Cheddar	133 d	-0.523	0.261	-1.605
7	Cheddar	182 d	-1.085	0.039	-1.328
8	Gouda	1 d	1.532	-0.034	1.158
9	Gouda	17 d	1.179	0.056	0.703
10	Gouda	31 d	0.941	0.135	0.551
11	Gouda	50 d	0.623	0.345	-0.039
12	Gouda	63 d	0.683	0.147	0.439
13	Gouda	91 d	0.196	0.185	0.194
14	Gouda	120 d	-0.046	0.076	0.200

15	Gouda	150 d	-0.362	-0.120	0.331
16	Gouda	182 d	-0.466	-0.109	0.148
17	Swiss	1 d	1.669	-0.264	1.246
18	Swiss	14 d	1.145	-0.845	0.697
19	Swiss	35 d	0.423	-1.457	0.018
20	Swiss	91 d	-0.409	-1.973	-0.253
21	Swiss	120 d	-0.627	-2.125	-0.168
22	Swiss	150 d	-0.818	-2.269	-0.348
23	Swiss	182 d	-1.003	-2.119	-0.395
24	Elsberg	2 d	1.473	0.116	0.575
25	Elsberg	24 d	-0.144	0.595	0.517
26	Elsberg	42 d	-0.697	0.988	0.033
27	Elsberg	63 d	-0.750	0.628	0.751
28	Elsberg	91 d	-0.912	0.998	0.481
29	Elsberg	120 d	-1.387	0.895	1.037
30	Elsberg	150 d	-1.623	0.900	1.404
31	Elsberg	182 d	-1.984	1.085	1.367

PC1 (70%) v PC2 (18%) v PC3 (7%)



7.8.4 RP-HPLC of the WSF

Peak data for PCA

	Cheese	Ripening time	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	Cheddar	1 d	39932	14701	18543	2894	16947	3338	11864	2669	9298	17704	14139	7688	12555	6061	7466	13186	25328
2	Cheddar	14 d	51463	14766	27287	4966	39128	9301	19034	8367	11586	21417	24511	29465	20382	10220	11037	17167	47714
3	Cheddar	30 d	53499	16439	40682	3808	71153	10559	17334	12313	12451	31184	33693	59439	34177	14895	20156	25332	67629
4	Cheddar	63 d	70187	22595	57369	9905	119365	10711	17275	24912	18078	41185	56362	60426	59134	22514	48770	31961	105593
5	Cheddar	91 d	56387	25362	76366	13293	143105	14034	21013	30141	21971	52613	70996	60980	88018	19532	66887	35908	128129
6	Cheddar	133 d	55497	30338	111638	14106	165989	15184	27106	34734	27503	63649	81960	69892	104339	19727	98966	36826	131873
7	Cheddar	182 d	51904	35203	140252	23000	188677	16259	39042	39962	31581	76898	93822	71647	143908	24044	75717	57564	140250
8	Elsberg	2 d	10300	12669	7275	1923	8385	2497	2139	4607	2824	1521	6628	4958	4571	3994	2557	2459	11333
9	Elsberg	24 d	24865	22505	27221	4600	45687	11544	5792	12639	7555	5492	13759	26934	31043	11177	5122	8544	30636
10	Elsberg	42 d	30541	26208	33750	4882	58835	13966	7641	15572	9484	8141	17511	33312	39539	13912	6205	10911	36149
11	Elsberg	63 d	33719	29667	38429	5835	68112	19741	10885	19452	11733	10193	27914	37415	45370	16665	7105	13284	45108
12	Elsberg	91 d	36774	32804	40641	5367	72285	20406	10136	19520	11034	9822	23782	35245	46534	17293	8570	12856	54204
13	Elsberg	120 d	49612	36362	49748	7630	93050	26732	13304	25229	14915	13995	29915	41759	56965	21177	9069	14356	44998
14	Elsberg	150 d	53931	36997	48623	7300	102031	18442	7018	25133	7652	6341	20054	30484	46953	12344	5691	14675	37504
15	Elsberg	182 d	57645	30661	50258	8679	106226	18475	8134	25647	10770	8504	23865	42215	55168	15768	6853	19707	39604
16	Gouda	1 d	10290	10543	6468	980	4392	1500	475	3101	2012	879	4514	1737	2490	3310	975	3388	9831
17	Gouda	17 d	10238	10235	7045	2101	7842	3698	1789	3310	1535	1079	3760	4022	4582	3081	1853	2254	14361
18	Gouda	31 d	13192	15304	10136	3313	15095	7840	4713	3512	2554	2219	6687	8420	6983	6322	3138	3909	25624
19	Gouda	50 d	14208	16776	11752	3901	19827	10466	6018	5693	3111	3033	8691	13611	9668	7050	3520	5442	37966
20	Gouda	63 d	14686	17929	12455	4224	21698	11736	6713	6417	3752	3286	9813	14252	14242	3978	3797	5626	36735
21	Gouda	91 d	17992	21610	18544	5212	31857	15142	8971	8481	4246	4947	9597	22630	19827	5924	4800	8482	46945
22	Gouda	120 d	20230	25843	19358	5842	37103	18255	8363	10190	7532	5523	15883	26750	23261	7398	5467	9869	52526
23	Gouda	150 d	21773	25921	21694	6493	42419	19312	10589	11970	5792	5908	17523	28869	25069	8525	6019	11191	54955
24	Gouda	182 d	25742	28293	28368	8386	49174	23459	11564	7154	8767	7389	21831	29742	40643	11059	6841	15122	62858
25	Mozzarella	1 d	20164	22622	9036	2126	1862	2031	1533	741	2306	3078	1582	433	2078	756	355	2884	6626
26	Mozzarella	14 d	19840	26959	8466	1712	1425	1074	1755	776	2186	3646	1928	539	1513	1830	354	5030	16844
27	Mozzarella	30 d	23071	27169	6685	1875	1546	1637	1119	1367	2364	4870	1449	492	2094	2049	1650	3899	25917
28	Mozzarella	63 d	27946	23643	7515	2076	2560	1162	1103	1027	1596	4362	3402	2294	2063	1087	998	5177	55857
29	Mozzarella	91 d	27068	24090	9433	3346	6889	1091	1755	1354	1879	4828	6395	14526	3776	3106	1735	6552	71626
30	Mozzarella	133 d	35859	22680	11039	2814	11038	750	1443	2619	2474	3936	5741	1356	3277	2126	5914	4815	76005
31	Mozzarella	182 d	44865	23675	22902	4115	34652	1167	4040	7053	6634	14559	14852	31347	10639	13609	6416	13125	107214
32	Swiss	1 d	10057	11797	1613	670	3060	1675	821	2831	854	3001	1107	515	1539	2704	3020	734	11337
33	Swiss	14 d	12796	14594	5931	2035	5737	5926	1203	4854	2245	1655	1507	1365	2597	4196	2942	2677	27444
34	Swiss	35 d	17769	15637	16247	3271	14429	18250	3286	6391	3199	2373	2842	3051	6031	4904	4725	3215	42678
35	Swiss	91 d	28862	20285	24810	3787	31611	18864	5489	9963	5446	2381	3929	4880	9580	8547	5482	9266	49397
36	Swiss	120 d	31550	22231	29176	1817	35844	21720	6627	10922	5135	3156	4537	5259	10825	9903	6350	11287	53874
37	Swiss	150 d	39847	22408	30882	1822	44831	18767	6879	11972	1968	6849	4908	5640	11463	10620	6744	7710	59752
38	Swiss	182 d	42779	23290	33320	1950	49254	17890	2560	9028	5577	6069	7661	7145	12074	9944	6665	12527	54990

	Cheese	Ripening time	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33
1	Cheddar	1 d	12080	7048	3956	5517	3609	2442	6261	6021	2042	18653	13829	6738	9146	5883	3558	2074
2	Cheddar	14 d	17664	12848	8618	8277	5383	7053	5887	8380	10951	25700	22683	8588	15179	15645	4056	2860
3	Cheddar	30 d	19792	23198	12467	10160	8382	11736	6335	12527	15781	31521	28122	8748	19488	23480	7528	4680
4	Cheddar	63 d	26775	33951	21036	15164	15214	16568	8686	21263	20252	43108	39401	11200	23567	37975	15056	8027
5	Cheddar	91 d	29603	34565	26008	17628	18911	18296	10688	23580	19639	50955	46350	11934	26582	42543	17804	9771
6	Cheddar	133 d	30608	32109	26731	23251	16600	21005	7275	24095	15209	47701	41791	10076	26855	41515	16371	10348
7	Cheddar	182 d	31491	32919	28700	21586	16445	22358	7810	33992	17103	55057	44077	9713	26518	39065	18379	11320
8	Elsberg	2 d	9656	7998	3283	5044	1510	2468	3340	1536	7459	6038	5914	2162	5849	2058	1129	1825
9	Elsberg	24 d	10053	15818	6608	8413	3885	5078	5579	10616	5901	14118	9905	7598	9513	6036	5556	2088
10	Elsberg	42 d	12685	20623	7067	9133	5152	5351	5855	10494	7754	16849	12496	9588	9934	7296	5794	2690
11	Elsberg	63 d	5889	21080	8625	10330	5945	5599	6639	11674	8173	18641	13706	12200	10475	7720	5626	3215
12	Elsberg	91 d	6397	13492	8579	11292	5216	6249	5881	11183	9157	18992	14086	10634	11297	8498	6375	4153
13	Elsberg	120 d	8016	14253	11566	9633	5871	6891	7374	14391	8156	18664	13865	14517	10428	7372	4513	3968
14	Elsberg	150 d	5754	10275	18516	14331	4360	3963	5744	10516	8458	23164	14543	4946	8354	4922	2493	7247
15	Elsberg	182 d	9394	9490	21462	15070	5729	5005	6411	12890	8878	25462	17476	6227	10363	7301	3523	8465
16	Gouda	1 d	2387	2145	1745	2761	1742	500	3740	1609	2163	5833	3859	1151	2429	3460	2308	2577
17	Gouda	17 d	7221	2359	3696	1125	746	746	1625	1350	6540	7257	5303	1520	3372	2541	1568	843
18	Gouda	31 d	12030	4201	7283	1742	1219	1219	2700	3664	8980	10684	8394	1869	5114	3997	2427	1398
19	Gouda	50 d	12447	6525	9295	5317	3249	1870	4634	10929	6428	14867	2397	1300	4546	5691	3085	1972
20	Gouda	63 d	18882	6906	10103	2076	3631	3423	3729	2998	11305	12033	10905	1956	6448	5659	3116	2125
21	Gouda	91 d	21639	10888	13376	3086	6190	4244	4645	4233	13845	14718	13311	1450	8976	7968	4198	3366
22	Gouda	120 d	22773	12761	15135	3785	7357	4715	5403	5458	13919	16471	14673	1805	10245	9931	4579	4108
23	Gouda	150 d	22406	15552	16101	3751	7695	4509	6130	5933	14125	17510	14507	1972	10558	10464	4849	4973
24	Gouda	182 d	23198	17306	17205	5203	10106	6024	6812	8258	14278	21174	17202	2628	13575	15373	5429	6005
25	Mozzarella	1 d	5845	1861	2029	1896	1499	2038	4398	2361	6454	4864	5995	2094	5444	6884	2438	1895
26	Mozzarella	14 d	960	2032	1452	936	3062	3443	4685	5596	16088	5169	7992	2728	6824	11766	2832	3434
27	Mozzarella	30 d	1312	2441	2854	1263	3462	4606	5407	10668	33686	3346	10817	5433	6662	19619	3864	5803
28	Mozzarella	63 d	2909	3073	4101	7090	4630	3803	7858	27797	57460	10369	18646	7977	9496	45791	8367	12221
29	Mozzarella	91 d	3838	9426	5888	9940	7061	4873	13319	36440	75809	5780	21882	9644	16335	37800	10656	15890
30	Mozzarella	133 d	6062	5494	8351	13358	13582	3347	10065	65634	76604	15768	30083	10259	22952	55864	17321	21141
31	Mozzarella	182 d	18012	19743	17256	25829	17401	17087	20130	120799	83188	28889	47805	23527	36805	62867	26786	28116
32	Swiss	1 d	3134	2746	5909	2214	864	864	1184	3523	5222	3411	3316	1856	2937	3357	1265	1906
33	Swiss	14 d	6498	5512	15573	3916	1608	1608	1115	6975	9979	8026	6732	2078	4509	5759	5062	2710
34	Swiss	35 d	14294	9809	25980	3227	3920	2146	1674	14048	19574	22254	9836	4394	6685	7355	7278	1923
35	Swiss	91 d	25212	13949	27511	3533	4423	3131	1974	19861	22423	25438	22573	6034	5939	8915	7286	2907
36	Swiss	120 d	28344	15009	28986	3823	9755	3629	2912	23389	24325	28915	26090	7318	6418	10882	8015	2962
37	Swiss	150 d	31550	16723	28838	3622	5926	4144	2495	25353	24525	32779	27820	7391	7022	11348	5615	2699
38	Swiss	182 d	33505	23758	26434	3119	6171	4366	2496	25752	23751	34858	28851	7714	6771	10865	2281	8413

	Cheese	Ripening time	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49
1	Cheddar	1 d	3113	1582	12530	3247	5534	3846	13221	19629	33516	31296	14695	59263	78639	27548	3809	23261
2	Cheddar	14 d	5592	4236	17392	7528	11069	6121	13533	22131	45744	42396	19263	91862	131897	24771	4451	21768
3	Cheddar	30 d	11405	9137	17693	10131	18583	16026	14591	22665	48969	47609	73684	88152	71701	24504	6094	22585
4	Cheddar	63 d	22294	16414	21593	17703	24427	31639	24755	33056	62975	61079	80529	81732	62638	28053	6895	24986
5	Cheddar	91 d	26993	19333	20614	19785	32280	27502	34220	38191	72274	68014	70661	84961	60238	25895	6470	26170
6	Cheddar	133 d	22850	16669	17715	17304	29773	28469	42653	43697	77638	67917	68948	50627	56495	19788	4667	25987
7	Cheddar	182 d	24007	15885	16818	16543	28066	24884	61978	47719	89561	69561	54629	26800	51582	16859	4522	27809
8	Elsberg	2 d	1199	1892	3412	4060	5351	2194	5334	6173	13390	14934	9424	9512	7591	13977	1645	14390
9	Elsberg	24 d	6120	3258	2060	7451	9714	7916	13386	22401	31485	32686	28466	16898	13591	13415	2392	17267
10	Elsberg	42 d	7648	3975	2835	8122	11422	10147	14831	24717	38954	42360	38381	28813	22716	20671	3244	18391
11	Elsberg	63 d	8293	4379	3119	7496	11555	10902	15002	22516	39831	41676	36239	22371	24229	9095	3307	18987
12	Elsberg	91 d	8085	5288	3435	7146	11383	10861	15667	22724	39953	42509	38755	29347	27521	9595	3359	18291
13	Elsberg	120 d	9606	3991	3728	6824	11998	11452	15822	23350	49671	43162	42296	25866	32853	10725	3914	20448
14	Elsberg	150 d	9228	16946	12542	7795	12542	19420	20202	27713	9140	66123	60367	61841	41333	17171	8736	19473
15	Elsberg	182 d	8210	18396	13919	8336	14949	22241	17577	46020	12992	64600	57289	52320	43184	15263	6505	21618
16	Gouda	1 d	1567	930	3935	2667	1951	3461	4963	11378	13927	11781	4072	11728	3972	7558	2184	12216
17	Gouda	17 d	2601	1560	6212	2352	4358	8656	6426	11474	16016	11891	6360	12995	5776	10673	5557	10326
18	Gouda	31 d	4765	2947	9868	4170	6832	15231	10522	16553	23759	17067	9311	17542	7513	11359	3670	13398
19	Gouda	50 d	6801	6077	9981	5489	8326	20222	14446	19032	27712	18628	12146	17443	7827	11137	3410	14172
20	Gouda	63 d	7326	6676	10000	5534	8426	20684	15632	19593	28721	19206	12397	14598	6702	9418	2744	13741
21	Gouda	91 d	10831	7339	14335	6948	10321	25102	21077	22903	35886	21295	17363	15426	6380	9154	2815	15083
22	Gouda	120 d	11933	8521	15398	8104	10999	26945	25123	25085	41474	23300	20868	18147	8227	9974	3035	15250
23	Gouda	150 d	14270	9064	15696	8229	11603	30342	30582	27320	48150	24880	24821	17894	7890	9408	3065	15347
24	Gouda	182 d	13619	10077	17326	10206	12138	30683	33196	28583	52035	27922	27098	21130	8706	9911	3201	16504
25	Mozzarella	1 d	7493	3677	5490	7904	6390	10646	15963	26816	33976	15660	4922	23901	32268	149518	7804	23166
26	Mozzarella	14 d	10955	3737	8069	9317	7150	22210	33278	48099	43928	16600	6602	21676	25300	158622	5124	24278
27	Mozzarella	30 d	14661	5767	11306	13424	9044	35635	55704	70777	51136	19375	8987	25976	35062	148702	4917	25692
28	Mozzarella	63 d	25274	10695	19951	22143	13409	108754	107850	75445	69269	26316	13941	33180	32133	83838	2686	24122
29	Mozzarella	91 d	33693	12938	23848	23809	17081	148359	111336	84336	78898	31511	14707	27002	21162	30728	1520	25812
30	Mozzarella	133 d	40825	20861	32067	34405	23452	197864	118149	60636	84325	36925	19409	37500	27313	71372	4858	28096
31	Mozzarella	182 d	52473	30451	46100	45120	50046	218813	87535	66463	104550	37122	16417	58664	44912	72000	4686	15532
32	Swiss	1 d	2454	667	4812	4592	3611	3237	4659	11072	17937	18023	4252	3136	5575	4887	1487	10722
33	Swiss	14 d	3208	2203	9153	8518	6743	7814	8234	24473	27802	38141	14278	7249	5450	15603	2135	11476
34	Swiss	35 d	5866	5000	13433	13856	11814	19072	14045	61010	33030	49441	16805	11024	7764	5522	1983	11588
35	Swiss	91 d	8317	6966	14941	16186	16576	21166	21801	84393	35513	73894	23030	24947	12815	12700	3282	13774
36	Swiss	120 d	8733	8554	22804	30538	19823	23358	23184	88413	41243	74663	23126	28082	13649	11600	3032	13266
37	Swiss	150 d	12915	9436	19484	18565	22042	22782	26876	90334	42907	76470	23812	30059	16063	11051	2932	14065
38	Swiss	182 d	11482	10772	18813	19961	22996	22573	27844	87833	42399	75142	24451	33068	18574	12830	3192	14081

7.8.4.1 PCA of the correlation matrix (RP-HPLC/WSF)

PCA: Peaks 1 - 48

Unrotated Factor Loadings (Factor-variable correlations) and Communalities

Peak	Factor 1	Factor 2	Factor 3	Communality
1	-0.835	-0.140	-0.059	0.721
2	-0.550	-0.119	-0.004	0.317
3	-0.851	-0.444	-0.001	0.921
4	-0.800	-0.414	-0.111	0.824
5	-0.847	-0.491	-0.015	0.958
6	-0.301	-0.553	0.562	0.713
7	-0.777	-0.501	-0.130	0.872
8	-0.777	-0.539	0.029	0.895
9	-0.845	-0.467	-0.164	0.959
10	-0.854	-0.317	-0.211	0.875
11	-0.858	-0.421	-0.194	0.951
12	-0.829	-0.420	-0.201	0.904
13	-0.801	-0.512	-0.117	0.917
14	-0.800	-0.457	0.039	0.851
15	-0.812	-0.329	-0.107	0.780
16	-0.888	-0.374	-0.097	0.938
17	-0.957	0.087	0.101	0.933
18	-0.589	-0.242	0.570	0.731
19	-0.861	-0.293	0.185	0.862
20	-0.616	-0.195	0.709	0.921
21	-0.893	0.092	-0.210	0.850
22	-0.936	0.170	0.052	0.908
23	-0.944	-0.086	-0.153	0.922
24	-0.648	0.552	-0.322	0.827
25	-0.570	0.737	0.101	0.879
26	-0.329	0.915	0.071	0.950
27	-0.890	-0.269	0.265	0.936
28	-0.936	0.203	0.150	0.940
29	-0.725	0.332	-0.054	0.638
30	-0.905	0.283	-0.203	0.940
31	-0.740	0.602	-0.163	0.935
32	-0.840	0.429	-0.020	0.889
33	-0.624	0.724	-0.081	0.921
34	-0.675	0.708	-0.062	0.961
35	-0.795	0.432	0.077	0.825
36	-0.589	0.676	0.224	0.854
37	-0.563	0.734	0.300	0.946
38	-0.866	0.374	0.212	0.936
39	-0.337	0.911	-0.007	0.943
40	-0.402	0.786	-0.078	0.786
41	-0.284	0.529	0.569	0.684
42	-0.754	0.515	-0.074	0.839
43	-0.665	-0.205	0.535	0.770
44	-0.779	-0.418	-0.049	0.784
45	-0.641	-0.010	-0.266	0.482
46	-0.539	-0.086	-0.441	0.492
47	0.081	0.477	-0.453	0.439
48	-0.401	-0.110	-0.392	0.327
Variance	25.78	10.46	3.21	39.45
% Variance	53.71	21.78	6.69	82.18

Factor Score Coefficients

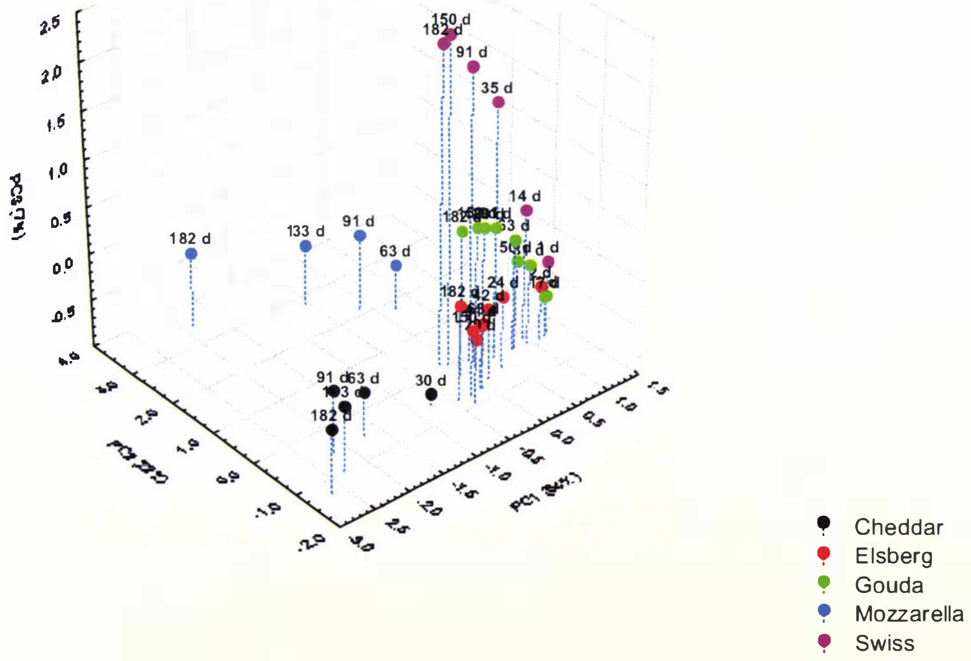
Peak	Factor 1	Factor 2	Factor 3
1	-0.032	-0.053	-0.046
2	-0.021	-0.021	0.035
3	-0.033	-0.024	0.057
4	-0.031	-0.017	0.049
5	-0.033	-0.046	0.104
6	-0.012	-0.040	0.074
7	-0.030	-0.047	0.125
8	-0.030	-0.039	0.122
9	-0.033	-0.033	0.075
10	-0.033	0.005	0.117
11	-0.033	-0.024	0.127
12	-0.032	-0.013	0.065
13	-0.031	-0.011	0.157
14	-0.031	-0.043	0.136
15	-0.032	-0.040	0.091
16	-0.034	-0.047	0.089
17	-0.037	-0.053	-0.035
18	-0.023	-0.048	-0.072
19	-0.033	-0.052	-0.002
20	-0.024	-0.045	-0.015
21	-0.035	-0.030	0.082
22	-0.036	-0.040	-0.019
23	-0.037	-0.040	-0.019
24	-0.025	-0.049	-0.001
25	-0.022	-0.044	-0.000
26	-0.013	-0.031	-0.035
27	-0.035	-0.036	-0.005
28	-0.036	0.008	0.175
29	-0.028	-0.023	-0.040
30	-0.035	-0.028	0.009
31	-0.029	-0.019	-0.051
32	-0.033	0.009	-0.066
33	-0.024	0.016	-0.060
34	-0.026	-0.008	-0.063
35	-0.031	0.053	-0.036
36	-0.023	0.070	0.012
37	-0.022	0.087	-0.033

Factor Scores

	Cheese	Ripening time	Factor 1	Factor 2	Factor 3
1	Cheddar	1 d	0.472	-0.333	-1.126
2	Cheddar	14 d	-0.171	-0.443	-1.183
3	Cheddar	30 d	-0.733	-0.551	-0.888
4	Cheddar	63 d	-1.709	-0.495	-0.534
5	Cheddar	91 d	-2.169	-0.529	-0.347
6	Cheddar	133 d	-2.302	-0.972	-0.318
7	Cheddar	182 d	-2.667	-1.294	-0.327
8	Elsberg	2 d	1.118	-0.288	-0.430
9	Elsberg	24 d	0.396	-0.492	-0.239
10	Elsberg	42 d	0.114	-0.590	-0.249
11	Elsberg	63 d	-0.061	-0.727	-0.294
12	Elsberg	91 d	-0.076	-0.705	-0.324
13	Elsberg	120 d	-0.315	-0.935	-0.312
14	Elsberg	150 d	-0.254	-0.749	-0.307
15	Elsberg	182 d	-0.430	-0.731	0.022
16	Gouda	1 d	1.268	-0.208	-0.607
17	Gouda	17 d	1.193	-0.274	-0.579
18	Gouda	31 d	0.948	-0.248	-0.146
19	Gouda	50 d	0.767	-0.220	-0.059

20	Gouda	63 d	0.725	-0.234	0.189
21	Gouda	91 d	0.450	-0.215	0.401
22	Gouda	120 d	0.262	-0.236	0.463
23	Gouda	150 d	0.159	-0.221	0.505
24	Gouda	182 d	-0.087	-0.250	0.552
25	Mozzarella	1 d	1.000	0.169	-1.487
26	Mozzarella	14 d	0.894	0.487	-1.255
27	Mozzarella	30 d	0.692	0.887	-1.030
28	Mozzarella	63 d	0.215	1.800	-0.525
29	Mozzarella	91 d	-0.112	2.163	-0.195
30	Mozzarella	133 d	-0.536	2.795	-0.356
31	Mozzarella	182 d	-1.849	3.489	-0.207
32	Swiss	1 d	1.292	-0.184	-0.249
33	Swiss	14 d	0.973	-0.106	0.386
34	Swiss	35 d	0.577	-0.019	1.611
35	Swiss	91 d	0.184	-0.025	2.097
36	Swiss	120 d	-0.048	0.203	2.538
37	Swiss	150 d	-0.053	0.108	2.452
38	Swiss	182 d	-0.128	0.173	2.358

PC1 (54%) v PC2 (22%) v PC3 (7%)



7.8.4.2 PCA of the correlation matrix - without Mozzarella cheese data (RP-HPLC/WSF)

PCA: Peaks 1 - 48

Unrotated Factor Loadings (Factor-variable correlations) and Communalities

Peak	Factor 1	Factor 2	Factor 3	Communality
1	-0.833	-0.110	-0.339	0.821
2	-0.630	-0.035	0.465	0.615
3	-0.935	-0.130	0.205	0.934
4	-0.864	-0.234	0.330	0.910
5	-0.945	-0.188	0.158	0.953
6	-0.427	0.415	0.389	0.507
7	-0.881	-0.263	0.057	0.849
8	-0.886	-0.193	0.245	0.882
9	-0.924	-0.309	0.108	0.961
10	-0.903	-0.248	-0.016	0.878
11	-0.925	-0.308	0.117	0.964
12	-0.873	-0.388	0.087	0.921
13	-0.897	-0.291	0.300	0.980
14	-0.877	-0.207	0.114	0.825
15	-0.880	-0.149	0.059	0.800
16	-0.953	-0.177	0.022	0.941
17	-0.963	0.086	0.075	0.941
18	-0.661	0.602	-0.095	0.808
19	-0.923	0.073	-0.007	0.857
20	-0.659	0.692	0.031	0.914
21	-0.867	-0.378	0.067	0.898
22	-0.947	0.069	0.036	0.902
23	-0.953	-0.180	0.033	0.942
24	-0.741	-0.464	0.013	0.764
25	-0.805	0.433	0.009	0.836
26	-0.520	0.802	-0.123	0.928
27	-0.958	0.204	-0.133	0.977
28	-0.941	0.204	-0.188	0.963
29	-0.689	-0.154	-0.105	0.510
30	-0.934	-0.237	-0.067	0.934
31	-0.929	-0.043	-0.097	0.874
32	-0.899	0.012	0.055	0.811
33	-0.884	0.016	0.104	0.793
34	-0.931	0.123	0.116	0.895
35	-0.833	0.169	-0.020	0.723
36	-0.596	0.550	-0.385	0.806
37	-0.622	0.687	-0.119	0.873
38	-0.924	0.326	-0.071	0.965
39	-0.538	0.617	0.104	0.681
40	-0.841	0.252	0.310	0.867
41	-0.344	0.860	-0.089	0.867
42	-0.865	0.067	0.116	0.765
43	-0.709	0.457	-0.192	0.748
44	-0.857	-0.197	-0.130	0.790
45	-0.615	-0.226	-0.707	0.930
46	-0.548	-0.384	-0.616	0.828
47	-0.559	-0.333	-0.671	0.873
48	-0.568	-0.260	-0.370	0.527
Variance	31.75	5.96	2.82	40.53
% Variance	66.14	12.42	5.88	84.44

Factor Score Coefficients

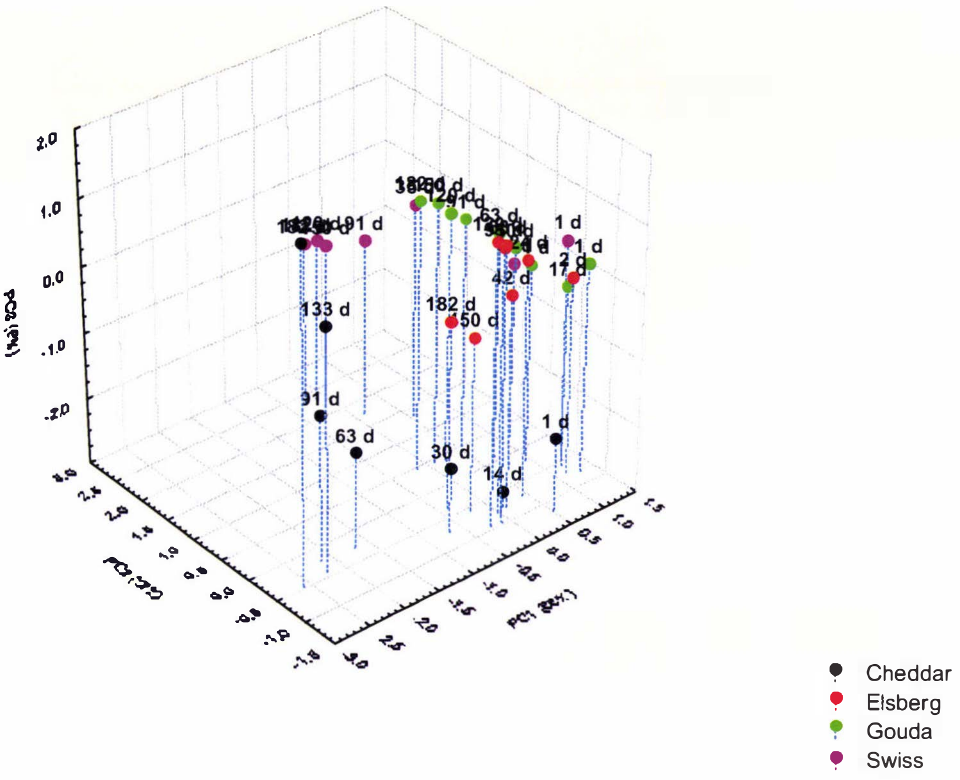
Peak	Factor 1	Factor 2	Factor 3
1	-0.026	-0.068	-0.071
2	-0.020	-0.065	-0.050
3	-0.029	-0.064	-0.036
4	-0.027	-0.068	-0.043
5	-0.030	-0.061	0.021
6	-0.013	-0.043	0.147
7	-0.028	-0.045	0.018
8	-0.028	-0.067	0.169
9	-0.029	-0.039	-0.092
10	-0.028	-0.061	0.017
11	-0.029	-0.025	-0.044
12	-0.028	-0.063	-0.113
13	-0.028	-0.052	0.106
14	-0.028	-0.062	0.195
15	-0.028	-0.045	0.050
16	-0.030	-0.040	0.050
17	-0.030	-0.041	-0.038
18	-0.021	-0.041	-0.058
19	-0.029	-0.018	-0.011
20	-0.021	-0.006	0.003
21	-0.027	-0.022	0.004
22	-0.030	-0.039	0.030
23	-0.030	-0.031	0.041
24	-0.023	0.070	0.134
25	-0.025	-0.044	0.167
26	-0.016	-0.032	0.079
27	-0.030	-0.052	0.150
28	-0.030	-0.042	0.061
29	-0.022	-0.052	0.210
30	-0.029	-0.065	0.016

Factor Scores

	Cheese	Ripening time	Factor 1	Factor 2	Factor 3
1	Cheddar	1 d	0.513	-1.166	-1.883
2	Cheddar	14 d	-0.093	-0.949	-2.525
3	Cheddar	30 d	-0.669	-0.711	-2.038
4	Cheddar	63 d	-1.689	-0.269	-1.550
5	Cheddar	91 d	-2.143	-0.213	-0.808
6	Cheddar	133 d	-2.216	-0.413	0.639
7	Cheddar	182 d	-2.591	-0.473	1.996
8	Elsberg	2 d	1.145	-0.697	-0.002
9	Elsberg	24 d	0.436	-0.703	0.573
10	Elsberg	42 d	0.146	-0.754	0.199
11	Elsberg	63 d	0.011	-0.812	1.026
12	Elsberg	91 d	-0.008	-0.779	0.978
13	Elsberg	120 d	-0.236	-0.927	1.243
14	Elsberg	150 d	-0.221	-0.552	-0.366
15	Elsberg	182 d	-0.385	-0.356	-0.156
16	Gouda	1 d	1.297	-0.784	0.181
17	Gouda	17 d	1.207	-0.551	-0.254
18	Gouda	31 d	0.946	-0.194	0.001
19	Gouda	50 d	0.742	-0.136	0.306
20	Gouda	63 d	0.716	0.096	0.422
21	Gouda	91 d	0.422	0.337	0.625
22	Gouda	120 d	0.227	0.382	0.775
23	Gouda	150 d	0.105	0.446	0.945
24	Gouda	182 d	-0.138	0.470	1.053
25	Swiss	1 d	1.324	-0.412	0.315
26	Swiss	14 d	0.958	0.118	-0.142

27	Swiss	35 d	0.551	1.350	0.261
28	Swiss	91 d	0.131	1.756	-0.314
29	Swiss	120 d	-0.116	2.340	-0.512
30	Swiss	150 d	-0.119	2.172	-0.487
31	Swiss	182 d	-0.252	2.381	-0.503

PC1 (66%) v PC2 (12%) v PC3 (6%)



7.8.4.3 PCA of the covariance matrix (RP-HPLC/WSF)

PCA: Peaks 1 – 48

Unrotated Factor Loadings (Factor-variable correlations) and Communalities

Peak	Factor 1	Factor 2	Factor 3	Communality
1	-0.840	0.047	0.169	0.737
2	-0.571	0.063	0.126	0.346
3	-0.938	-0.229	0.033	0.934
4	-0.882	-0.205	0.021	0.821
5	-0.941	-0.287	0.003	0.967
6	-0.397	-0.464	-0.391	0.525
7	-0.869	-0.301	0.105	0.857
8	-0.883	-0.353	-0.051	0.907
9	-0.930	-0.259	0.124	0.947
10	-0.911	-0.113	0.197	0.881
11	-0.938	-0.214	0.110	0.937
12	-0.898	-0.233	0.046	0.862
13	-0.906	-0.305	0.044	0.916
14	-0.867	-0.285	-0.081	0.839
15	-0.878	-0.137	0.112	0.802
16	-0.951	-0.159	0.109	0.942
17	-0.915	0.293	-0.133	0.940
18	-0.598	-0.123	-0.309	0.468
19	-0.886	-0.113	-0.132	0.815
20	-0.626	-0.075	-0.403	0.560
21	-0.855	0.260	-0.018	0.800
22	-0.863	0.357	-0.074	0.877
23	-0.931	0.114	0.126	0.895
24	-0.502	0.661	0.056	0.692
25	-0.378	0.809	-0.193	0.835
26	-0.130	0.969	-0.139	0.975
27	-0.915	-0.081	-0.104	0.854
28	-0.857	0.387	-0.056	0.887
29	-0.617	0.456	-0.018	0.588
30	-0.810	0.455	0.045	0.865
31	-0.599	0.752	0.066	0.928
32	-0.715	0.578	-0.086	0.853
33	-0.456	0.834	-0.098	0.913
34	-0.505	0.838	-0.052	0.960
35	-0.676	0.577	-0.120	0.804
36	-0.409	0.762	-0.178	0.780
37	-0.376	0.822	-0.174	0.847
38	-0.742	0.523	-0.149	0.846
39	-0.134	0.957	-0.174	0.965
40	-0.250	0.896	-0.011	0.865
41	-0.164	0.606	-0.130	0.411
42	-0.621	0.689	0.057	0.864
43	-0.688	-0.061	-0.208	0.521
44	-0.854	-0.241	0.048	0.789
45	-0.613	0.108	0.339	0.503
46	-0.538	0.039	0.518	0.559
47	0.189	0.505	0.796	0.923
48	-0.406	-0.005	0.568	0.487
Variance	8.69E+09	5.59E+09	1.53E+09	1.58E+10
% Variance	46.91	30.16	8.25	85.32

Factor Score Coefficients

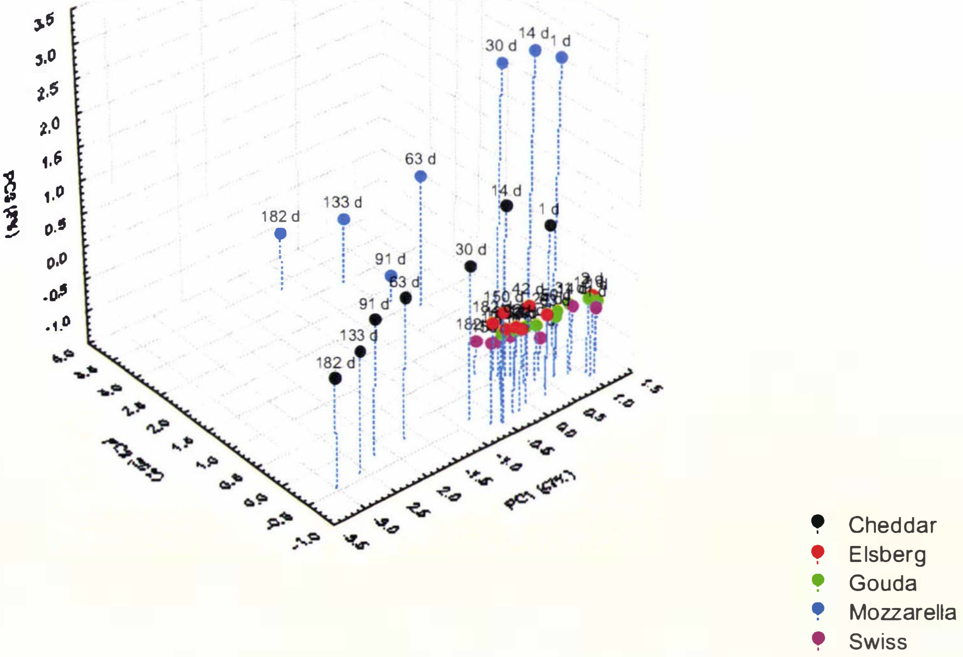
Peak	Factor 1	Factor 2	Factor 3
1	-1.60E-06	-5.97E-07	1.64E-06
2	-4.66E-07	-2.02E-06	3.20E-06
3	-3.11E-06	-2.08E-06	1.32E-06
4	-4.39E-07	-2.63E-06	2.32E-06
5	-5.16E-06	-2.07E-06	2.63E-06
6	-3.58E-07	-2.07E-06	1.71E-06
7	-8.14E-07	1.10E-06	1.63E-05
8	-1.03E-06	-1.01E-07	9.06E-06
9	-7.70E-07	1.38E-07	5.27E-06
10	-1.86E-06	7.95E-08	5.36E-06
11	-2.41E-06	-1.18E-06	-4.38E-07
12	-2.21E-06	-1.59E-07	-1.77E-06
13	-3.30E-06	-2.45E-06	8.73E-07
14	-6.55E-07	-6.51E-07	3.53E-07
15	-2.23E-06	-4.38E-07	6.97E-06
16	-1.28E-06	-6.41E-07	-2.77E-09
17	-3.53E-06	-3.33E-07	1.84E-06
18	-6.88E-07	-3.58E-07	5.85E-07
19	-9.59E-07	-8.55E-07	6.22E-07
20	-6.52E-07	-8.92E-07	6.02E-08
21	-6.37E-07	-1.73E-06	1.09E-07
22	-4.90E-07	-3.35E-07	-2.01E-06
23	-6.04E-07	-5.41E-07	5.61E-07
24	-2.08E-07	-3.31E-07	-3.40E-07
25	-9.32E-07	1.76E-06	5.85E-07
26	-3.01E-07	-2.21E-07	2.29E-06
27	-1.40E-06	-1.91E-07	1.61E-06
28	-1.23E-06	-1.21E-07	6.40E-07
29	-3.37E-07	3.02E-07	9.04E-07
30	-7.46E-07	3.15E-07	-3.49E-07
31	-1.12E-06	1.14E-07	1.61E-06
32	-4.77E-07	4.27E-07	8.34E-07
33	-3.02E-07	3.10E-06	-2.91E-06
34	-6.47E-07	3.50E-06	-2.02E-06
35	-5.21E-07	-1.92E-07	-8.11E-07
36	-4.20E-07	8.67E-07	-2.39E-06
37	-4.04E-07	3.88E-07	-7.43E-08
38	-8.15E-07	6.52E-07	-2.40E-07
39	-7.70E-07	2.20E-06	4.65E-07
40	-8.49E-07	5.99E-07	1.32E-07

Factor Scores

	Cheese	Ripening time	Factor 1	Factor 2	Factor 3
1	Cheddar	1 d	0.423	-0.475	0.951
2	Cheddar	14 d	-0.222	-0.395	1.451
3	Cheddar	30 d	-0.791	-0.412	0.832
4	Cheddar	63 d	-1.682	-0.194	0.618
5	Cheddar	91 d	-2.202	-0.227	0.547
6	Cheddar	133 d	-2.590	-0.390	0.343
7	Cheddar	182 d	-2.994	-0.431	0.135
8	Elsberg	2 d	1.039	-0.678	-0.248
9	Elsberg	24 d	0.291	-0.628	-0.269
10	Elsberg	42 d	-0.001	-0.596	0.001
11	Elsberg	63 d	-0.180	-0.669	-0.250
12	Elsberg	91 d	-0.257	-0.649	-0.195
13	Elsberg	120 d	-0.506	-0.729	-0.071
14	Elsberg	150 d	-0.505	-0.700	0.152
15	Elsberg	182 d	-0.642	-0.654	0.032
16	Gouda	1 d	1.127	-0.670	-0.387

17	Gouda	17 d	1.052	-0.595	-0.365
18	Gouda	31 d	0.841	-0.466	-0.424
19	Gouda	50 d	0.688	-0.376	-0.513
20	Gouda	63 d	0.651	-0.388	-0.571
21	Gouda	91 d	0.417	-0.301	-0.647
22	Gouda	120 d	0.267	-0.250	-0.624
23	Gouda	150 d	0.161	-0.189	-0.667
24	Gouda	182 d	-0.058	-0.175	-0.644
25	Mozzarella	1 d	1.104	0.126	2.858
26	Mozzarella	14 d	1.018	0.515	2.821
27	Mozzarella	30 d	0.854	0.919	2.516
28	Mozzarella	63 d	0.404	1.922	0.527
29	Mozzarella	91 d	0.158	2.238	-1.086
30	Mozzarella	133 d	-0.030	3.000	-0.485
31	Mozzarella	182 d	-0.774	3.379	-0.597
32	Swiss	1 d	1.143	-0.641	-0.519
33	Swiss	14 d	0.918	-0.432	-0.485
34	Swiss	35 d	0.595	-0.177	-0.978
35	Swiss	91 d	0.246	-0.029	-0.882
36	Swiss	120 d	0.115	0.066	-0.959
37	Swiss	150 d	-0.005	0.069	-0.935
38	Swiss	182 d	-0.074	0.284	-0.983

PC1 (47%) v PC2 (30%) v PC3 (8%)



7.8.4.4 PCA of the covariance matrix – without Mozzarella cheese data (RP-HPLC/WSF)

Unrotated Factor Loadings (Factor-variable correlations) and Communalities

Peak	Factor 1	Factor 2	Factor 3	Communality
1	-0.836	-0.145	-0.429	0.904
2	-0.646	0.170	0.199	0.485
3	-0.964	0.072	0.153	0.957
4	-0.895	-0.020	0.333	0.912
5	-0.973	-0.009	0.115	0.960
6	-0.377	0.493	-0.045	0.387
7	-0.907	-0.162	0.086	0.856
8	-0.921	0.022	0.165	0.876
9	-0.958	-0.146	0.135	0.958
10	-0.929	-0.126	0.092	0.888
11	-0.958	-0.143	0.198	0.977
12	-0.908	-0.265	0.142	0.915
13	-0.940	-0.059	0.296	0.974
14	-0.896	-0.051	0.022	0.806
15	-0.898	0.003	0.159	0.831
16	-0.968	-0.058	0.043	0.943
17	-0.939	0.190	0.027	0.918
18	-0.564	0.544	-0.270	0.687
19	-0.889	0.160	-0.074	0.822
20	-0.579	0.712	-0.279	0.919
21	-0.920	-0.206	0.106	0.900
22	-0.913	0.147	0.001	0.855
23	-0.964	-0.055	0.092	0.941
24	-0.765	-0.388	0.077	0.741
25	-0.757	0.528	-0.235	0.908
26	-0.406	0.717	-0.409	0.845
27	-0.923	0.241	-0.248	0.971
28	-0.900	0.215	-0.268	0.928
29	-0.701	-0.075	-0.237	0.553
30	-0.942	-0.171	0.005	0.916
31	-0.913	0.016	-0.029	0.835
32	-0.883	0.153	0.069	0.808
33	-0.882	0.128	0.033	0.796
34	-0.895	0.222	0.058	0.853
35	-0.806	0.207	-0.126	0.709
36	-0.501	0.358	-0.524	0.653
37	-0.527	0.666	-0.381	0.867
38	-0.874	0.376	-0.218	0.954
39	-0.445	0.592	-0.136	0.566
40	-0.808	0.398	0.146	0.833
41	-0.248	0.822	-0.481	0.969
42	-0.834	0.160	0.050	0.725
43	-0.661	0.466	-0.484	0.888
44	-0.869	-0.133	-0.129	0.789
45	-0.612	-0.432	-0.632	0.961
46	-0.578	-0.556	-0.533	0.928
47	-0.565	-0.463	-0.472	0.757
48	-0.592	-0.317	-0.286	0.533
Varaince	9.72E+09	1.27E+09	1.11E+09	1.21E+10
% Variance	73.12	9.54	8.35	91.00

Factor Score Coefficients

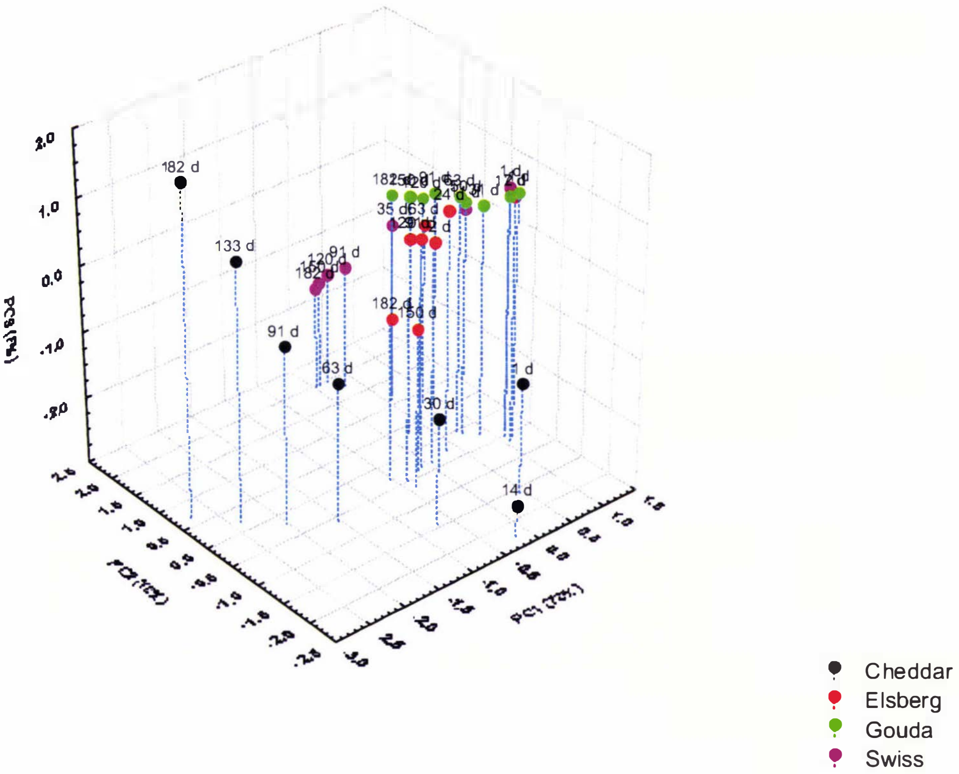
Peak	Factor 1	Factor 2	Factor 3
1	-0.0000015	-0.0000014	-0.0000003
2	-0.0000005	-0.0000028	-0.0000010
3	-0.0000030	-0.0000017	0.0000002
4	-0.0000004	-0.0000045	0.0000006
5	-0.0000049	-0.0000041	0.0000003
6	-0.0000003	-0.0000055	0.0000012
7	-0.0000008	-0.0000045	0.0000010
8	-0.0000010	-0.0000049	0.0000019
9	-0.0000007	-0.0000010	0.0000036
10	-0.0000018	-0.0000003	0.0000025
11	-0.0000023	-0.0000020	0.0000054
12	-0.0000020	0.0000010	0.0000041
13	-0.0000032	0.0000017	0.0000166
14	-0.0000006	-0.0000001	0.0000026
15	-0.0000022	-0.0000003	0.0000086
16	-0.0000012	0.0000026	-0.0000025
17	-0.0000032	-0.0000011	-0.0000093
18	-0.0000006	0.0000002	-0.0000138
19	-0.0000009	-0.0000009	-0.0000025
20	-0.0000005	-0.0000019	-0.0000005
21	-0.0000006	-0.0000027	-0.0000069
22	-0.0000004	-0.0000046	0.0000014
23	-0.0000006	-0.0000015	0.0000042
24	-0.0000002	-0.0000003	0.0000014
25	-0.0000007	0.0000000	0.0000050
26	-0.0000003	-0.0000006	-0.0000003
27	-0.0000013	0.0000050	0.0000006
28	-0.0000011	0.0000041	0.0000015
29	-0.0000003	0.0000012	0.0000009
30	-0.0000007	0.0000050	0.0000016
31	-0.0000011	-0.0000010	0.0000042
32	-0.0000004	0.0000006	0.0000028
33	-0.0000003	-0.0000003	0.0000088
34	-0.0000006	-0.0000007	0.0000001
35	-0.0000005	0.0000036	0.0000034
36	-0.0000003	0.0000037	0.0000005
37	-0.0000004	0.0000025	0.0000008
38	-0.0000007	0.0000021	-0.0000023

Factor Scores

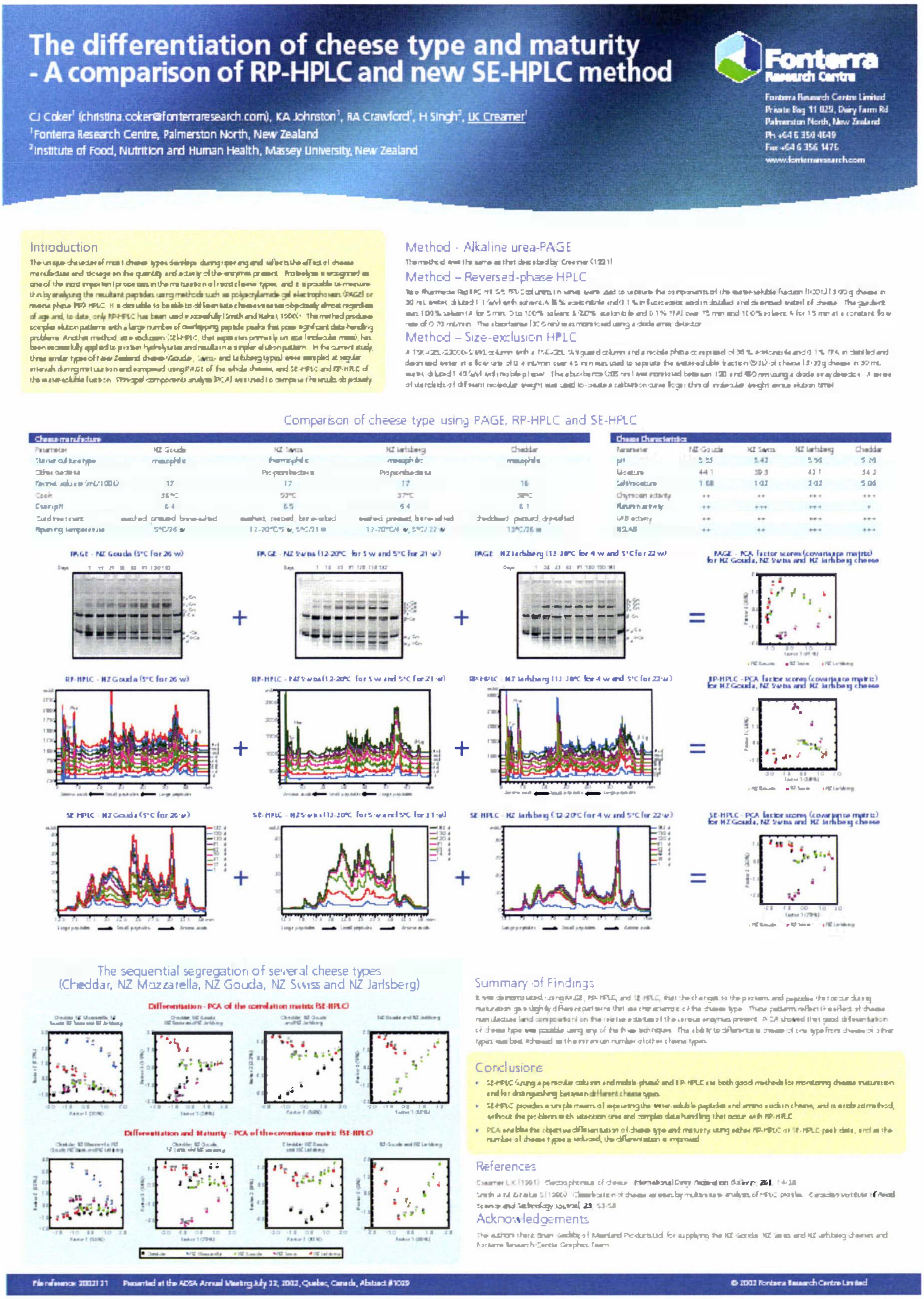
	Cheese	Ripening time	Factor 1	Factor 2	Factor 3
1	Cheddar	1 d	0.449	-1.592	-1.334
2	Cheddar	14 d	-0.162	-2.280	-2.528
3	Cheddar	30 d	-0.683	-1.440	-1.397
4	Cheddar	63 d	-1.524	-0.575	-0.902
5	Cheddar	91 d	-2.018	-0.202	-0.320
6	Cheddar	133 d	-2.385	0.196	0.905
7	Cheddar	182 d	-2.768	0.685	1.963
8	Elsberg	2 d	1.067	-0.624	0.739
9	Elsberg	24 d	0.360	-0.264	0.671
10	Elsberg	42 d	0.074	-0.360	0.363
11	Elsberg	63 d	-0.081	-0.338	0.680
12	Elsberg	91 d	-0.155	-0.372	0.527
13	Elsberg	120 d	-0.395	-0.471	0.680
14	Elsberg	150 d	-0.397	-0.638	-0.566
15	Elsberg	182 d	-0.524	-0.311	-0.534
16	Gouda	1 d	1.157	-0.567	0.738
17	Gouda	17 d	1.084	-0.498	0.676
18	Gouda	31 d	0.885	-0.257	0.513

19	Gouda	50 d	0.744	-0.092	0.543
20	Gouda	63 d	0.710	-0.008	0.621
21	Gouda	91 d	0.491	0.195	0.651
22	Gouda	120 d	0.347	0.252	0.617
23	Gouda	150 d	0.247	0.364	0.642
24	Gouda	182 d	0.039	0.432	0.707
25	Swiss	1 d	1.176	-0.389	0.729
26	Swiss	14 d	0.954	0.192	0.241
27	Swiss	35 d	0.664	1.227	-0.365
28	Swiss	91 d	0.324	1.767	-1.141
29	Swiss	120 d	0.203	1.985	-1.300
30	Swiss	150 d	0.090	1.991	-1.374
31	Swiss	182 d	0.028	1.992	-1.446

PC1 (73%) v PC2 (10%) v PC3 (8%)



7.8.5 Poster Presentation - ADSA Symposium 2002



The differentiation of cheese type and maturity - A comparison of RP-HPLC and new SE-HPLC method

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Introduction

Method - Alkaline urea-PAGE

Method – Reversed-phase HPLC

Method – Size-exclusion HPLC

Comparison of cheese type using PAGE, RP-HPLC and SE-HPLC

Cheese manufacture

Cheese Characteristics

The sequential segregation of several cheese types (Cheddar, NZ Mozzarella, NZ Gouda, NZ Swiss and NZ Jarlsberg)

Differentiation - PCA of the correlation matrix (SE-HPLC)

Differentiation and Maturity - PCA of the covariance matrix (SE-HPLC)

Summary of Findings

Conclusions

References

Acknowledgements

File reference: 2002131 Presented at the ADSA Annual Meeting July 22, 2002, Quebec, Canada, Abstract #1029

8.8 APPENDIX – CHAPTER 8

8.8.1 Sensory evaluation of cheese

Twelve members of the trained cheese panel at NZDRI evaluated the sensory properties of the cheese.

Each cheese sample was evaluated twice. At each sensory evaluation session, all of the panellists evaluated the same set of six samples. The random function in Minitab (v. 12) was used to select the samples for evaluation in each sensory session. Each panellist received the 6 samples in a different randomised order designed using the random function in Minitab (v. 12). Every sample that was presented to every panellist had a unique three digit random code. These random codes were assigned using Compusense version 5.

The panellists were asked to rate the intensity of 3 texture attributes, 16 flavour attributes and an overall maturity attribute. The panel had been previously trained to evaluate all of these attributes.

The data was collected using Compusense 5.

Sample preparation

A 1-2 kg portion of cheese was cut from the 20 kg block, vacuum-sealed, labelled and stored at –2°C. Samples required for each day were stored at 4°C overnight. The outer 0.5 cm layer of each cheese portion was removed and the samples were cut into approximately 2.0 cm cubes using a cheese cutter (Nemco model N55300A, Nemco INC, Hicksville, Ohio, USA). The cubes were placed in a plastic bag (ziplock 200 mm x 255 mm x 40 μm), sealed and stored at 12°C for at least one hour until required for sensory analysis. Three cubes were presented to each panellist in plastic cups (Lily, 21106-60T) labelled with the appropriate three digit random code. After each sample the panellists had a time delay of 2 minutes before evaluating the next sample. During this time delay the panellists were provided with filtered water at 24°C, peeled carrot sticks and soda water at room temperature to rinse their mouths.

All sensory evaluations took place in the individual sensory booths at the FRC sensory evaluation facility using red lights to mask any colour differences between the samples and a constant air temperature of approximately 20°C.

8.8.2 Sample code, factory, age, ripening temperature, MI, SMS, pH, TN, WSN, WSN/TN, NPN, and NPN/TN for each of 77 Cheddar cheeses

Sample	Factory	Age (months)	Ripening Temp (°C)	Maturity Index	SMS	pH	TN (%w/w)	WSN (%w/w)	WSN/TN	NPN (%w/w)	NPN/TN
4311	Southland	3	5	15	2.79	5.17	3.78	0.44	0.12	0.44	0.12
4314	Stirling	3	5	15	4.23	5.17	3.75	0.47	0.12	0.43	0.12
4317	Stirling	3	5	15	4.50	5.11	3.77	0.45	0.12	0.37	0.10
4320	Stirling	3	5	15	3.04	5.29	3.75	0.43	0.12	0.32	0.08
4343	Marlborough	3	5	15	2.21	5.59	4.17	0.50	0.12	0.43	0.10
4344	Marlborough	3	5	15	2.27	5.57	4.10	0.38	0.09	0.41	0.10
4347	Marlborough	3	5	15	2.42	5.6	4.06	0.39	0.10	0.37	0.09
4312	Southland	3	10	30	3.74	5.34	3.90	0.50	0.13	0.50	0.13
4315	Stirling	3	10	30	5.08	4.99	3.81	0.58	0.15	0.45	0.12
4318	Stirling	3	10	30	4.88	5.1	3.81	0.54	0.14	0.41	0.11
4321	Stirling	3	10	30	4.29	5.12	3.75	0.53	0.14	0.40	0.11
4335	Waitoa	6	5	30	3.56	5.24	3.77	0.53	0.14	0.45	0.12
4337	Waitoa	6	5	30	3.05	5.5	3.89	0.54	0.14	0.48	0.12
4338	Waitoa	6	5	30	4.36	5.32	3.73	0.58	0.16	0.49	0.13
4342	Marlborough	3	10	30	2.58	5.61	4.11	0.54	0.13	0.48	0.12
4345	Marlborough	3	10	30	1.83	5.58	4.18	0.49	0.12	0.44	0.10
4348	Marlborough	3	10	30	2.35	5.34	4.05	0.57	0.14	0.43	0.11
4356	Kiwi	3	10	30	3.08	5.5	4.00	0.67	0.17	0.56	0.14
4357	Kiwi	3	10	30	3.52	5.5	3.99	0.67	0.17	0.56	0.14
4364	Marlborough	6	5	30	1.96	5.53	4.23	0.51	0.12	0.61	0.14
4365	Marlborough	6	5	30	2.42	5.63	4.06	0.55	0.14	0.61	0.15
4366	Marlborough	6	5	30	2.54	5.48	4.16	0.58	0.14	0.64	0.15
4369	Hautapu	3	10	30	3.75	5.34	3.67	0.59	0.16	0.44	0.12
4370	Hautapu	3	10	30	3.83	5.23	3.75	0.61	0.16	0.42	0.11
4371	Hautapu	3	10	30	3.92	5.18	3.80	0.58	0.15	0.49	0.13
4372	Hautapu	3	10	30	4.08	5.32	3.86	0.59	0.15	0.50	0.13
4373	Hautapu	3	10	30	3.68	5.4	3.92	0.61	0.16	0.47	0.12
4374	Hautapu	3	10	30	3.18	5.39	3.95	0.57	0.14	0.46	0.12
4423	Southland	3	10	30	4.17	5.22	3.74	0.50	0.13	0.50	0.13
4424	Southland	3	10	30	3.38	5.45	3.77	0.48	0.13	0.49	0.13
4313	Southland	3	13	39	3.71	5.25	3.75	0.57	0.15	0.54	0.14
4316	Stirling	3	13	39	4.45	5.05	3.84	0.61	0.16	0.47	0.12
4319	Stirling	3	13	39	4.25	5.17	3.67	0.59	0.16	0.44	0.12
4322	Stirling	3	13	39	4.52	5.13	3.79	0.61	0.16	0.44	0.12
4341	Marlborough	3	13	39	3.80	5.48	4.25	0.64	0.15	0.53	0.13
4346	Marlborough	3	13	39	2.68	5.51	4.10	0.63	0.15	0.47	0.11
4349	Marlborough	3	13	39	2.28	5.57	4.14	0.57	0.14	0.48	0.12
4358	Kiwi	6	10	60	5.96	5.31	3.88	0.86	0.22	0.67	0.17
4359	Kiwi	6	10	60	3.69	5.32	3.99	0.86	0.22	0.69	0.17
4063	Southland	12	5	60	6.08	5.19	3.85	0.83	0.22	0.67	0.17
4066	Southland	12	5	60	4.96	5.41	3.85	0.75	0.19	0.62	0.16
4075	Waitoa	12	5	60	4.39	5.54	3.76	0.80	0.21	0.65	0.17
4078	Waitoa	12	5	60	5.12	5.38	3.76	0.85	0.23	0.67	0.18
4323	Stirling	6	10	60	3.92	5.28	3.87	0.85	0.22	0.78	0.20
4324	Stirling	6	10	60	5.30	5.22	3.88	0.82	0.21	0.74	0.19
4325	Stirling	6	10	60	3.00	5.47	3.88	0.88	0.23	0.66	0.17
4332	Southland	6	10	60	4.91	5.28	3.88	0.78	0.20	0.78	0.20
4333	Southland	6	10	60	4.88	5.29	3.86	0.82	0.21	0.73	0.19
4334	Southland	6	10	60	5.00	5.26	3.84	0.80	0.21	0.65	0.17
4336	Waitoa	6	10	60	4.57	5.22	3.75	0.68	0.18	0.50	0.13
4360	Waitoa	6	10	60	4.52	5.2	3.76	0.72	0.19	0.53	0.14
4361	Lichfield	6	10	60	3.63	5.38	3.86	0.82	0.21	0.82	0.21
4362	Lichfield	6	10	60	5.50	5.17	3.54	0.86	0.24	0.72	0.20
4363	Waitoa	6	10	60	3.29	5.27	3.77	0.61	0.16	0.45	0.12
4368	Lichfield	6	10	60	4.83	5.2	3.86	0.79	0.20	0.79	0.21
4420	Alpine	6	10	60	4.04	5.2	3.90	0.74	0.19	0.60	0.15
4421	Alpine	6	10	60	3.46	5.51	3.86	0.81	0.21	0.59	0.15
4422	Alpine	6	10	60	3.58	5.3	3.91	0.78	0.20	0.60	0.15
4326	Southland	15	5	75	5.00	5.51	3.87	0.88	0.23	0.74	0.19
4327	Southland	15	5	75	5.16	5.39	3.90	0.85	0.22	0.71	0.18
4339	Waitoa	15	5	75	5.84	5.19	3.87	0.90	0.23	0.70	0.18
4340	Waitoa	15	5	75	5.71	5.1	3.77	0.88	0.23	0.67	0.18
4309	Southland	6	13	78	6.16	5.22	3.77	0.82	0.22	0.86	0.23
4310	Southland	6	13	78	4.72	5.21	3.84	0.75	0.19	0.76	0.20
4074	Waitoa	12	10	120	6.53	5.29	3.78	1.02	0.27	0.82	0.22
4077	Waitoa	12	10	120	6.78	5.2	3.75	1.03	0.27	0.84	0.23
4079	Southland	12	10	120	6.92	5.29	3.86	1.01	0.26	0.85	0.22
4367	Waitoa	15	10	150	5.00	5.49	3.65	0.93	0.26	0.67	0.18
4061	Southland	12	13	156	7.60	5.35	3.88	1.12	0.29	0.98	0.25
4064	Southland	12	13	156	7.96	5.37	3.87	1.13	0.29	1.01	0.26
4081	Waitoa	12	13	156	7.73	5.58	3.82	1.16	0.30	0.95	0.25
4083	Waitoa	12	13	156	7.79	5.44	3.77	1.14	0.30	0.94	0.25
4328	Waitoa	12	13	156	7.38	5.32	3.77	1.17	0.31	1.08	0.29
4329	Waitoa	12	13	156	7.17	5.23	3.73	1.13	0.30	1.08	0.29
4330	Waitoa	12	13	156	7.61	5.07	3.76	1.14	0.30	1.09	0.29
4331	Southland	12	13	156	7.54	5.27	3.91	1.07	0.27	0.97	0.25
4350	Southland	12	13	156	7.96	5.21	3.88	1.13	0.29	0.98	0.25

8.8.3 RP-HPLC

8.8.3.1 RP-HPLC peak area data

	Factory	Storage (T°C)	Storage time	SMS	pH	TN	P1	P2	P3	P4	P5	P6	P7	P8	P9
1	Southland	13	12	7.60	5.35	3.88	77196	53489	14698	73514	19214	5775	4769	13372	11976
2	Southland	5	12	6.08	5.19	3.85	47507	27137	12824	51984	10250	8225	7377	12876	6225
3	Southland	13	12	7.96	5.37	3.87	79801	50602	16422	71303	23867	4112	6387	13649	12816
4	Southland	5	12	4.96	5.41	3.85	46681	26803	10459	49946	10818	7157	6350	12931	6234
5	Waitoa	10	12	6.53	5.29	3.78	56409	31256	11623	58179	8562	7289	7662	14411	7638
6	Waitoa	5	12	4.39	5.54	3.76	35607	18168	6308	41077	6518	6561	5974	9923	4885
7	Waitoa	10	12	6.78	5.20	3.75	55896	28719	14284	51451	8501	9713	8362	13986	8852
8	Waitoa	5	12	5.12	5.38	3.76	37098	20053	8091	43942	5324	8123	6311	10227	5639
9	Southland	10	12	6.92	5.29	3.86	63115	39962	12442	65239	13612	5384	6735	14917	6959
10	Waitoa	13	12	7.73	5.58	3.82	67642	37943	19081	60760	12226	9346	9229	16145	10828
11	Waitoa	13	12	7.79	5.44	3.77	67341	38301	16882	63569	11889	8173	9086	17258	9795
12	Southland	13	6	6.16	5.22	3.77	56676	33131	11778	57422	13868	5305	5205	15753	6483
13	Southland	13	6	4.72	5.21	3.84	58399	32280	9281	54700	15400	3962	4738	11884	6767
14	Southland	5	3	2.79	5.17	3.78	23609	10435	5113	25389	2682	5627	3926	6908	3512
15	Southland	10	3	3.74	5.34	3.90	29591	12738	5245	31297	2578	5148	3286	7516	4168
16	Southland	13	3	3.71	5.25	3.75	34620	17778	6296	38147	4958	5685	3457	10068	4508
17	Stirling	5	3	4.23	5.17	3.75	22859	12404	4611	30088	3428	4481	8049	6337	3485
18	Stirling	10	3	5.08	4.99	3.81	31598	18545	7854	40669	6540	5749	11401	9155	5044
19	Stirling	13	3	4.45	5.05	3.84	27622	14684	5777	36295	4666	4305	8795	7400	4243
20	Stirling	5	3	4.50	5.11	3.77	20399	10485	5109	27111	2648	4678	8434	5682	3134
21	Stirling	10	3	4.88	5.10	3.81	28652	16905	6786	40342	3804	5999	8774	8724	4839
22	Stirling	13	3	4.25	5.17	3.67	39135	22724	7267	48580	8223	5322	8593	10272	5778
23	Stirling	5	3	3.04	5.29	3.75	22465	11536	2211	26798	2842	4117	4493	5165	3559
24	Stirling	10	3	4.29	5.12	3.75	29481	16825	6284	38816	4443	5165	8700	8292	4790
25	Stirling	13	3	4.52	5.13	3.79	35899	24001	4033	47339	6935	4703	7139	9706	5832
26	Stirling	10	6	3.92	5.28	3.87	47309	32824	5281	50698	7372	3408	9517	12237	7636
27	Stirling	10	6	5.30	5.22	3.88	47230	32278	7348	57257	8451	4236	8505	13203	7077
28	Stirling	10	6	3.00	5.47	3.88	45790	29085	4330	54148	10422	2807	5170	10442	7208
29	Southland	5	15	5.00	5.51	3.87	52618	30216	4726	53020	10353	4001	4698	9831	6673
30	Southland	5	15	5.16	5.39	3.90	54954	31551	4842	54300	12252	2777	4559	9282	6266
31	Waitoa	13	12	7.38	5.32	3.77	66311	40119	13472	64450	11964	6881	7814	16563	9559
32	Waitoa	13	12	7.17	5.23	3.73	67555	36960	12853	60214	14386	5765	8410	16675	8970
33	Waitoa	13	12	7.61	5.07	3.76	68845	38421	16723	60627	15904	7739	9044	17992	9636
34	Southland	13	12	7.54	5.27	3.91	74631	52342	14166	70613	20234	5655	5334	14264	11669
35	Southland	10	6	4.91	5.28	3.88	50125	27648	9700	52272	11798	4730	4825	13113	6606
36	Southland	10	6	4.88	5.29	3.86	53905	28684	11585	53142	11425	5792	5954	15491	6239
37	Southland	10	6	5.00	5.26	3.84	53937	30205	11003	54479	12400	5381	5164	15656	6307
38	Waitoa	5	6	3.56	5.24	3.77	24787	13039	4144	29617	3356	7653	5168	6797	3977
39	Waitoa	10	6	4.57	5.22	3.75	35549	19922	7303	41493	7098	6663	7340	11053	6181
40	Waitoa	5	6	3.05	5.50	3.89	29511	15700	6079	34091	5446	7837	6812	8882	4477
41	Waitoa	5	6	4.36	5.32	3.73	30201	18745	8248	39921	6452	9160	7591	10869	4897
42	Waitoa	5	15	5.84	5.19	3.87	42621	24676	10740	46228	7585	7686	11341	12155	7197
43	Waitoa	5	15	5.71	5.10	3.77	42776	25351	11906	49883	5538	9283	12054	12079	6756
44	Marlborough	13	3	3.80	5.48	4.25	41772	22920	7385	52819	4479	6518	6377	10453	6634
45	Marlborough	10	3	2.58	5.61	4.11	32138	16500	3620	41130	3906	4559	4445	6876	5400
46	Marlborough	5	3	2.21	5.59	4.17	21705	10776	1988	28091	3225	4699	3935	4638	3561
47	Marlborough	5	3	2.27	5.57	4.10	20073	7952	3667	24652	2761	5008	3611	5200	3378
48	Marlborough	10	3	1.83	5.58	4.18	31424	16169	5266	39578	6042	5095	5174	8683	4892
49	Marlborough	13	3	2.68	5.51	4.10	37301	20016	6626	45996	4793	6523	5812	9948	6352
50	Marlborough	5	3	2.42	5.60	4.06	18554	6618	3044	21601	1823	4278	2910	4001	3105
51	Marlborough	10	3	2.35	5.34	4.05	30754	14930	4967	38534	3350	5889	4783	7472	5146
52	Marlborough	13	3	2.28	5.57	4.14	35953	20152	3531	45672	5419	5915	5778	10132	5799
53	Southland	13	12	7.96	5.21	3.88	74271	50892	13995	70951	17815	6589	4973	14107	11648
54	Kiwi	10	3	3.08	5.50	4.00	35506	19570	6880	42801	7406	7446	6289	10628	5641
55	Kiwi	10	3	3.52	5.50	3.99	38429	20919	7362	45145	7745	7316	5785	10822	5962
56	Kiwi	10	6	5.96	5.31	3.88	50766	29406	14879	52816	7776	11277	8098	15589	7505
57	Kiwi	10	6	3.69	5.32	3.99	48954	29734	13469	52591	10744	9827	7825	14911	7054
58	Waitoa	10	6	4.52	5.20	3.76	36896	22585	8912	46006	7934	7489	8277	11878	6278
59	Lichfield	10	6	3.63	5.38	3.86	42455	22890	9956	49194	4699	9158	6036	11489	7153
60	Lichfield	10	6	5.50	5.17	3.54	45278	23635	12154	42919	4690	8599	8736	11088	9055
61	Waitoa	10	6	3.29	5.27	3.77	29985	16768	5974	37277	5501	7431	6250	9662	4842
62	Marlborough	5	6	1.96	5.53	4.23	29459	11969	3983	28979	4561	4025	3349	6305	3762
63	Marlborough	5	6	2.42	5.63	4.06	26112	10476	1932	26862	3387	3438	3037	4505	3804
64	Marlborough	5	6	2.54	5.48	4.16	28521	13961	3497	33050	3068	5481	3166	5752	4167
65	Waitoa	10	15	5.00	5.49	3.65	44410	24731	4420	48832	10963	3815	7354	11423	6084
66	Lichfield	10	6	4.83	5.2	3.86	37137	20732	9759	45815	4329	8407	7175	10746	6923
67	Hautapu	10	3	3.75	5.34	3.67	30697	16098	6563	38815	4862	6608	5708	9512	5024
68	Hautapu	10	3	3.83	5.20	3.75	27730	14647	6390	36200	3825	6885	6043	8507	4927
69	Hautapu	10	3	3.92	5.18	3.80	24711	12812	6082	32334	3498	6474	5604	7682	4503
70	Hautapu	10	3	4.08	5.32	3.86	26163	14728	7219	35879	3946	7400	5561	8649	4697
71	Hautapu	10	3	3.68	5.40	3.92	23911	13138	5497	32631	3719	5915	5339	7493	4554
72	Hautapu	10	3	3.18	5.39	3.95	24044	12743	5664	32558	3462	6081	5163	7445	4360
73	Alpine	10	6	4.04	5.20	3.90	39222	24244	7906	48077	9467	4906	10360	12296	6327
74	Alpine	10	6	3.46	5.51	3.86	38718	19986	6294	42677	7816	6367	5791	12141	5687
75	Alpine	10	6	3.58	5.30	3.91	32687	19409	6208	42947	5624	6335	6915	11229	5786
76	Southland	10	3	4.17	5.22	3.74	29001	14133	5496	32356	2389	5763	2835	7548	4809
77	Southland	10	3	3.38	5.45	3.77	27608	12845	5536	30817	2890	5672	3751	7734	4263

	P10	P11	P12	P13	P14	P15	P16	P17	P18	P19	P20	P21	P22	P23	P24
1	10405	7023	11699	18668	17559	8789	14579	63067	39002	17275	16345	7391	14123	7727	5553
2	7278	5453	7193	14332	12447	7019	29970	41210	31172	18248	17399	15429	15353	8797	6078
3	10780	7304	12122	20090	15962	8006	21345	61291	40085	19100	18238	10086	15173	7249	5717
4	10277	5066	5050	15377	11800	6820	21936	38474	27937	18020	16950	15605	14179	9111	4914
5	8235	5111	10201	15524	15882	8603	36378	38707	33488	23090	22881	15646	19899	12372	6468
6	9380	4138	5011	12258	9744	5419	21839	24248	18554	18378	19638	18534	18590	12083	7006
7	6060	5934	13337	15213	16122	9213	39764	44808	32952	25462	25957	17997	22100	12994	9663
8	7369	4443	6374	14648	10705	5565	26123	30487	20559	20484	20765	19501	21488	11505	9978
9	17216	3757	6532	18767	22037	10434	14608	50413	39036	23073	20663	10642	12497	13823	4816
10	7258	6949	17591	18036	18250	10842	42484	52878	39447	27805	25348	15489	21734	13008	6778
11	9211	6356	14449	18043	18144	11698	38707	47845	36685	26299	25500	16469	20573	13638	6109
12	12771	5517	6169	17391	12430	6473	20414	41995	30986	17609	17663	11754	13438	7886	5156
13	15330	3048	5153	14609	12026	6397	11995	38122	31334	14784	14658	7953	8915	8730	4670
14	5901	1975	3151	7974	5495	3873	13460	16500	10580	11233	10954	9217	11118	7135	3972
15	6672	2751	2758	9044	5756	3796	13864	17431	11897	11956	11413	8700	11254	7425	3911
16	9450	3736	3514	11697	7918	4346	14629	22083	16875	13920	13672	9422	12155	7971	4067
17	3778	2740	3561	3691	9436	3262	24273	15619	12848	11951	11660	12711	12371	7632	7521
18	4214	3996	8309	10237	9326	5057	33194	25661	22182	16463	14998	15323	15904	9956	7331
19	3812	3140	5289	3961	11424	4037	28132	19766	17046	13580	12197	12815	13501	8649	7072
20	3308	2609	4205	3556	9007	3511	25418	16923	11925	12203	12056	12798	13320	7063	10661
21	4585	3665	6142	10461	8239	4913	30240	24036	20494	16164	15473	14776	16052	9592	7955
22	5576	3935	6978	5285	16802	4892	31710	23996	25992	17432	14152	12449	15499	10133	5358
23	4033	2371	2425	3477	7526	2434	16896	11489	9388	10927	9701	9172	11014	7286	5152
24	4009	3289	6427	4488	13194	4443	30261	23145	20552	15113	14151	12528	15133	8966	7645
25	6013	3391	5745	5225	15964	3878	28515	21038	24349	16721	13576	11016	14963	9371	4803
26	4181	4636	6998	10503	15176	5498	38619	31961	28769	17692	16189	8826	18705	6159	5595
27	4940	2677	7551	10904	15242	5123	32532	28160	29762	15953	13318	8865	14424	6276	3926
28	6827	2467	2128	11740	18201	3118	17027	19609	24485	15420	11218	7195	13210	4454	2727
29	11410	2513	5472	13484	13069	4888	17705	29996	29924	16968	14050	10138	11326	9290	4438
30	14685	3122	8755	7507	16612	4359	1908	34372	31614	16292	13802	7388	9838	9166	3836
31	9230	5278	11171	18266	18929	10502	37412	41160	41319	26588	21678	14475	19113	14512	5721
32	9835	4889	10877	16618	19131	9445	36110	36131	40702	26623	21869	14444	18627	14431	5522
33	10274	5957	15153	17820	18488	11454	37358	46001	38687	27318	25327	16001	20535	13815	6177
34	9604	5456	9652	19036	17203	8793	26258	58252	41570	19392	16832	8551	10897	13100	5371
35	12509	4428	5304	16110	11353	5712	18921	35187	27790	16615	16137	10972	12954	7301	4671
36	11583	5286	6422	18315	11743	6879	21571	40473	25813	19142	19859	14794	15616	10039	5334
37	12148	4683	6026	17246	11546	6458	21604	39227	28499	17588	18797	11972	13027	8353	5925
38	4383	1980	4777	6756	7231	3536	20243	18428	8676	14816	13678	12580	14465	6241	6481
39	5752	4214	6705	11019	8878	6236	28434	26278	17977	18571	19506	16882	17213	10590	6563
40	6853	2420	4783	4940	13336	5031	18099	19207	11445	15232	15096	15896	12248	8750	3841
41	7669	2658	5503	12840	9297	5592	21137	22231	15118	15346	14856	16613	12520	7733	3463
42	5162	5259	9557	14154	12951	7051	39264	34062	27855	22613	22592	20413	20736	12026	9607
43	5326	5332	9968	16190	13737	7062	38243	34118	25703	21998	21811	18838	20736	10716	8430
44	9233	5520	6462	15257	11234	4321	24960	22619	20536	18669	17446	14163	18402	12711	5750
45	6413	3441	4191	7796	8758	3076	23700	15887	12358	14268	12315	11902	17677	10359	8069
46	5267	2977	2658	3691	7882	2475	17924	9855	7661	12496	11349	10127	15117	8883	9762
47	6287	3164	1899	3831	7545	2322	12062	10211	6768	10153	9857	8869	10628	7601	3895
48	9944	4901	3660	6403	13217	3030	17460	14298	16585	13629	13195	12955	15573	11125	4476
49	8052	4614	6153	12004	11745	4672	26233	20693	17250	18487	18528	16165	22495	12787	9137
50	5020	2294	2113	3687	5962	2195	11088	10609	5021	11256	10540	7346	13481	6538	8556
51	6012	3197	5173	9423	9418	3910	19956	20412	10986	18189	16555	12610	25226	10349	15461
52	9245	4836	5287	11826	10685	4718	23754	19309	17623	16835	17465	16091	20828	12656	7178
53	10846	5915	9115	21795	18936	8923	27152	61531	40174	21306	19395	9618	12439	13306	5651
54	11672	5197	5097	12626	10851	6759	19640	23221	17592	17474	19934	17107	17245	11961	5181
55	11447	5134	4688	15009	10515	5959	19710	25004	20085	17169	18001	15230	16633	11078	4882
56	9313	6716	9113	18152	14029	8579	31066	41227	24263	23947	22767	23982	20957	12322	5435
57	10591	6842	5295	18795	13347	7951	25618	34749	25301	21032	18857	23249	18838	11363	3760
58	5678	4747	7115	12146	9848	6443	30861	28068	22711	17995	17308	15745	16580	9859	5238
59	8084	5097	7417	15984	13399	6592	31635	32021	16105	21099	21612	18166	24749	12829	9748
60	5173	4755	11023	17252	17444	7135	40131	39028	19239	23312	23371	16487	25755	12790	18592
61	6466	2602	5499	10256	7039	4617	22138	20554	13578	15526	15874	14939	15269	8487	4487
62	8015	3460	2158	4934	8926	2314	11994	10780	10954	10446	9459	9587	9866	6905	4542
63	6223	2695	2054	3520	6883	2167	15131	8958	6925	11152	9755	8705	12052	8482	5571
64	6039	1843	4059	3879	10877	2835	18582	15398	10198	12852	10772	10310	12900	8947	6355
65	9866	4517	5926	8887	14935	5231	26142	20303	25430	18717	18544	18229	18520	14367	5182
66	6117	4752	6994	16824	11499	6050	35467	29554	17410	20080	20039	17099	23499	12191	10035
67	8856	4036	5656	13142	9483	5826	15499	25990	15888	18660	19897	17343	26774	12986	9201
68	6024	3943	6642	13475	8388	5874	16287	27394	15025	19798	22447	15505	32031	12502	17843
69	5630	3578	5919	12515	7571	5293	14796	24702	11793	20054	20834	13978	30589	11479	17730
70	7226	3781	4982	12803	9656	5081	15306	25675	15063	18286	18513	16558	26910	11610	10855
71	6343	3528	4546	10686	8392	4468	14063	21920	13586	17096	17967	14556	26309	10373	12702
72	6691	3413	4316	10673	8482	4355	13787	21352	13100	16531	16798	14400	24890	11012	11111
73	5782	4405	5743	9283	15735	5493	32653	21976	24202	16672	15432	15089	14813	9362	4901
74	9499	3415	4937	10460	11487	3754	20592	18006	16534	15864	18389	15894	16779	9455	3571
75	6511	2894	6124	10594	9276	4568	26179	21409	18481	16931	17677	15537	17788	9836	4957
76	6057	2403	3077	10163	6919	4081	19737	20817	13506	13152	13112	8063	12739	8263	5677
77	7764	3118	2964	9648	6695	4095	12645	19088	12106	12450	12411	10179	13371	8561	4132

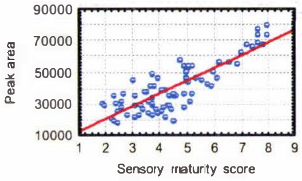
	P25	P26	P27	P28	P29	P30	P31	P32	P33	P34	P35	P36	P37	P38	P39
1	11964	1264	2510	5479	3761	1787	10280	2985	7637	1327	5433	3276	2040	6399	4222
2	10948	3742	2638	7706	8613	1928	8855	2194	8974	3212	9149	4596	3610	7479	5222
3	12153	1633	1796	7608	3737	2095	10874	2545	7423	978	5853	2798	2511	5732	4390
4	11556	4840	3336	4984	8429	2035	8054	2176	8541	2791	7084	4826	3279	7999	6125
5	18088	9404	3304	10607	8808	3458	10379	4770	12884	9374	13974	9001	5509	11073	6743
6	18700	17417	3795	7310	10167	2049	11290	3884	12120	5996	11394	8705	4625	10748	8549
7	17968	8905	3879	13719	10936	4168	10486	6345	13734	11955	17941	10020	5406	12603	6973
8	17944	12989	4121	8527	9062	2645	11955	3347	12988	7730	13577	9891	4569	12597	10016
9	12887	6285	3574	5796	5604	2759	11298	2200	11278	3753	8039	5859	2586	8344	5492
10	17115	5672	3161	14278	9970	3597	10092	5834	11850	7759	15025	5302	5113	10308	4950
11	17591	7105	3626	12214	9726	3509	10669	5716	13156	7579	13869	6580	5840	10723	5726
12	9815	3084	2996	3990	5735	1625	8419	1910	6961	1509	4232	3913	2563	7283	3782
13	8756	2336	2410	3337	4915	1114	8009	1999	6084	1520	3331	3066	2394	6679	3844
14	8396	3983	3725	2239	6587	1038	6059	1076	6684	2264	4340	4764	2077	7547	5183
15	8925	5175	2842	2751	4974	1489	6036	1231	6899	2183	4113	4817	1881	7284	4968
16	9293	4827	3082	3073	4619	1735	7130	1628	7702	1991	4742	4738	1757	8628	5936
17	10396	5202	2437	5452	6422	3233	8386	1967	7936	7018	8821	8360	3326	7908	7289
18	12636	5294	2582	9133	7557	1941	12658	2342	10884	10230	14035	9007	4338	10323	8035
19	11358	4940	2367	6921	6659	1555	11281	2056	9419	7589	10797	8144	3668	8784	8059
20	10065	4271	2790	6202	9267	3269	10417	1802	8823	7578	9269	9025	3678	9116	8108
21	12987	6195	3014	7790	7522	1960	11461	2325	10405	8644	12511	9189	3920	10854	8490
22	13953	6186	3146	7924	6000	2590	10168	2369	10797	9030	13021	9364	3933	10078	7604
23	10787	6566	2324	3669	4645	973	7323	1566	7920	3950	6639	7047	2505	6714	6201
24	12512	5710	2789	7334	6642	1943	11211	2341	9888	8550	11814	8966	3964	10158	8109
25	14839	8224	3425	6074	4869	2482	8650	2476	10512	7892	11498	12909	1574	8265	7559
26	12944	3095	3959	7085	6555	2573	8815	2113	11354	10126	11082	7968	3722	9691	7566
27	11195	3016	3487	4879	5066	2096	7864	2029	9002	8527	8575	7275	2978	7822	5537
28	13735	5114	4481	3053	3268	2896	7194	2076	10125	6247	6519	7630	2353	7125	5804
29	15914	6608	3230	4354	3163	3786	9616	2490	9398	3707	6678	5693	2630	8013	5916
30	15684	6127	3551	3011	1950	4466	10461	2428	8962	2707	4486	4811	1223	7991	4661
31	19925	9912	3841	10407	7829	4110	11151	4839	13411	9132	14150	7066	7059	10992	5846
32	21294	12026	3965	10245	7601	4208	13142	3659	13251	10841	14372	8304	6696	11706	6361
33	18524	8309	3533	12611	9524	3778	13832	3342	13241	8661	13825	7743	5745	11128	5138
34	12531	3082	2657	6645	4736	1531	10285	2185	9171	2697	7226	3939	2523	7493	3539
35	9847	3841	3199	3259	5154	1877	7984	1840	7098	1694	4313	3915	2372	7570	4534
36	11477	4583	3512	4699	7746	1876	8432	2105	8199	1711	6305	4463	3163	8408	5349
37	9615	3142	3028	4048	6346	1705	8607	1978	6941	1395	4301	3697	2781	7323	3988
38	12766	10607	2585	4776	6160	1140	9562	2020	8276	4847	6570	6982	2940	8733	6746
39	15550	12239	3624	6106	8192	1769	9435	2747	9860	6872	8763	8548	3490	10000	7159
40	13940	9418	2985	4926	9436	1139	10737	1966	9122	5105	6875	6178	4073	7518	5910
41	13151	6941	3547	4091	7172	1702	8897	1571	8776	5909	6345	6721	2528	7797	4874
42	18678	10454	3040	12834	9433	3173	9953	6454	14200	12705	16307	10589	5140	11993	8360
43	16975	8806	3665	12011	7786	2902	9177	5896	13638	12167	15367	10132	4603	10901	7241
44	16975	9885	4379	7311	6133	3207	10661	3178	12823	4926	14839	8017	3916	10981	10211
45	17276	10365	3173	6078	5021	1582	11019	3516	12753	6102	12184	7780	3518	9507	7113
46	13589	9210	2314	5365	6269	3467	9894	2882	11086	4187	10825	9132	1326	10247	7883
47	11101	7012	2450	3031	4759	1108	7151	1924	8191	2056	7817	7796	1542	6756	6329
48	15781	9719	4004	4883	5117	2101	8782	3069	11238	3947	11523	6222	3161	9660	7859
49	19337	14027	4470	7717	6883	3695	12145	4218	14515	6766	15186	9577	4112	13105	10104
50	11731	7846	2074	3313	5311	2599	7985	2440	9648	2340	7724	9126	3210	6445	6142
51	17214	12216	4036	6178	7232	4325	10666	4028	13982	5877	14192	9278	3535	12510	10401
52	18179	12967	4142	6643	6761	1724	11927	4087	13097	5704	12313	7950	3965	11670	8529
53	13123	3373	2735	7205	5595	1019	10876	2489	9482	1842	6820	3992	2686	8139	3861
54	15763	11833	4853	5437	9678	1929	10197	2564	11493	3328	10598	7829	4029	11247	8753
55	14741	9591	4627	4568	7590	1997	9543	2196	10691	3376	9740	7474	2886	11154	8637
56	16451	9514	4684	7659	11014	2516	9585	2494	11857	6089	11258	7933	4056	10635	7596
57	15104	10628	4028	6113	9889	2458	8401	2258	11773	6686	10547	7382	4508	10045	6881
58	13866	8604	3286	6488	6964	2112	8606	2209	10236	7530	9198	8153	3363	9140	6264
59	16253	9819	4148	8876	9205	2042	11136	3167	13264	7498	14217	9088	4840	12445	9972
60	17469	5674	4750	12424	11628	3692	9947	2282	17065	10771	16768	13691	4068	13019	10146
61	13987	11301	3176	4607	7092	2025	7435	2098	8829	5382	6748	7989	3004	9460	6844
62	12723	7164	2645	2983	3832	1792	6359	2200	7858	1944	5625	4127	1153	7163	5281
63	14602	9332	2372	3951	3580	1561	8013	3196	10117	2916	6816	5190	2275	6373	4332
64	13169	6777	3161	4497	4575	1953	8145	2153	9882	3644	8211	6298	2212	8187	7202
65	25751	22562	3563	8373	7186	3528	9978	6347	13966	10542	12765	8883	4820	11099	6937
66	15601	7849	3867	9103	9100	2283	11058	2925	13308	9952	14283	10319	4560	12287	9899
67	16399	9663	5093	6129	12372	1662	10132	3234	12259	4482	11906	8763	4777	13334	10091
68	16552	7988	4627	7773	15571	5235	10385	3401	13435	6850	12470	10934	4361	15075	10934
69	16052	7045	4734	6411	16196	5002	10222	3208	12253	5855	11582	9265	4756	14584	11231
70	16344	7206	4685	5484	11258	1539	10823	2799	10834	5317	11493	7961	3786	14078	11666
71	16600	9170	4529	5518	11543	3679	9254	3204	11776	5267	11863	8496	3821	13555	11318
72	15751	8122	4377	5116	10664	1405	11177	2918	10856	4522	11209	7416	3524	13336	10723
73	14570	7813	2713	6352	5572	2346	7873	3734	9934	10963	10306	8178	4095	8706	8112
74	16430	19373	3428	5091	5753	2963	7992	3323	10615	6242	7797	7067	3648	9512	7252
75	15669	16830	3131	5801	5909	2014	8411	2936	10313	7898	8766	7875	3481	10124	7684
76	8298	4534	3704	2526	5129	1569	7315	1634	7242	1959	4908	5009	2153	7352	5846
77	9598	5280	3295	3150	6098	1320	6469	1734	6629	1936	5196	5013	1984	8780	6248

	P40	P41	P42	P43	P44	P45	P46	P47	P48	P49	P50	P51	P52	P53	P54
1	2100	5448	1342	2833	2602	3696	2624	2462	2106	6591	6138	17054	9929	11269	4919
2	2720	5071	1931	1561	3513	3793	2547	4575	826	5022	4545	11878	5008	7349	3268
3	1956	5888	1708	2711	3013	3728	2721	2414	1561	7291	6866	14994	9876	9527	4578
4	3460	4386	1924	1393	3626	2155	2774	2792	2259	4611	4163	9698	4446	5916	3021
5	4693	8566	3733	5119	7890	3340	1881	5685	5485	10149	14700	19572	18619	16306	5679
6	5766	8099	4001	8725	10962	3023	3822	2293	4534	8771	13289	15909	18042	15283	5897
7	4668	10030	3864	4842	9277	7182	3556	2979	2278	10691	12504	19431	16764	16237	5290
8	5434	8828	4124	7700	10766	3114	3731	2513	4798	7889	11156	15989	16859	16440	5475
9	2739	5581	1915	2012	3907	2320	2732	2246	2806	5832	7184	14883	9749	10138	4415
10	3034	8742	2143	3772	6886	7525	3604	1834	3646	10328	12742	21281	17936	16890	6040
11	3589	8277	2618	3681	5625	4633	3297	1675	2234	11727	14462	20938	18775	15865	5912
12	3558	3105	1587	1343	2611	1536	2556	1881	1583	4682	3806	8191	4002	4917	2574
13	2664	3386	1472	1913	1985	1202	2385	1971	1502	5240	4542	9666	4694	5459	2822
14	4509	2648	1345	2546	3007	1012	2268	2355	1810	3211	2142	5250	1702	3419	1864
15	4129	3113	1560	2671	2502	1580	2285	2501	1821	3824	2426	6692	2197	4045	1850
16	5173	2948	1556	1735	2865	2154	2560	3122	2502	3862	4212	10836	3810	6509	2551
17	2431	7826	4279	6795	7195	3676	3413	3144	2976	4578	6006	7461	8561	8742	2086
18	3400	10296	4840	6461	9031	6914	2774	1960	5151	5500	8032	8663	13580	10858	2537
19	3178	9280	4255	6521	8314	5873	2677	2432	4633	5029	7472	9926	10936	11107	2351
20	3473	7653	3954	6111	8590	3988	2394	3030	3123	4554	4373	6566	8404	8362	2044
21	3931	9362	4568	6898	11112	4399	3045	2317	4777	5756	9234	11293	13941	12212	2667
22	3599	7955	6270	7038	9048	5965	2307	1890	5058	5477	10194	12530	13500	12516	2840
23	2868	5389	3145	6687	8609	981	2897	2730	2886	4935	6423	7265	8766	7538	2087
24	3714	9241	4547	6950	9269	6411	2649	2412	4910	5931	8538	10941	12632	11504	2706
25	3917	8849	4902	8466	9949	4407	4012	998	4993	6446	14117	14832	16614	12689	4664
26	2523	9064	3971	4466	7595	1849	3608	2488	4246	7386	10077	10327	14047	10504	2631
27	2026	7678	3207	4042	6659	1512	3529	2163	3837	5974	8760	10120	11063	9381	2722
28	2800	6578	3483	5804	8517	1924	3649	1522	4340	6801	14378	12893	12826	12560	4583
29	3635	5218	2280	3097	6537	1181	2753	2145	1989	6834	7089	13238	8062	6559	5354
30	2749	5094	1726	1217	4489	1661	2597	1744	1263	6211	5584	11288	7198	7224	3883
31	4020	8580	3403	4691	6623	2519	2266	606	5183	11287	20230	24079	23211	18209	6499
32	4261	10137	4202	6103	6509	3185	2403	1083	5139	11890	21452	23781	24520	17916	6511
33	3793	8532	2742	4174	5744	4396	3199	1998	2203	13126	17359	22819	20775	15926	6234
34	2297	5592	1416	2691	2507	3175	2776	2343	1930	8531	8593	20446	10243	11934	4664
35	3966	3264	1674	1626	2526	1840	2545	2051	1753	4948	3943	9463	3375	5190	2621
36	4931	3377	1802	1851	2647	2028	2819	2503	1967	5148	4528	10308	4241	5802	2468
37	3673	3390	1845	1059	2931	2040	2612	2066	1946	5228	4017	8930	4305	5112	2753
38	3162	6302	2991	7915	5888	1168	3042	2447	3030	4553	5652	6761	5416	9342	2495
39	3893	7660	3349	7050	8331	651	3202	2244	3627	5656	7526	11099	9097	9928	3123
40	2450	5904	2197	3768	4863	2759	3091	1902	2534	3461	4099	5658	2985	3109	2633
41	1903	5399	1844	3006	3726	1932	2858	1854	2317	2636	3083	4531	3339	3924	2334
42	3614	10886	3953	5807	8277	4430	3798	1776	4880	8788	10906	15348	19303	16044	4525
43	2907	9545	3290	4486	5897	5282	3502	1205	4907	7100	9174	13231	15013	13961	3955
44	4930	8388	4098	7445	12671	3014	3797	3050	5442	6821	19436	19086	23899	18019	6917
45	4720	8604	4930	11411	12652	3200	4671	2218	5425	9294	19992	19966	22375	17000	6589
46	4236	6883	4142	10267	12814	4836	2947	3112	3428	7981	12844	12170	15714	12853	3133
47	3929	3860	2499	6470	8321	818	2680	3006	2290	6282	5368	11934	4016	7932	2768
48	5544	6044	3583	8344	12012	1610	2595	3571	3562	7236	16870	17296	18642	13307	5393
49	6400	9508	5163	9971	14666	3544	4103	3257	5877	9159	23850	23730	25913	18038	7759
50	3803	4905	2798	7303	8796	1690	2385	3897	3223	8250	7525	11231	10224	9256	2848
51	6609	8499	4900	10162	15222	3364	4459	2956	4786	8424	18702	19234	22301	17303	5731
52	6501	7945	4014	8692	11835	2096	3732	2897	4765	8266	20369	20942	19305	16084	4336
53	2528	5376	1586	2733	2716	2871	2908	2289	1006	10131	9631	20773	13311	13523	5385
54	4879	6997	2621	4762	7559	3483	3080	3233	4018	5032	8267	12425	12006	12110	4613
55	4930	6224	2290	4072	6338	2787	3139	3109	3538	4594	7252	11713	10165	10940	4091
56	4071	6794	2645	4448	7474	735	3198	3547	3824	5552	8613	13593	13206	14061	4085
57	4286	6255	2888	5461	8024	516	3379	3102	3940	5221	10068	13315	14230	14100	3768
58	2952	7642	3227	5840	7358	1043	3145	2091	3666	4988	6995	9030	10543	10090	2906
59	5284	7801	3375	6229	8720	2840	3811	2123	4564	6101	11102	14790	9176	19635	4037
60	5588	8281	2851	4488	7773	11358	3539	1174	5183	6769	6460	19788	7036	14415	4711
61	3717	6279	2793	6454	6090	2048	3236	1188	3862	4460	5960	8351	7272	7699	2881
62	3655	4116	2049	4831	5525	1346	2469	2883	1997	4532	7817	10231	6294	6490	2743
63	3778	5826	3057	7999	9174	824	3045	2737	1718	9989	13162	14328	13589	10949	4054
64	3850	5905	2898	6231	8844	652	2954	3387	3242	5392	8795	10681	11476	9755	3100
65	5865	11514	5440	13053	9802	2387	4666	1079	3747	13709	27187	22556	31834	21992	7490
66	4464	8597	3975	6748	8977	2915	3618	2532	4496	5679	9399	12572	9416	16968	3418
67	8830	4789	3036	5718	10212	2171	2177	4693	3223	6071	8457	14514	11067	12832	4402
68	9330	6957	3314	5637	10300	5734	2988	3454	4060	6255	8173	13381	13086	14076	3935
69	9209	6293	3136	5606	9790	4862	2878	3981	3813	5889	7148	11877	11515	12609	3559
70	9136	4239	2828	5683	8917	2662	1997	4884	3375	5540	7407	11575	11764	12479	3686
71	7578	6325	3594	7932	12248	2533	1896	5432	4235	6969	9764	13439	15367	14809	4211
72	8671	4565	3083	6741	11229	1974	1774	5348	3907	6167	8898	12450	13947	13909	4031
73	2131	10011	4534	6993	6002	3452	4429	2527	3066	7384	10676	9982	14990	11341	3234
74	4445	7302	3795	9465	9499	1858	3970	1527	4408	9396	16893	17146	20352	15118	4856
75	3992	8609	4571	10613	9389	2432	3907	2543	4946	8049	15544	15127	18077	15179	3582
76	4731	3098	1500	1644	2526	2549	2606	3381	2566	3410	3646	9255	3495	6045	2413
77	5508	3068	1543	2764	2973	1904	2501	3029	2235	4027	2828	8274	2707	5234	2162

8.8.3.2 RP-HPLC peak area v sensory maturity score

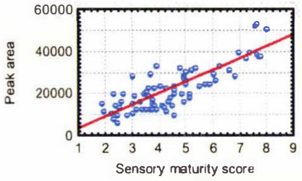
Peak 1

R^2 = 0.7131; R = 0.8445, p = 0.0000



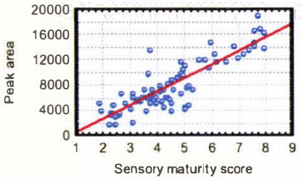
Peak 2

R^2 = 0.7031; R = 0.8385, p = 0.0000



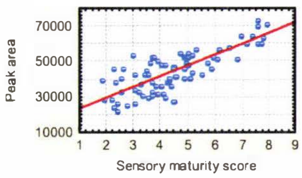
Peak 3

R^2 = 0.7482; R = 0.8650, p = 0.0000



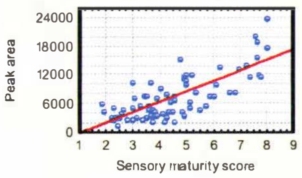
Peak 4

R^2 = 0.6473; R = 0.8046, p = 0.0000



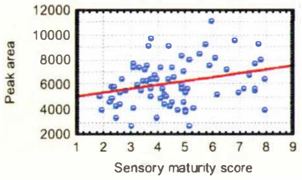
Peak 5

R^2 = 0.5794; R = 0.7612, p = 0.0000



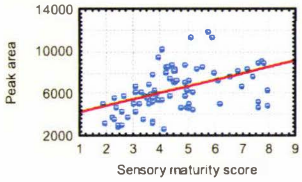
Peak 6

R^2 = 0.0803; R = 0.2834, p = 0.0125



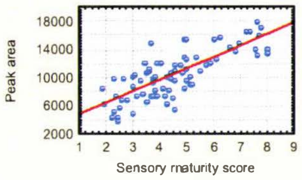
Peak 7

R^2 = 0.2197; R = 0.4687, p = 0.00002



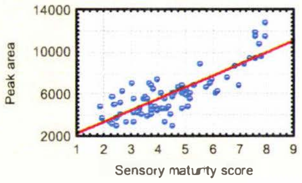
Peak 8

R^2 = 0.6090; R = 0.7804, p = 0.0000



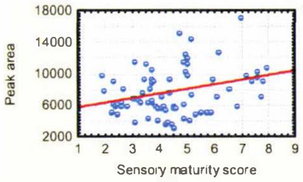
Peak 9

R^2 = 0.6720; R = 0.8198, p = 0.0000



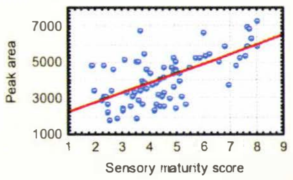
Peak 10

R^2 = 0.1066; R = 0.3265, p = 0.0038



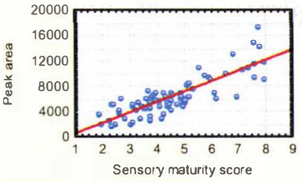
Peak 11

R^2 = 0.4128; R = 0.6425, p = 0.0000



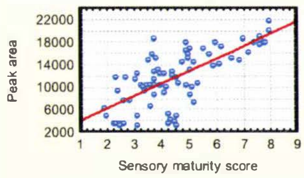
Peak 12

R^2 = 0.7107; R = 0.8430, p = 0.0000



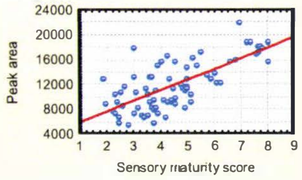
Peak 13

R^2 = 0.5136; R = 0.7167, p = 0.0000



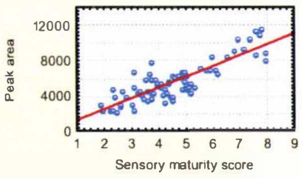
Peak 14

R^2 = 0.5074; R = 0.7123, p = 0.0000



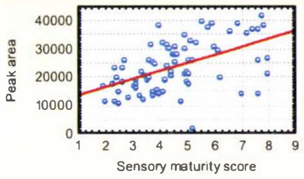
Peak 15

R^2 = 0.7465; R = 0.8640, p = 0.0000



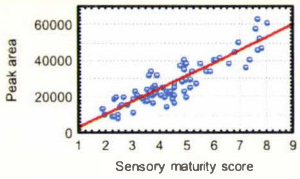
Peak 16

R^2 = 0.2735; R = 0.5229, p = 0.000001



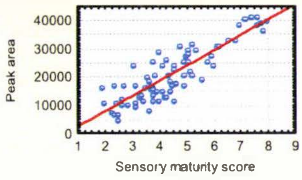
Peak 17

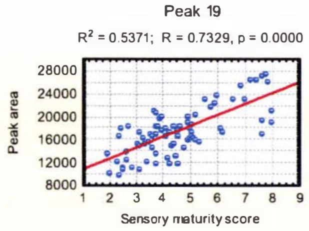
R^2 = 0.8092; R = 0.8996, p = 0.0000



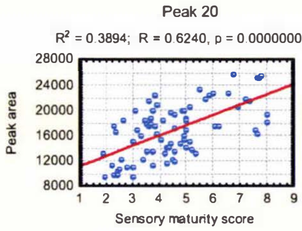
Peak 18

R^2 = 0.7852; R = 0.8861, p = 0.0000

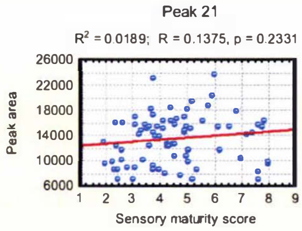



Peak 19

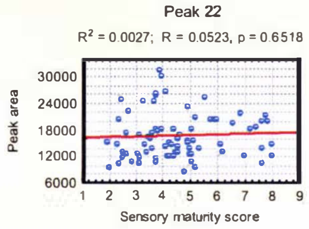
$R^2 = 0.5371$; R = 0.7329, p = 0.0000


Peak 20

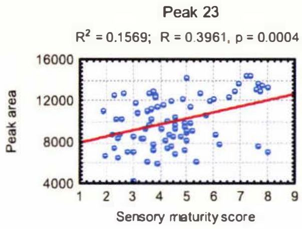
$R^2 = 0.3894$; R = 0.6240, p = 0.00000000


Peak 21

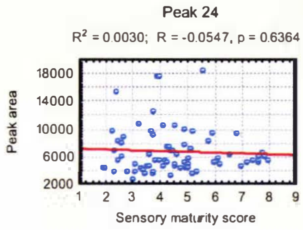
$R^2 = 0.0189$; R = 0.1375, p = 0.2331


Peak 22

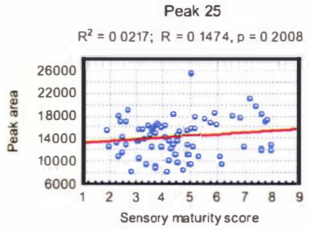
$R^2 = 0.0027$; R = 0.0523, p = 0.6518


Peak 23

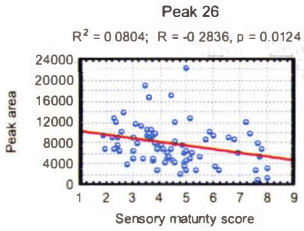
$R^2 = 0.1569$; R = 0.3961, p = 0.0004


Peak 24

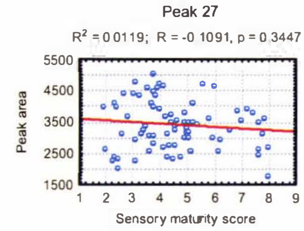
$R^2 = 0.0030$; R = -0.0547, p = 0.6364


Peak 25

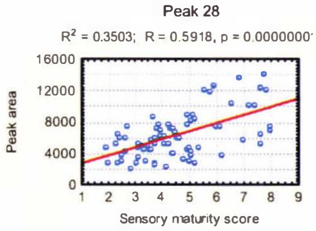
$R^2 = 0.0217$; R = 0.1474, p = 0.2008


Peak 26

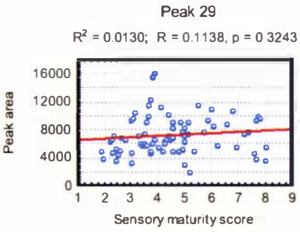
$R^2 = 0.0804$; R = -0.2836, p = 0.0124


Peak 27

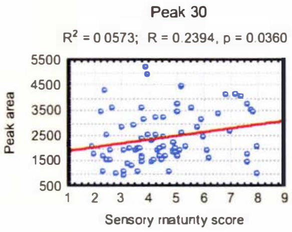
$R^2 = 0.0119$; R = -0.1091, p = 0.3447


Peak 28

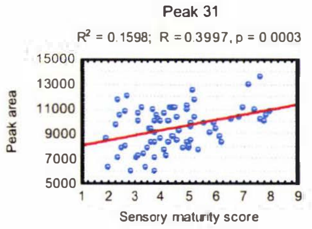
$R^2 = 0.3503$; R = 0.5918, p = 0.0000000


Peak 29

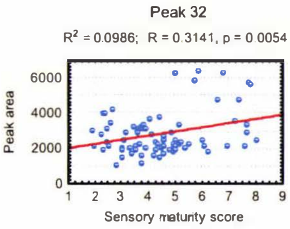
$R^2 = 0.0130$; R = 0.1138, p = 0.3243


Peak 30

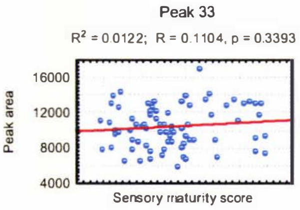
$R^2 = 0.0573$; R = 0.2394, p = 0.0360


Peak 31

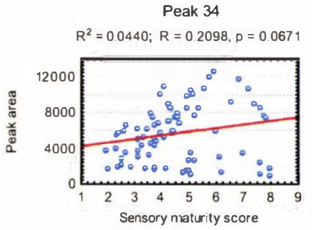
$R^2 = 0.1598$; R = 0.3997, p = 0.0003


Peak 32

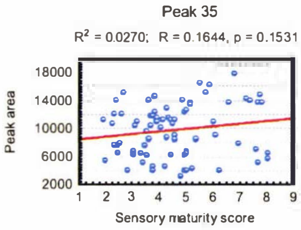
$R^2 = 0.0986$; R = 0.3141, p = 0.0054


Peak 33

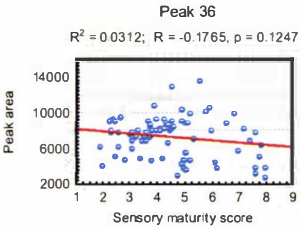
$R^2 = 0.0122$; R = 0.1104, p = 0.3393


Peak 34

$R^2 = 0.0440$; R = 0.2098, p = 0.0671


Peak 35

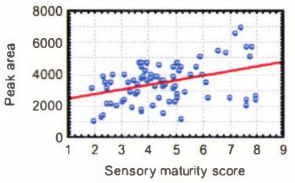
$R^2 = 0.0270$; R = 0.1644, p = 0.1531


Peak 36

$R^2 = 0.0312$; R = -0.1765, p = 0.1247

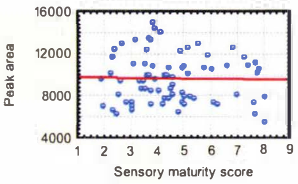
Peak 37

R^2 = 0.1446; R = 0.3803, p = 0.0006



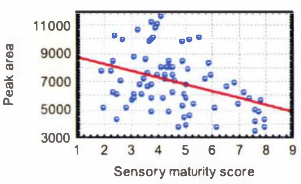
Peak 38

R^2 = 0.0003; R = -0.0186, p = 0.8723



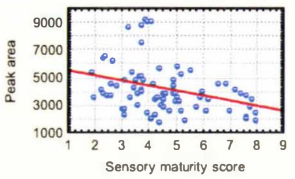
Peak 39

R^2 = 0.1447; R = -0.3804, p = 0.0006



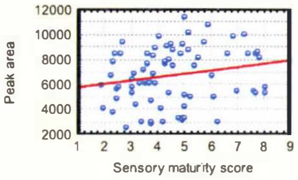
Peak 40

R^2 = 0.1170; R = -0.3421, p = 0.0023



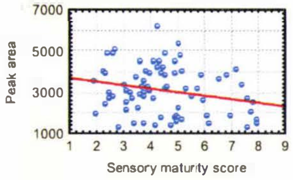
Peak 41

R^2 = 0.0397; R = 0.1992, p = 0.0824



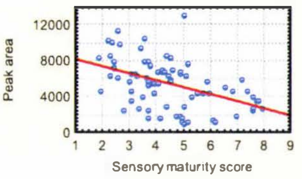
Peak 42

R^2 = 0.0563; R = -0.2372, p = 0.0378



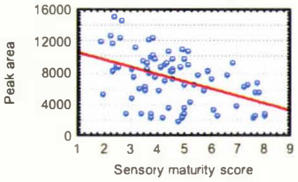
Peak 43

R^2 = 0.2187; R = -0.4677, p = 0.00002



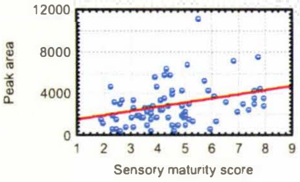
Peak 44

R^2 = 0.2096; R = -0.4578, p = 0.00003



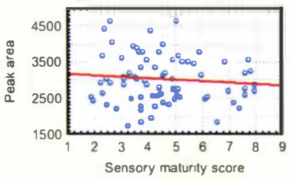
Peak 45

R^2 = 0.1166; R = 0.3414, p = 0.0024



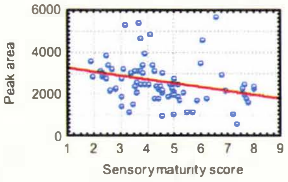
Peak 46

R^2 = 0.0099, R = -0.0997, p = 0.3882



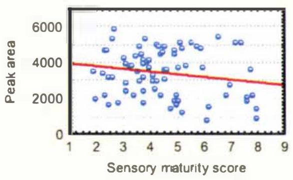
Peak 47

R^2 = 0.0850; R = -0.2916, p = 0.0101



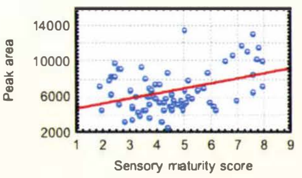
Peak 48

R^2 = 0.0354; R = -0.1882, p = 0.1013



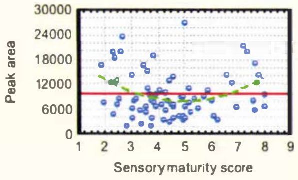
Peak 49

R^2 = 0.1490; R = 0.3859, p = 0.0005



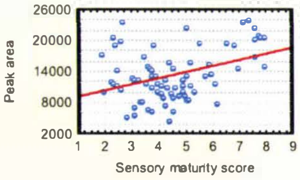
Peak 50

R^2 = 0.0000; R = 0.0044, p = 0.9697



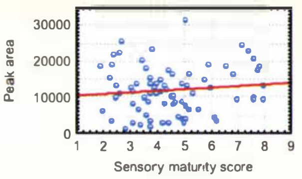
Peak 51

R^2 = 0.1523; R = 0.3902, p = 0.0005



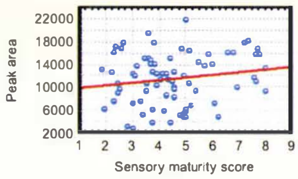
Peak 52

R^2 = 0.0121; R = 0.1100, p = 0.3409



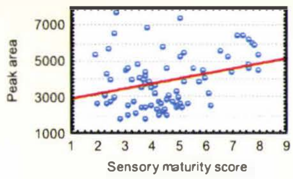
Peak 53

R^2 = 0.0292; R = 0.1709, p = 0.1372



Peak 54

R^2 = 0.0971; R = 0.3115, p = 0.0058



8.8.3.3 PCA (correlation matrix) of the RP-HPLC peak data with MLR of the principal component scores (SMS v TN and PC1, PC2 and PC3)

8.8.3.3.1 PCA : PEAKS 1 – 54

Unrotated Factor Loadings (Factor-variable correlations) and Communalities

Variable	Factor 1	Factor 2	Factor 3	Communality
1	-0.503	0.835	0.143	0.969
2	-0.469	0.828	0.168	0.934
3	-0.603	0.708	-0.238	0.922
4	-0.558	0.753	0.170	0.907
5	-0.318	0.859	0.193	0.876
6	-0.527	0.030	-0.553	0.584
7	-0.633	0.028	0.008	0.401
8	-0.613	0.694	-0.049	0.861
9	-0.616	0.700	0.103	0.881
10	-0.078	0.625	-0.012	0.396
11	-0.672	0.497	-0.154	0.723
12	-0.770	0.483	-0.051	0.829
13	-0.571	0.632	-0.324	0.830
14	-0.613	0.561	0.267	0.761
15	-0.695	0.639	-0.219	0.939
16	-0.732	0.083	0.089	0.551
17	-0.501	0.806	-0.169	0.930
18	-0.543	0.778	0.145	0.921
19	-0.898	0.327	-0.197	0.952
20	-0.860	0.226	-0.372	0.929
21	-0.651	-0.240	-0.357	0.608
22	-0.681	-0.429	-0.479	0.876
23	-0.825	-0.034	-0.155	0.706
24	-0.363	-0.495	-0.468	0.595
25	-0.814	-0.337	0.170	0.806
26	-0.388	-0.566	0.256	0.536
27	-0.441	-0.314	-0.443	0.490
28	-0.902	0.030	-0.048	0.816
29	-0.523	-0.294	-0.717	0.874
30	-0.597	-0.187	0.007	0.391
31	-0.707	-0.079	-0.005	0.507
32	-0.778	-0.157	0.174	0.660
33	-0.850	-0.423	-0.018	0.902
34	-0.676	-0.380	0.156	0.625
35	-0.841	-0.438	-0.044	0.900
36	-0.463	-0.724	-0.035	0.739
37	-0.836	-0.148	-0.144	0.742
38	-0.666	-0.544	-0.411	0.908
39	-0.330	-0.789	-0.347	0.852
40	-0.202	-0.577	-0.499	0.622
41	-0.728	-0.374	0.383	0.816
42	-0.409	-0.701	0.402	0.820
43	-0.231	-0.798	0.435	0.880
44	-0.336	-0.837	0.170	0.842
45	-0.523	-0.099	-0.135	0.302
46	-0.398	-0.277	0.443	0.431
47	0.157	-0.293	-0.500	0.360
48	-0.510	-0.631	0.147	0.679
49	-0.722	0.001	0.453	0.727
50	-0.647	-0.333	0.549	0.832
51	-0.800	0.052	0.288	0.726
52	-0.733	-0.357	0.459	0.875
53	-0.839	-0.358	0.205	0.874
54	-0.761	-0.020	0.303	0.671
Variance	21.170	13.970	4.950	39.210
% Variance	39.21	25.86	9.16	74.23

Factor Score Coefficients

Variable	Factor 1	Factor 2	Factor 3
1	-0.024	0.060	0.029
2	-0.022	0.059	0.034
3	-0.029	0.051	-0.048
4	-0.026	0.054	0.034
5	-0.015	0.062	0.039
6	-0.025	0.002	-0.112
7	-0.030	0.002	0.002
8	-0.029	0.050	-0.010
9	-0.029	0.050	0.021
10	-0.004	0.045	-0.002
11	-0.032	0.036	-0.031
12	-0.036	0.035	-0.010
13	-0.027	0.045	-0.066
14	-0.029	0.040	0.054
15	-0.033	0.046	-0.044
16	-0.035	0.006	0.018
17	-0.024	0.058	-0.034
18	-0.026	0.056	0.029
19	-0.042	0.023	-0.040
20	-0.041	0.016	-0.075
21	-0.031	-0.017	-0.072
22	-0.032	-0.031	-0.097
23	-0.039	-0.003	-0.031
24	-0.017	-0.035	-0.095
25	-0.039	-0.024	0.035
26	-0.018	-0.041	0.052
27	-0.021	-0.023	-0.090
28	-0.043	0.002	-0.010
29	-0.025	-0.021	-0.145
30	-0.028	-0.013	0.001
31	-0.033	-0.006	-0.001
32	-0.037	-0.011	0.035
33	-0.040	-0.030	-0.004
34	-0.032	-0.027	0.032
35	-0.040	-0.031	-0.009
36	-0.022	-0.052	-0.007
37	-0.040	-0.011	-0.029
38	-0.031	-0.039	-0.083
39	-0.016	-0.057	-0.070
40	-0.010	-0.041	-0.101
41	-0.034	-0.027	0.078
42	-0.019	-0.050	0.081
43	-0.011	-0.057	0.088
44	-0.016	-0.060	0.034
45	-0.025	-0.007	-0.027
46	-0.019	-0.020	0.090
47	0.007	-0.021	-0.101
48	-0.024	-0.045	0.030
49	-0.034	0.000	0.092
50	-0.031	-0.024	0.111
51	-0.038	0.004	0.058
52	-0.035	-0.026	0.093
53	-0.040	-0.026	0.042
54	-0.036	-0.001	0.061

Factor Scores

Sample	Factor 1	Factor 2	Factor 3
1	0.006	2.363	0.516
2	0.277	1.114	-1.057
3	-0.111	2.484	0.624
4	0.500	1.013	-0.902

5	-1.334	0.163	-0.190
6	-0.612	-0.884	0.294
7	-1.913	0.106	-0.531
8	-0.896	-0.878	-0.326
9	-0.258	1.635	0.006
10	-1.930	1.268	-0.091
11	-1.821	1.162	-0.016
12	0.764	1.690	-0.492
13	1.158	1.667	0.212
14	1.916	0.078	-0.790
15	1.820	0.237	-0.428
16	1.367	0.439	-0.587
17	0.945	-0.725	0.438
18	-0.229	-0.570	0.159
19	0.471	-0.655	0.497
20	0.832	-0.911	-0.311
21	-0.043	-0.770	0.104
22	-0.180	-0.390	0.740
23	1.570	-0.607	0.715
24	0.124	-0.690	0.413
25	-0.016	-0.618	1.672
26	-0.103	0.097	0.727
27	0.398	0.498	0.967
28	0.594	0.122	1.783
29	0.535	0.678	0.742
30	0.810	1.086	0.898
31	-1.850	0.764	0.826
32	-1.906	0.476	1.120
33	-1.939	1.150	0.212
34	-0.249	2.194	0.644
35	0.961	1.325	-0.363
36	0.434	1.250	-1.036
37	0.743	1.493	-0.553
38	1.058	-0.562	0.033
39	0.025	-0.271	-0.198
40	0.866	0.000	-0.392
41	0.919	0.380	-0.686
42	-1.488	-0.443	0.129
43	-1.184	-0.099	-0.217
44	-0.791	-0.708	0.646
45	-0.195	-1.271	1.920
46	0.672	-1.366	0.842
47	1.623	-0.540	0.434
48	0.217	-0.680	0.755
49	-1.223	-1.392	0.894
50	1.392	-0.958	0.508
51	-0.622	-1.685	0.490
52	-0.582	-0.941	0.574
53	-0.461	2.191	0.495
54	-0.157	-0.255	-0.877
55	0.124	0.013	-0.809
56	-0.910	0.450	-1.450
57	-0.586	0.407	-0.798
58	0.082	0.083	-0.070
59	-0.911	-0.456	-0.874
60	-1.508	-0.497	-1.611
61	0.679	-0.297	-0.073
62	1.662	-0.101	0.513
63	1.203	-0.692	1.567
64	1.077	-0.620	0.490
65	-1.499	-1.064	2.728
66	-0.759	-0.618	-0.792
67	-0.271	-0.844	-1.959
68	-0.722	-1.341	-2.313
69	-0.389	-1.352	-2.462
70	-0.036	-0.924	-2.203
71	-0.108	-1.374	-1.464
72	0.156	-1.150	-1.633
73	-0.158	-0.109	1.172

74	-0.155	-0.576	1.369
75	-0.141	-0.773	1.077
76	1.532	0.156	-0.741
77	1.559	0.095	-0.824

8.8.3.3.2 Regression Analysis: SMS versus TN and PC1, PC2 and PC3 of the (RP-HPLC) correlation matrix (Model F)

MLR Results

	SMS v PC1, PC2, PC3	SMS v TN, PC1, PC2, PC3)
Multiple R	0.8986	0.9341
Multiple R²	0.8074	0.8726
Adjusted R²	0.7995	0.8655
For (3,73) or (4,72)	102.03	123.25
p-level	0.0000	0.0000
Std.Error of prediction	0.7227	0.5920

Regression summary (SMS v TN, PC1, PC2, PC3)

	Beta	Std.Error of Beta	B	Std.Erorr of B-coefficient	t(72)	*p*-level
Intercept			16.79540	2.020756	8.3114	0.000000
TN	-0.276715	0.045618	-3.16884	0.522397	-6.0660	0.000000
PC1	-0.492492	0.043192	-0.79332	0.069574	-11.4025	0.000000
PC2	0.664461	0.042958	1.07824	0.069709	15.4677	0.000000
PC3	0.025252	0.043813	0.04068	0.070574	0.5764	0.566161

Regression equation

Cheddar cheese maturity = 16.79540 - 3.16884 TN - 0.79332 PC1 + 1.07824 PC2 + 0.04068 PC3 (*Equation 6*)

Analysis of Variance

	Sums of Squares	df	Mean Squares	F	*p*-level
Regress.	172.7775	4	43.19439	123.2463	0.000000
Residual	25.2340	72	0.35047		
Total	198.0115				

8.8.3.4 PCA (covariance matrix) of the RP-HPLC peak data with MLR of the principal component scores (SMS v TN and PC1, PC2 and PC3)

8.8.3.4.1 PCA : Peaks 1 - 54

Unrotated Factor Loadings (Factor-variable correlations) and Communalities

Variable	Factor 1	Factor 2	Factor 3	Communality
1	-15207.27	1644.85	-1532.82	2.36E+08
2	-10435.31	1428.27	-1124.26	1.12E+08
3	-3574.20	-209.55	1431.81	1.49E+07
4	-11891.97	225.92	-1553.78	1.44E+08
5	-4209.35	1278.42	-833.67	2.00E+07
6	-441.54	-697.51	1078.24	1.84E+06
7	-795.65	-1062.32	791.25	2.39E+06
8	-3045.13	-280.25	387.15	9.50E+06
9	-2065.11	-141.03	-7.76	4.28E+06
10	-1556.64	1021.56	-887.05	4.25E+06
11	-1063.02	-322.49	240.45	1.29E+06
12	-2623.80	-1011.97	786.20	8.53E+06
13	-4141.04	-192.58	1255.36	1.88E+07
14	-3286.34	-557.19	-590.84	1.15E+07
15	-2049.00	-355.00	667.63	4.77E+06
16	-4309.07	-5266.60	2892.50	5.47E+07
17	-12251.59	1549.43	2999.55	1.61E+08
18	-9583.21	393.37	-472.65	9.22E+07
19	-3260.80	-2084.03	1058.99	1.61E+07
20	-2791.44	-2192.25	1606.71	1.52E+07
21	-521.73	-2498.49	1526.05	8.84E+06
22	-329.31	-3724.61	1753.18	1.71E+07
23	-1055.88	-1538.35	88.28	3.49E+06
24	536.71	-1631.35	1371.65	4.83E+06
25	-761.84	-2774.37	-797.54	8.91E+06
26	873.22	-2645.51	-1398.46	9.72E+06
27	-9.27	-357.15	102.54	1.38E+05
28	-1517.03	-2009.29	884.18	7.12E+06
29	-53.45	-1436.20	1725.18	5.04E+06
30	-225.35	-534.28	-40.75	3.38E+05
31	-640.47	-936.55	21.91	1.29E+06
32	-398.67	-871.93	-126.01	9.35E+05
33	-417.97	-2126.07	73.18	4.70E+06
34	-328.08	-2434.99	536.70	6.32E+06
35	-562.80	-3375.19	627.36	1.21E+07
36	637.57	-1761.66	423.40	3.69E+06
37	-430.33	-931.81	298.82	1.14E+06
38	79.21	-1765.55	590.75	3.47E+06
39	829.49	-1378.13	436.72	2.78E+06
40	576.37	-745.91	177.35	9.20E+05
41	-373.42	-1828.78	-167.03	3.51E+06
42	288.56	-891.40	-268.71	9.50E+05
43	1194.51	-1876.30	-1101.18	6.16E+06
44	1331.12	-2477.44	-776.54	8.51E+06
45	-417.48	-835.90	733.30	1.41E+06
46	-31.91	-342.58	-151.57	1.41E+05
47	323.14	-17.56	180.61	1.37E+05
48	191.39	-1007.62	-77.50	1.06E+06
49	-1074.41	-1435.65	-972.21	4.16E+06

50	-936.22	-4085.78	-3218.30	2.79E+07
51	-2708.12	-3009.00	-1753.14	1.95E+07
52	-1319.45	-5320.32	-3046.44	3.93E+07
53	-738.43	-3656.81	-739.78	1.45E+07
54	-685.14	-881.34	-631.69	1.65E+06
Variance	872077141	215542726	78150135	1.17E+09
% Variance	66.40	16.40	5.90	88.70

Factor Score Coefficients

Variable	Factor 1	Factor 2	Factor 3
1	-0.270	0.118	-0.303
2	-0.128	0.071	-0.154
3	-0.017	-0.004	0.074
4	-0.167	0.013	-0.243
5	-0.023	0.028	-0.050
6	-0.001	-0.006	0.024
7	-0.002	-0.010	0.021
8	-0.012	-0.004	0.017
9	-0.005	-0.001	0.000
10	-0.005	0.014	-0.033
11	-0.002	-0.002	0.004
12	-0.010	-0.015	0.032
13	-0.024	-0.004	0.080
14	-0.015	-0.010	-0.029
15	-0.005	-0.004	0.020
16	-0.044	-0.216	0.327
17	-0.182	0.093	0.497
18	-0.109	0.018	-0.060
19	-0.016	-0.041	0.057
20	-0.014	-0.043	0.088
21	-0.002	-0.044	0.074
22	-0.002	-0.087	0.113
23	-0.003	-0.017	0.003
24	0.002	-0.025	0.057
25	-0.003	-0.043	-0.034
26	0.004	-0.048	-0.070
27	0.000	-0.001	0.001
28	-0.005	-0.026	0.032
29	0.000	-0.018	0.060
30	0.000	-0.002	-0.001
31	-0.001	-0.007	0.000
32	-0.001	-0.005	-0.002
33	-0.001	-0.023	0.002
34	-0.001	-0.035	0.021
35	-0.002	-0.057	0.029
36	0.002	-0.018	0.012
37	-0.001	-0.005	0.005
38	0.000	-0.018	0.017
39	0.002	-0.013	0.011
40	0.001	-0.006	0.004
41	-0.001	-0.019	-0.005
42	0.000	-0.005	-0.004
43	0.004	-0.023	-0.038
44	0.005	-0.037	-0.032

45	-0.001	-0.007	0.018
46	0.000	-0.001	-0.001
47	0.000	0.000	0.002
48	0.000	-0.006	-0.001
49	-0.003	-0.016	-0.029
50	-0.006	-0.102	-0.222
51	-0.015	-0.068	-0.110
52	-0.010	-0.159	-0.251
53	-0.004	-0.076	-0.043
54	-0.001	-0.006	-0.012

Factor Scores

Sample	Factor 1	Factor 2	Factor 3
1	-2.198	1.747	-0.796
2	-0.609	0.716	1.291
3	-2.245	1.526	-0.403
4	-0.420	1.068	0.822
5	-1.058	-1.052	-0.012
6	0.321	-0.904	-0.499
7	-1.072	-1.416	1.261
8	0.066	-1.061	0.389
9	-1.506	1.095	-0.527
10	-1.830	-1.005	1.014
11	-1.726	-0.927	0.347
12	-0.884	1.617	0.350
13	-0.725	2.037	-0.687
14	1.320	1.483	0.622
15	1.055	1.483	0.272
16	0.596	1.300	0.054
17	1.150	0.294	0.430
18	0.329	-0.501	0.945
19	0.766	-0.067	0.366
20	1.251	0.164	1.009
21	0.493	-0.526	0.610
22	0.009	-0.374	-0.194
23	1.401	0.720	-0.378
24	0.537	-0.333	0.422
25	0.176	-0.464	-1.037
26	-0.479	-0.183	0.229
27	-0.475	0.427	-0.336
28	-0.095	0.586	-2.064
29	-0.444	1.028	-1.055
30	-0.515	1.781	-1.722
31	-1.654	-0.913	-0.799
32	-1.511	-1.377	-1.354
33	-1.744	-1.077	-0.103
34	-2.149	1.157	-0.654
35	-0.442	1.537	0.171
36	-0.667	1.142	0.773
37	-0.691	1.455	0.471
38	1.127	0.452	0.517
39	0.279	-0.205	0.673
40	0.859	0.793	0.606
41	0.586	0.903	0.935
42	-0.407	-1.497	1.056
43	-0.427	-1.058	1.328

44	-0.066	-1.116	-1.411
45	0.669	-1.054	-1.736
46	1.388	-0.347	-0.905
47	1.571	0.881	-0.392
48	0.697	-0.410	-1.699
49	0.195	-1.813	-1.505
50	1.680	0.491	-0.509
51	0.683	-1.380	-0.871
52	0.299	-1.132	-1.308
53	-2.202	0.884	-0.483
54	0.321	-0.128	0.028

8.8.3.4.2 Regression Analysis: SMS versus TN and PC1, PC2 and PC3 of the (RP-HPLC) covariance matrix (Model G)

MLR Results

	SMS v PC1, PC2, PC3	SMS v TN, PC1, PC2, PC3
Multiple R	0.9149	0.9368
Multiple R^2	0.8370	0.8776
Adjusted R^2	0.8303	0.8708
F(3,73)	124.95	129.04
p-level	0.0000	0.0000
Std.Error of prediction	0.6649	0.5802

Regression summary

	Beta	Std.Err. of Beta	B	Std.Err. of B-coefficient	t(72)	*p*-level
Intercept			15.14657	2.170746	6.9776	0.000000
TN	-0.239467	0.049016	-2.74229	0.561314	-4.8855	0.000006
PC1	-0.830414	0.043062	-1.34888	0.069947	-19.2843	0.000000
PC2	0.013820	0.041256	0.02216	0.066159	0.3350	0.738606
PC3	0.115408	0.047572	0.18720	0.077165	2.4260	0.017775

Regression equation

Cheddar cheese maturity = 15.14657 – 2.74229 TN – 1.34888 PC1 + 0.02216 PC2 + 0.18720 PC3

(Equation 7)

Analysis of Variance

	Sums of Squares	df	Mean Squares	F	*p*-level
Regress.	173.7710	4	43.44275	129.0351	0.000000
Residual	24.2405	72	0.33667		
Total	198.0115				

8.8.4 SE-HPLC

8.8.4.1 SE-HPLC 'peak' area data

	Factory	Storage (T°C)	Storage time	SMS	pH	TN	P1	P2	P3	P4	P5	P6	P7	P8	P9
1	Southland	13	12	7.60	5.35	3.88	221	201	1061	854	1921	2060	977	989	1896
2	Southland	5	12	6.08	5.19	3.85	175	146	906	524	2030	2486	1001	762	1629
3	Southland	13	12	7.96	5.37	3.87	187	189	880	694	1721	2077	1104	1052	2003
4	Southland	5	12	4.96	5.41	3.85	204	100	846	580	1958	2110	820	784	1536
5	Waitoa	10	12	6.53	5.29	3.78	278	186	629	756	3913	3127	1686	686	2318
6	Waitoa	5	12	4.39	5.54	3.76	327	208	884	859	4305	2554	1321	853	1846
7	Waitoa	10	12	6.78	5.20	3.75	271	183	1002	642	3884	3399	1739	923	2541
8	Waitoa	5	12	5.12	5.38	3.76	264	149	901	751	4185	2827	1417	857	1886
9	Southland	10	12	6.92	5.29	3.86	261	78	751	668	2780	2518	772	1127	1909
10	Waitoa	13	12	7.73	5.58	3.82	243	184	1154	368	3773	3124	1713	1010	2642
11	Waitoa	13	12	7.79	5.44	3.77	296	110	888	405	3982	3028	1653	986	2609
12	Southland	13	6	6.16	5.22	3.77	286	54	616	516	1478	2003	915	865	1667
13	Southland	13	6	4.72	5.21	3.84	271	76	601	703	1706	1700	671	898	1421
14	Southland	5	3	2.79	5.17	3.78	140	163	619	481	1305	1355	544	443	769
15	Southland	10	3	3.74	5.34	3.90	140	158	555	627	1587	1445	569	478	894
16	Southland	13	3	3.71	5.25	3.75	135	588	834	667	1816	1557	600	603	1116
17	Stiirling	5	3	4.23	5.17	3.75	162	193	707	752	3062	2019	762	578	997
18	Stiirling	10	3	5.08	4.99	3.81	141	257	1225	625	3511	2603	1027	700	1567
19	Stiirling	13	3	4.45	5.05	3.84	112	541	1150	586	3339	2178	848	577	1196
20	Stiirling	5	3	4.50	5.11	3.77	109	166	707	561	2705	2056	772	540	939
21	Stiirling	10	3	4.88	5.10	3.81	148	669	1298	719	3671	2622	1076	674	1526
22	Stiirling	13	3	4.25	5.17	3.67	157	487	1179	736	3876	2597	1014	731	1690
23	Stiirling	5	3	3.04	5.29	3.75	126	246	723	858	2622	1546	606	464	777
24	Stiirling	10	3	4.29	5.12	3.75	134	605	1233	647	3415	2407	988	602	1396
25	Stiirling	13	3	4.52	5.13	3.79	175	557	1095	955	4203	2391	996	625	1534
26	Stiirling	10	6	3.92	5.28	3.87	231	104	703	906	3521	2904	1317	594	1952
27	Stiirling	10	6	5.30	5.22	3.88	203	131	588	765	2951	2377	879	544	1593
28	Stiirling	10	6	3.00	5.47	3.88	347	219	721	1021	3847	1956	680	482	1420
29	Southland	5	15	5.00	5.51	3.87	229	114	704	824	2547	1913	716	783	1408
30	Southland	5	15	5.16	5.39	3.90	270	135	732	1016	2026	1475	530	856	1310
31	Waitoa	13	12	7.38	5.32	3.77	342	152	784	679	4435	3197	1875	651	2461
32	Waitoa	13	12	7.17	5.23	3.73	384	142	742	722	4679	3172	1861	631	2411
33	Waitoa	13	12	7.61	5.07	3.76	331	131	736	685	3590	3064	1973	726	2682
34	Southland	13	12	7.54	5.27	3.91	252	180	934	730	2431	2360	1288	830	1925
35	Southland	10	6	4.91	5.28	3.88	277	62	574	622	1646	1938	827	827	1527
36	Southland	10	6	4.88	5.29	3.86	260	65	586	565	1718	2159	980	777	1740
37	Southland	10	6	5.00	5.26	3.84	243	59	530	519	1381	1998	924	772	1650
38	Waitoa	5	6	3.56	5.24	3.77	141	93	504	661	2555	1810	835	499	1036
39	Waitoa	10	6	4.57	5.22	3.75	240	72	614	671	3051	2442	1156	628	1573
40	Waitoa	5	6	3.05	5.50	3.89	145	66	539	545	2037	1604	670	621	1144
41	Waitoa	5	6	4.36	5.32	3.73	152	117	473	461	1799	1731	686	607	1157
42	Waitoa	5	15	5.84	5.19	3.87	178	135	894	697	4641	3174	1471	953	2176
43	Waitoa	5	15	5.71	5.10	3.77	183	153	826	733	4341	3251	1518	970	2300
44	Marlborough	13	3	3.80	5.48	4.25	198	738	1175	1326	4855	2550	1158	921	1843
45	Marlborough	10	3	2.58	5.61	4.11	153	559	906	1453	4890	2371	1041	674	1434
46	Marlborough	5	3	2.21	5.59	4.17	199	270	995	1193	3792	1901	744	696	897
47	Marlborough	5	3	2.27	5.57	4.10	154	374	735	783	2244	1420	635	547	749
48	Marlborough	10	3	1.83	5.58	4.18	219	692	903	1386	4299	2130	874	833	1358
49	Marlborough	13	3	2.68	5.51	4.10	226	775	1111	1452	5527	2842	1279	863	1860
50	Marlborough	5	3	2.42	5.60	4.06	138	267	875	1038	2577	1591	654	523	743
51	Marlborough	10	3	2.35	5.34	4.05	182	682	1032	1410	5043	2850	1292	789	1552
52	Marlborough	13	3	2.28	5.57	4.14	301	181	677	1326	4638	2671	1224	821	1791
53	Southland	13	12	7.96	5.21	3.88	276	208	855	417	2813	2600	1338	798	2064
54	Kiwi	10	3	3.08	5.50	4.00	210	514	1147	798	3001	2062	918	893	1546
55	Kiwi	10	3	3.52	5.50	3.99	212	517	1120	826	3110	2086	899	847	1498
56	Kiwi	10	6	5.96	5.31	3.88	200	286	1339	726	3328	2765	1231	939	1946
57	Kiwi	10	6	3.69	5.32	3.99	155	392	1278	779	3641	2447	1026	944	1757
58	Waitoa	10	6	4.52	5.20	3.76	200	160	816	549	2925	2441	1095	598	1499
59	Lichfield	10	6	3.63	5.38	3.86	242	176	1043	813	3749	2931	1478	859	2047
60	Lichfield	10	6	5.50	5.17	3.54	273	261	1158	556	3056	3591	1757	1115	2515
61	Waitoa	10	6	3.29	5.27	3.77	216	103	610	650	2734	1993	929	564	1222
62	Marlborough	5	6	1.96	5.53	4.23	244	196	553	881	2374	1408	644	574	964
63	Marlborough	5	6	2.42	5.63	4.06	245	239	612	1053	3125	1641	806	474	1034
64	Marlborough	5	6	2.54	5.48	4.16	174	154	678	932	2929	1756	825	484	1039
65	Waitoa	10	15	5.00	5.49	3.65	338	211	980	1293	5621	2678	1530	589	1833
66	Lichfield	10	6	4.83	5.2	3.86	208	198	871	749	3616	2911	1437	761	1918
67	Hautapu	10	3	3.75	5.34	3.67	229	552	1120	799	3494	2542	1226	872	1738
68	Hautapu	10	3	3.83	5.20	3.75	162	512	1172	689	3704	2891	1312	852	1752
69	Hautapu	10	3	3.92	5.18	3.80	134	451	1083	650	3272	2611	1104	746	1446
70	Hautapu	10	3	4.08	5.32	3.86	164	536	1161	817	3466	2546	1147	747	1422
71	Hautapu	10	3	3.68	5.40	3.92	155	677	1149	922	3862	2494	1128	764	1384
72	Hautapu	10	3	3.18	5.39	3.95	151	710	1145	893	3484	2313	1059	705	1281
73	Alpine	10	6	4.04	5.20	3.90	183	67	957	798	3729	2428	968	656	1589
74	Alpine	10	6	3.46	5.51	3.86	466	383	1040	1084	4947	2536	1226	807	1738
75	Alpine	10	6	3.58	5.30	3.91	239	242	888	987	4581	2522	1182	610	1557
76	Southland	10	3	4.17	5.22	3.74	101	401	842	580	1412	1596	707	410	978
77	Southland	10	3	3.38	5.45	3.77	134	518	860	660	1744	1493	621	520	942

	Factory	Storage (T°C)	Storage time	SMS	pH	TN	P10	P11	P12	P13	P14	P15
1	Southland	13	12	7.60	5.35	3.88	2199	1787	1288	4217	2279	239
2	Southland	5	12	6.08	5.19	3.85	2310	1746	1142	2416	1503	215
3	Southland	13	12	7.96	5.37	3.87	2537	1887	1419	4016	2207	241
4	Southland	5	12	4.96	5.41	3.85	2066	1779	1031	2255	1427	202
5	Waitoa	10	12	6.53	5.29	3.78	2574	1718	1189	2843	1545	272
6	Waitoa	5	12	4.39	5.54	3.76	2569	1530	1028	1848	1090	239
7	Waitoa	10	12	6.78	5.20	3.75	3090	1678	1382	2604	1574	243
8	Waitoa	5	12	5.12	5.38	3.76	2560	1585	999	1821	1148	82
9	Southland	10	12	6.92	5.29	3.86	1886	2093	1238	3234	1861	269
10	Waitoa	13	12	7.73	5.58	3.82	3229	1936	1349	3389	1870	231
11	Waitoa	13	12	7.79	5.44	3.77	3076	1559	1513	3348	1820	234
12	Southland	13	6	6.16	5.22	3.77	1851	1814	1061	2984	1777	266
13	Southland	13	6	4.72	5.21	3.84	1476	1514	1229	2807	1766	250
14	Southland	5	3	2.79	5.17	3.78	1277	922	484	1016	689	1
15	Southland	10	3	3.74	5.34	3.90	1226	997	599	1440	856	37
16	Southland	13	3	3.71	5.25	3.75	1411	1230	735	1647	1065	47
17	Stiirling	5	3	4.23	5.17	3.75	1808	911	871	1410	1155	428
18	Stiirling	10	3	5.08	4.99	3.81	2374	982	1146	1664	1153	85
19	Stiirling	13	3	4.45	5.05	3.84	1851	918	870	1416	1024	1
20	Stiirling	5	3	4.50	5.11	3.77	1790	735	799	1035	824	42
21	Stiirling	10	3	4.88	5.10	3.81	2228	1012	1138	1646	1154	36
22	Stiirling	13	3	4.25	5.17	3.67	2156	1388	921	2068	1277	147
23	Stiirling	5	3	3.04	5.29	3.75	1263	816	582	1171	791	46
24	Stiirling	10	3	4.29	5.12	3.75	1941	916	1057	1561	1072	14
25	Stiirling	13	3	4.52	5.13	3.79	1905	1302	901	1987	1221	47
26	Stiirling	10	6	3.92	5.28	3.87	1600	1468.5	983.4	2699	1359	201
27	Stiirling	10	6	5.30	5.22	3.88	1498	1418	909	2588	1355	124
28	Stiirling	10	6	3.00	5.47	3.88	1135	1392	903	2691	1264	46
29	Southland	5	15	5.00	5.51	3.87	1258	1722	913	2732	1579	102
30	Southland	5	15	5.16	5.39	3.90	1041	1828	1072	2842	1591	111
31	Waitoa	13	12	7.38	5.32	3.77	2540	1888	1280	3593	1834	46
32	Waitoa	13	12	7.17	5.23	3.73	2541	1988	1189	3255	1754	164
33	Waitoa	13	12	7.61	5.07	3.76	3044	1678	1577	3367	1860	176
34	Southland	13	12	7.54	5.27	3.91	2118	1951	1212	4231	2259	212
35	Southland	10	6	4.91	5.28	3.88	1657	1699	1031	2622	1523	129
36	Southland	10	6	4.88	5.29	3.86	2032	1849	1001	2577	1548	229
37	Southland	10	6	5.00	5.26	3.84	1754	1777	1047	2626	1598	250
38	Waitoa	5	6	3.56	5.24	3.77	1852	998	791	1215	789	1
39	Waitoa	10	6	4.57	5.22	3.75	2228	1394	1025	1810	1079	118
40	Waitoa	5	6	3.05	5.50	3.89	2082	1323	939	1334	934	1
41	Waitoa	5	6	4.36	5.32	3.73	2046	1426	1004	1527	1004	41
42	Waitoa	5	15	5.84	5.19	3.87	2955	1344	1341	2025	1245	60
43	Waitoa	5	15	5.71	5.10	3.77	3409	1217	1427	2125	1364	94
44	Marlborough	13	3	3.80	5.48	4.25	2412	1574	1092	2149	1222	66
45	Marlborough	10	3	2.58	5.61	4.11	1774	1154	768	1783	990	95
46	Marlborough	5	3	2.21	5.59	4.17	1463	927	545	1133	722	1
47	Marlborough	5	3	2.27	5.57	4.10	1276	801	460	953	701	1
48	Marlborough	10	3	1.83	5.58	4.18	1870	1339	773	1617	975	70
49	Marlborough	13	3	2.68	5.51	4.10	2439	1509	873	1840	1028	43
50	Marlborough	5	3	2.42	5.60	4.06	1286	680	435	858	713	1
51	Marlborough	10	3	2.35	5.34	4.05	2177	1318	692	1558	915	42
52	Marlborough	13	3	2.28	5.57	4.14	2220	1526	935	2112	1048	338
53	Southland	13	12	7.96	5.21	3.88	2271	1547	1490	4204	2225	242
54	Kiwi	10	3	3.08	5.50	4.00	2265	1455	1045	1745	930	1
55	Kiwi	10	3	3.52	5.50	3.99	2088	1663	862	2008	1118	208
56	Kiwi	10	6	5.96	5.31	3.88	3091	1865	1223	2260	1329	221
57	Kiwi	10	6	3.69	5.32	3.99	2832	1650	1247	2254	1162	47
58	Waitoa	10	6	4.52	5.20	3.76	2144	1416	977	1852	888	1
59	Lichfield	10	6	3.63	5.38	3.86	2440	1462	983	2428	1220	91
60	Lichfield	10	6	5.50	5.17	3.54	2537	1570	1006	2417	1240	83
61	Waitoa	10	6	3.29	5.27	3.77	1945	1301	855	1596	960	68
62	Marlborough	5	6	1.96	5.53	4.23	1321	987	720	1498	845	1
63	Marlborough	5	6	2.42	5.63	4.06	1283	987	415	1306	728	1
64	Marlborough	5	6	2.54	5.48	4.16	1353	1075	547	1562	888	1
65	Waitoa	10	15	5.00	5.49	3.65	2272	1686	844	2142	702	1
66	Lichfield	10	6	4.83	5.2	3.86	2175	1292	979	2169	1105	44
67	Hautapu	10	3	3.75	5.34	3.67	2324	1712	684	1602	1100	275
68	Hautapu	10	3	3.83	5.20	3.75	2548	1532	748	1456	1034	169
69	Hautapu	10	3	3.92	5.18	3.80	2210	1255	708	1255	887	42
70	Hautapu	10	3	4.08	5.32	3.86	2112	1371	755	1405	875	70
71	Hautapu	10	3	3.68	5.40	3.92	2028	1378	590	1389	863	77
72	Hautapu	10	3	3.18	5.39	3.95	1928	1381	523	1260	845	62
73	Alpine	10	6	4.04	5.20	3.90	1994	1540	937	2065	1103	1
74	Alpine	10	6	3.46	5.51	3.86	2537	1743	1075	2441	1477	745
75	Alpine	10	6	3.58	5.30	3.91	2339	1535	925	1981	998	241
76	Southland	10	3	4.17	5.22	3.74	1211	1058	575	1388	853	45
77	Southland	10	3	3.38	5.45	3.77	1357	980	660	1290	847	33

8.8.4.2 PCA (correlation matrix) of the SE-HPLC peak data with MLR of the principal components (SMS v TN and PC1, PC2 and PC3)

8.8.4.2.1 PCA : 'Peaks' 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15

Unrotated Factor Loadings (Factor-variable correlations) and Communalities

Variable	Factor 1	Factor 2	Factor 3	Communality
1	-0.652	-0.172	-0.635	0.858
2	0.223	0.765	0.101	0.645
3	-0.252	0.730	0.366	0.731
4	0.158	0.570	-0.685	0.818
5	-0.367	0.795	-0.361	0.897
6	-0.797	0.459	0.088	0.854
7	-0.815	0.353	-0.003	0.788
8	-0.749	0.091	0.186	0.604
9	-0.964	0.111	0.054	0.944
10	-0.806	0.342	0.284	0.846
11	-0.809	-0.223	-0.141	0.724
12	-0.865	-0.218	0.210	0.840
13	-0.809	-0.410	-0.082	0.829
14	-0.782	-0.468	0.047	0.834
15	-0.575	-0.243	-0.275	0.465
SMS(supp)	-0.741	-0.350	0.275	0.746
Variance	7.136	3.121	1.421	11.678
% Variance	47.57	20.81	9.47	77.85

Factor Score Coefficients

Variable	Factor 1	Factor 2	Factor 3
1	-0.091	-0.055	-0.447
2	0.031	0.245	0.071
3	-0.035	0.234	0.257
4	0.022	0.183	-0.482
5	-0.051	0.255	-0.254
6	-0.112	0.147	0.062
7	-0.114	0.113	-0.002
8	-0.105	0.029	0.131
9	-0.135	0.036	0.038
10	-0.113	0.110	0.200
11	-0.113	-0.071	-0.099
12	-0.121	-0.070	0.148
13	-0.113	-0.131	-0.058
14	-0.110	-0.150	0.033
15	-0.081	-0.078	-0.194

Factor Scores

Sample	Factor 1	Factor 2	Factor 3
1	-1.077	-1.146	0.371
2	-0.383	-0.824	1.002
3	-1.238	-1.350	0.961
4	-0.120	-1.068	0.479
5	-1.210	-0.169	-0.773
6	-0.638	0.406	-1.002
7	-1.685	0.387	0.522
8	-0.585	0.478	-0.110

9	-1.059	-1.315	-0.214
10	-1.990	0.034	1.419
11	-1.872	-0.319	0.730
12	-0.496	-1.911	-0.227
13	-0.135	-1.883	-0.502
14	1.896	-1.050	0.593
15	1.629	-1.110	0.171
16	1.208	-0.299	0.721
17	0.707	-0.528	-0.429
18	0.115	0.629	1.517
19	0.914	0.747	1.475
20	1.289	-0.262	0.931
21	0.225	1.317	1.473
22	-0.040	0.801	0.741
23	1.723	-0.262	-0.162
24	0.577	1.002	1.484
25	0.301	1.000	0.011
26	-0.325	-0.311	-1.024
27	0.267	-0.857	-0.621
28	0.437	-0.525	-2.192
29	0.169	-1.187	-0.692
30	0.203	-1.457	-1.202
31	-1.576	-0.099	-0.694
32	-1.600	-0.105	-1.424
33	-1.854	-0.325	-0.216
34	-1.193	-1.150	-0.035
35	-0.071	-1.650	-0.338
36	-0.362	-1.543	-0.197
37	-0.213	-1.871	-0.098
38	1.227	-0.628	0.252
39	0.039	-0.501	-0.244
40	0.938	-1.051	0.752
41	0.798	-1.271	0.798
42	-0.922	0.728	0.986
43	-1.131	0.691	1.115
44	-0.381	1.835	-0.411
45	0.554	1.487	-1.309
46	1.258	0.792	-0.917
47	1.828	-0.140	0.019
48	0.498	1.333	-1.294
49	-0.268	2.363	-1.111
50	1.852	0.248	-0.293
51	0.202	2.151	-0.964
52	-0.482	0.454	-2.427
53	-1.364	-1.225	0.534
54	0.162	0.685	0.821
55	0.031	0.420	0.118
56	-1.007	0.634	1.165
57	-0.478	0.842	1.436
58	0.279	-0.174	0.612
59	-0.672	0.554	0.151
60	-1.288	0.734	1.105
61	0.651	-0.655	-0.179
62	1.336	-0.731	-0.933
63	1.454	-0.066	-1.589
64	1.303	-0.279	-0.721
65	-0.309	1.386	-2.236
66	-0.272	0.403	0.278
67	-0.257	0.883	-0.037
68	-0.201	1.212	0.967
69	0.463	0.922	1.196
70	0.360	1.150	0.646
71	0.491	1.493	0.314
72	0.726	1.366	0.382
73	0.150	0.060	0.117
74	-1.335	0.315	-3.128
75	-0.140	0.554	-1.112
76	1.615	-0.489	0.886
77	1.551	-0.207	0.724

8.8.4.2.2 Regression Analysis: SMS versus TN and PC1, PC2, PC3 of the (SE-HPLC) correlation matrix (Model H)

MLR Results

	SMS v PC1, PC2, PC3	SMS v TN, PC1, PC2, PC3
Multiple R	0.9061	0.9098
Multiple R²	0.8129	0.8277
Adjusted R²	0.8053	0.8181
F(4,72)	105.75	86.48
P	0.0000	0.0000
SE of prediction	0.7123	0.6883

Regression summary

	Beta	Std.Error of Beta	B	Std.Error of B-coefficient	t(72)	*p*-level
Intercept			10.72339	2.488556	4.3091	0.000051
TN	-0.139579	0.056186	-1.59841	0.643423	-2.4842	0.015310
PC1	-0.735728	0.051264	-1.19119	0.082999	-14.3519	0.000000
PC2	-0.337626	0.050314	-0.54234	0.080820	-6.7104	0.000000
PC3	0.256771	0.052938	0.41501	0.085561	4.8504	0.000007

Regression equation

Cheddar cheese maturity = 10.72339 - 1.59841 TN - 1.19119 PC1 - 0.54234 PC2 + 0.41501 PC3

(Equation 8)

Analysis of Variance

	Sums of Squares	df	Mean Squares	F	*p*-level
Regress.	163.8969	4	40.97423	86.47746	0.000000
Residual	34.1146	72	0.47381		
Total	198.0115				

8.8.4.3 PCA (covariance matrix) of the SE-HPLC peak data with MLR of the principal components (SMS v TN and PC1, PC2 and PC3)

8.8.4.3.1 PCA: 'Peaks' 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15

Unrotated Factor Loadings (Factor-variable correlations) and Communalities

Variable	Factor 1	Factor 2	Factor 3	Communality
1	-37.913	-21.840	24.977	2.53E+03
2	-18.108	112.770	8.313	1.31E+04
3	-100.916	61.200	-45.671	1.60E+04
4	-52.828	147.870	154.560	4.84E+04
5	-853.756	610.070	181.652	1.13E+06
6	-499.502	-7.110	-153.489	2.74E+05
7	-316.407	-36.140	-85.630	1.09E+05
8	-95.625	-68.250	-29.234	1.46E+04
9	-424.161	-199.360	-78.796	2.26E+05
10	-450.628	-70.330	-271.367	2.81E+05
11	-184.104	-209.610	35.025	7.43E+04
12	-170.793	-172.970	-38.683	6.35E+04
13	-406.829	-661.430	223.854	6.53E+05
14	-169.343	-348.180	66.245	1.54E+05
15	-42.189	-49.890	9.382	4.37E+03
SMS*1000	-728.736	-1175.930	-202.791	
Variance	1744410	1096756	228570	3069737
% Variance	52.79	33.19	6.92	9.29E+01

Factor Score Coefficients

	Factor 1	Factor 2	Factor 3
1	-0.000022	-0.000020	0.000109
2	-0.000010	0.000103	0.000036
3	-0.000058	0.000056	-0.000200
4	-0.000030	0.000135	0.000676
5	-0.000489	0.000556	0.000795
6	-0.000286	-0.000006	-0.000672
7	-0.000181	-0.000033	-0.000375
8	-0.000055	-0.000062	-0.000128
9	-0.000243	-0.000182	-0.000345
10	-0.000258	-0.000064	-0.001187
11	-0.000106	-0.000191	0.000153
12	-0.000098	-0.000158	-0.000169
13	-0.000233	-0.000603	0.000979
14	-0.000097	-0.000317	0.000290
15	-0.000024	-0.000045	0.000041

Factor Scores

	Factor 1	Factor 2	Factor 3
1	-0.065	-2.498	1.285
2	0.339	-1.079	-1.106
3	-0.065	-2.586	0.319
4	0.654	-0.947	-0.627
5	-1.222	-0.453	-0.192
6	-0.783	0.693	-0.295
7	-1.475	-0.443	-1.554
8	-0.822	0.595	-0.701
9	-0.243	-1.390	0.995
11	-1.638	-1.172	-0.979
12	-1.601	-0.969	-0.589
13	0.723	-1.825	0.056
14	0.951	-1.452	0.935
15	2.174	0.137	-0.792
16	1.852	-0.056	-0.056
17	1.465	-0.183	-0.010
18	0.687	0.599	-0.009
19	-0.151	0.514	-1.019
20	0.428	0.806	-0.289
21	1.032	0.780	-0.905
22	-0.203	0.687	-0.681
23	-0.433	0.414	0.122
24	1.401	0.807	0.501
25	0.162	0.685	-0.461
26	-0.387	0.772	0.951
27	-0.461	-0.276	0.956
28	0.217	-0.430	1.089
29	0.045	0.160	2.893
30	0.575	-0.816	1.730
31	1.003	-1.157	2.192
32	-1.788	-0.778	0.844
33	-1.802	-0.411	0.801
34	-1.499	-1.216	-0.739
35	-0.434	-2.263	1.468
36	0.889	-1.343	0.182
37	0.610	-1.385	-0.526
38	0.917	-1.584	-0.280
39	1.071	0.527	-0.669
40	0.111	0.093	-0.780
41	1.233	-0.017	-1.109
42	1.252	-0.330	-1.175
43	-1.393	0.593	-1.085
44	-1.453	0.285	-1.840
45	-1.108	0.913	0.969
46	-0.577	1.477	1.785
47	0.589	1.495	0.977
48	1.690	0.783	-0.011
49	-0.183	1.226	1.259
50	-1.431	1.592	1.001
51	1.497	1.080	0.161
52	-0.930	1.653	0.704
53	-0.909	0.829	0.983
54	-0.733	-2.073	1.030
55	0.255	0.231	-0.657
56	0.171	0.079	0.061
57	-0.707	-0.306	-1.547
58	-0.576	0.045	-0.686
59	0.231	0.094	-0.860
60	-0.802	-0.016	-0.442
61	-0.869	-0.565	-2.032
62	0.689	0.227	-0.427
63	1.384	0.344	0.632
64	0.995	0.982	0.981
65	0.940	0.603	0.837

66	-1.449	1.475	1.477
67	-0.515	0.171	-0.474
68	-0.220	0.485	-0.807
69	-0.441	0.704	-1.474
70	0.161	0.774	-1.324
71	0.045	0.809	-0.759
72	-0.084	1.107	-0.205
73	0.259	1.023	-0.334
74	-0.216	0.354	0.407
75	-1.274	0.581	1.225
76	-0.756	0.920	0.601
77	1.848	-0.110	-0.474
78	1.714	0.155	-0.350

8.8.4.3.2 Regression Analysis: SMS versus TN and PC1, PC2, PC3 of the (SE-HPLC) covariance matrix (Model I)

MLR Results

	SMS v PC1, PC2 and PC3	SMS v TN, PC1, PC2 and PC3
Multiple R	0.9024	0.9224
Multiple R^2	0.8143	0.8508
Adjusted R^2	0.8067	0.8425
F(4,72)	106.70	102.63
p	0.0000	0.0000
SE of prediction	0.7097	0.6406

Regression summary

	Beta	Std.Error of Beta	B	Std Error of B-coefficient	t(72)	*p*-level
Intercept			14.62095	2.402859	6.0848	0.000000
TN	-0.227670	0.054255	-2.60719	0.621312	-4.1963	0.000076
PC1	-0.445386	0.045905	-0.71571	0.073766	-9.7024	0.000000
PC2	-0.695563	0.048204	-1.12658	0.078074	-14.4297	0.000000
PC3	-0.054075	0.051742	-0.08739	0.083616	-1.0451	0.299479

Regression equation

Cheddar cheese maturity = 14.62095 - 2.60719 TN - 0.71571 PC1 - 1.12658 PC2 - 0.08739 PC3

(Equation 9)

Analysis of Variance

	Sums of Squares	df	Mean Squares	F	*p*-level
Regress.	168.4661	4	42.11651	102.6346	0.000000
Residual	29.5455	72	0.41035		
Total	198.0115				

8.8.5 MLR AND MODELS J-O

8.8.5.1 MLR: SMS versus TN and RP-HPLC peaks 1 – 54 (Model J)

MLR Results

	SMS v RP-HPLC peaks 1 - 54	SMS v TN and RP-HPLC peaks 1 - 54
Multiple R	0.9932	0.9960
Multiple R^2	0.9865	0.9922
Adjusted R^2	0.9533	0.9717
F(55,21)	29.7495	48.4782
p	0.00000	0.00000
SE of prediction	0.3487	0.2714

Regression summary

	Beta	Std.Error of Beta	B	Std Error of B-coefficient	t(72)	*p*-level
Intercept			15.188316	3.211454	4.72942	0.000114
TN	-0.259058	0.066220	-2.966638	0.758328	-3.91208	0.000801
P1	-0.612544	0.419025	-0.000064	0.000044	-1.46183	0.158591
P2	0.428888	0.546087	0.000065	0.000083	0.78538	0.441000
P3	0.623583	0.332644	0.000251	0.000134	1.87463	0.074823
P4	0.702471	0.349341	0.000093	0.000046	2.01085	0.057355
P5	-0.569791	0.230446	-0.000197	0.000080	-2.47256	0.022045
P6	-0.396173	0.144402	-0.000362	0.000132	-2.74355	0.012173
P7	-0.404980	0.166543	-0.000311	0.000128	-2.43169	0.024065
P8	0.201311	0.370693	0.000097	0.000178	0.54307	0.592803
P9	0.070407	0.315567	0.000053	0.000236	0.22311	0.825604
P10	0.079489	0.137928	0.000044	0.000077	0.57631	0.570532
P11	-0.085284	0.099537	-0.000102	0.000119	-0.85681	0.401223
P12	0.017788	0.239457	0.000009	0.000122	0.07428	0.941487
P13	0.141447	0.224778	0.000046	0.000073	0.62927	0.535956
P14	-0.185416	0.184818	-0.000077	0.000076	-1.00324	0.327166
P15	-0.349561	0.178085	-0.000247	0.000126	-1.96289	0.063042
P16	0.033128	0.168266	0.000006	0.000031	0.19688	0.845816
P17	-0.143292	0.421834	-0.000018	0.000053	-0.33969	0.737466
P18	0.082629	0.400528	0.000014	0.000066	0.20630	0.838544
P19	0.437708	0.264279	0.000169	0.000102	1.65624	0.112541
P20	-0.273885	0.386531	-0.000105	0.000148	-0.70857	0.486382
P21	0.135081	0.199720	0.000057	0.000085	0.67635	0.506199
P22	0.024650	0.217576	0.000008	0.000069	0.11329	0.910874
P23	0.032542	0.112601	0.000022	0.000076	0.28900	0.775413
P24	0.032931	0.136440	0.000016	0.000067	0.24136	0.811618
P25	0.005805	0.235449	0.000003	0.000114	0.02466	0.980562
P26	0.103343	0.157312	0.000042	0.000064	0.65693	0.518359
P27	0.093424	0.123077	0.000199	0.000262	0.75907	0.456245
P28	0.249002	0.357390	0.000143	0.000205	0.69672	0.493617
P29	0.349978	0.129901	0.000210	0.000078	2.69418	0.013584
P30	0.079973	0.066683	0.000128	0.000107	1.19932	0.243763
P31	0.307273	0.086037	0.000296	0.000083	3.57140	0.001801
P32	0.122774	0.101810	0.000163	0.000136	1.20590	0.241266
P33	-0.373235	0.247257	-0.000256	0.000170	-1.50950	0.146070
P34	0.138710	0.249013	0.000072	0.000130	0.55704	0.583388
P35	0.297569	0.331396	0.000131	0.000146	0.89793	0.379405
P36	0.041652	0.138265	0.000030	0.000099	0.30125	0.766190
P37	-0.299644	0.109727	-0.000391	0.000143	-2.73082	0.012523
P38	-0.469170	0.180634	-0.000339	0.000131	-2.59735	0.016813
P39	0.225018	0.151475	0.000177	0.000119	1.48551	0.152268
P40	-0.283864	0.203905	-0.000266	0.000191	-1.39213	0.178450
P41	0.314310	0.205556	0.000229	0.000150	1.52907	0.141172
P42	-0.196504	0.155368	-0.000274	0.000217	-1.26477	0.219810

P43	0.250268	0.221908	0.000151	0.000133	1.12780	0.272124
P44	-0.381326	0.150355	-0.000190	0.000075	-2.53617	0.019213
P45	-0.060000	0.094007	-0.000051	0.000080	-0.63826	0.530207
P46	-0.235983	0.078269	-0.000581	0.000193	-3.01503	0.006591
P47	-0.017036	0.052383	-0.000027	0.000084	-0.32522	0.748234
P48	-0.241762	0.093807	-0.000302	0.000117	-2.57722	0.017570
P49	-0.007162	0.170987	-0.000005	0.000117	-0.04189	0.966983
P50	-0.597244	0.225803	-0.000178	0.000067	-2.64498	0.015144
P51	0.422343	0.216498	0.000140	0.000072	1.95080	0.064552
P52	0.558352	0.210178	0.000139	0.000053	2.65657	0.014762
P53	-0.150976	0.164905	-0.000055	0.000060	-0.91553	0.370307
P54	-0.121169	0.133145	-0.000135	0.000148	-0.91006	0.373120

Regression equation

Predicted cheese maturity = 15.188316 - 2.966638 TN - 0.000064 P1 + 0.000065 P2 + 0.000251 P3 + 0.000093 P4 - 0.000197 P5 - 0.000362 P6 - 0.000311 P7 + 0.000097 P8 + 0.000053 P9 + 0.000044 P10 - 0.000102 P11 + 0.000009 P12 + 0.000046 P13 - 0.000077 P14 - 0.000247 P15 + 0.000006 P16 - 0.000018 P17 + 0.000014 P18 + 0.000169 P19 - 0.000105 P20 + 0.000057 P21 + 0.000008 P22 + 0.000022 P23 + 0.000016 P24 + 0.000003 P25 + 0.000042 P26 + 0.000199 P27 + 0.000143 P28 + 0.000210 P29 + 0.000128 P30 + 0.000296 P31 + 0.000163 P32 - 0.000256 P33 + 0.000072 P34 + 0.000131 P35 + 0.000030 P36 - 0.000391 P37 - 0.000339 P38 + 0.000177 P39 - 0.000266 P40 + 0.000229 P41 - 0.000274 P42 + 0.000151 P43 - 0.000190 P44 - 0.000051 P45 - 0.000581 P46 - 0.000027 P47 - 0.000302 P48 - 0.000005 P49 - 0.000178 P50 + 0.000140 P51 + 0.000139 P52 - 0.000055 P53 - 0.000135 P54

(Equation 10)

Analysis of variance

	Sums of Squares	df	Mean Squares	F	*p*-level
Regress.	196.4642	55	3.572076	48.47820	0.000000
Residual	1.5474	21	0.073684		
Total	198.0115				

8.8.5.2 MLR: SMS versus TN and 32 RP-HPLC peaks (Model K)

MLR Results

	SMS v TN and 32 RP-HPLC peaks
Multiple R	0.9944
Multiple R²	0.9889
Adjusted R²	0.9803
F(33,43)	115.5844
p	0.0000
Std.Err. of prediction	0.2266

Regression summary

	Beta	Std.Err. of Beta	B	Std.Err of B.	t(43)	*p*-level
Intercept			15.08415	1.547548	9.74713	0.000000
TN	-0.246473	0.032419	-2.82251	0.371245	-7.60283	0.000000
P1	-0.606161	0.212341	-0.00006	0.000022	-2.85466	0.006605
P2	0.970593	0.283364	0.00015	0.000043	3.42525	0.001362
P3	0.785408	0.138207	0.00032	0.000056	5.68283	0.000001
P4	0.522302	0.143846	0.00007	0.000019	3.63099	0.000747
P5	-0.585390	0.096422	-0.00020	0.000033	-6.07110	0.000000
P6	-0.407013	0.077388	-0.00037	0.000071	-5.25936	0.000004
P7	-0.305097	0.075020	-0.00023	0.000058	-4.06689	0.000199
P10	0.097587	0.065104	0.00005	0.000036	1.49895	0.141193
P11	-0.123748	0.054513	-0.00015	0.000065	-2.27005	0.028274
P14	-0.326482	0.055900	-0.00013	0.000023	-5.84050	0.000001
P15	-0.382819	0.105308	-0.00027	0.000074	-3.63524	0.000737
P17	-0.299819	0.155140	-0.00004	0.000020	-1.93257	0.059894
P19	0.628643	0.103733	0.00024	0.000040	6.06020	0.000000
P21	0.165596	0.077637	0.00007	0.000033	2.13296	0.038677
P26	0.070853	0.066832	0.00003	0.000027	1.06017	0.294986
P29	0.341875	0.071177	0.00020	0.000043	4.80319	0.000019
P31	0.230386	0.042542	0.00022	0.000041	5.41545	0.000003
P32	0.082541	0.042450	0.00011	0.000057	1.94441	0.058406
P33	-0.263844	0.074881	-0.00018	0.000051	-3.52354	0.001024
P34	0.177543	0.106786	0.00009	0.000056	1.66261	0.103664
P35	0.271186	0.123522	0.00012	0.000054	2.19545	0.033576
P37	-0.333171	0.060202	-0.00043	0.000078	-5.53425	0.000002
P38	-0.442715	0.102400	-0.00032	0.000074	-4.32337	0.000089
P39	0.185641	0.081331	0.00015	0.000064	2.28255	0.027463
P40	-0.230970	0.103176	-0.00022	0.000097	-2.23860	0.030411
P41	0.348837	0.134178	0.00025	0.000098	2.59981	0.012732
P44	-0.340321	0.063609	-0.00017	0.000032	-5.35017	0.000003
P46	-0.249248	0.035535	-0.00061	0.000087	-7.01424	0.000000
P48	-0.267895	0.043681	-0.00034	0.000055	-6.13305	0.000000
P50	-0.437185	0.117828	-0.00013	0.000035	-3.71037	0.000590
P51	0.380262	0.088577	0.00013	0.000029	4.29300	0.000098
P52	0.365019	0.099609	0.00009	0.000025	3.66450	0.000676

Regression equation

Predicted cheese maturity = 15.08415 - 2.82251 TN - 0.00006 P1 + 0.00015 P2 + 0.00032 P3 + 0.00007 P4 - 0.00020 P5 - 0.00037 P6 - 0.00023 P7 + 0.00005 P10 - 0.00015 P11 - 0.00013 P14 - 0.00027 P15 - 0.00004 P17 + 0.00024 P19 + 0.00007 P21 + 0.00003 P26 + 0.00020 P29 + 0.00022 P31 + 0.00011 P32 - 0.00018 P33 + 0.00009 P34 + 0.00012 P35 - 0.00043 P37 - 0.00032 P38 + 0.00015 P39 - 0.00022 P40 + 0.00025 P41 - 0.00017 P44 - 0.00061 P46 - 0.00034 P48 - 0.00013 P50 + 0.00013 P51 + 0.00009 P52

(Equation 11)

Analysis of variance

	Sums of Squares	df	Mean Squares	F	*p*-level
Regress.	195.8042	33	5.933459	115.5844	0.000000
Residual	2.2074	43	0.051334		
Total	198.0115				

8.8.5.3 MLR: SMS versus TN and 7 RP-HPLC peaks (Model L)

MLR Results

	SMS v TN and 7 RP-HPLC peaks
Multiple R	0.9617
Multiple R²	0.9249
Adjusted R²	0.9161
F(8,68)	104.6878
p	0.0000
Std.Err. of prediction	0.4676

Regression summary

	Beta	Std.Err. of Beta	B	Std. Err. of B	t(68)	*p*-level
Intercept			9.61716	1.849821	5.19897	0.000002
TN	-0.174162	0.042078	-1.99444	0.481858	-4.13906	0.000098
P1	0.310724	0.188999	0.00003	0.000020	1.64405	0.104781
P4	-0.464822	0.144215	-0.00006	0.000019	-3.22312	0.001948
P16	0.100602	0.045534	0.00002	0.000008	2.20941	0.030517
P17	0.476870	0.127687	0.00006	0.000016	3.73469	0.000387
P18	0.493576	0.149495	0.00008	0.000024	3.30162	0.001534
P50	-0.264408	0.156685	-0.00008	0.000047	-1.68751	0.096087
P52	0.297287	0.142114	0.00007	0.000036	2.09190	0.040184

Regression equation

Predicted cheese maturity = 9.61716 - 1.99444 TN + 0.00003 P1 - 0.00006 P4 + 0.00002 P16 + 0.00006 P17 + 0.00008 P18 - 0.00008 P50 + 0.00007 P52

(Equation 12)

Analysis of variance

	Sums of Squares	df	Mean Squares	F	*p*-level
Regress.	183.1416	8	22.89270	104.6878	0.000000
Residual	14.8700	68	0.21868		
Total	198.0115				

8.8.5.4 MLR: SMS versus TN and SE-HPLC 'peaks' 1 – 15 (Model M)

MLR Results

	SMS v TN and SE-HPLC 'peaks' 1 - 15
Multiple R	0.95848
Multiple R²	0.91869
Adjusted R²	0.89701
F(16,60)	42.36929
p	0.00000
Std.Err. of prediction	0.51802

Regression summary

	Beta	Std.Err. of Beta	B	Std.Err. of B	t(60)	*p*-level
Intercept			9.53621	2.485784	3.83630	0.000303
TN	-0.191996	0.055991	-2.19866	0.641193	-3.42902	0.001101
Peak 1	-0.218997	0.093841	-0.00503	0.002155	-2.33371	0.022979
Peak 2	-0.134724	0.074860	-0.00106	0.000592	-1.79969	0.076937
Peak 3	0.073118	0.077504	0.00052	0.000554	0.94341	0.349254
Peak 4	-0.182372	0.098035	-0.00118	0.000636	-1.86028	0.067749
Peak 5	0.159585	0.134090	0.00024	0.000202	1.19013	0.238684
Peak 6	-0.198194	0.189713	-0.00059	0.000563	-1.04470	0.300352
Peak 7	0.564227	0.207023	0.00257	0.000944	2.72543	0.008404
Peak 8	0.039246	0.091413	0.00037	0.000869	0.42933	0.669221
Peak 9	-0.311477	0.255582	-0.00104	0.000854	-1.21870	0.227730
Peak 10	-0.088134	0.165240	-0.00026	0.000486	-0.53337	0.595747
Peak 11	0.098132	0.088204	0.00047	0.000420	1.11256	0.270336
Peak 12	0.185066	0.134838	0.00110	0.000803	1.37251	0.175015
Peak 13	0.098988	0.222394	0.00020	0.000443	0.44510	0.657849
Peak 14	0.629878	0.203277	0.00252	0.000814	3.09862	0.002958
Peak 15	-0.079960	0.066603	-0.00104	0.000866	-1.20055	0.234645

Regression equation

Predicted cheese maturity = 9.53621 - 2.19866 TN - 0.00503 P1 - 0.00106 P2 + 0.00052 P3 - 0.00118 P4 + 0.00024 P5 - 0.00059 P6 + 0.00257 P7 + 0.00037 P8 - 0.00104 P9 - 0.00026 P10 + 0.00047 P11 + 0.00110 P12 + 0.00020 P13 + 0.00252 P14 - 0.00104 P15

(Equation 13)

Analysis of variance

	Sums of Squares	df	Mean Squares	F	*p*-level
Regress.	181.9110	16	11.36944	42.36929	0.000000
Residual	16.1005	60	0.26834		
Total	198.0115				

8.8.5.5 MLR: SMS versus TN and 7 SE-HPLC (Model N)

MLR Results

	SMS v TN and 7 SE-HPLC 'peaks'
Multiple R	0.95327
Multiple R²	0.90873
Adjusted R²	0.89799
F(8,68)	84.63023
p	0.00000
Std.Err. of prediction	0.51553

Regression summary

	Beta	Std.Err. of Beta	B	Std.Err. of B	t(68)	*p*-level
Intercept			9.31131	2.189933	4.25187	0.000066
TN	-0.181164	0.051333	-2.07463	0.587851	-3.52917	0.000753
Peak 1	-0.179155	0.052686	-0.00411	0.001210	-3.40042	0.001130
Peak 4	-0.166944	0.056415	-0.00108	0.000366	-2.95919	0.004242
Peak 7	0.425188	0.114124	0.00194	0.000520	3.72567	0.000398
Peak 9	-0.308600	0.159228	-0.00103	0.000532	-1.93810	0.056764
Peak 12	0.193847	0.085594	0.00115	0.000509	2.26473	0.026721
Peak 13	0.377218	0.153529	0.00075	0.000306	2.45698	0.016568
Peak 14	0.373773	0.151030	0.00150	0.000605	2.47482	0.015829

Regression equation

Cheddar cheese maturity = 9.31131 - 2.07463 TN - 0.00411 P1 - 0.00108 P4 + 0.00194 P7 - 0.00103 P9 + 0.00115 P12 + 0.00075 P13 + 0.00150 P14

(Equation 14)

Analysis of variance

	Sums of Squares	df	Mean Squares	F	*p*-level
Regress.	179.9390	8	22.49238	84.63023	0.000000
Residual	18.0725	68	0.26577		
Total	198.0115				

8.8.6 MODEL VALIDATION RESULT SET

Validation: 90% of the samples (randomly selected) were used to derive the regression equation used to predict the maturity scores of the remaining 10% of samples. These scores were regressed against SMS. This was repeated 5 times (Sets A – E) and the mean of the values obtained for each statistical parameter was reported in the final column.

SMS v All RP-HPLC peaks and TN											
	100% samples	A 90%	A 10%	B 90%	B 10%	C 90%	C 10%	D 90%	D 10%	E 90%	E 10%	Mean for 10%
R	0.9961	0.9976	0.9334	0.9964	0.9503	0.9969	0.9594	0.9965	0.9820	0.9968	0.9663	0.9583
R^2	0.9922	0.9952	0.8713	0.9928	0.9030	0.9938	0.9205	0.9929	0.9644	0.9936	0.9336	0.9186
R^2 (Adjusted)	0.9717	0.9762	0.8456	0.9625	0.8868	0.9675	0.9072	0.9631	0.9585	0.9640	0.9242	0.9045
p-value	0.0000	0.0000	0.0021	0.0000	0.0003	0.0000	0.0002	0.0000	0.0000	0.0000	0.0000	0.0005
error	0.2714	0.2466	0.7210	0.3095	0.5780	0.2990	0.3629	0.3025	0.3988	0.3114	0.3997	0.4921
d.f	55, 21	55, 14	1, 5	55, 13	1, 6	55, 13	1, 6	55, 13	1, 6	55, 12	1, 7	
F	48.48	52.51	33.86	32.71	55.86	37.79	69.45	33.27	162.49	33.64	98.49	84.03
Outliers	-	-	-	-	4347	-	-	-	-	-	-	4347
SMS v 32 RP-HPLC peaks and TN												
	100% samples	A 90%	A 10%	B 90%	B 10%	C 90%	C 10%	D 90%	D 10%	E 90%	E 10%	Mean for 10%
R	0.9944	0.9943	0.9917	0.9941	0.9941	0.9948	0.9871	0.9937	0.9975	0.9952	0.9768	0.9894
R^2	0.9889	0.9886	0.9834	0.9882	0.9883	0.9896	0.9743	0.9874	0.995	0.9903	0.9542	0.9790
R^2 (Adjusted)	0.9803	0.9782	0.9801	0.977	0.9864	0.9799	0.97	0.9754	0.9941	0.981	0.9476	0.9756
p-value	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
error	0.2266	0.236	0.259	0.2424	0.2005	0.2353	0.2062	0.2468	0.1497	0.2266	0.332	0.2295
d.f	33, 43	33, 36	1, 5	33, 35	1, 6	33, 35	1, 6	33, 55	1, 6	33, 34	1, 7	
F	115.58	94.94	296.04	88.46	508.17	101.32	227.68	82.79	1190.1	105.57	145.83	473.5720
Outliers	4309	4309	-	4309	-	4309	4362	4309	-	4309	4309	4309, 4362
SMS v 7 RP-HPLC peaks and TN												
	100% samples	A 90%	A 10%	B 90%	B 10%	C 90%	C 10%	D 90%	D 10%	E 90%	E 10%	Mean for 10%
R	0.9617	0.9633	0.9479	0.9590	0.9765	0.9613	0.9613	0.9589	0.9733	0.9621	0.9668	0.9652
R^2	0.9249	0.9279	0.8985	0.9197	0.9535	0.9242	0.9241	0.9194	0.9473	0.9256	0.9347	0.9316
R^2 (Adjusted)	0.9161	0.9184	0.8781	0.9089	0.9458	0.9141	0.9114	0.9087	0.9386	0.9155	0.9253	0.9198
p-value	0.0000	0.0000	0.0012	0.0000	0.0000	0.0000	0.0001	0.0000	0.0000	0.0000	0.0000	0.0003
error	0.4646	0.4568	0.6404	0.4821	0.4001	0.4861	0.3546	0.4758	0.4850	0.4773	0.3966	0.4553
d.f	8, 68	8, 61	1,5	8, 60	1, 6	8, 60	1, 6	8, 60	1, 6	8, 59	1, 7	
F	104.69	98.13	44.24	85.85	123.13	91.43	73.04	85.60	107.93	91.71	100.14	89.70
Outliers	4323, 4359	4323	-	4323, 4359	-	4323, 4359	-	4323, 4359	-	4323, 4359	-	4323, 4359
SMS v All SE-HPLC peaks and TN												
	100% samples	A 90%	A 10%	B 90%	B 10%	C 90%	C 10%	D 90%	D 10%	E 90%	E 10%	Mean for 10%
R	0.9585	0.9586	0.9359	0.9562	0.9794	0.9570	0.9774	0.9545	0.9735	0.9581	0.9617	0.9656
R^2	0.9187	0.9138	0.8760	0.9143	0.9593	0.9158	0.9552	0.9111	0.9477	0.9180	0.9248	0.9326
R^2 (Adjusted)	0.8970	0.8943	0.8511	0.8879	0.9525	0.8899	0.9478	0.8837	0.9389	0.8922	0.9141	0.9209
p-value	0.0000	0.0000	0.0020	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0004
error	0.5180	0.5199	0.7078	0.5350	0.3746	0.5502	0.2722	0.5370	0.4835	0.5389	0.4254	0.4527
d.f	16, 60	16, 53	1, 5	16, 52	1, 6	16, 52	1, 6	16, 52	1, 6	16, 51	1, 7	
F	42.37	37.50	35.31	34.60	141.31	35.36	128.06	33.80	108.63	35.68	86.12	99.89
Outliers	4079, 4361, 4367	4079, 4361	-	4079, 4361	4364	4361	-	4079, 4361, 4367	4358	4361	-	4079, 4361, 4358, 4364, 4367
SMS v 7 SE-HPLC peaks and TN												
	100% samples	A 90%	A 10%	B 90%	B 10%	C 90%	C 10%	D 90%	D 10%	E 90%	E 10%	Mean for 10%
R	0.9533	0.9541	0.9334	0.9500	0.9792	0.9527	0.9587	0.9492	0.9769	0.9512	0.9739	0.9644
R^2	0.9087	0.9103	0.8713	0.9024	0.9588	0.9076	0.9191	0.9010	0.9544	0.9048	0.9486	0.9304
R^2 (Adjusted)	0.8980	0.8986	0.8455	0.8894	0.9519	0.8953	0.9056	0.8878	0.9468	0.8919	0.9472	0.9194
p-value	0.0000	0.0000	0.0021	0.0000	0.0000	0.0000	0.0002	0.0000	0.0000	0.0000	0.0001	0.0005
error	0.5155	0.5094	0.7211	0.5313	0.3767	0.5366	0.3660	0.5273	0.4513	0.5398	0.3519	0.4534
d.f	8, 68	8, 61	1, 5	8, 60	1, 6	8, 60	1, 6	8, 60	1, 6	8, 59	1, 7	
F	84.63	77.40	33.84	69.37	139.66	73.70	68.18	68.29	125.54	70.10	129.09	99.26
Outliers	4079, 4323, 4361, 4367	4079, 4323, 4361, 4367	-	4079, 4323, 4361, 4367	-	4079, 4323, 4361, 4367	-	4079, 4323, 4361, 4367	-	4079, 4323, 4361, 4367	4319	4079, 4319, 4323, 4361, 4367

8.8.7 Cheddar cheese – SMS, and the maturity scores predicted by maturity index and the equations derived from models A - N

Code	Factory	SMS	MI	ModelA	Model B	Model C	Model D	Model E	Model F	ModelG	Model H	Model I	Model J	Model K	Model L	Model M	Model N
4345	Marlborough	1.83	3.52	2.61	2.04	2.10	2.48	2.19	3.50	2.90	2.46	2.86	1.97	1.81	2.45	1.97	2.10
4364	Marlborough	1.96	3.52	2.45	2.89	2.09	2.11	1.68	2.91	2.47	2.83	2.91	1.94	2.00	1.97	1.99	2.05
4343	Marlborough	2.21	3.03	2.20	2.05	2.21	1.72	1.75	2.29	2.20	2.06	2.01	2.36	2.27	1.98	1.92	1.63
4344	Marlborough	2.27	3.03	2.42	2.21	1.71	1.87	1.98	2.44	2.14	2.37	2.30	2.10	2.11	1.84	2.07	2.08
4349	Marlborough	2.28	3.80	3.00	2.48	2.72	3.01	2.82	3.92	3.61	3.68	3.96	2.20	1.97	2.58	2.52	2.81
4348	Marlborough	2.35	3.52	3.02	2.57	3.00	2.95	3.18	3.10	3.21	2.55	3.03	2.29	2.34	2.84	2.78	2.88
4347	Marlborough	2.42	3.03	2.55	2.13	1.89	1.91	2.13	2.15	1.93	1.96	2.04	2.64	2.55	2.21	2.08	1.91
4365	Marlborough	2.42	3.52	2.99	3.59	2.87	2.53	1.96	2.55	2.00	2.01	2.34	2.37	2.67	2.07	1.83	1.79
4366	Marlborough	2.54	3.52	2.67	3.37	2.72	2.32	2.35	2.83	2.82	2.74	2.88	2.54	2.33	2.47	2.80	2.62
4342	Marlborough	2.58	3.52	2.83	2.61	2.64	2.82	2.54	3.09	2.92	2.31	2.71	2.41	2.46	2.42	2.78	2.83
4346	Marlborough	2.68	3.80	3.13	2.59	3.21	3.28	3.07	3.94	3.68	2.91	3.41	2.77	2.91	3.00	2.70	2.79
4311	Southland	2.79	3.03	3.44	3.72	3.07	3.45	3.51	3.00	2.89	3.11	2.93	2.88	2.86	3.34	2.95	2.99
4325	Stirling	3.00	4.48	4.44	4.89	5.33	4.87	3.56	4.02	3.92	3.20	3.59	2.96	2.99	3.34	3.44	3.54
4320	Stirling	3.04	3.03	3.54	3.12	3.14	3.48	3.07	2.39	2.39	2.47	2.36	2.96	2.98	3.13	3.28	3.19
4337	Waitoa	3.05	3.52	3.53	3.56	3.35	3.49	3.49	3.81	3.54	4.33	3.91	3.06	3.12	3.04	3.86	3.66
4356	Kiwi	3.08	3.52	3.18	3.56	3.72	3.54	3.61	4.45	4.09	4.29	4.23	2.89	3.01	3.59	3.32	3.38
4374	Hautapu	3.18	3.52	3.34	3.16	3.32	2.80	3.55	3.19	3.31	3.04	3.17	3.25	3.33	3.46	3.05	3.08
4363	Waitoa	3.29	4.48	4.79	3.86	4.15	4.02	3.96	3.60	3.68	4.02	3.85	3.37	3.37	3.60	4.13	4.06
4424	Southland	3.38	3.52	3.91	4.10	3.35	3.82	3.81	3.33	3.20	3.07	3.14	3.52	3.43	3.44	3.23	3.45
4421	Alpine	3.46	4.48	4.51	4.33	5.01	4.37	3.45	3.91	3.42	4.48	4.48	3.13	3.09	3.54	3.67	4.08
4357	Kiwi	3.52	3.52	3.21	3.57	3.78	3.82	3.82	4.51	4.31	4.29	4.30	3.41	3.32	3.81	3.53	3.64
4335	Waitoa	3.56	3.52	3.91	3.83	3.66	3.59	3.74	2.95	3.11	3.50	3.25	3.37	3.40	3.23	3.81	3.75
4422	Alpine	3.58	4.48	4.35	4.21	4.65	3.67	3.67	3.69	3.69	3.84	3.83	3.75	3.83	3.63	3.83	3.88
4361	Lichfield	3.63	4.48	4.51	5.72	5.05	4.67	4.91	4.88	5.16	5.16	5.28	3.85	3.99	3.90	5.00	4.97
4373	Hautapu	3.68	3.52	3.44	3.37	3.64	2.91	3.69	3.15	3.34	3.22	3.29	3.65	3.63	3.62	3.27	3.32
4359	Kiwi	3.69	4.48	4.09	4.40	4.91	4.68	4.84	5.60	5.48	5.29	5.09	3.83	3.92	4.90	4.65	4.60
4313	Southland	3.71	3.80	4.24	4.51	3.98	4.48	4.17	3.88	3.72	3.54	3.66	3.79	3.96	3.84	3.74	3.94
4312	Southland	3.74	3.52	3.50	3.60	3.06	3.46	3.28	3.24	3.14	3.26	3.23	3.53	3.53	3.12	3.09	3.20
4369	Hautapu	3.75	3.52	4.23	4.21	4.34	4.48	4.80	3.95	4.03	4.35	4.31	3.86	3.96	4.20	4.14	4.11
4341	Marlborough	3.80	3.80	2.65	2.33	2.75	3.04	2.86	4.37	4.10	3.68	4.01	3.87	3.87	2.98	3.27	3.29
4370	Hautapu	3.83	3.52	3.98	3.76	4.17	3.91	4.73	3.80	4.16	4.59	4.37	3.86	3.82	4.31	4.39	4.36
4371	Hautapu	3.92	3.52	3.82	3.96	3.86	3.46	4.31	3.51	3.83	4.03	3.79	3.87	3.90	3.86	3.92	3.91
4323	Stirling	3.92	4.48	4.48	5.45	5.23	5.03	4.88	4.70	5.32	5.15	5.01	4.09	4.17	5.54	4.87	5.09
4420	Alpine	4.04	4.48	4.38	4.24	4.46	4.25	3.75	4.47	4.44	4.37	4.17	4.18	4.26	4.32	4.50	4.12
4372	Hautapu	4.08	3.52	3.63	3.78	3.71	3.34	4.25	3.67	3.88	3.74	3.70	3.84	3.85	3.90	3.71	3.74
4423	Southland	4.17	3.52	4.01	4.30	3.54	4.06	4.07	3.42	3.48	3.26	3.40	4.21	4.23	3.80	3.68	3.85
4314	Stirling	4.23	3.03	3.54	3.84	3.33	3.51	3.50	2.83	3.05	3.76	3.28	3.97	3.99	3.62	4.10	4.29
4319	Stirling	4.25	3.80	4.49	4.24	4.30	5.17	4.59	4.19	4.46	4.48	4.33	4.32	4.50	4.92	5.01	5.01
4321	Stirling	4.29	3.52	3.98	3.60	3.74	4.06	4.28	3.60	3.92	3.93	3.68	4.36	4.33	4.59	4.66	4.75
4338	Waitoa	4.36	3.52	4.04	4.28	4.10	4.20	4.24	4.23	4.06	4.65	4.29	4.53	4.48	3.67	4.38	4.40
4075	Waitoa	4.39	4.48	4.82	5.14	5.26	4.52	4.37	4.04	3.88	4.81	4.35	4.55	4.54	4.41	4.22	4.10
4316	Stirling	4.45	3.80	3.95	3.69	3.88	3.54	3.69	3.32	3.57	3.67	3.29	4.53	4.56	3.94	4.30	4.26
4317	Stirling	4.50	3.03	3.47	3.36	3.20	3.23	3.58	2.77	3.12	3.55	3.02	4.57	4.42	3.83	3.88	3.89
4322	Stirling	4.52	3.80	4.11	3.69	4.04	4.42	3.96	3.71	3.90	3.56	3.64	4.52	4.52	4.20	4.50	4.40
4360	Waitoa	4.52	4.48	4.82	4.37	4.78	4.62	4.77	4.57	4.73	4.59	4.46	4.57	4.43	4.82	4.40	4.52
4336	Waitoa	4.57	4.48	4.86	4.22	4.59	4.55	4.61	4.22	4.39	4.67	4.54	4.63	4.61	4.39	4.56	4.59
4310	Southland	4.72	5.06	5.10	5.47	4.71	6.06	5.60	5.44	5.36	5.53	5.38	4.86	4.93	5.54	5.55	5.47
4368	Lichfield	4.83	4.48	4.51	5.59	4.89	4.24	4.65	4.56	5.02	4.81	4.84	4.70	4.63	4.11	4.79	4.86
4318	Stirling	4.88	3.52	3.79	3.41	3.60	3.75	4.21	3.68	4.06	4.17	4.01	4.86	4.84	4.44	4.65	4.81
4333	Southland	4.88	4.48	4.51	5.18	5.09	5.61	5.79	5.70	5.81	5.81	5.92	5.07	5.00	5.29	5.06	5.13
4332	Southland	4.91	4.48	4.44	5.45	4.79	5.22	5.18	5.26	5.27	5.42	5.49	4.99	4.93	4.95	4.94	4.92
4066	Southland	4.96	4.48	4.54	4.56	4.69	5.06	5.61	5.35	5.47	5.54	5.37	4.74	4.71	5.37	4.99	4.88
4326	Southland	5.00	4.96	4.92	5.24	5.39	5.47	4.67	4.81	4.80	4.67	4.69	5.06	4.97	4.95	5.20	4.85
4334	Southland	5.00	4.48	4.57	4.77	5.02	5.69	5.71	5.67	5.74	5.86	5.88	5.23	5.29	5.44	5.39	5.45
4367	Waitoa	5.00	7.38	7.81	5.71	6.41	5.68	4.26	4.43	3.69	3.01	3.45	5.02	4.93	4.77	3.92	3.66
4315	Stirling	5.08	3.52	3.79	3.71	3.82	3.99	4.38	4.08	4.42	4.76	4.27	4.82	4.92	4.87	4.92	4.83
4078	Waitoa	5.12	4.48	4.82	5.25	5.55	4.64	5.02	4.34	4.58	4.95	4.60	5.28	5.17	4.98	5.06	4.65
4327	Southland	5.16	4.96	4.82	4.91	5.09	5.54	5.04	5.02	4.67	4.49	4.67	5.15	5.18	5.05	4.80	4.44
4324	Stirling	5.30	4.48	4.44	5.18	5.01	4.98	4.45	4.70	5.11	4.42	4.64	5.16	5.08	4.73	4.44	4.68
4362	Lichfield	5.50	4.48	5.52	6.50	6.34	6.20	6.51	5.43	6.04	6.33	6.40	5.45	5.34	6.05	5.69	5.89
4340	Waitoa	5.71	4.96	5.23	5.22	5.73	5.06	5.37	5.50	5.65	6.09	5.74	5.82	5.80	5.66	5.75	5.80
4339	Waitoa	5.84	4.96	4.92	4.97	5.50	4.65	5.09	5.34	5.51	5.74	5.13	5.80	5.83	6.05	5.57	5.34
4358	Kiwi	5.96	4.48	4.44	4.73	5.23	5.27	5.81	5.98	6.07	5.97	5.84	5.82	5.90	5.61	5.19	5.13
4063	Southland	6.08	4.48	4.54	4.84	5.15	5.13	5.89	5.67	5.92	5.98	5.89	6.21	6.19	5.86	5.60	5.68
4309	Southland	6.16	5.06	5.32	6.40	5.34	6.20	6.19	5.87	5.98	6.18	6.24	5.69	5.55	5.82	5.91	5.93
4074	Waitoa	6.53	6.41	6.52	6.12	6.50	6.14	5.82	5.93	6.06	5.79	6.07	6.39	6.55	6.27	5.96	6.21
4077	Waitoa	6.78	6.41	6.62	6.37	6.66	6.22	6.54	6.41	6.55	6.68	6.59	6.68	6.75	7.15	6.34	6.38
4079	Southland	6.92	6.41	6.27	5.94	6.19	6.37	6.83	6.67	6.54	6.51	6.20	6.85	6.88	6.58	5.76	5.68
4329	Waitoa	7.17	7.57	7.74	7.90	7.31	7.25	5.69	6.71	6.21	5.93	6.22	7.31	7.26	6.94	7.03	6.78
4328	Waitoa	7.38	7.57	7.61	7.73	7.42	6.99	6.11	7.03	6.63	6.25	6.65	7.21	7.08	6.94	7.58	7.37
4331	Southland	7.54	7.57	7.16	6.44	6.38	7.11	7.51	7.27	7.42	6.69	7.30	7.51	7.59	7.35	7.93	7.84
4061	Southland	7.60	7.57	7.26	6.67	6.77	7.44	8.09	7.24	7.45	6.72	7.35	7.76	7.65	7.35	7.40	7.43
4330	Waitoa	7.61	7.57	7.64	7.84	7.27	7.24	6.64	7.59	7.02	6.94	7.36	7.66	7.63	7.40	7.56	7.60
4081	Waitoa	7.73	7.57	7.45	6.73	7.20	6.90	7.19	7.74	7.56	7.73	7.47	7.84	7.76	7.95	7.69	7.49
4083	Waitoa	7.79	7.57	7.61	6.88	7.24	7.07	6.80	7.52	7.16	7.44	7.09	7.68	7.70	7.20	7.31	7.33
4350	Southland	7.96	7.57	7.26	6.66	6.83	7.20	7.93	7.46	7.56	7.22	7.40	7.94	8.03	7.62	8.05	8.35
4064	Southland	7.96	7.57	7.29	6.88	6.86	7.69	7.93	7.48	7.66	7.32	7.70	7.86	7.90	7.63	7.76	7.80